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LE ROLE DES GLYCANNES N-GLYCOSIDIQUEMENT LIES DE LA SURFACE CELLULAIRE DANS L'INVASION TUMORALE

Présentée le 10 juillet 1989 devant la Commission d'Examen

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Préface

Ce mémoire contient huit articles publiés ou en cours de publication. Le travail exposé ici a été réalisé à l'Unité de Recherche sur la Chimie Biologique (Unité Mixte de Recherche du CNRS, N° 111) de l'Université des Sciences et Techniques de Lille Flandres-Artois, au Laboratoire de la Cancérologie Expérimentale à l'Hôpital Universitaire de Gand et au Laboratoire de Cytologie Expérimentale à l'Institut Néerlandais du Cancer d'Amsterdam. La simple énumération des quatorze co-auteurs des publications ne peut pas révéler les liens profonds qui m'attachent à plusieurs d'entre eux.

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ERIK BRUYNEEL.

TABLE DES MATIERES

Abbréviations

ADN : acide désoxyribonucléique

AMP : adénosine monophosphate

Asn : asparagine

ATP : adénosine triphosphate

Endo-H : endo-β-N-acétylglucosaminidase H

ET-18-OCH_3 : 1-O-octadécyl-2-O-méthylglycéro-3-phosphocholine

FCP : fragment de coeur précultivé d'un embryon de poulet de 9 jours

Fuc : L-fucose

Gal : D-galactose

GlcNAc : N-acétyl-D-glucosamine

IgD : immunoglobuline D

IgM : immunoglobuline M

Man : D-mannose

PBS : tampon phosphate 10 mM, NaCl 0.154 M, pH 7.2

RER : réticulum endoplasmique rugueux

Ser : sérine

Thr : thréonine

Les inhibiteurs de la glycosylation

CS : castanospermine

dGlc : 2-désoxy-D-glucose

DMDP : 2,5-dihydroxyméthyl-3,4-dihydroxypyrrolidine

dMM : 1-désoxymannojirimycine

dNM : 1-désoxynojirimycine

HNV : hydroxynorvaline

MdNM : N-méthyl-1-désoxynojirimycine

MON : monensine

SW : swainsonine.

TM : tunicamycine

INTRODUCTION GENERALE

Le processus de la cancérogénèse se déroule en plusieurs étapes : l'initiation, la promotion, la perte de contrôle de croissance, l'invasion et la formation de métastases (Foulds, 1969; Nicolson et coll., 1977, fig. 1). Le cancer, qui peut être défini comme une maladie cellulaire, n'est pas la règle mais l'exception. Il est donc peu probable que la transformation d'une cellule normale en cellule cancéreuse soit un phénomène fréquent, provoqué par un agent cancérigène de manière identique sur de nombreuses cellules. Il vaut mieux définir le cancer comme une maladie à point de départ unicellulaire et expliquer l'hétérogénéité du tissu cancéreux par des phénomènes de différenciation, accentués par la croissance et la motilité accrues des cellules malignes (Foulds, 1969; Gabbert, 1985). En pathologie humaine il est possible d'observer in vivo la cancérisation d'une zone tissulaire entière, d'une part, et des cancers multicentriques, d'autre part. L'origine unicellulaire de la leucémie myéloïde chronique est établie puisque toutes les cellules malignes portent la même aberration (chromosome de Philadelphie). De plus, une étude chez des personnes ayant deux types de cellules génétiquement différentes a démontré que toute tumeur maligne résulte de la transformation d'une seule cellule (Fialkov, 1976). Une propriété très importante de cellules malignes est la perte de l'inhibition de contact de la croissance (Abercrombie et Ambrose, 1976). Les anomalies mitotiques les plus précoces sont les mitoses à chromosomes polaires, qui conduisent à des anomalies de la forme des noyaux. Le nombre de figures mitotiques permettent de quantifier **la croissance tumorale** (Dustin, 1966).

Les néoplasies très tôt détectables sont celles qui se situent dans une partie du corps, qui peut être facilement controlée : c'est le cas pour la peau, le col utérin, l'estomac et le gros intestin. Quelques-unes de ces lésions sont dans un état pré-invasif et appelées des **cancers in situ, cancers pré-invasifs ou du stade 0;** elles ont un diamètre de quelques millimètres, représentant environ un million de cellules. L'oxygénation et la nutrition sont assurées par diffusion et les lésions peuvent

rester dormantes pendant plusieurs années (Ruddon, 1981). La vascularisation, qui a été associée à la sécrétion de la molécule TAF ("Tumor Angiogenesis Factor") augmente la nutrition et favorise la croissance des tumeurs primaires (Folkman et Cotran, 1976; Folkman et Klagsbrun, 1987).

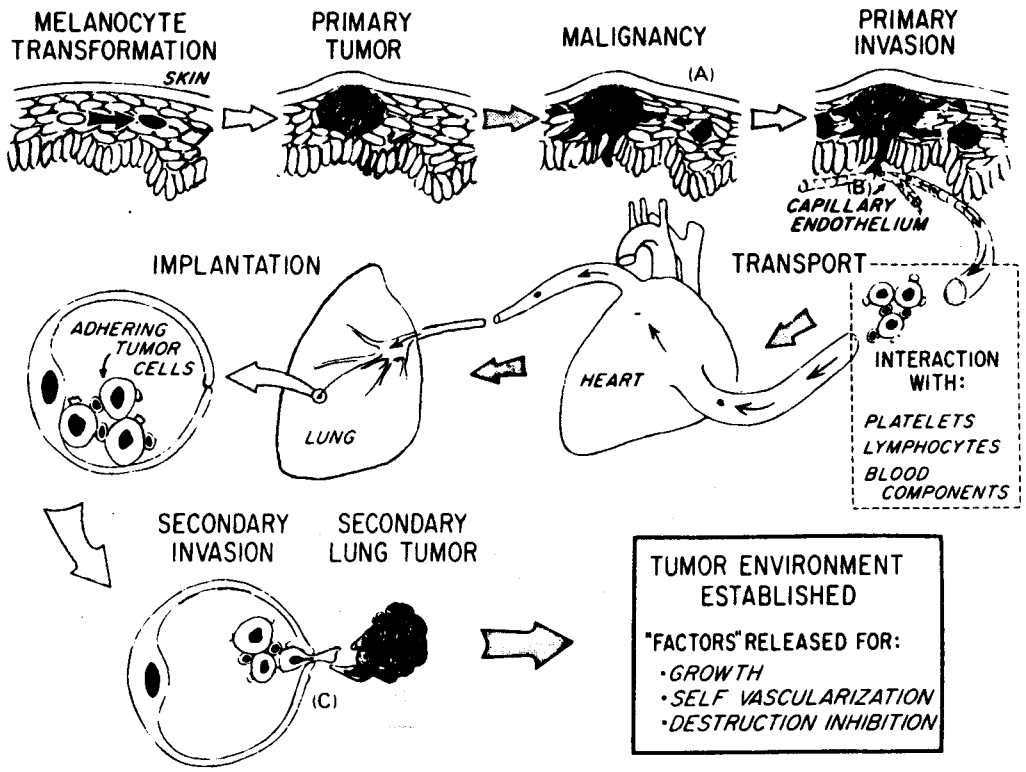


Fig. 1 : Schéma du processus métastatique d'après Nicolson et coll. (1977) : (A) l'invasion tumorale primaire; (B) l'intravasation; (C) l'extravasation et la formation d'une tumeur secondaire ou métastase.

Les tumeurs bénignes ont tendance à croître comme une masse immobile, délimitées par une membrane fibreuse naturelle ou néoplasique. Ces tumeurs ne sont pas invasives et ne donnent jamais lieu à des métastases distantes.

Les tumeurs malignes se manifestent par une anomalie très néfaste vis-à-vis de leur entourage, appelée **invasion tumorale.** Si on peut considérer la cellule invasive comme une cellule asociale, le processus de l'invasion tumorale est un trouble d'intégration qui est hystologiquement bien défini. Dans le sens le plus commun, l'invasion tumorale peut être décrite comme cette activité cellulaire qui mène à l'occupation des tissus, composé dans des circonstances habituelles par des cellules

normales et d'origine tissulaire parfois différente de celle des cellules invasives (Abercrombie et Heaysman, 1976; Armstrong, 1977). L'occupation par des cellules invasives se fait au détriment de l'organisation tissulaire des organes envahis. De plus, **l'occupation et la destruction** du tissu conjonctif et de la matrice extracellulaire **sont progressives dans l'espace et le temps.** Ces phénomènes d'invasion, soutenus par des changements enzymatiques dans le micro-environnement (Liotta et coll., 1980; Bernacki et coll., 1985) sont indispensables pour la dissémination de la tumeur primaire maligne (Mareel, 1980; Poste et Fidler, 1980).

Il existe des processus biologiques non-cancéreux qui montrent quelques aspects de l'invasion tumorale, p.ex. : 1) la progression dans le temps est limitée quand le trophoblaste de carnivores, de rongeurs ou de l'homme envahit l'endomètre lors de l'implantation de l'embryon (Denker, 1979); 2) la progression dans l'espace est limitée dans le cas du pannus rhumatoïde qui occupe le cartilage articulaire (Sokoloff, 1966; Shiozawa et coll., 1983; Hamilton, 1983) et dans le cas des granulocytes neutrophyles infiltrant le tissu normale et détruisant les micro-organismes (Grant, 1973).

L'invasion tumorale donne lieu à une symbiose serrée entre la plupart des néoplasies (à l'exception de sarcomes du tissu conjonctif lui-même) et le tissue qui lui procure soutien et vascularisation, le stroma. Celui-ci conditionne en grande partie la croissance de la tumeur primaire ou des métastases et n'en diffère que par les mitoses normales de ces fibroblastes et cellules endothéliales (Robbins, 1957; Ruddon, 1981).

L'invasion locale d'organes vitaux (p.ex.: le cerveau) peut mener à la mort du malade sans que des métastases se soient formées. Bien que l'invasion soit indispensable pour la formation de métastases - et des corrélations positives ont été trouvées entre le degré d'invasion et la survie dans le carcinome du col utérin (Stendahl et coll., 1980) et du sein (Roses et coll., 1982) -, la relation précise entre les caractères de la tumeur primaire et la capacité métastatique n'est pas évidente.

Il est donc important de ne pas confondre l'invasion tumorale avec **le processus métastatique** qui consiste en une séquence d'évènements durant lesquels des cellules tumorales se détachant de la tumeur primaire iront se fixer, envahir et croître dans des tissus les plus divers en formant des tumeurs secondaires.

L'intravasation de cellules malignes, originaires de tumeurs épithéliales, se fait le plus souvant par pénétration des vaisseaux lymphatiques : le réseau lymphatique de la plupart des organes est vaste et selon certains auteurs la paroi de ces vaisseaux serait discontinue. La pénétration directe des vaisseaux sanguins (p.ex.: par les sarcomes ou mélanomes malins) et le transport par le liquide céphalo-rachidien (p.ex.: des tumeurs du système nerveux ou de certaines leucémies) sont des phénomènes moins fréquents. Une autre possibilité est la dissémination par les cavités pleurales, péricardiques et péritonéales (Dustin, 1966).

La plupart des cellules malignes circulantes ne survit pas et moins de 0.1% possède les caractéristiques multiples nécessaires pour la formation des métastases (Weiss, 1982; Fidler, 1985; Nicolson, 1984 et 1987). Dans la circulation, les cellules tumorales subiront une série d'interactions sélectives comme : 1) aggrégation aux plaquettes, lymphocytes, neutrophiles ou macrophages; 2) adhésion à l'endothélium capillaire ou à la matrice extracellulaire - à ces phénomènes participent des glycannes et des lectines endogènes de cellules normales ou tumorales (Gabius et coll., 1986; Raz et Lotan, 1987; Monsigny et coll., 1988) -; 3) extravasation et 4) croissance spécifique de la métastase (Ruddon, 1981).

L'étude des facteurs influençant la dissémination métastatique a mené à l'hypothèse du **"germe et terrain"** par Paget qui, en 1889 proposait que certaines cellules tumorales ("germe") se multiplient dans certains tissus ("terrain"), mais pas dans d'autres. Des expériences faites à l'aide de cellules de mélanomes B16 plaident en faveur de cette hypothèse : il est en effet possible de sélectionner des sous-populations de cellules B16 ayant des capacités métastatiques accrues soit dans le poumon (Fidler, 1973 et 1975), soit dans le cerveau, soit dans l'ovaire ou la surrénale (Nicolson et coll., 1978). De plus, il a été démontré <u>in vivo</u> que

l'installation d'un shunt entre le péritoine et les veines n'augmentait pas nécessairement la fréquence de métastases chez l'homme. L'identification des cellules tumorales par des marqueurs cytologiques a montré leur distribution dans tous les organes, mais elles se multiplient uniquement dans certains tissus (Tarin et coll., 1984).

A l'heure actuelle, l'hypothèse du "germe et terrain" est décrite en trois étapes : 1) le processus de la métastase n'est pas un hasard; 2) les néoplasies ne sont pas des entités uniformes, mais contiennent des cellules aux capacités métastatiques hétérogènes; 3) l'effet de la formation des métastases dépend des propriétés des cellules tumorales, des facteurs de croissance et des facteurs du tissu-hôte. Le résultat de ces contributions individuelles dépend donc aussi bien de la nature de la tumeur primaire ("germe") que du tissu-hôte ("terrain") (Fidler et Price, 1988).

En résumé, la malignité est caractérisée par trois phénotypes dont l'ordre n'est pas bien établi et qui peuvent exister en différentes combinaisons : la croissance **(C)**, l'invasion **(I)** et la formation de métastases **(M)**. La croissance seule n'est pas cruciale pour le développement fatal d'un cancer. L'expression de ces phénotypes malins peut être absente **(-)** ou présente **(+)** in vivo, p.ex. : 1) les tumeurs bénignes dont les polypes intestinaux sont un bon exemple **($C^+I^-M^-$)**; 2) les tumeurs primaires du cerveau et les épithéliomas basocellulaires **($C^+I^+M^-$)**; 3) la plupart des tumeurs malignes ($C^+I^+M^+$). La croissance est démontrée par l'augmentation en volume de la tumeur. Elle dépend du nombre de cellules malignes et de la quantité du matériel intercellulaire. L'invasion et les métastases sont étudiées par hystologie et différenciées par le site d'interaction : un exemple d'invasion est la tumeur du toit de la vésicule biliaire envahissant le parenchyme hépatique voisin, alors que la présence de vésicules thyroïdiennes dans un ganglion cervical voisin est un phénomène métastatique (Dustin, 1966; Mareel et coll., 1988). Le phénotype des cellules malignes peut être influencé par l'environnement du tissu-hôte : ces interactions ont lieu, pour la plupart, par l'intermédiaire de la surface cellulaire (Nicolson, 1982; Monsigny et coll., 1983); le cytosquelette contrôle la distribution

et la motilité des récepteurs de la surface cellulaire (Raz et Ben Ze'ev, 1987); la laminine et la fibronectine qui participent dans l'établissement de la matrice extracellulaire, peuvent influencer l'adhésion, la migration et la différenciation de cellules malignes (Ruoslati, 1984; McCarthy et coll., 1985).

Notre connaissance des mécanismes moléculaires de l'invasion tumorale est basée sur des observations de tumeurs humaines et expérimentales.

Les possibilités limitées d'utiliser des tumeurs humaines et l'hétérogénéité de fragments de biopsie ont mené au développement d'essais expérimentaux pour étudier l'invasion. **Trois types d'essais ont été développés :**

i) La confrontation de cellules malignes avec des fibroblastes normaux sur un substrat artificiel (Abercrombie et Heaysman, 1976). Le but de ces expériences était de tester si la perte d'inhibition de contact entre des cellules hétérologues corresponderait avec la capacité d'invasion de ces cellules. Plus récemment, des cultures primaires d'hépatocytes (Roos et al., 1981) ou des cellules fibroblastiques de souris embryonnaires 10 T 1/2 en monocouche (Verschueren et al., 1987) ont été confrontées avec des cellules malignes.

ii) Des essais utilisant une chambre à deux compartiments : les cellules à étudier doivent pénétrer une barrière naturelle. La membrane chorioallantoidienne de l'oeuf de poulet a été utilisée comme diaphragme afin de pouvoir quantifier l'invasion. Après le marquage des cellules malignes par un isotope radioactif, celles-ci sont ensemencées sur la membrane. La proportion de cellules invasives, qui franchissent la membrane, est déterminée par une mesure de radioactivité sur une plaque photographique (Hart et Fidler, 1978) ou après la récolte des cellules invasives dans une matrice d'agar, qui se trouve en dessous du diaphragme (Poste et coll., 1980). Russo et collaborateurs (1982) ont démontré que l'amnion humain est un substrat tout à fait différent pour tester la capacité d'invasion de cellules malignes. L'épithélium est enlevé de l'amnion afin de permettre l'attachement direct des cellules en confrontation avec la membrane basale. Les cellules invasives sont récupérées sur un filtre Millipore, qui se trouve en dessous de l'amnion, et comptées après une coloration à l'hématoxyline-éosine.

iii) Des systèmes de confrontation en trois dimensions sont probablement plus proches de la situation in vivo. Dans ces méthodes les cellules cancéreuses sont confrontées aux fragments d'organes qui conservent leur structure histiotypique. L'examination histologique de coupes sériées de ces cultures en confrontation semble la technique la plus sûre et la plus réproductrice de toutes les méthodes développées et testées in vitro. Wolff et collaborateurs (1952) étaient les premiers à utiliser la culture organotypique pour tester l'invasion tumorale. Ils confrontaient des fragments de tumeurs humaines et animales, ou des cellules tumorales cultivées in vitro, avec des organes embryonnaires de poulet à la surface d'un milieu d'agar sémi-solide. Ces expériences démontrent en effet le comportement parasitaire de cellules tumorales au détriment du tissu normal. Si Wolff et collaborateurs mettent l'accent sur la croissance des cellules tumorales, Easty et Easty (1963) considèrent le fait que les cellules en confrontation ne se multiplient pas extensivement comme un avantage afin de pouvoir analyser la motilité et le comportement de contact selon des degrés différents d'invasion. Ces chercheurs cultivent les tissus en confrontation sur une bande en soie artificielle soutenue par un filet métallique placé au-dessus du milieu de culture. Leighton et collaborateurs (1956) ont confronté des petits agglomérats de cellules HeLa à une matrice contenant des fibroblastes humains. Schleich et collaborateurs (1976) ont étudié l'invasion de cellules tumorales humaines sur des cellules déciduales humaines en incubant leur cultures sur un agitateur. Ceci permet de submerger la culture organotypique dans un milieu de culture fluide et évite l'attachement des fragments et la perte subséquente de la structure histiotypique tridimensionelle.

Ce dernier type de culture est préférable pour l'étude de l'invasion in vitro. La méthode que nous avons employée, utilise des fragments de coeur d'embryon de poulet comme tissu-hôte. Elle a été mise au point par Mareel et collaborateurs (1979, fig. 2) et son importance pour certains aspects de l'invasion in vivo a été discutée (Mareel, 1982).

I. TECHNIQUE D'INVASION (Figure 2)

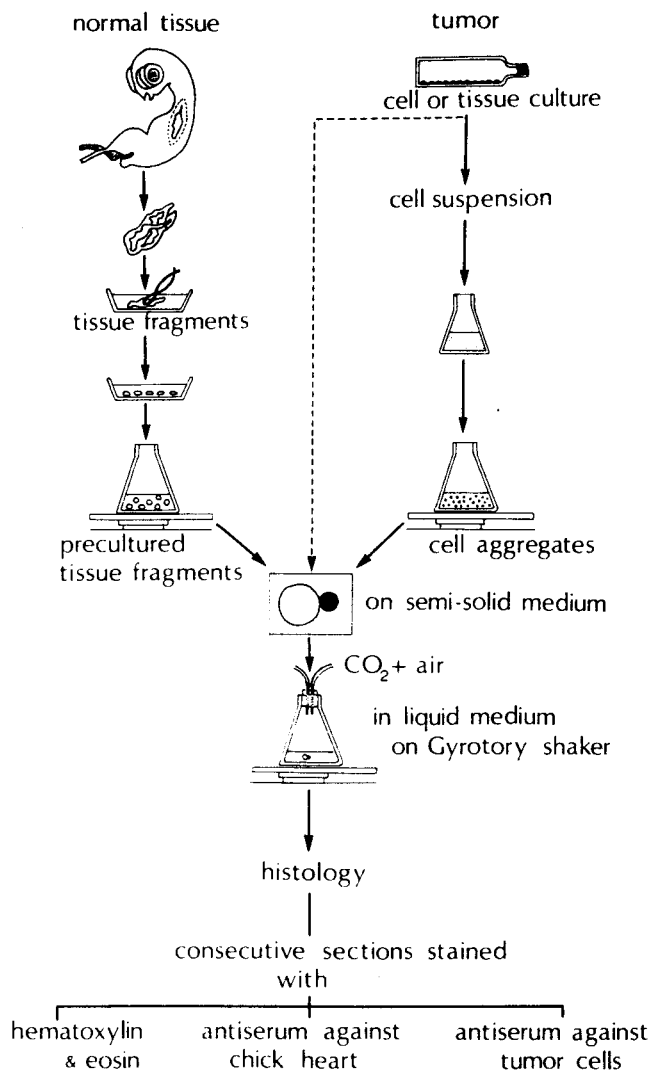


Figure 2 : Schéma de la technique d'invasion in vitro (Mareel et coll., 1979).

a. Préparation des Fragments de Coeur Précultivés (FCPs)

1. Le coeur d'un embryon de poulet de 9 jours est disséqué dans un milieu de tampon PBS. Puis, il est placé dans une boîte Petri bactériologique (ne permettant pas l'adhésion cellulaire) contenant du milieu de culture, afin d'enlever la partie auriculaire, les vaisseaux et le péricarde. Les ventricules sont incisés dans une dizaines de morceaux pour éliminer l'excès de sang.
2. Ces morceaux de ventricules sont transférés dans une autre boîte Petri bactériologique, contenant du milieu de culture frais, et coupés en fragments d'environ 0.4 mm de diamètre sous un Macroscope^{R} (Wild, FRG).

3. Des fragments éffilés et des impuretés sont écartés, avant de transférer les fragments jugés adéquats dans un flacon conique de 50 ml. Celui-ci contient un minimum de milieu de culture (2-3 ml) pour éviter le regroupement et la nécrose des FCPs pendant l'incubation de 24 hrs sur un agitateur Gyrotory^R (New Brunswick Scientific Co, New Brunswick, N.Y.) à 70 rpm et à 37°C sous un mélange d'air et de CO_2 (5 ou 10% selon le type de milieu utilisé).
4. Après 24 hrs d'incubation les blessures de la préparation des FCPs se sont cicatrisées et une couche minimale de cellules fibroblastiques a été formée et enveloppe les myoblastes. Ceci permet d'incuber les fragments utiles (une centaine par coeur d'embryon de 9 jours) dans un volume plus important de milieu de culture (6 ml) sans qu'ils se regroupent pendant l'incubation supplémentaire de 3 jours.
5. Après 4 jours, les FCPs ayant un diamètre de 0.4 mm, bien arrondis, actifs (démontrant des battements cardiaques) et ayant une capsule uniforme de cellules fibroblastiques sont sélectionnés.

Dans le cas de prétraitement pharmacologique du tissu cardiaque, les substances peuvent être ajoutées au milieu de culture après les premières 24 hrs d'incubation. Des incubations prolongées du tissu-hôte nécessite un remplacement du milieu de culture tous les 3 à 4 jours. Ceci évite la formation d'aggrégats secondaires d'origine fibroblastique et capsulaire, ce qui explique pourquoi certains FCPs ont une tendance à diminuer en volume pendant des expériences à long terme.

b. Préparation des aggrégats cellulaires

Les cellules à tester sont détachées de leur substrat artificiel et mises en suspension à une densité de 6.10^5 cellules par 6 ml de milieu de culture. Cette suspension est transférée dans un flacon conique de 50 ml et incubée pendant 3 jours à 37°C sur un agitateur Gyrotory^R, comme pour les FCPs. Les aggrégats arrondis et viables, ayant un diamètre de 0.2 mm, sont alors sélectionnés sous un Macroscope^R avant d'être confronté aux FCPs.

Dans le cas ou l'on ne peut pas obtenir des aggrégats cellulaires, il est possible de confronter des fragments de couches cellulaires ou des suspensions denses, qu'on obtient par centrifugation des cellules en suspension après l'aspiration maximale du milieu. La dernière méthode ne permet pas d'évaluer la quantité de cellules en confrontation.

c. Culture organotypique en confrontation

1. Les FCPs (8 au maximum) et les aggrégats cellulaires sont placés sur un support d'agar sémi-solide. L'excès de milieu de culture est aspiré une première fois à l'aide d'une pipette Pasteur courbée.
2. Les FCPs et les aggrégats sont déplacés en paires vers la périphérie du support d'agar en utilisant des aiguilles en acier inoxydable. Le milieu superflu est alors absorbé une deuxième fois à l'aide d'un petit filtre triangulaire en papier.
3. Fermer le récipient à l'aide d'un couvercle et de la paraffine pour qu'il soit bien étanche, et incuber les paires à 37°C pendant 2 à 4 hrs. Dans le cas de fragments de couches cellulaires ou de suspensions denses, le temps peut varier jusqu'à 24 hrs. Ceci permet l'attachement des cellules à tester aux FCPs.
4. Les paires confrontées sont submergées prudemment du milieu de culture et transférées dans une boîte Petri bactériologique. Ceci permet d'enlever les bulles d'air qui peuvent s'attacher aux FCPs pendant l'incubation sur agar.
5. A ce moment, chaque paire est transférée à l'aide d'une pipette Pasteur courbée dans un flacon conique de 5 ml contenant 1.5 ml de milieu. Le tout est incubé à 37°C sur un agitateur Gyrotory à 120 rpm sous un mélange air-CO_2 (5 ou 10%).

d. Analyse de l'invasion

Après des temps d'incubation, variant entre 2 à 28 jours selon la vitesse d'invasion, les cultures organotypiques sont fixées dans une solution Bouin-Hollande et incluées dans la paraffine. Toute la paire est alors coupée en tranches de 8 µm d'épaisseur, qui sont déposées alternativement sur trois lames de microscope.

Les coupes sont colorées à l'aide d'hématoxyline-éosine, d'un antisérum dirigé contre le fragment de coeur du poulet (Mareel et coll., 1981) ou d'un antisérum contre les cellules en confrontation. Les interactions entre les cellules en confrontation et le tissu cardiaque sont évaluées à l'aide d'un microscope optique et classifiées selon une graduation convenue (figure 3) :

grade 0 : les cellules à tester ne sont plus présentes sur le FCP.

grade I : les cellules fibroblastiques sont encore présentes entre les myoblastes et les cellules en confrontation.

grade II_a : les cellules en confrontation ont occupé les cellules capsulaires et sont en contact direct avec les myoblastes.

grade II_b : les cellules en confrontation sont encerclées par les myoblastes, couvrant une surface de contact minimale entre les deux partenaires.

grade III : les cellules en confrontation ont occupé et détruit moins de la moitié du tissu cardiaque.

grade IV : les cellules en confrontation ont occupé et détruit plus de la moitié du tissu cardiaque.

Ces grades d'interactions constituent des évaluations de vues momentanées et statiques d'une activité dynamique. Pour l'évaluation du phénomène de l'invasion, il nous faut une mesure de la progression de l'occupation et de la destruction du tissu-hôte, ce qui implique une analyse des interactions en fonction du temps. Les grades III et IV conviennent aux critères d'invasion, selon Bracke et collaborateurs (1984, fig. 3).

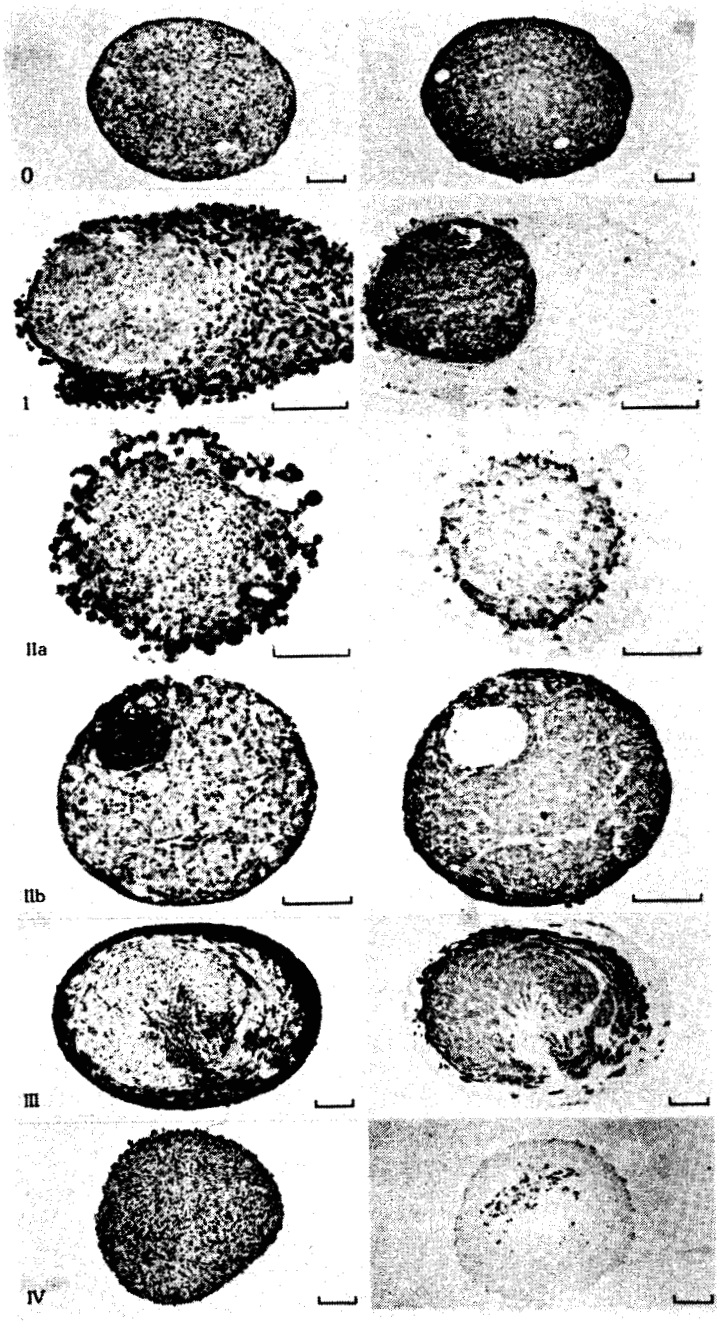


Figure 3 : Exemples des différents grades de l'invasion, d'après Bracke et collaborateurs (1984). A gauche, les coupes sont colorées à l'aide d'hématoxyline-éosine; à droite, à l'aide d'un antisérum contre le tissu cardiaque du poulet.

II. LA SPECIFICITE DE L'ESSAI D'INVASION ET SON IMPORTANCE POUR L'INVASION IN VIVO

Il est assez évident qu'un essai d'invasion tumorale in vitro implique le risque de spécificité par rapport à l'invasion in vivo. L'innervation, l'approvisionnement sanguin, les réactions lymphocytaires et la néovascularisation sont des facteurs qui sont absents in vitro. Les cellules malignes et le tissu-hôte se rencontrent pour la première fois quand ils sont associés artificiellement in vitro. In vivo, les cellules malignes normalement ne sont pas confrontées à une zône de blessures du tissu-hôte comme elles le sont dans la culture organotypique in vitro. En plus, le tissu-hôte n'est pas embryonnaire et par son micro-environnement spécifique, il peut avoir assisté au développement des cellules malignes, ce qui n'est pas le cas in vitro. Toutes ces différences qualitatives ne sont pas nécessairement désavantageuses. L'explantation in vitro peut aussi augmenter ou diminuer les activités cellulaires, impliquant des différences quantitatives. Pour toutes ces raisons, il est nécessaire que chaque modification des cellules malignes ou du tissu-hôte impliquant une inhibition ou une augmentation de l'invasion in vitro, soit confirmée par des observations in vivo.

Quelques questions qui restent à résoudre sont : 1) Quelles sont les premières étapes de l'invasion ?; 2) Quelles sont les activités les plus importantes dans l'invasion ?; 3) Quelle est l'événement-clef initiant l'invasion ?; 4) Quelles sont les activités cellulaires responsables de la progression de l'invasion?; 5) Comment pouvons-nous arrêter l'invasion ?

Pour répondre à ces questions, il importe d'utiliser un essai rapide et reproductible, dont l'analyse est relativement facile et qui reflète autant que possible la situation in vivo. D'autres arguments morphologiques en faveur de la méthode utilisée sont :

1) l'occupation et la destruction progressive du tissu-hôte par les cellules malignes se produisent de manière très semblable in vivo et in vitro;

2) l'histiotypie du processus d'invasion in vivo est retrouvée dans le FCP : l'image morphologique des cellules MO_4 en confrontation avec le FCP est celui d'un fibrosarcome invasif comme on le retrouve après l'injection des cellules MO_4 dans les souris C_3H (Mareel et coll., 1979). D'autre part les cellules MDCK, qui sont des cellules non-invasives de rein de chien, déposent une membrane basale en contact avec le FCP (Schroyens et coll., 1984) comme c'est le cas in vivo. Un pathologiste pourrait donc facilement reconnaître les cellules tumorales ou normales au moyen de leur morphologie.

La confrontation avec des organes embryonnaires en culture évite le problème des réactions immunitaires après transplantation et rend l'étude des cellules tumorales humaines plus facile. Cette méthode permet aussi l'étude de l'influence de la température, des hormones ou des altérations métaboliques.

Finalement, le facteur le plus important en faveur de l'utilisation des FCPs comme tissu-hôte est qu'il y existe une très bonne corrélation entre le phénotype invasif in vitro et la formation de tumeurs invasives dans les animaux syngéniques ou immunodéficients.

III. LES INHIBITEURS DE L'INVASION IN VITRO

a. Les inhibiteurs de microtubules

Les inhibiteurs du fonctionnement microtubulaire perturbent l'équilibre de l'assemblage-désassemblage des microtubules et interfèrent ainsi avec la locomotion des cellules, qui est une activité nécessaire des cellules invasives (revue par Mareel et De Mets, 1984). Quel que soit leur mécanisme d'action au niveau moléculaire, toutes les classes d'inhibiteurs de microtubules inhibent l'invasion de cellules malignes in vitro. Ils peuvent être divisés en trois classes :

i) les substances comme la colchicine, qui se lient à la tubuline et interviennent au niveau de l'assemblage, provoquent le désassemblage des microtubules;

ii) le taxol diminue la concentration critique de tubuline nécessaire à l'assemblage et cause un taux d'assemblage exagéré et désordonné;

iii) les molécules comme l'estramustine stabilisent la fonction des protéines associées aux microtubules (MAPs), ce qui donne lieu à des changements en longueur des microtubules individueles sans affecter l'équilibre de l'assemblage-désassemblage de la tubuline (Mareel et coll., 1988).

Les inhibiteurs microtubulaires bloquent aussi la croissance cellulaire parce qu'ils interviennent dans la formation de l'appareil mitotique. Les expériences utilisant le 5-fluoro-uracile, qui permet l'invasion mais inhibe la croissance, ont démontré que l'invasion et la croissance sont des activités cellulaires indépendantes. La seule activité cellulaire qui soit systématiquement affectée par les inhibiteurs microtubulaires est la migration directionelle. Elle peut être considérée comme une activité-clef de cellules invasives.

Les inhibiteurs de microtubules possèdent aussi une activité anti-invasive in vivo (Distelmans et coll., 1985) : ceci est un argument supplémentaire démontrant la validité de l'essai d'invasion.

b. (+)-Catéchine

La (+)-catéchine est un flavonoïde naturel, qu'on peut extraire d'Uncaria gambir Roxb. Elle possède une activité anti-invasive pour un nombre de cellules malignes sans affecter la croissance des cellules en confrontation (Bracke et coll., 1987). L'activité anti-invasive de ce flavonoïde est différente de celle des autres substances décrites jusqu'ici par le fait qu'il faut prétraiter le FCP pour obtenir une inhibition maximale de l'invasion. L'observation au microscope électronique à transmission indique que la matrice extracellulaire du FCP non-traité est amorphe, tandis qu'elle est fibrilaire et décorée de granules dans le cas des FCPs traités par la (+)-catéchine. Ce résultat indique que la cible moléculaire de la (+)-catéchine

se trouve dans le tissu cardiaque. Plus récemment, des expériences ont démontré que la (+)-catéchine se lie à la laminine mais pas aux collagènes du type I ou IV ni à la fibronectine. De plus, après prétraitement à la (+)-catéchine, les cellules MO_4 ne sont plus capables d'adhérer à ou de s'étaler sur la membrane basale de l'amnion humain ou sur une couche de laminine appliquée sur une lame de verre. Au contraire, les cellules d'un réticulosarcome murin (M5076) ne sont pas sensibles aux activités anti-invasives et anti-adhésives de la (+)-catéchine. Une explication possible est que l'invasion des cellules M5076 ne dépend pas de la laminine.

Ces expériences démontrent un autre avantage de l'essai d'invasion in vitro : il est donc possible d'identifier les cibles moléculaires d'agents anti-invasifs en prétraitant l'un ou l'autre ou les deux partenaires en confrontation.

c. Tangéritine

Le fait que la (+)-catéchine peut inhiber l'invasion des cellules MO_4, a stimulé l'étude d'un certain nombre de flavonoïdes. Une inhibition nette de l'invasion a été démontré pour la tangéritine, un flavonoïde extrait des citronniers. Pour obtenir cet effet, il faut préincuber les FCPs, et le flavonoïde doit rester présent dans le milieu de culture pendant la confrontation du tissu cardiaque aux cellules MO_4 (Bracke et coll., 1989).

Le mécanisme de l'action anti-invasive de la tangéritine n'est pas connu. Ni des altérations ultrastructurales de la matrice extracellulaire du FCP, ni une perturbation du complexe microtubulaire ou des changements des glycannes N-glycosidiquement liés de la surface des cellules MO_4 n'ont pu être démontrés. L'explication la plus plausible est que la tangéritine affecte le taux d'ATP et ainsi un nombre d'activités cellulaires comme la motilité (et par là l'invasion) des cellules MO_4. Les concentrations anti-invasives de la tangéritine arrêtent aussi la prolifération des cellules MO_4.

Trois flavonoïdes proches de la tangéritine (l'hespéritine, la nobilétine et la naringine) inhibent aussi la croissance des cellules MO_4, mais ils n'ont pas d'influence sur l'invasion. Ceci démontre une fois de plus l'indépendance de ces deux activités cellulaires.

d. Dipyridamole

Le dipyridamole et ses dérivés sont connus pour leur effet sur la vasodilatation et l'agglutination des plaquettes sanguines. Les cibles moléculaires du dipyridamole sont l'actine et l'AMP cyclique, qui règle la phosphodiestérase. Le dipyridamole et un de ses dérivés ont un effet sur la motilité cellulaire, qui est différent de celui des inhibiteurs de microtubules. Ils sont moins toxiques que la cytochalasine D (Verschueren et coll., 1983). Ces substances inhibent l'invasion des cellules MO_4, des cellules de vessie de rat (NBTII), des cellules du glioblastome humain (SA_4), des cellules de carcinome de Lewis (LLC-H61) et des hybrides mélanome-lymphocytes murins (F87Cl.6T2) dans le tissu cardiaque en culture organotypique. En cultivant en l'absence de sérum foetal bovin, selon le protocole de Bracke et collaborateurs (1986), il est possible de diminuer la dose efficace du dipyridamole de 10 fois par raport aux tests réalisés en présence de 10% de sérum (Van Larebeke et coll., 1989).

Les expériences utilisant le dipyridamole ou la tangéritine soulignent le rôle de la motilité cellulaire dans l'invasion, bien que les cibles moléculaires de ces deux inhibiteurs de l'invasion ne soient pas encore bien définies.

Le nombre d'agents anti-invasifs et la connaissance de leur mécanisme d'action sont donc limités. C'est pourquoi nous sommes intéressés à élucider le rôle de la glycosylation des protéines membranaires dans l'invasion tumorale.

Des observations ultrastructurales ont démontré que la formation d'extensions dans les cellules malignes était déclinchée par le contact avec le tissu normal (Bruyneel

et Mareel, 1981; Steinsvag, 1985). Par cette technique et après une série de manipulations, il était possible d'analyser les premières étapes de l'invasion des cellules MO_4 dans le tissu cardiaque in vitro (Figure 4).

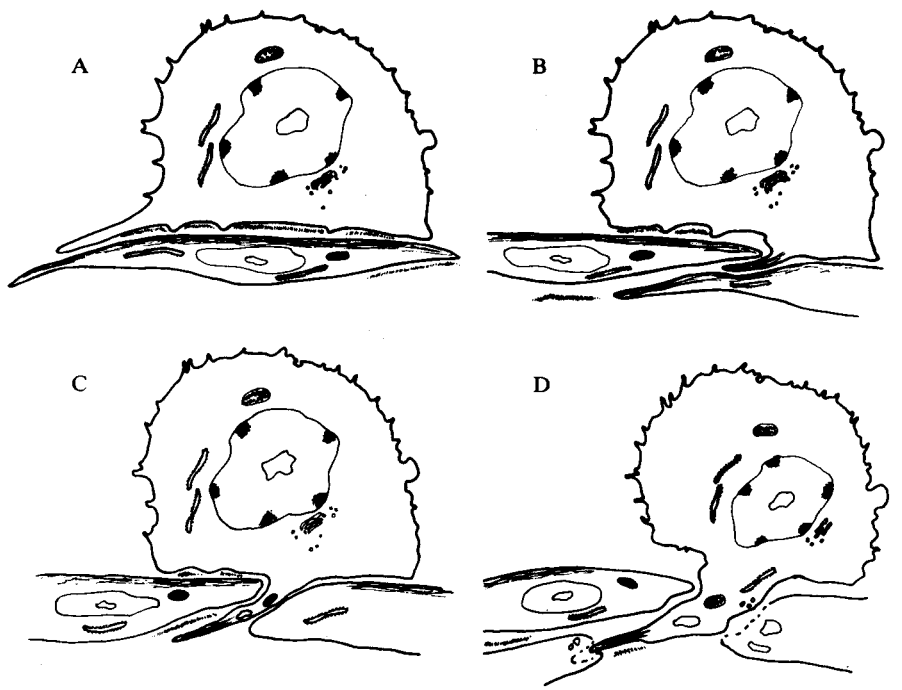


Figure 4. Représentation schématique des premières étapes de l'invasion. A : l'attachement; B : l'ancrage; C : la pénétration; D : la destruction; d'après Bruyneel et Mareel (1981).

A : les cellules MO_4 s'attachent aux cellules fibroblastiques du FCP directement ou par interposition d'une matrice extracellulaire.

B : les cellules MO_4 produisent des extensions cytoplasmiques qui s'ancrent entre les cellules fibroblastiques de la périphérie du tissu cardiaque.

C : les parties étendues du cytoplasme des cellules MO_4 pénètrent en même temps que les cellules fibroblastiques se rétractent.

D : la destruction du tissu cardiaque par les cellules malignes.

Dans l'hypothèse en trois étapes de Liotta (1986) la pénétration et la destruction sont inversées : 1) l'attachement se fait par l'action de la laminine ou d'autres facteurs adhésifs, qui forment une liaison entre le récepteur de la laminine, présent à la surface de la cellule maligne, et le collagène du type IV de la membrane

basale; 2) les protéinases des cellules tumorales détériorent la matrice extracellulaire et les facteurs adhésifs; 3) la migration des cellules tumorales se fait dans la zone de la matrice extracellulaire modifiée, probablement sous l'influence de facteurs chemotactiques. En plus, Liotta a proposé que la progression de l'invasion se fait par une répétition cyclique de ces trois étapes.

La dernière hypothèse nous semble plus réaliste compte tenu de la situation in vivo: 1) la matrice extracellulaire du tissu-hôte ne contient normalement pas de passages pré-existants pour les cellules, ce qui nécessite d'abord sa dégradation avant que les cellules tumorales puissent se déplacer dans cet espace; 2) la répétition cyclique est nécessaire pour expliquer la continuité des phénomènes de l'invasion. Néanmoins, il reste à discuter si les cellules tumorales pourraient s'attacher directement à la matrice extracellulaire sans que les cellules endothéliales se retirent d'un endothélium intact. Cette collaboration du tissu-hôte peut être retrouvé dans la première hypothèse (Bruyneel et Mareel, 1981) et non dans la dernière (Liotta, 1986).

L'attachement, la destruction de la matrice extracellulaire et la migration directionnelle sont des activités-clef de cellules invasives. Néanmoins, des cellules normales peuvent également exprimer ces activités. Une explication possible serait que ces activités cellulaires seraient contrôlées par le contact direct entre les cellules malignes et le tissu-hôte normal. Les glycannes N-glycosidiquement liés de la surface cellulaire sont parmi les candidats pour la médiation entre la perception et la transmission de tels signaux.

Le but de cette étude est d'explorer dans quelles activités cellulaires et comment les glycannes N-glycosidiquement liés de la surface cellulaire sont impliqués dans l'invasion tumorale. Trois types d'expériences ont été considérés :

1) Quelles sont les modifications des glycannes N-glycosidiquement liés de la surface cellulaire après l'induction du phénotype invasif dans des cellules normales. Cette induction est obtenue d'une part par la transformation

consécutive à la transfection d'oncogènes ou d'autre part par un prétraitement par le 1-O-octadécyl-2-O-méthylglycéro-3-phosphocholine (Chapitre I).

2) En diminuant la température d'incubation des cultures organotypiques, nous avons cherché à créer une situation d'invasion minimale. Ceci nous permet d'évaluer les activités-clef de cellules invasives au moment ou l'invasion démarre (Chapitre II).

3) Les inhibiteurs de la biosynthèse ou du processus de maturation de la N-glycosylation ont été utilisés en cultures organotypiques entre des cellules malignes MO_4 et des FCPs. La première raison de ces expériences est de voir si un changement de la glycosylation était nécessaire pour que l'invasion démarre. La deuxième raison est de chercher des structures de glycannes N-glycosidiquement liés nécessaires et/ou suffisantes pour l'invasion par les cellules malignes (Chapitre III).

Avant de présenter les résultats de nos recherches, une description de la biosynthèse et du processus de maturation des glycannes N-glycosidiquement liés est indiquée pour mieux comprendre l'utilisation et le site d'action des inhibiteurs de la glycosylation, que nous avons utilisés.

IV. LA GLYCOSYLATION DES PROTEINES

Les chaînes glycanniques sont attachées par des liaisons covalentes aux squelettes polypeptidiques. Il existe deux sites d'attachement, fournissant deux types de liaisons chimiques.

1) **Les liaisons O-glycosidiques** reliant la sérine (Ser), la thréonine (Thr), l'hydroxylysine ou l'hydroxyproline le plus souvent à la N-acétyl-galactosamine (GalNAc). La plupart des liaisons impliquent les groupements -OH de la Ser ou de la Thr. Après le transfert du GalNAc par la N-acétylgalactosaminyltransférase sur la Ser ou la Thr, la glycosylation des acides aminés hydroxylés est obtenue par des additions successives de monosaccharides.

Ces réactions sont catalysées par les différentes glycosyltransférases. La taille des glycannes O-glycosidiquement liés varie entre un seul jusqu'à plusieurs dizaines d'unités monosaccharidiques, ce qui peut mener à une immense hétérogénéité (Schachter et Roseman, 1980).

Les glycannes O-glycosidiquement liés sont présents dans le mucus de l'appareil digestif, de la bouche jusqu'au rectum, formant une protection efficace pour les tissus sous-jacents (Puchelle et coll., 1980). Seuls ou accompagnés de glycannes N-glycosidiquement liés, ils se retrouvent sur de nombreuses autres molécules.

2) **Les liaisons N-glycosidiques** reliant exclusivement l'asparagine (Asn) par son groupement aminé à la N-acétylglucosamine (GlcNAc). Pour qu'un résidu Asn soit glycosylé il est nécessaire, mais pas suffisant que l'Asn se trouve dans une séquence tétrapeptidique Asn-X-Ser/Thr-Y; X et Y peuvent être n'importe qu'elle acide aminé à l'exception de la proline ou de l'acide aspartique. Pour les protéines membranaires ou sécrétées, la plupart des sites satisfaisant aux conditions susmentionnées est effectivement glycosylée. Le processus de la N-glycosylation se déroule dans deux sites cytoplasmiques différents, le réticulum endoplasmique rugueux et l'appareil de Golgi, comme présenté dans la figure 5 (d'après Verbert et coll., 1987).

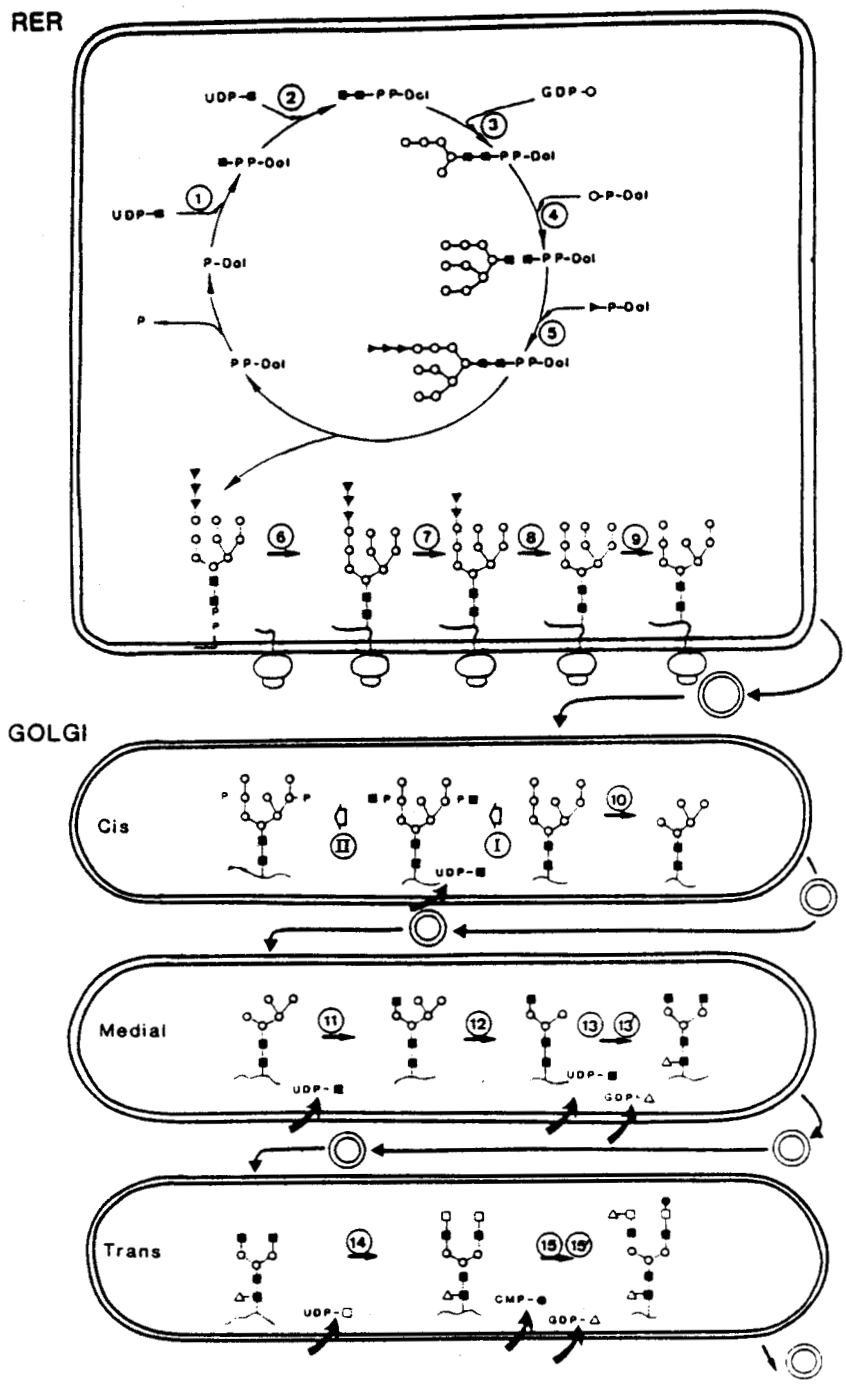


Figure 5. Représentation schématique de la biosynthèse des oligosaccharides dans le réticulum endoplasmique rugueux (RER) et du processus de maturation des glycannes N-glycosidiquement liés dans l'appareil de Golgi; d'après Verbert et coll. (1987). Les symboles représentent : ■ : N-acétylglucosamine; ○ : mannose; ▼ glucose;
□ : galactose; ● acide sialique.

A. Le "cycle du dolicholphosphate" se passe dans **le réticulum endoplasmique rugueux** (RER) et démarre par le transfert du GlcNAc-P de l'UDP-GlcNAc vers le dolichol-P afin de former le Dol-P-P-GlcNAc (étape 1, figure 5). Puis les différentes molécules glycosidiques sont transférées par l'intervention de leur nucléotides correspondantes ou de leurs intermédiaires lipidiques, le Dol-P-mannose ou le Dol-P-glucose (étapes 2-5, figure 5). Ceci aboutit à la formation du $Glc_3Man_9GlcNAc_2$-P-P-dolichol, nommé oligosaccharide G, lié au dolichol par une liaison de pyrophosphate.

L'oligosaccharide G est alors transféré "en bloc" par l'oligosaccharide G-transférase vers les sites d'Asn dans des séquences tétrapeptidiques Asn-X-Thr/Ser-Y de la protéine (étape 6, figure 5).

Après son transfert sur l'Asn, l'oligosaccharide G subit l'élagage des trois résidus glucosidiques par l'action des glucosidases I (α-1,2) et II (α-1,3) (étapes 7 et 8, figure 5). Une mannosidase (α-1,2), active dans le RER, mène à la formation du glycanne $Man_8GlcNAc_2$ (étape 9, figure 5). La glycoprotéine est alors transportée du RER vers l'appareil de Golgi dans des vésicules.

B. Dans **l'appareil de Golgi,** 3 résidus mannosidiques sont élagués par l'action de la mannosidase I du Golgi (étape 10, figure 5), ce qui résulte dans la formation du $Man_5GlcNAc_2$. Cette molécule forme un point crucial dans la maturation des glycannes N-glycosidiquement liés. A partir de cette structure oligomannosidique il est possible d'allonger les glycannes en ajoutant un résidu GlcNAc au mannose α-1,3 par l'action de la N-acétylglucosaminyltransférase I (étape 11, figure 5). Le transfert de cette première GlcNAc est un préalable indispensable au déroulement des étapes suivantes.

La mannosidase II termine l'élagage (étape 12, figure 5). Si cette enzyme n'intervient pas, des structures hybrides peuvent être formées en élongant la branche GlcNAc(α-1,2)Man. Leur structure est intermédiaire entre les glycannes du type oligomannosidique et les glycannes complexes, qui contiennent aucun résidu mannosidique terminal.

L'action des différentes GlcNAc-transférases introduit la formation des structures poly-antennaires (étape 13, figure 5). Le branchement en β-1,6 par l'action de la GlcNAc-transférase V a été mis en corrélation directe avec le pouvoir métastatique des cellules MDAY-D_2 (Dennis et coll., 1987).

La décoration de la structure centrale par un résidu Fuc, lié en α-1,6 à la GlcNAc-Asn, est due à l'action de l'α-1,6 fucosyltransférase (étape 13', figure 5).

L'élongation des différentes antennes se fait par l'addition du galactose ou de l'acide sialique (étapes 14 et 15, figure 5). En plus, il est possible d'obtenir des unités répétitives de GlcNAc-Gal dans une ou plusieures antennes : ce sont des structures du type poly-N-acétyl-lactosaminique. L'addition du fucose lié en α-1,3 au galactose peut terminer le processus de maturation des glycannes N-glycosidiquement liés (Kornfeld et Kornfeld, 1985).

Les enzymes lysosomiales ont une glycosylation particulière à partir de l'étape 9 (figure 5). Le transfert de phosphate au glycanne $Man_8GlcNAc_2$ se fait en deux temps : d'abord le transfert du GlcNAc-1-P sur un ou plusieurs résidus mannosidiques (voie I, figure 5); ensuite l'hydrolyse du GlcNAc, exposant le mannose-6-P. En absence de la phosphoryl-N-acétyl-glucosaminyltransférase, la première réaction n'a pas lieu et il y a une accumulation de lipides (les mucolipidoses II et III). La deuxième réaction est incomplète dans l'endomètre gravide, ce qui a pour conséquence la sécrétion de la phosphatase acide, l'utéroferrine (Baumbach et coll., 1984).

Une glycoprotéine peut contenir des structures du type oligomannosidique ensemble avec des glycannes complexes, ce qui résulte en une microhétérogénéité. Les facteurs qui contrôlent la structure des glycannes sont pour la plupart inconnus. Swiedler et coll. (1985) ont proposé que la structure primaire de la glycoprotéine détermine les sites de la glycosylation et probablement aussi la taille des glycannes. Autres facteurs qui peuvent moduler plus directement la glycosylation sont : les taux en dolichol-P, en oligosaccharide G-

transférase et en autres enzymes impliquées dans la glycosylation. Quelques exemples : la dexaméthasone augmente l'activité de certaines enzymes du cycle dolicholphosphate (Sarkar et Mookerjea, 1985); dans le parotide, l'addition du dolichol-P stimule la glycosylation sans affecter sa sécrétion (Kousvelari et coll., 1983); la dopamine stimule l'incorporation du fucose et du mannose dans le cerveau (Lössner et coll., 1984). Néanmoins, une question intéressante reste à résoudre: est-ce que la stimulation par la voie calcique a le même effet sur la glycosylation que la stimulation par la voie de l'AMP cyclique ?

V. LES INHIBITEURS DE LA GLYCOSYLATION

Afin d'intervenir artificiellement dans l'évolution normale de la glycosylation, un nombre de substances a été testé. Les références ci-dessous rapportent le travail d'autres laboratoires, réalisé à l'aide de ces inhibiteurs de la glycosylation, qui seront utilisés dans nos études sur l'invasion (voir Chapitre III). Pour un aperçu plus général, les articles de revue de Schwartz et Datema (1982 et 1984), de Fuhrmann et coll. (1985) et d'Elbein (1987) sont à recommander.

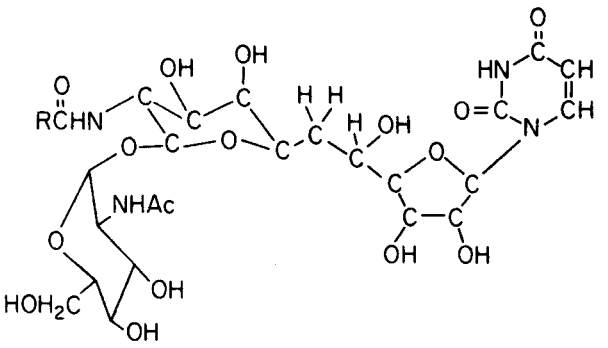
A. Les inhibiteurs de la biosynthèse

Tunicamycine (TM)

La tunicamycine, un antibiotique nucléosidique, est le nom qu'on donne à un nombre de substances caractérisées et isolées pour la première fois des Streptomyces lysosuperificus (Takatsuki et coll., 1971) (figure 6).

Dans des systèmes acellulaires et dans les cellules intactes, la TM est capable d'inhiber la première étape du cycle de dolichol-P (voir figure 5) : le transfert de la GlcNAc-1-P de l'UDP-GlcNAc vers le dolichol-P est bloqué (Lehle et Tanner, 1976; Heifetz et coll., 1979; Schwartz et Datema, 1982).

La découverte de la TM était due au fait qu'en présence de cette substance il n'était plus possible de répliquer certains virus; p.ex. le virus d'Hantaan dans les cellules Vero-E6 (Schmaljohn et coll., 1986).



I : R = $CH_3)_2CH(CH_2)_7CH=CH-$ VI : R = $(CH_3)_2CH(CH_2)_{11}-$

II : $(CH_3)_2CH(CH_2)_8CH=CH-$ VII : $(CH_3)_2CH(CH_2)_{10}CH=CH-$

III : $CH_3(CH_2)_{10}CH=CH-$ VIII : $CH_3(CH_2)_{12}CH=CH-$

IV : $CH_3(CH_2)_{11}CH=CH-$ IX : $CH_3(CH_2)_{13}CH=CH-$

V : $(CH_3)_2CH(CH_2)_9CH=CH-$ X : $(CH_3)_2CH(CH_2)_{11}CH=CH-$

Figure 6. La structure de la tunicamycine et ses différents homologues.

L'incorporation de mannose en présence de TM est fortement réduite dans les rétines de grenouille (Fliesler et Basinger, 1985) et dans les cellules BHK, sans affecter l'incorporation de leucine (Damsky et coll., 1979; Butters et coll., 1980). Après 2 hrs d'incubation en présence de la TM, l'incorporation de [^{3}H]-glucosamine est fortement réduite dans le RER et l'appareil de Golgi des gonadotrophes de souris par rapport aux cellules non-traitées (Hurbain-Kosmath et coll., 1987).

Il n'est pas exclu que la TM peut affecter la glycosylation des molécules autres que des protéines. La TM inhibe la biosynthèse des glycosaminoglycannes dans les cellules F9 du tératocarcinome (Romero et coll., 1986) et dans les embryons de souris (Iwakura et coll., 1985). La sécrétion d'acide hyaluronique est partiellement retardée dans les chondrocytes de rat (Bansal et Mason, 1987). Le transport de l'UDP-Gal par les vésicules du Golgi dans le foie de rat est affecté et mène à l'inhibition de la biosynthèse du ganglioside GM1 (Yusuf et coll., 1983), ce qui est en accord avec les observations faites dans les cellules hybrides gliome-neuroblastome (Guarnaccia et coll., 1983).

La TM inhibe la glycosylation et la croissance de cellules leucémiques (Morin et coll., 1983), d'hépatocytes normaux et malins (Struck et coll., 1978; Savage et Bauer, 1983), et de cellules HT29 d'adénocarcinome du côlon humain (El-Battari et coll., 1986). En plus de l'inhibition de croissance, les cellules 3T3 changent leur morphologie, probablement à la suite d'une expression réduite de la fibronectine (Duksin et Bornstein, 1977; Duksin et coll., 1978). La formation de myotubes par les cellules d'une lignée de myoblastes de rat peut être inhibée par des concentrations non-toxique de TM (Gilfix et Sanwal, 1980).

La TM inhibe la formation de blastocystes dans les embryons de souris (Webb et Duksin, 1981; Atienza-Samols et coll., 1980; Iwakura et Nozaki, 1985), tandis qu'elle n'a pas d'effet sur la morphogénèse de l'oursin (Schneider et coll., 1978). La différentiation cellulaire et la cohésion du Dictyostelium discoideum sont réduites en présence de TM (Lam et Siu, 1982).

Après traitement par la TM, le récepteur à l'insuline n'est plus fonctionnel dans les cellules de chondrosarcome (Stevens et coll., 1982), dans les fibroblastes murins (Kohno et coll., 1980), et dans les lymphocytes humains IM9 (Keefer et De Meyts, 1981). Dans les cellules B16 de mélanome murin, la production de la mélanine est arrêtée après l'inhibition de la glycosylation (Imokawa et coll., 1986).

Le virus d'Epstein-Barr, modifié par la TM, a une capacité réduite d'induire la sécrétion d'immunoglobulines par des lymphocytes humains (Lam et Siu, 1982). L'IgG, modifiée par la TM, provenant d'une lignée d'hybridome M31 n'est plus sécrétée sous sa forme non-glycosylée : elle a subi une modification post-translationelle induisant la sulfatation de la tyrosine dans le fragment Fc (Baeuerle et Huttner, 1984). Dans les cellules d'hépatome humain ($HepG_2$), la TM bloque complètement la glycosylation de la globuline liant la tyroxine, et retarde la sécrétion de cette glycoprotéine (Bartelena et Robbins, 1984). Dans les cellules de mélanomes, l'expression de certains antigènes tumoraux ne dépend pas de la glycosylation (Khosravi et coll., 1984; Johnston et Bystryn,

1985), mais l'expression d'autres antigènes est inhibée selon Morgan et coll. (1981). Dans l'hépatocyte, la distribution du récepteur de mannose-6-P est perturbé après traitement par la TM : le récepteur non-glycosylé est sécrété au lieu de rejoindre les lysosomes (Brown et coll., 1984).

In vivo, le pouvoir métastatique des cellules B16 de mélanome murin est significativement réduit après prétraitement des cellules par la TM (Irimura et coll., 1981; Humphries et coll., 1986).

2-Désoxy-D-glucose (dGlc)

Comme c'était le cas pour la TM, le dGlc inhibe la multiplication de différents virus enveloppés (Schwarz et Datema, 1982). Il est maintenant clair que le dGlc et ses analogues doivent être métabolisés afin d'excercer leur effet inhibiteur sur la glycosylation. Le dGlc est converti en UDP-dGlc et GDP-dGlc, puis en dolichol-P-dGlc; il est alors dégagé pour former le doiichol-P-P-$GlcNAc_2Man_9dGlc$, qui ne peut pas être reconnu comme substrat par les glycosyltransférases (étape 5, figure 4) (Datema et Schwarz, 1979).

En général, la glycosylation est bloquée sans affecter la synthèse des protéines, p.ex. de l'hémagglutinine dans les cellules HeLa infectées par le virus de l'influenza (Ghandi et coll., 1972) et le mannan chez les protoplastes de levures (Farkas et coll., 1970).

Aux concentrations effectives pour l'inhibition de la glycosylation (10-100 mM) le dGlc bloque la glycolyse par la réduction du taux d'ATP et par l'inhibition du métabolisme respiratoire (Wick et coll., 1956; Ibsen et coll., 1962). Ceci fait que le dGlc a été largement remplacé par la TM comme inhibiteur de la glycosylation.

β-Hydroxynorvaline (HNV)

La HNV est un acide aminé qui peut remplacer la thréonine au site de glycosylation (-Asn-X-Thr) de telle façon que l'oligosaccharide G n'est plus

transféré sur le polypeptide présenté (étape 6, figure 5). Pour obtenir un effet maximal, il faut exclure la thréonine du milieu de culture.

La synthèse des glycoprotéines virales est complètement inhibé par la HNV dans les cellules BHK infectées par le virus d'Herpes simplex du type 1 (Kumarasamy et Blough, 1984) et par des virus de leucémie murine (Polonoff et coll., 1982). La HNV induit des changements soit dans la glycosylation, soit dans la séquence peptidique de la glycoprotéine acide-α1 d'hépatocytes et intervient ainsi dans le transport intracellulaire de cette glycoprotéine (Docherty et Aronson, 1985).

B. Les inhibiteurs du transport des glycoprotéines

Entre la synthèse et le processus de maturation des glycannes N-glycosidiquement liés, les glycoprotéines sont transportées du RER vers les différents sites de l'appareil de Golgi. Bien que les signaux impliqués dans ce trafic ne sont pas connus, un nombre de substances, comme p.ex. les ionophores, ont une influence sur ce trafic et bloquent ainsi la conversion de structures oligomannosidiques en structures complexes. Un de ces ionophores est la monensine (figure 7).

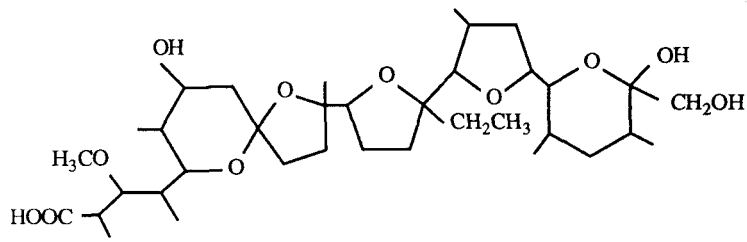


Figure 7. La structure de la monensine.

Monensine (MON)

La MON est un antibiotique lipophilique, isolée de Streptomyces cinnamonensis. Elle est utilisée dans l'élevage de volailles et comme supplément de la nourriture pour animaux en général.

La MON perturbe les taux du Na^+ et du K^+ intra- et extracellulaire, résultant en une dilatation rapide des citernes golgiennes et un ralentissement du transport intracellulaire (Tartakoff, 1983; Boss et coll., 1984). Lors d'un traitement par la MON, certaines glycoprotéines membranaires ne sont plus capables de s'incorporer dans la membrane plasmique, p.ex. les molécules IgM et H-2 (Tartakoff et coll., 1979 et 1981), des précurseurs du facteur von Willebrand (Wagner et coll., 1985), le précurseur des trois sousunités de la laminine dans les cellules F9 du tératocarcinome (Morita et coll., 1985), la protéine G du virus de la stomatite vésiculaire dans les cellules MDCK (Alonso-Caplen et Compans, 1983). Par contre, la globuline liant la thyroxine dans les cellules Hep G_2 d'hépatome (Bartalena et Robbins, 1984), l'hémagglutinine dans les cellules MDCK infectées par le virus de l'influenza (Alonso-Caplen et Compans, 1983), la galactosyltransférase dans les cellules HeLa (Strous et coll., 1985), et l'inhibiteur de la protéinase-α1 dans les hépatocytes de rat (Oda et coll., 1983) sont normalement transportées vers la membrane en présence de MON.

La sécrétion de la matrice extracellulaire et l'étalement des cellules mésodermales sont inhibés par la MON dans l'embryon de poulet (Sanders et Chokka, 1987). Le précollagène du type I n'est plus sécrété par les fibroblastes du tendon de lapin en présence de la MON (Kay et coll., 1984). La MON inhibe aussi la sécrétion du précollagène et de la fibronectine par des fibroblastes humains (Ledger et coll., 1980 et 1983); ainsi que la sécrétion du collagène du type II par des chondrocytes d'embryons d'oiseaux (Nishimoto et coll., 1982). L'activité lysyloxidase, une enzyme initiante la formation de cross-links dans le collagène et dans l'élastine à l'extérieur de la cellule, est inhibée d'une façon efficace par la MON au niveau de l'appareil de Golgi (Kuivaniemi et coll., 1986). La MON inhibe la croissance et l'étalement de cellules HT29 d'adénocarcinome du côlon humain (El-Battari et coll., 1986), ainsi que le pouvoir d'agrégation et l'étalement de fibroblastes distrophiques ou normaux (Pizzey et coll., 1983 et 1984; Jones et coll., 1985).

La MON inhibe la réplication du virus d'Hantaan dans les cellules Vero E6 (Schmaljohn et coll., 1986). La pénétration du virus de la stomatite vésiculaire ou de Semliki est diminuée de 100 fois dans les cellules de souris Balb/3T3 résistantes à la MON (Ohno et coll., 1985). Le transport intracellulaire des glycoprotéines membranaires, codées par le virus de Semliki Forest ou de Sindbis, est inhibé dans les cellules infectées d'embryon de poulet (Kääriänen et coll., 1980). La maturation du virus d'Uukuniemi est inhibé par la MON dans l'appareil de Golgi des fibroblastes embryonnaires de poulet et dans des cellules BHK (Kuismanen et coll., 1985). La MON bloque la sécrétion de la dopamine-β-hydroxylase sans affecter l'enveloppement dans les cellules PC12 (Kuhn et coll., 1986).

Outre son action sur l'appareil de Golgi, la MON inhibe aussi certaines activités lysosomiales. Ainsi, l'endocytose et la dégradation de l'albumine traité à la formaldéhyde étaient fortement réduites dans les cellules endothéliales du foie en présence de la MON (Eskild et Berg, 1988); elle provoque une accumulation de l'insuline dans les adipocytes de rat (Ueda et coll., 1985).

Il n'est pas surprenant que la MON influence la glycosylation autres que celle des glycannes N-glycosidiquement liés. Les glycannes O-glycosidiquement liés des antigènes HLA-DR sont également affectés (Machamer et Cresswell, 1984). La MON a une influence sur la biosynthèse des glycosphingolipides et provoque l'accumulation de la glucosyl- et de la lactosylcéramide à l'intérieur des fibroblastes humains (Saito et coll., 1984). La MON intervient dans la sulfatation des protéoglycannes de chondrocytes (Tajiri et coll., 1980; Kajiwara et Tanzer, 1981; Mitchell et Hardingham, 1982); elle inhibe aussi la synthèse de l'acide hyaluronique dans les cellules de fibrosarcome de rat (Goldberg et Toole, 1983).

C. Les inhibiteurs du processus de maturation des glycannes N-glycosidiquement liés

Un nombre de substances possédant des sites d'action spécifiques a été caractérisé ou synthétisé. Ils interviennent dans la glycosylation à une certaine phase de la maturation par inhibition des glucosidases et mannosidases I et/ou II.

La structure de certains de ces inhibiteurs est représentée dans la figure 8.

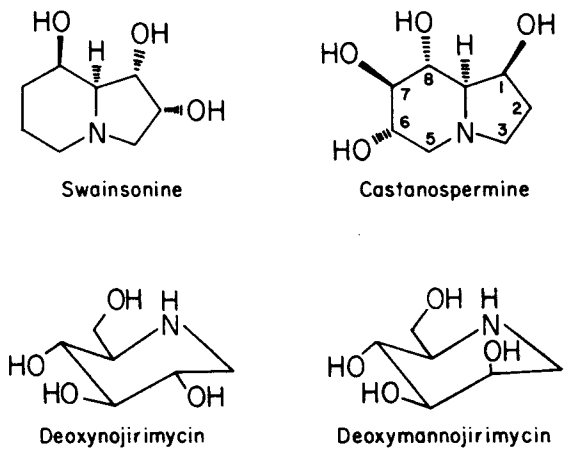


Figure 8. La structure de certains inhibiteurs du processus de maturation des glycannes N-glycosidiquement liés.

1-Désoxynojirimycine (dNM)

La dNM est un homologue du glucose; elle est dérivée de la nojirimycine par réduction du groupe -OH du premier atome de carbone (figure 8). La nojirimycine est un antibiotique produit par certaines familles de Streptomyces. La dNM inhibe les glucosidases I et II (Hettkamp et coll., 1982).

La dNM empêche l'élagage des résidus glucosidiques de l'antitrypsine-α1 et de l'antichymotrypsine-α1; elle bloque ainsi le transport de ces glycoprotéines du RER vers l'appareil de Golgi (Lodish et Kong, 1984).

Pour les cellules IEC et MDCK, la glycosylation de certaines glycoprotéines membranaires est profondément altérée par la dNM sans que leur expression ne soit changée (Saunier et coll., 1982; Burke et coll., 1984; Schwarz et Elbein, 1985; Romero et coll., 1985). La dNM cause une diminution des structures glycanniques complexes et une augmentation des structures oligomannosidiques dans la thyroglobuline du porc (Franc et coll., 1986), dans l'inhibiteur de la protéinase-α1 et dans la glycoprotéine acide-α1 (Gross et coll., 1986).

En présence de dNM, la glycoprotéine codée par l'oncogène v-erb-B est normalement sécrétée, mais il s'agit d'une forme moins glycosylée (68 kDa au lieu de 74 kDa). Elle conserve son activité transformante comme il a été démontré par la formation de colonies dans l'agar sémi-solide et par l'inhibition de la différentiation d'érythroblastes (Schmidt et coll., 1985).

Pour certaines glycoprotéines membranaires, un raccourcissement de la demi-vie a été démontré pour les antigènes HLA-A,B,C mais pas pour les antigènes HLA-DR en présence de dNM (Peyreiras et coll., 1983).

N-méthyl-1-désoxynojirimycine (MdNM)

La MdNM est une substance sémi-synthétique obtenue par méthylation en -N de la dNM (figure 8); elle est avant tout un inhibiteur de la glucosidase I (Romero et coll., 1986).

L'inhibition partielle de l'incorporation du mannose dans les glycannes complexes et lactosaminiques des cellules F9 de tératocarcinome résulte dans une accumulation du $Glc_3Man_{7-9}GlcNAc_2$-P-P-dolichol (Romero et coll., 1986). Contrairement à la dNM, la MdNM permet la sécrétion de l'inhibiteur de la protéinase-α1 et de la glycoprotéine acide-α1 par les hépatocytes de rat (Gross et coll., 1986).

Dans l'agar sémi-solide et dans un milieu exempt de sérum bovin, la MdNM inhibe la croissance des cellules embryonnaires Fe-EC transformées par l'oncogène v-fms, mais reste sans influence sur les cellules transformées par

l'oncogène v-fos. Les glycoprotéines gp125$^{v\text{-}fms}$ et gp85$^{v\text{-}fos}$ sont normalement exprimées en présence de la MdNM (Hadwiger et coll., 1986).

2,5-Dihydroxyméthyl-3,4-dihydroxypyrrolidine (DMDP)

Cet alcaloïde a été isolé des plantes Lonchocarpus sericeus et Derris elliptica. Le DMDP est un inhibiteur de la glucosidase I. L'effet du DMDP sur l'expression et l'activité transformante de l'oncogène v-erb-B est semblable à celui du dNM, produisant une forme immature (68 kDa au lieu de 74 kDa) mais toujours capable de démontrer des activités transformantes (Schmidt et coll., 1985).

Les cellules MDCK, infectées par le virus de l'influenza, produisent une hémagglutinine qui est beaucoup plus sensible à l'endo-β-N-acétylglucosaminidase H (l'endo-H) : 80% pour les cellules traitées contre 20% pour les cellules non-traitées (Elbein et coll., 1984; Schwarz et Elbein, 1985).

Castanospermine (CS)

La CS est la 1,6,7,8-tétrahydroxyindolizidine (figure 8), isolée des noix d'un arbre australien, le Castanospermum Australe (Hohenschutz et coll., 1981). Cet alcaloïde est toxique pour les animaux qui mangent ces noix et cause une affection gastro-intestinale, qui peut mener à la mort.

La CS est un inhibiteur de la glucosidase I, causant une diminution de structures complexes et une augmentation en structures hybrides et oligomannosidiques. Un nombre de glycosidases lysosomiales ne semble pas être affecté par la CS; par contre, la β-glucosidase de lysosomes et la β-glucocérébrosidase sont bloquées (Saul et coll., 1983).

L'incorporation du mannose n'est pas affectée dans les cellules B16 de mélanome murin (Humphries et coll., 1986b) et même augmentée dans les rétines de Xenopus (Fliesler et coll., 1986).

La CS, comme la MdNM, permet le transport de l'inhibiteur de la protéinase-α1 et de la glycoprotéine acide-α1 dans des hépatocytes de rat (Gross et coll., 1983).

La CS provoque une accumulation des chaînes glycanniques exprimant un mannose terminal. Le récepteur, spécifique pour le mannose, des macrophages est ainsi constamment saturé et ne peut plus assurer l'endocytose de glycoprotéines portant des chaînes oligomannosidiques (Chung et coll., 1984).

Une glycosylation anormale provoquée par la CS inhibe la séparation protéolytique de l'oncogène env-sea. Les érythroblastes et fibroblastes transformés et traités à la CS reprennent une morphologie normale. Ceci n'est pas le cas pour des cellules transformées par l'oncogène v-src ou v-erb-B (Knight et coll., 1988). Dans les cellules embryonnaires, la CS modifie la glycosylation de la glycoprotéine codée par l'oncogène v-fms, et retarde ainsi la croissance de ces cellules dans l'agar sémi-solide (Hadwiger et coll., 1986).

In vivo, la CS peut inhiber la colonisation des cellules B16 de mélanome murin dans les poumons de souris syngénique. Par contre, elle est sans effet pour la tumorigénicité subcutanée et pour l'adhésion des cellules B16 sur la fibronectine et la laminine (Humphries et coll., 1986b).

1-Désoxymannojirimycine (dMM)

La dMM a été synthétisée par voie chimique; c'est un inhibiteur de la mannosidase I (Legler et Jülich, 1984).

La dMM réduit d'une façon considérable l'incorporation du mannose dans les glycannes complexes et hybrides des cellules F9 du tératocarcinome; par contre, l'incorporation du mannose dans les glycannes $Man_8GlcNAc$ et $Man_9GlcNAc$ est plus élevée (Romero et coll., 1986).

La dMM change considérablement la structure glycannique des glycoprotéines membranaires sans affecter leur expression ou leur sécrétion : des exemples sont l'inhibiteur de la protéinase-α1 et la glycoprotéine acide-α1 (Gross et coll.,

1985); l'IgM et l'IgD (Fuhrmann et coll., 1984); l'hémagglutinine du virus de l'influenza et la protéine G dans les cellules MDCK infectées par le virus de la stomatite vésiculaire (Burke et coll., 1984; Schwarz et Elbein, 1985).
L'inhibition de la conversion des structures oligomannosidiques en structures complexes par la dMM bloque la production d'immunoglobulines par des lymphocytes B humains (Tulp et coll., 1986).

Swainsonine (SW)

La SW (figure 8) est un alcaloïde d'indolizidine, qu'on peut isoler des plantes légumineuses comme p.ex. la Swainsona canescens et l'Astragulus sp., ou d'un champignon, le Rhizoctonia leguminicola (Colegate et coll., 1979; Hartley, 1978).
La SW est un inhibiteur des mannosidases α-lysosomiales dans les macrophages (Dorling et coll., 1980; Greenaway et coll., 1983) et dans les fibroblastes humains (Di Bello et coll., 1983). Dans l'appareil de Golgi, seule la mannosidase II est affectée et la SW prévient ainsi la formation de glycannes complexes (Di Bello et coll., 1983; Tulsiani et coll., 1982; Tulsiani et Tauster, 1983a et b).
Les cellules MDCK, CHO ou B16 expriment un taux élevé en glycannes hybrides et oligomannosidiques après le traitement par la SW, mais ni la croissance, ni la taille ou la morphologie de ces cellules ne sont affectées (Elbein et coll., 1981 et 1983; Schwarz et Elbein, 1984).
Sous l'effet de la SW, les glycannes de la thyroglobuline du porc acquièrent seulement des structures oligomannosidiques (Franc et coll., 1986). La fibronectine, sécrétée par des fibroblastes humains en présence de la SW, est du type hybride (au lieu de complexe) et sensible (au lieu de résistant) à l'endo-H (Arumugham et Tanzer, 1983b).
La SW cause une inhibition modérée du transport et de l'expression de l'aminopeptidase-N. En plus, un type différent de processus a été observé, impliquant l'expression de glycannes O-glycosidiquement liés (Danielsen et coll., 1983).

Les cellules BHK normales, traitées par la SW, agglutinent en présence de la concanavaline A, mais pas en présence de l'agglutinine du germe de blé, de Ricinus communis, d'Erythrina cristagalli ou d'Erythrina corallodendron, qui peuvent reconnaître des structures hybrides non-sialylées (Foddy et Hughes, 1986; Monis et coll., 1987). Des structures hybrides et oligomannosidiques des cellules BHK traitées à la SW ont été caractérisées par résonance magnétique nucléaire (500 MHz) (Fody et coll., 1986).

La SW inhibe l'endocytose et la dégradation d'un oligosaccharide mannosidique par des macrophages pulmonaires de rat (Arumugham et Tanzer, 1983a) ou par des macrophages de la moelle osseuse de rat (Chung et coll., 1984). Après l'endocytose de certaines glycoprotéines, la dégradation par des lysosomes ou par des cellules parenchymales, tous les deux isolés du foie de rat, est inhibé en présence de la SW (Winkler et Segal, 1984a et b).

En présence de la SW, les fibroblastes NIH-3T3 transfectés à l'aide de l'ADN tumorale humaine ne croissent plus dans l'agar sémi-solide et n'expriment plus les oligosaccharides complexes, caractéristiques des cellules malignes (DeSantis et coll., 1987).

Le titre en hémagglutination des cellules MDCK, infectées par le virus de la stomatite vésiculaire, ne change pas en présence de la SW (Elbein et coll., 1981).

In vivo, l'administration de la SW aux rats, aux cochons d'Inde et aux moutons provoque la sécrétion de glycoprotéines ayant des glycannes du type oligo-mannosidique (Abraham, 1983; Daniel et coll., 1984). Une vacuolisation, d'origine lysosomiale, dans le foie et le rein de rat peut être observée après l'addition chronique de SW à la nourriture (Novikoff et coll., 1985); chez les porcs et les moutons, des structures hybrides apparaissent dans les glycoprotéines de cerveau (Tulsiani et coll., 1988).

Les cellules MDAY-D2, dérivées de tumeurs lymphoides, et les cellules B16 F10 de mélanome démontrent une capacité métastatique réduite, quand elles sont

traitées pendant 48 hrs à 0.3 µg SW/ml avant l'injection intraveineuse dans la queue de souris (Dennis, 1986; Humphries et coll., 1986a). Une explication possible est que la SW stimulerait la prolifération des cellules immuno-surveillantes (White et coll., 1988).

En conclusion, nous en sommes loin du temps ou les glycannes étaient considérés comme des substances de réserve ou des entrepôts d'énergie, sans avoir un rôle spécifique dans la biologie cellulaire. Les progrès des recherches faites sur la structure, le métabolisme et la fonction des glycannes de glycoprotéines ont été résumés par Montreuil (1984).

Nous considérons les glycoprotéines comme des cibles attrayantes pour les substances anti-invasives. Plusieurs molécules, probablement impliquées dans le processus de l'invasion et de la métastase, sont des glycoprotéines membranaires, tel que les facteurs de locomotion et leurs récepteurs, les protéinases et leurs inhibiteurs, les molecules d'adhésion cellulaire (CAMs), les composantes de la matrice extracellulaire et leur récepteurs de la surface cellulaire.

Un exemple : la laminine consiste en trois polypeptides N-glycosylés dont la biosynthèse se déroule dans le RER et l'appareil de Golgi, avant l'insertion dans des granules sécrétoires et le dépôt dans la matrice extracellulaire (Hogan et coll., 1980; Laurie et coll., 1982).

Il n'est pas établi si la partie glycannique joue un rôle dans l'invasion tumorale. Nous avons choisi d'étudier l'expression des glycannes N-glycosidiquement liés de la surface cellulaire dans des cellules invasives et non-invasives, d'analyser les glycannes N-glycosidiquement liés dans une situation minimale et transitoire de l'invasion, et finalement de contrôler l'expression des glycannes N-glycosidiquement liés, en utilisant des inhibiteurs de la glycosylation, et de tester le phénotype invasif des cellules MO_4 malignes.

CHAPITRE I : LA CORRELATION ENTRE LA COMPLEXITE DES GLYCANNES N-GLYCOSIDIQUEMENT LIES DE LA SURFACE CELLULAIRE ET L'INVASION TUMORALE

INTRODUCTION

Les glycannes des glycoprotéines membranaires dérivés de cellules malignes et de cellules normales sont typiquement différentes dans la distribution de leur taille : on observe, dans les cellules malignes, un accroissement de la multi-antennarisation ou une élongation des antennes, accompagnée par une augmentation en teneur d'acide sialique (Warren et coll., 1978; Van Beek et coll., 1979). L'expression de certains types de glycannes sialylés a été mise en corrélation avec l'acquisition du phénotype métastatique en cas de cellules tumorales murines (Schirrmacher et coll., 1982), de cellules T activées (Collard et coll., 1986), et de cellules mutées et sélectionnées de la lignée MDAY-D2 (Dennis et Laferte, 1987), mais pas en cas des cellules de mélanomes B16 (Passaniti et Hart, 1988); Un métabolisme modifié du fucose a été démontré pour des cellules tumorales murines ayant une capacité réduite de formation de métastases (Dennis et Kerbel, 1981; Schwartz et coll., 1984). L'adhésion entre les cellules du carcinome de Lewis et les cellules normales de poumon a été attribuée à l'action d'une lectine présente dans les cellules normales et spécifique pour des résidus α-Fuc, et une lectine présente dans les cellules malignes et spécifique pour des résidus α-Glc. En injectant par voie intravéneuse des cellules du carcinome de Lewis avec des néoglycoprotéines, portant des résidus α-L-Fucose, le nombre de métastases trouvées dans les poumons est réduit d'une façon significative (Monsigny et coll., 1988). L'adhésion cellulaire a pu être bloquée par des anticorps monoclonaux, qui peuvent réagir avec l'α-L-fucose, dans Polysphondylium pallidum (Toda et coll., 1984). Par le fait que les glycannes de la surface cellulaire sont impliquées dans l'établissement et le maintien de contacts fonctionels entre les cellules, des changements de ces glycannes pourraient lever certains contrôles et fournir la capacité d'envahir le

tissu normal. L'invasion peut être caractérisée par l'occupation et la destruction du tissu normal par des cellules malignes; ces deux phénomènes sont progressifs dans l'espace et le temps (Mareel, 1980).

Dans cette étude, les N-glycosylpeptides de la surface cellulaire provenant de cellules invasives et non-invasives ont été comparés par chromatographie sur gel afin de tester si l'expression des "glycannes malins" est correlée avec l'acquisition du phénotype invasif, induit par une transformation maligne ou par un lysophospholipide alkylé, le 1-O-octadécyl-2-O-méthylglycéro-3-phosphocholine (ET-18-OCH_3).

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Effect of Cancer-related and Drug-induced Alterations in Surface Carbohydrates on the Invasive Capacity of Mouse and Rat Cells[1]

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ABSTRACT

The effect of alterations in cell surface carbohydrates on invasion of mouse and rat cells into embryonic chick heart fragments in organ culture was studied. Matching pairs of malignant and nonmalignant cells, including all categories of carcinogenic induction (*i.e.*, viral, chemical, or oncogenic), were compared for their alterations in cell surface carbohydrates and invasive behavior. Glycopeptides derived from the surface of malignant cells expressed cancer-related changes in carbohydrate composition, demonstrated by gel filtration chromatography as a shift in size distribution in comparison with those from nonmalignant counterparts. This phenotypic property strictly correlated with the acquisition of the invasive capacity. Morphological transformation of cells without simultaneous alteration in surface carbohydrates was, however, insufficient for invasion.

To test the possible mechanistic role of altered surface carbohydrates in the invasive capacity of cells, the surface molecules of noninvasive cells were modified by incubation with an alkyl-lysophospholipid (racemic 1-*O*-octadecyl-2-*O*-methyl glycero-3-phosphocholine). Alkyl-lysophospholipid induced an increase in surface sialylation resembling the changes found in malignant and invasive cells. After pretreatment with alkyl-lysophospholipid, morphologically transformed but nonmalignant and noninvasive cells became able to invade chick heart tissue.

These findings indicate that alterations in cell surface carbohydrates, induced by entirely different mechanisms, endowed cells with invasive capacity.

INTRODUCTION

The fucose containing carbohydrate moieties of membrane glycoproteins from malignant cells are characteristically different in their gel filtration size distribution in comparison with those from nonmalignant cells. The carbohydrates from malignant cells contain more highly branched or elongated chains with increased amounts of sialic acid residues (for reviews see Refs. 1–5). The most striking feature is their general occurrence in all malignant tissues and cell lines, independent of transforming principle and cellular origin.

Since carbohydrates are known to be involved in establishing and maintaining functional contacts between cells, alterations in surface carbohydrates may enable tumor cells to overrule normal control functions, *e.g.*, endowment of the capacity to invade normal tissue. Invasion includes infiltration and subsequent destruction of normal tissue, both being progressive in time and space (6), and is an important step in the sequence of events leading to metastasis. In several cell systems metastatic capacity is influenced and regulated by the degree of sialylation of specific cellular binding sites (7–13). In addition, differences in invasive capacity between T-cell lymphoma variants (7) and hybrids of noninvasive T-lymphoma (BW 5417) and activated T-cells (14) have been ascribed to differences in sialylation of cell surface carbohydrates. Also in normal cells a correlation between cell surface carbohydrate expression and functional aspects related to invasion has been described. Mature granulocytes transiently express leukemic-like carbohydrates during egression from the bone marrow (15). Temporal changes in cell surface carbohydrate composition and lectin binding of mouse trophoblasts have been connected with infiltration in the uterine wall (16–17).

To further investigate the apparent participation of cell surface carbohydrates in tissue invasion, an organ culture model was used which meets the above-mentioned criteria of invasion (18, 19). Matched pairs of malignant and nonmalignant cells were studied for the existence of a possible correlation between the occurrence of cancer-related carbohydrate changes and the invasive capacity. Moreover, to go beyond correlative observations and to obtain a direct indication of a functional relationship it was attempted to transiently modify the surface carbohydrates of noninvasive, nontumorigenic cells. In view of its reported activity on invasion (20) the ether analogue ET-18-OCH_3[4] of the naturally occurring 2-lysophosphatidylcholine was tested for possible effects on cell surface carbohydrates. This ALP transiently modified the carbohydrates to an extent that they resembled those found on cancer cell surfaces.

MATERIALS AND METHODS

Cells. The cell lines R1C and HSU, established from baby rat kidney cells by transfection with genomic fragments of the oncogenic Ad12, were identical to those described by De Leij *et al.* (21). The most left-hand fragment (0–7.2%) of the Ad12 genome caused stable morphological transformation but no tumorigenic potential (HSU). Transfection with a larger fragment (0–16%) resulted in fully transformed, tumorigenic R1C cells (22).

Malignant rat brain cells (RB14-T) and their nonmalignant precursors (RB14-N and RB22) were obtained and cultured in the same way as described by Laerum and Rajewski (23). Briefly, neonatal rat brain cells exposed *in vivo* to the carcinogen ENU underwent continuous morphological transformation during serial culture *in vitro*, followed by malignant transformation (tested in syngeneic animals) after about 12 passages (RB14-T). The RB14-N cells were from cultures prior to tumorigenic transformation, while the RB22 cells were normal controls from animals not exposed to the carcinogen.

The mouse MO cell family has been previously described by Mareel *et al.* (24). The MO_4 cells, transformed with KiSV, are invasive *in vitro* (18) and produce invasive and metastasizing tumors in syngeneic mice (19), in contrast to the nontransformed counterpart (MOH).

The NIH/3T3 cells and its transformant induced by human tumor DNA have been described by Collard *et al.* (25). The malignant T13

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[4] The abbreviations used are: ET-18-OCH_3, racemic 1-*O*-octadecyl-2-*O*-methyl glycero-3-phosphocholine; ALP, alkyl-lysophospholipid; ENU, ethylnitrosourea; Ad12, adenovirus type 12; KiSV, Kirsten sarcoma virus.

transformant was established by transfection with T24 bladder carcinoma DNA.

All cells were cultured in Dulbecco's modified Eagle's medium (Flow Laboratories, Ltd., Irvine, Scotland) supplemented with 10% fetal calf serum (Gibco Europe, England) and antibiotics (penicillin, 100 IU/ml; streptomycin, 50 μg/ml) in a humidified incubator at 37°C in 5% CO_2.

Invasion Assay. The capacity of cells to invade normal tissue was assayed in confrontations between test cell aggregates and fragments of 9-day-old embryonic chick heart in organ culture as described earlier (18). Briefly, the aggregates (diameter, 0.2 mm) were brought into contact with precultured heart fragments (diameter, 0.4 mm) on top of a semisolid agar medium. After attachment of the aggregates to the heart fragment the confronting pairs were further incubated individually in fluid culture medium on a gyratory shaker at 120 rpm. The confronting pairs were fixed in Bouin-Hollande's solution after 1–28 days, followed by embedding in paraffin or Histowax and complete serial sectioning (8 μm thick), and stained with hematoxylin-eosin or with an antiserum against chick heart tissue (26). For semiquantitative evaluation the interaction of test cells with chick heart was classified as described before (27): Grades I and II (confronting cells found at the periphery of, or intermixed with, the outer fibroblastic layer of the heart fragment, respectively) were combined to indicate the absence of invasion (denoted as −); Grade III, when confronting cells replaced the cardiac muscle to less than 50% (denoted as +); and Grade IV, when confronting cells replaced the cardiac muscle to more than 50% (denoted as ++). According to Mareel *et al.* (19) Grades III and IV meet the criteria of invasion *in vivo*. In assays with ALP-treated cells, suspensions of cells were confronted with heart fragments on top of a semisolid agar medium to provide a more rapid and maximal interaction. After 4 h the excess of unattached cells was removed, and the heart fragments with attached cells were incubated individually in fluid culture medium as usual.

Cell Surface Glycopeptide Isolation and Analysis. Cells or cell aggregates were metabolically labeled with either L-[5,6-^{3}H]fucose (1 μCi/ml, 60 Ci/mmol) or L-[1-^{14}C]fucose (0.5 μCi/ml, 60 mCi/mmol) for 20 h at 37°C. Radiochemicals were obtained from New England Nuclear, Boston, MA, or The Radiochemical Center, Amersham, United Kingdom. Glycopeptides were isolated from the cell surface by proteolytic enzymes as described (28) and desalted by gel filtration-centrifugation on Bio-Gel P-2 columns (29) or dialysis against bidistilled water. Glycopeptides were analyzed as such or following treatment with mild acid or neuraminidase (*Vibrio cholerae*, EC. 3.2.1.18; Behringwerke AG, Marburg, West Germany) to remove terminal sialic acids as described earlier (28). Gel filtration was performed on Bio-Gel P-10: Sephadex G-50 (2:1, w/w) columns, eluted with 0.1 M Tris-HCl buffer (pH 8.0) containing sodium dodecyl sulfate (0.1%, w/v), EDTA (0.01%, w/v), and 2-mercaptoethanol (0.1%, v/v) with a flow rate of 4 ml/h and 0.7-ml fraction size.

Modification of Cell Surface Carbohydrates by ET-18-OCH_3. Cells were incubated with ALP (kindly provided by Dr. W. E. Berdel, Technical University, Munich, Federal Republic of Germany) 48 h prior to the invasion assay or glycopeptide analysis. The drug was used at concentrations which permitted growth to at least 75% of that of controls. These concentrations varied between 5 and 30 μg/ml, depending on the cell type.

RESULTS

Comparison of Malignant and Nonmalignant Cells. The possible involvement of cancer-related changes in cell surface carbohydrates in malignant invasion was studied in four matched pairs of malignant and nonmalignant cells. The panel included viral and chemical transformants as well as human- and viral-DNA transfectants.

The first cell system consisted of R1C and HSU rat kidney cells tranformed by specific Ad12 genome fragments (22). To test whether the differences in carbohydrate composition between these transfectants observed in monolayer cultures (21) were also present when grown in aggregates, HSU and R1C cell aggregates were metabolically labeled with [^{3}H]- or [^{14}C]-fucose. The size distribution pattern (Fig. 1) showed that the glycopeptides derived from tumorigenic R1C cell aggregates were of a higher apparent molecular weight relative to those from HSU cell aggregates, comparable to the differences in R1C and HSU cells grown in monolayers (*cf.* Ref. 21). The invasive capacity of HSU and R1C cells was studied in parallel. Multiple confronting pairs of cell aggregates and precultured heart fragments were incubated and fixed after 4 and 7 days. Characteristic stages of the invasive process are illustrated in Fig. 2, and the results are included in Table 1. The tumorigenic R1C cells were highly invasive (++, Grade IV). The histology of all cultures met the criteria of malignant invasion since R1C cells progressively replaced the cardiac muscle (Fig. 2). Counterstaining with an antiserum against chick heart tissue revealed that only remnants of the cardiac muscle were left (Fig. 2*d*). However, morphological transformation alone appeared insufficient for invasion since the interaction between HSU cells and heart tissue was at most limited to the occupation of the outer fibroblastic layer of the heart fragment (Fig. 3). Even after 28 days when HSU cells had totally surrounded the heart fragment, these cells were still incapable of infiltrating and degrading the chick heart fragment (Fig. 3, *e* and *f*). Apparently, the additional information in the larger Ad12 genome fragment, responsible for alterations in cell surface carbohydrates (21), was causal in the acquisition of a tumorigenic and invasive phenotype.

Neonatal rat brain cells exposed *in vivo* to the carcinogen ENU undergo continuous transformation during serial culture *in vitro* (*cf.* 23). The acquisition of the tumorigenic potential of these cells coincides with the capacity to invade into chick heart tissue (30). In a similar series of rat brain cells the surface carbohydrates were analyzed before and after transformation to tumorigenic cells. Glycopeptides derived from the rat brain cells after tumorigenic transformation (cell line RB14-T) expressed cancer-related changes in cell surface carbohydrates when compared with those from cells before malignant transformation (RB14-N) (Fig. 4). The surface carbohydrate composition of the latter cells was comparable with that of RB22 cells derived from untreated animals (data not shown).

In the third system glycopeptides derived from the tumori-

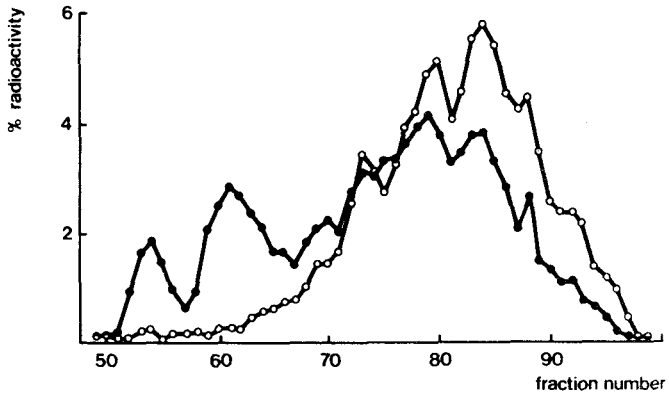

Fig. 1. Gel-filtration profiles of surface glycopeptides derived from malignant R1C (●) and morphologically transformed nonmalignant HSU rat kidney cells (○). The glycopeptides were isolated from the cell surface by proteolytic digestion as described in "Materials and Methods," followed by cochromatography on a Bio-Gel P-10:Sephadex G-50 (2:1, w/w) (200 x 1 cm) column, eluted with 0.1 M Tris-HCl buffer (pH 8.0) containing sodium dodecyl sulphate (0.1%, w/v), EDTA (0.01%, w/v), and 2-mercaptoethanol (0.1%, v/v) with a flow rate of 4 ml/h and 0.7-ml fraction size. Cochromatography of size markers derived from vesicular stomatitis virus suggests the elution of fully sialylated triantennary structures between Fractions 70 and 75 and monosialobiantennary structures just before Fraction 90 (results unpublished).

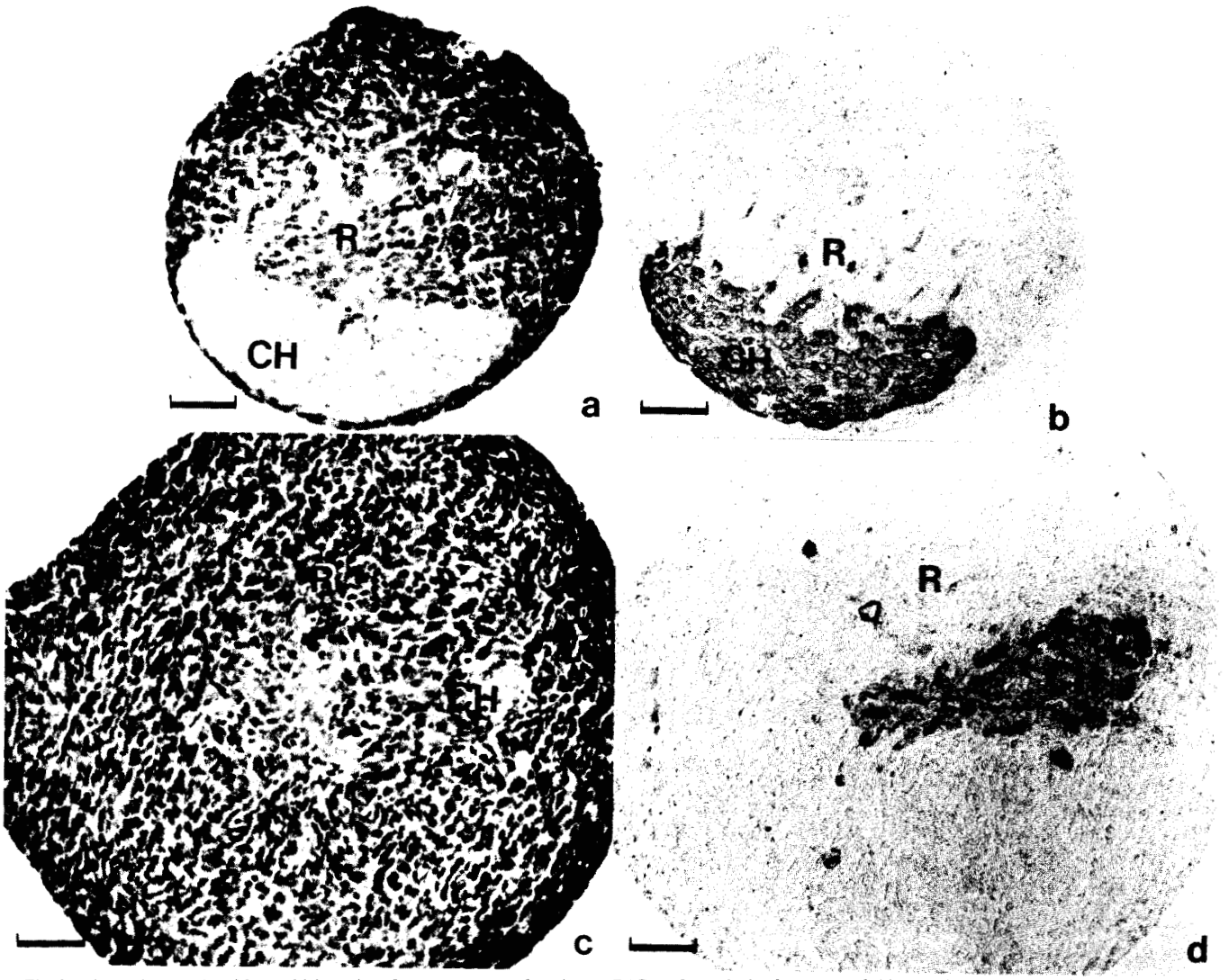

Fig. 2. Light micrographs of 8-μm-thick sections from aggregates of malignant R1C confronted with fragments of chick cardiac muscle. Fixation after 7 (*a*, *b*) and 14 (*c*, *d*) days; *a* and *c* were stained with H & E; *b* and *d* with an antiserum against chick heart. *R*, R1C; *CH*, chick heart tissue. *Bars*, 50 μm.

Table 1 *Semiquantitative analysis of invasion into chick heart tissue*

	Cells	Grading[a] after	
		Day 4	Day 7
HSU	Nonmalignant rat kidney cells transfected with (0–7.2%) Ad12 DNA	− (5)[b]	− (7)
RIC	Malignant rat kidney cells transfected with (0–16%) Ad12 DNA	+ (3)	++ (4)
NIH/3T3	Nonmalignant mouse cells	− (3)	− (3)
T13	Malignant transformant of NIH/3T3 induced by T24 bladder carcinoma DNA	+ (6)	+ (1); ++ (4)

[a] Grading of invasion was according to Bracke *et al.* (27). −, no invasion (Grades I and II); +, less than 50% invasion (Grade III); ++, over 50% invasion (Grade IV). For details see "Materials and Methods."

[b] Numbers in parentheses, number of confronted pairs analyzed.

genic and invasive MO_4 (18, 19) cells and the nontransformed counterparts (MOH) were analyzed as usual. The size distribution pattern (data not shown) revealed that the tumorigenic MO_4 cells expressed cancer-related glycopeptide changes. The invasive capacity of MO_4 cells and the absence of this property in MOH cells were confirmed in parallel experiments (data not shown).

The fourth cell system consisted of NIH/3T3 cells and its transformant established by transfection with T24 bladder carcinoma DNA (T13). Similarly, as published previously (25, 31), T13 cells expressed cancer-related carbohydrate changes relative to the parent NIH/3T3 cells (data not shown). The T13 and NIH/3T3 cells were analyzed in parallel for their ability to invade chick heart tissue. As included in Table 1, the T13 cells were invasive (++, Grade IV) while parent NIH/3T3 cells were not.

The combined observations in the various cell systems (summarized in Table 2) demonstrate a strict correlation between cancer-related carbohydrate changes and the invasive capacity of transformed cells.

Modulation of Cell Surface Carbohydrates and Invasion. It has been recently reported that ALP interfers with the invasive process when present during the assay (20). We therefore investigated whether ALP may mediate invasion by affecting the carbohydrate moieties of surface glycoproteins. The tumorigenic R1C cells and the morphologically transformed but nontumorigenic HSU cells were incubated with ALP during 48 h in 5 and 20 μg of ALP per ml, respectively (the highest concen-

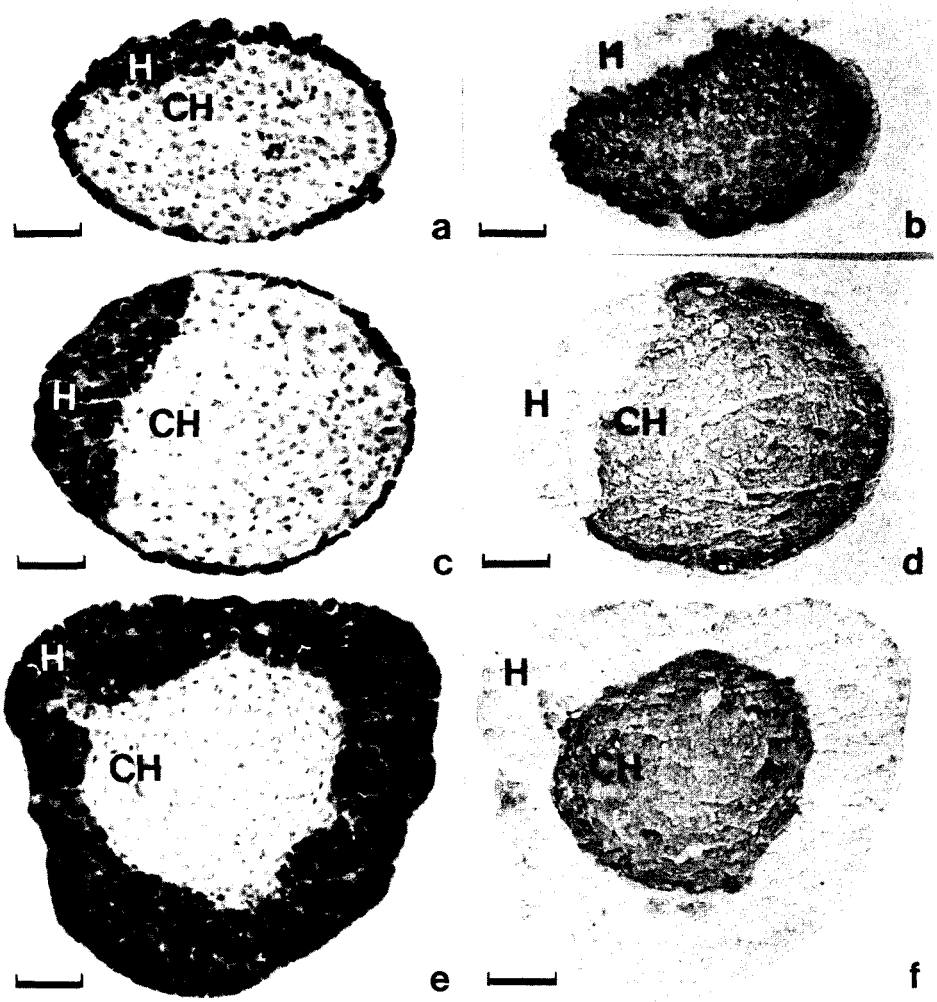

Fig. 3. Light micrographs of 8-μm-thick sections from aggregates of morphologically transformed but nonmalignant HSU rat kidney cells confronted with fragments of chick cardiac muscle. Fixation after 7 (*a*, *b*), 14 (*c*, *d*), and 28 (*e*, *f*) days; *a*, *c*, and *e* were stained with H & E; *b*, *d*, and *f* with an antiserum against chick heart. *H*, HSU; *CH*, chick heart tissue. *Bars*, 50 μm.

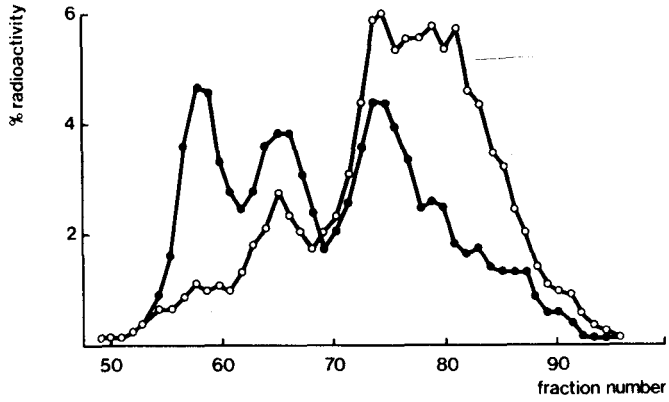

Fig. 4. Gel filtration profiles of surface glycopeptides derived from ENU-treated, malignant RB14-T (●) and ENU-treated, premalignant RB14-N rat brain cells (○). For conditions, see legend to Fig. 1.

trations which permitted growth to at least 75% of that of controls). ALP had hardly any effect on the size distribution of glycopeptides from the R1C cells (Fig. 5*a*), while those derived from ALP-treated HSU cells clearly showed a shift towards higher apparent molecular weight (Fig. 5*b*). Comparison of this latter profile with that of R1C-derived glycopeptides revealed almost identical size distributions. The duration of this ALP effect was tested at 16, 40, and 64 h after removal of the drug, preceded by a 16-h fucose labeling period. The phenotypic change decreased with time but was still present after 64 h (Fig. 5*d*). Removal of sialic acid by neuraminidase or mild acid

Table 2 *Relation between cancer-related alterations in cell surface carbohydrates and invasiveness in vitro*

	Cells	Invasiveness[a]	Cell surface carbohydrates[b]
HSU	Nonmalignant rat kidney cells transfected with (0–7.2%) Ad12 DNA	Noninvasive	Normal
RIC	Malignant rat kidney cells transfected with (0–16%) Ad12 DNA	Invasive	Altered
RB22	Untreated rat brain cells	Noninvasive[c]	Normal
RB14-N	Premalignant, ENU-treated rat brain cells	Noninvasive[c]	Normal
RB14-T	Malignant, ENU-treated rat brain cells	Invasive[c]	Altered
MOH	Nonmalignant C3H mouse cells	Noninvasive	Normal
MO_4	Malignant C3H mouse cells transformed by KiSV	Invasive	Altered
NIH/3T3	Nonmalignant mouse cells	Noninvasive	Normal
T13	Malignant transformant of NIH/3T3 induced by T24 bladder carcinoma DNA	Invasive	Altered

[a] Invasion was measured in an organ culture assay into precultured chick heart fragments as described in "Materials and Methods."

[b] Glycopeptides derived from matching pairs of cells were analyzed by cochromatography as described in "Materials and Methods"; "altered" means that the cells expressed cancer-related alterations in carbohydrate moieties of membrane glycoproteins relative to nonmalignant counterparts (normal).

[c] From De Ridder and Laerum (30); measured in similar series of rat brain cells using the same assay.

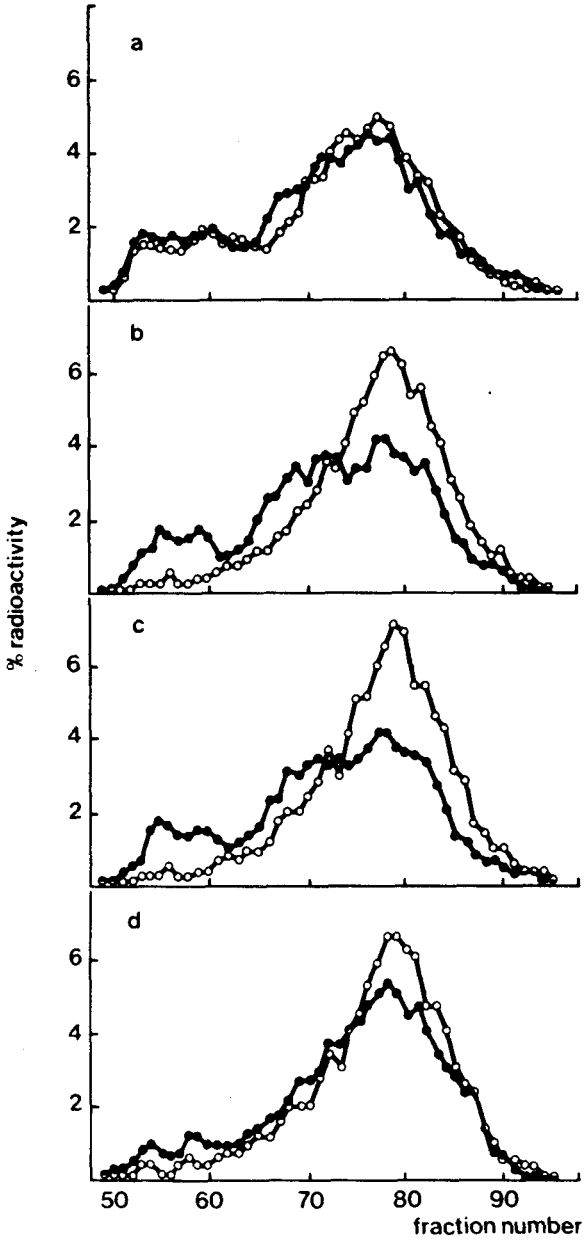

Fig. 5. Gel filtration profiles of surface glycopeptides derived from malignant R1C (*a*) and nonmalignant HSU rat kidney cells (*b* to *d*), pretreated with (●) or without (○) ALP. Effect of removal of the drug and subsequent culturing during 16 or 64 h on the glycopeptide size distribution is shown in *c* and *d*, respectively. For conditions, see legend to Fig. 1.

treatment completely abolished the ALP effect (data not shown), suggesting that increased surface sialylation was the underlying cause of this shift in size distribution. A full account of the biochemical effects of ALP will be reported separately.[5] To study the effect of ALP pretreatment on invasion, HSU cells were confronted with the heart tissue as a single cell suspension on semisolid agarose medium to provide a rapid and maximal interaction. After 2, 4, and 7 days histological examination indicated a clear occupation (+, Grade III) of the heart tissue by ALP-treated HSU cells, whereas in control cultures both cardiac muscle and HSU cells remained sharply delineated (Fig. 6; Table 3). Occupation and penetration of the host tissue were to some extent progressive in time, meeting the criteria of at least Grade III of invasion. However, after 7 days the chick heart fragment was not totally abolished by the ALP-treated HSU cells in contrast to the very progressive invasion of R1C (++, Grade IV). Pretreatment of R1C cells with ALP had no effects on the invasive capacity.

The same ALP-mediated induction of invasion was achieved with NIH/3T3 cells (Table 3) tested as suspended cells. Unlike the results with NIH/3T3 cells in aggregates (Table 1), occasionally some untreated NIH/3T3 cells appeared to be able to penetrate the chick heart muscle. The background invasion in this assay is as yet unexplained but possibly due to selection of variant cells facilitated by the increased interaction area.

DISCUSSION

The biological consequences of cancer-related alterations in cell surface carbohydrates have been discussed in several reviews (1–5). A prevailing conclusion was that such changes may not affect a single cellular function but rather provoke a general infidelity in various cell surface-mediated processes controlling normal cell behavior. In the present study it was attempted to associate specific surface carbohydrate expressions with the capacity to invade normal tissue. It was observed in four different cell systems (Table 2) that only cell lines with altered carbohydrates were capable of invading normal tissue. Immortalization and morphological transformation without simultaneous alteration in surface carbohydrates, as in the HSU and RB14-N cells, were in themselves insufficient for invasion. Thus, a previously established correlation between invasive capacity *in vitro* and tumorigenicity *in vivo* (18, 30) can be extended with the concomitant occurrence of cell surface carbohydrate changes. Accordingly, cancer-related changes in cell surface carbohydrates may be conditional if not causative in the invasive potential.

Supportive evidence for the latter contention follows from the studies with ALP. The ALP-induced epigenetic change in the degree of sialylation of surface molecules transiently endowed nontumorigenic HSU cells with invasive capacity (Fig. 6) and stimulated that of NIH/3T3 cells (Table 3). Quantitatively, the invasion by ALP-treated HSU cells was less than obtained with permanently malignant cell lines. This is ascribed to the disappearance of the effect of ALP 64 h after its removal (Fig. 5*d*). On the other hand the limited invasion by ALP-treated cells was persistent after 7 days (Table 3; Fig. 6) and to some extent progressive in time (*cf.* Table 3, Days 2 and 4). It is assumed that proliferation of already invaded cells accounts for these observations. The precise mechanisms through which ALP alters the level of surface sialylation are as yet unknown, but probably different from those operational after malignant transformation as will be reported in detail separately.[5]

How alteration in glycosylation could be instrumental in tissue invasion might be considered in the context of cellular adhesion and contact inhibition of movement (reviewed in Refs. 32–35). Abercrombie (32) concluded from studies with confronting colonies that pairs of malignant cells do not intermix and that a polarity in contact inhibition of movement between malignant and normal tissue is required to bring about the invasion-like growth pattern in this system. The differential adhesion hypothesis proposed by Steinberg (36), based on thermodynamic considerations of homotypic and heterotypic adhesive forces, predicts that cells will move into an environment with maximal cellular interaction. Interaction between cells is mediated by cell adhesion molecules (*cf.* Ref. 37), and the strength of intercellular adhesion can be strongly influenced by

[5] D. C. C. Schallier, J. G. M. Bolscher, H. van Rooy, G. A. Storme, and L. A. Smets. The effect of an alkyl-lysophospoholipid on the expression of cell surface carbohydrates and invasive behavior, submitted for publication.

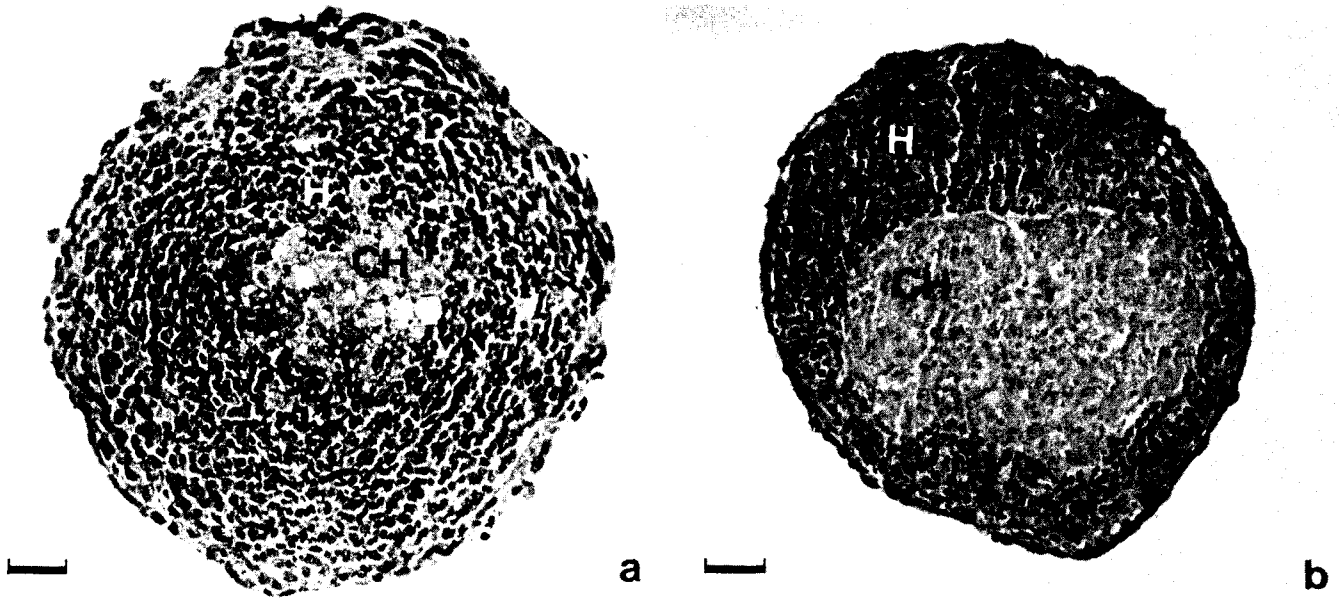

Fig. 6. Light micrographs of 8-μm-thick sections of nonmalignant HSU rat kidney cells after pretreatment with (*a*) or without (*b*) ALP, confronted as cell suspensions with fragments of chick cardiac muscle. Fixation after 7 days and staining with H & E. *H*, HSU; *CH*, chick heart tissue. *Bars*, 50 μm.

Table 3 *Semiquantitative analysis of ALP-induced invasion into chick heart tissue*

Cells were pretreated with ALP and confronted as cell suspensions with chick heart fragments in the absence of the drug as described in "Materials and Methods."

Cells	ALP (μg/ml)	Grading[a] after Day 2	Day 4	Day 7
HSU	0	−(3)[b]	−(4)	−(2)
HSU	20	−(3); +(2)	+(5)	+(2)
NIH(3T3)	0	−(3)	−(3)	−(4); +(2)
NIH(3T3)	30	−(4)	+(4)	+(4)

[a] See Table 1, Footnote *a*.
[b] Numbers in parentheses, number of confronted pairs analyzed.

the degree of sialylation of these cell adhesion molecules (37, 38). If such mechanisms are also relevant in the invasive process, it is conceivable that the increased carbohydrate sialylation in malignant cells or in ALP-treated HSU and NIH/3T3 cells could alter in a similar fashion the adhesive forces to meet the conditions for penetration into the heart tissue.

Paradoxically, ALP, which stimulated invasiveness in HSU and NIH/3T3 cells, is antiinvasive when present during the confrontation of malignant MO_4 cells and chick heart tissue (20). In the latter experiments, however, the drug was continuously present during the invasion assay, thus acting on both the malignant cells and the heart tissue. As already demonstrated for R1C and HSU cells (Fig. 5), increased sialylation by ALP is most pronounced in the nontumorigenic HSU cells. Also in normal chick heart fibroblasts and myoblasts large increases in surface carbohydrate sialylation have been observed.[5] As a result, preexisting differences in the degree of sialylation (and consequently in the relative intercellular adhesion) may diminish or disappear during confrontation of malignant cells and chick cardiac tissue in the continuous presence of ALP and abolish the invasive process.

In conclusion cancer-related and drug-induced alterations in cell surface carbohydrates are associated with the invasive capacity and may play a mechanistic role in this process. However, whether increased sialylation is the crucial alteration remains to be established. Moreover, transient carbohydrate changes may have physiological functions in normal cell migration as well. Van Beek *et al.* (15) demonstrated that metamyelocytes during bone marrow egress temporarily express surface carbohydrate properties such as those permanently expressed on myeloid leukemic cells, but absent on mature granulocytes and promyelocytic precursors. They concluded that these changes assisted in the penetration through the sinus epithelium. Corresponding observations have been made in implanting trophoblastic cells (16, 17). Accordingly, tumorigenic cells could acquire invasive capacity by a permanent expression of the gene(s) responsible for the generation of cancer-related carbohydrates. In all invasive cell lines in this study (Table 2) transformation involved introduction or activation of *ras* genes or functional analogous DNA sequences, namely the 0–16% fragment of the Ad12 genome in R1C cells (39), the v-Ki-*ras* gene in MO_4 cells transformed with KiSV (24), and c-Ha-*ras* in the bladder carcinoma DNA-transfected T13 cells (25, 31). Finally, in the tumorigenic rat brain cells (RB14-T) N-*ras* was activated as proved in the NIH/3T3 focus assay.[6] Accordingly, it would appear that in the cell lines studied (activated) oncogenes of the *ras* family accompanied the acquisition of invasive capacity, associated with specific alterations in surface carbohydrates.

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Mémoire 2

EFFECT OF RACEMIC 1-O-OCTADECYL-2-O-METHYLGLYCERO-3-PHOSPHOCHOLINE (ET-18-OCH_3) ON INVASION IN VITRO AND ON N-LINKED SURFACE GLYCOSYLATION

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ABSTRACT

The effect of ET-18-OCH_3, an alkyl-lysophospholipid, has been studied on invasion in vitro and on N-linked surface glycosylation.

In presence of ET-18-OCH_3, invasion into precultured embryonic chick heart fragments (PHF) of malignant baby rat kidney (12R1C-RK) cells or malignant mouse (MO_4) cells are inhibited, whereas highly metastatic Lewis lung carcinoma (LLC-H61) cells are still invasive. No correlation is found between the invasive phenotype and the ET-18-OCH_3 induced changes of N-linked fucosylated glycopeptides.

Pretreatment of the host tissue (PHF) with ET-18-OCH_3 inhibits the invasion of MO_4 cells when tested in absence of the drug. This invasion resistance of the PHF is still present 11 days after treatment. The surface glycopeptide profiles from PHF treated with ET-18-OCH_3 shift towards apparently higher molecular weights, when compared with those of untreated PHF. This effect is irreversible within 64 hr of culture in absence of the drug. The shift in the profiles is abolished after desialylation of the isolated glycopeptides.

ET-18-OCH_3 pretreatment induces temporarily the invasive phenotype in non-malignant baby rat kidney (HSU) cells, immortalized mouse (3T3) cells and epithelial canine kidney (MDCK) cells. This effect can not be demonstrated on normal epithelial mouse (NMuMG) cells or on non-invasive human mammary carcinoma (MCF-7) cells. Increased sialylation and malignant changes in the glycosylation profile are found on ET-18-OCH_3 treated HSU and 3T3 cells. The ET-18-OCH_3 effect on glycosylation and invasive behavior of HSU cells is reversible after 4 days.

ET-18-OCH_3 has been shown to inhibit or induce epigenetically the invasive phenotype in vitro and to cause concomitantly cancer related changes of N-linked glycoproteins.

INTRODUCTION

Invasion marks the difference between a benign and malignant tumor (1). Using a three-dimensional organ culture assay (2), invasion of MO_4 cells is tested in vitro in presence of different alkyl-lysophospholipids. ET-18-OCH_3 can inhibit the invasion of MO_4 cells into precultured embryonic chick heart or lung fragments (3); a thioether phospholipid (BM 41,440) has the same anti-invasive capacity, while the naturally occuring 2-lysophosphatidylcholine (2-LPC) or an alkyl-linked lipoidal amine (CP-46,665) have no effect (4).

ET-18-OCH_3 has been demonstrated to inhibit tumorigenicity and metastasis in human and animals (5,6). Possible explanations are selective killing of the tumor cells for lack of the O-alkyl cleaving enzyme (6-9), activation of macrophages (10-12) and enhancement of tumor cell differentiation (13).

Increased branching or elongated chains together with increased sialylation of N-linked glycans are related to malignant transformation (14-17). Comparable changes in glycosylation are found between couples of invasive and non-invasive cells after treatment with ET-18-OCH_3 (3,4,18-20).

Therefore, the possible correlation between ET-18-OCH_3 induced changes of invasion and altered glycosylation on different cell lines is extended and further discussed.

MATERIAL AND METHODS

Cell lines

MO_4 cells (obtained through Dr. M. De Brabander, Janssen Life Sciences, Beerse, Belgium) are C_3H mouse cells transformed by Kirsten murine sarcoma virus (21); they are invasive in vitro (2) and produce invasive and metastasizing sarcomas in syngeneic mice (22).

HSU and 12R1C-RK cells (obtained through Dr. L. Smets, The Netherlands Cancer Institute, Amsterdam, The Netherlands) are baby WAG RIJ rat kidney cells transfected respectively with a smaller (0-7.2%) and a larger left hand (0-16%) fragment of the adenovirus type 12 genome. HSU cells are morphologically transformed but non-tumorigenic (23), while 12R1C-RK cells are fully transformed and tumorigenic in nude mice (24).

LLC-H61 cells (obtained from Dr. G. Vaes, Institute of Cellular and Molecular Pathology, Brussels, Belgium) are derived from a highly metastatic subclone of a C57B1/6 mouse Lewis lung carcinoma cell line (25).

NMuMG cells (obtained through Dr. F. Van Roy, Laboratory of Molecular Biology, Ghent, Belgium) are mouse epithelial cells derived from normal murine mammary gland (26).

3T3 cells (obtained from NIH, Bethesda, MD, USA) are immortalized non-malignant mouse cells (27).

MCF-7 cells (obtained from Dr. P. Briand, the Fibiger Institute, Copenhagen, Denmark) are obtained by pleural effusion from a human breast carcinoma (28); they are non-invasive in vitro (29).

MDCK cells (obtained from Dr. J. Leighton, Department of Pathology, Medical College of Pennsylvania, Philadelphia, USA) are Madin-Darby canine kidney cells; they are non-invasive in vitro (30) and produce non-invasive tumors in vivo (31).

HSU, 12R1C-RK, LLC-H61, NMuMG and 3T3 cells are maintained in Dulbecco's Modification of Eagle's Medium (DMEM, Flow Laboratories, Irvine, Scotland); MO_4 and MCF-7 cells are cultured in Eagle's modified Minimal Essential Medium with Earle's salts and non-essential amino acids (EMEM, Flow Laboratories). Both culture media are supplemented with 10% (v/v) fetal bovine serum, 0.05% (w/v) L-glutamine, 250 IU/ml penicillin and 100 µg/ml streptomycin (hereafter called culture medium).

Invasion assay

Cell aggregates with a diameter of 0.2 mm, monolayer fragments or cell clumps containing a comparable amount of cells are confronted with precultured embryonic chick heart fragments (PHF; diameter 0.4 mm) on top of a semi-solid agar medium (2). After attachment, the confronting pairs are transferred into an Erlenmeyer flask containing 2 ml of culture medium and further incubated on a Gyrotory shaker (New Brunswick, N.J., U.S.A.) at 120 rpm and 37°C; they are fixed after different times of incubation in Bouin-Hollande's solution, embedded in paraffin and completely sliced into 8 µm thick sections. Consecutive sections are stained with hematoxylin-eosin or with an antiserum against chick heart or against the confronting cells (32). Invasion of the confronting cells into the PHF is controlled by histological examination of the sections, and is scored as based on occupation and degeneration of the PHF : Grade I, when the confronting cells are found at the periphery of the outer fibroblastic cells of the PHF; Grade II when the confronting cells occupy the outer fibroblastic cell layers; Grades III and IV when the confronting cells occupy less or more than half the PHF. According to Mareel et al. (33), Grades III and IV meet the criteria of invasion. The specificity of this assay and its relevance for tumorigenicity have been discussed (34).

Isolation and analysis of cell surface glycopeptides

The technique to isolate cell surface glycopeptides has been described in detail (35). Briefly, cells in monolayer or PHF in suspension are metabolically labeled with either L-[1-^{3}H] fucose (1 µCi/ml; 60 Ci/mmol) or L-[1-^{14}C] fucose (0.5 µCi/ml; 60 mCi/mmol) for 20 h at 37°C (Radiochemicals are obtained from New England Nuclear, Boston, MA, USA or the Radiochemical Center, Amersham, Buckinghamshire, U.K.). Cell surface glycopeptides are isolated through mild trypsinization, exhaustively digested with pronase and dialysed against water. Gelfiltration is carried out on a Bio-Gel P-10 : Sephadex G50 (2:1, w/w) column (200 x 1 cm), eluted with 0.1 M Tris-HCl buffer (pH 8.0) containing sodium

dodecylsulfate (0.1%, w/v), EDTA (0.01%, w/v) and 2-mercapto-ethanol (0.1%, v/v). Removal of terminal sialic acid is performed by mild acid treatment (0.1 M H_2SO_4, 80°C, 90 minutes) or incubation with Vibrio Cholerae neuraminidase (0.1 unit/ml, 16 h; Behringwerke AG, Marburg, Federal Republic of Germany).

Drug treatment

ET-18-OCH_3 (kindly supplied by Dr. W. Berdel, Technical University, Munich, Federal Republic of Germany) is dissolved in the culture medium at 100 µg/ml, sterilized and stored at -20°C. This stock solution is diluted to the desired concentration at the onset of the experiment. The chemical structure of this compound has been described by Berdel et al. (6).

For the invasion assay in presence of ET-18-OCH_3, the drug is added to the semi-solid medium after attachment of the malignant cells to the PHF and also during incubation in the Erlenmeyer flask for 4 days. PHF or non-invasive cells are pretreated with ET-18-OCH_3 for 48 or 72 hr; they are extensively washed and further tested for invasion in absence of the drug.

For the isolation of glycopeptides, malignant cells, PHF or non-invasive cells are pretreated for 48 hr prior to fucose labeling in presence of ET-18-OCH_3. The concentrations used vary between 3 and 30 µg/ml ET-18-OCH_3.

RESULTS

Effect of ET-18-OCH3 on malignant cells

Invasion of MO_4 cells into the PHF is completely inhibited at 10 µg/ml ET-18-OCH_3; this concentration of the drug has no effect on directional migration and permit growth to at least 75% of controls (3). Confronting cultures between 12R1C-RK and PHF show complete inhibition of invasion at 3 µg/ml, whereas the LLC-H61 cells are still invasive in presence of 30 µg/ml ET-18-OCH_3 (4).

Gelfiltration of the N-linked surface glycopeptides obtained from these three untreated cell lines demonstrates an elution profile corresponding with the "malignant" phenotype. ET-18-OCH_3 treatment during 48h at the lowest anti-invasive concentration results in a shift towards higher apparent molecular weights for MO_4 cells while this is not obvious for 12R1C-RK or LLC-H61 cells (4).

Pretreatment of PHF with ET-18-OCH_3

Pretreatment of the host tissue (PHF) with 10 µg/ml ET-18-OCH_3 for 48h is sufficient to inhibit the invasion of MO_4 cells (20). This invasion resistance persists after washing out the drug and further incubation of the ET-18-OCH_3 pretreated PHF on a Gyrotory shaker for 4 or 7 days : confrontations of these PHF with MO_4 cells shows complete absence of invasion, whereas control cultures invariably demonstrate grade III or IV of invasion (see table I). Explantation on tissue culture plastic substrate of such confronting pairs (PHF_{ALP} + 4 d/MO_4) after 4 days of incubation shows migration and proliferation of both heart and MO_4 cells, indicating that absence of invasion is not due to cytotoxicity. The gelfiltration profiles of fucosylated surface glycopeptides from control versus ET-18-OCH_3 treated PHF shift from the "normal" to the "malignant" phenotype. This effect is abolished after desialylation, indicating that ET-18-OCH_3 enhances the degree of sialylation and not the branching or elongation of the N-linked oligo-saccharides. The induced "malignant" changes in ET-18-OCH_3 pretreated PHF remain unchanged after washing and further incubation in absence of the drug for another 64 hr (20).

Pretreatment of non-invasive cells with ET-18-OCH_3

Previous experiments demonstrate that ET-18-OCH_3 induces the invasive phenotype in HSU and 3T3 cells (18,19). We report here on the extension and the reversibility of this kind of experiments. Cells are seeded and cultured in presence of 10 µg/ml ET-18-OCH_3 for 3 days. The growth of these cells is only minimally

Table 1 : Analysis of invasion : reversibility of ET-18-OCH_3 pretreatment of the host tissue (PHF).

Confrontation culture	Grading[a] of invasion after 4 days			
	I	II	III	IV
Reversibility[b]				
$PHF_{ALP + 4\ d}/MO_4$	0	4[d]	0	0
$PHF_{ALP + 7\ d}/MO_4$	0	4	0	0
Controls[c]				
$PHF_{+ 4\ d}/MO_4$	0	0	3	1
$PHF_{+ 7\ d}/MO_4$	0	0	0	4

[a] as described in Material and Methods : Grades III and IV meet the criteria of invasion.

[b] $PHF_{ALP + 4\ d\ or\ +\ 7\ d}$ are PHF pretreated with 10 µg/ml ET-18-OCH_3 for 48 h, extensively washed and further incubated on a Gyrotory shaker in absence of the drug for 4 or 7 days prior to confrontation with untreated MO_4 cells.

[c] $PHF_{+ 4d\ or\ +\ 7\ d}$ are untreated PHF, washed and further incubated in the same way as under **b.**

[d] number of cultures examined histologically.

affected by the drug treatment as demonstrated by a slightly lower number of cells in these cultures after three days as compared to control cultures. Treated and control cells are washed 3 x with culture medium and associated as a monolayer fragment with PHF. Pretreatment with ET-18-OCH_3 of HSU and MDCK cells results in invasion of those cells into the PHF (fig. 1), but has no effect in NMuMG and MCF-7 cells. Washing of the pretreated HSU cells and further incubation in absence of the drug for another 4 days reverses the ET-18-OCH_3 mediated induction of invasion (table 2).

Glycopeptide profiles of HSU and 3T3 cells treated with ET-18-OCH_3 shift to apparently higher molecular weights when compared with those of untreated cells. These "malignant" changes of surface glycopeptides are abolished after removal of sialic acid and strongly decreased 64h after washing out the drug before labeling with fucose (18).

DISCUSSION

Membrane phospholipds are found to play an important role in the transduction of biological signal molecules (36) such as hormones, neurotransmitters and growth factors. They affect also activities of sialyltransferase (37) and fucosyltransferase (38) which are key enzymes in the "malignant" changes of cell-surface glycosylation. Increased incorporation of fucose into MO_4 cells at reduced temperature (39) and malignant changes of N-glycosylation related to increased sialylation (18,19) are found to coincide with the acquisition of invasiveness.

The present experiments show a complete correlation between modulation of invasion and alterations of cell surface glycoproteins. i) ET-18-OCH_3 pretreatment of the PHF is found to induce "malignant" alterations in the cell surface carbohydrates and invasion resistance when these PHF are confronted with constitutively invasive MO_4 cells; ii) This effect is irreversible as found on the invasion and the glycosylation pattern; iii) The non-invasive HSU or 3T3 cells

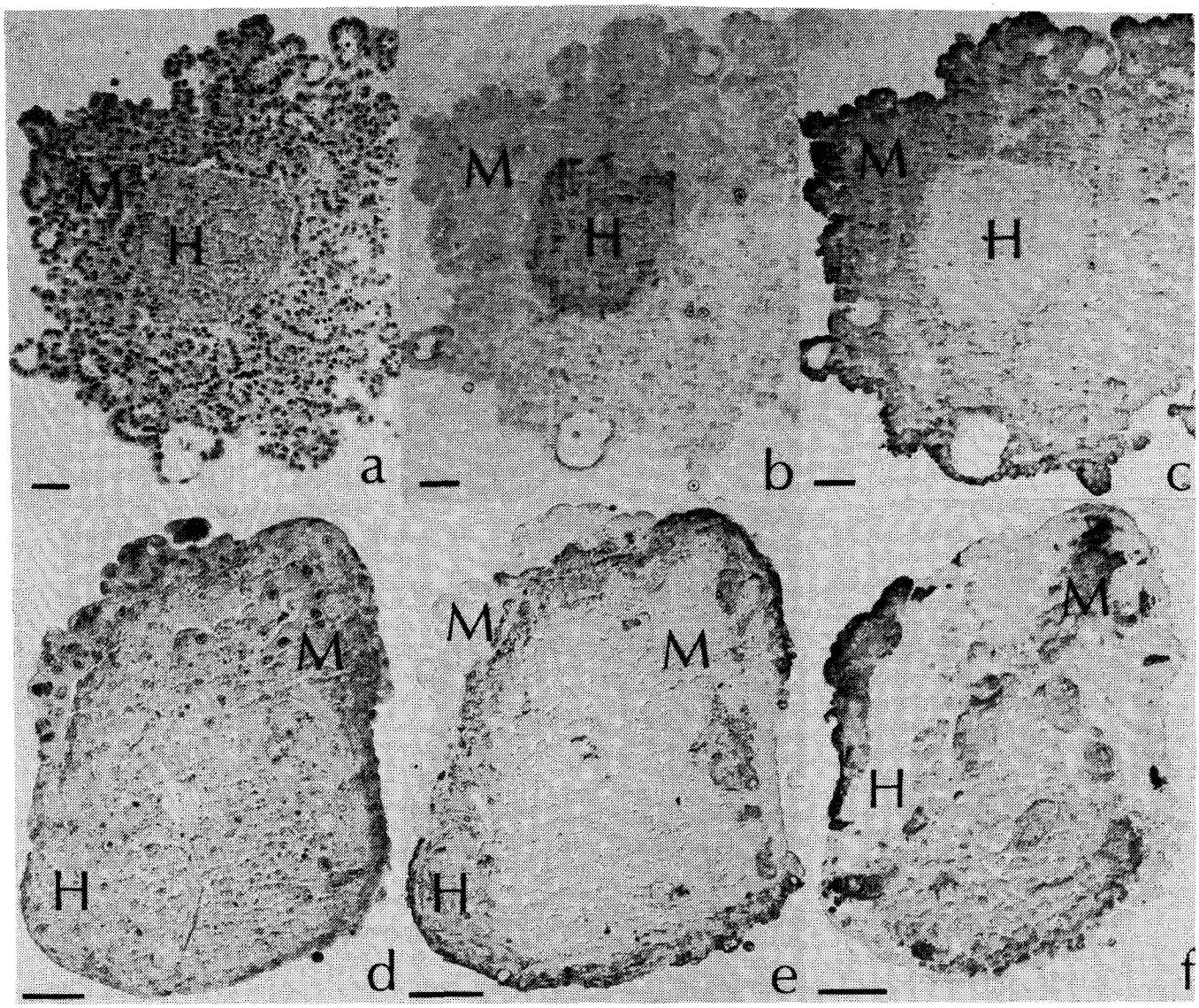

Fig. 1 : Photomicrographs of consecutive sections from confronting cultures between embryonic chick heart (H) and untreated MDCK cells (M) (1a, 1b and 1c) or MDCK cells pretreated with 10 µg/ml ET-18-OCH_3 for 72 h (1d, 1e and 1f). Fixation after 4 days; staining with haematoxylin and eosin (1a and 1d), with an antiserum against chick heart (1b and 1e) or with an antiserum against MDCK cells (1c and 1f). Scale bars = 50 µm.

Table 2. Analysis of invasion : ET-18-OCH_3 pretreatment of non-invasive cells and reversibility

Confrontation culture	Grading[a] of invasion after 4 days I	II	III	IV
Controls[b]				
PHF/MDCK	1[e]	4	0	0
PHF/NMuMG	0	4	0	0
PHF/MCF-7	0	5	0	0
PHF/HSU	0	4	0	0
Pretreatment[c]				
$PHF/MDCK_{ALP}$	0	0	5	0
$PHF/NMuMG_{ALP}$	0	4	0	0
$PHF/MCF\text{-}7_{ALP}$	0	5	0	0
PHF/HSU_{ALP}	0	1	4	0
Reversibility[d]				
PHF/HSU_{ALP} + 4 d	0	5	0	0

a as described in Material and Methods : Grades III and IV meet the criteria of invasion.
b neither the host (PHF) nor the confronting cells are treated.
c the confronting cells are pretreated with 10 µg/ml ET-18-OCH_3 for 72 h, washed extensively and then confronted with untreated PHF.
d the HSU cells are pretreated with ET-18-OCH_3 as under **c**, washed extensively, further incubated on a Gyrotory shaker in absence of the drug for 4 days and confronted with untreated PHF.
e number of cultures examined histologically.

acquire the invasive phenotype after ET-18-OCH_3 pretreatment, concomitantly expressing cancer-related changes of the cell surface glycans; iv) The ET-18-OCH_3 effect on invasion and glycosylation of HSU cells is reversible after 4 days of incubation in absence of the drug.

In contrast, the invasion experiments between malignant cells and PHF in presence of ET-18-OCH_3 can hardly be explained by changes in the cell surface glycosylation alone : i) The anti-invasive ranking of ET-18-OCH_3 treatment : 12R1C-RK > MO_4 >> LLC-H61 is not reflected in the ET-18-OCH_3 induced changes of N-linked glycopeptide profiles of the three malignant cell lines; ii) ET-18-OCH_3 induces also the "malignant" carbohydrate changes in the PHF. Cochromatography should demonstrate that the cell surface glycopeptides from ET-18-OCH_3 treated MO_4 cells have an apparently higher molecular weight than those of ET-18-OCH_3 treated PHF; these differences should be less obvious between the glycopeptides of treated 12R1C-RK or LLC-H61 cells and treated PHF. Therefore, it is unlikely that the relative alterations of glycosylation or sialylation between treated PHF and treated malignant cells can be responsible for the ET-18-OCH_3 effect on invasion.

Finally, we still have to investigate the alterations of N-linked glycosylation and its relationship with the induction of invasiveness on MDCK, NMuMG and MCF-7 cells.

We conclude that inhibition of invasion through alterations of cell surface glycoconjugates may be an alternative mechanism for the anti-tumor effect of ET-18-OCH_3.

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Mémoire 3

ALTERED GLYCOSYLATION IN MADIN-DARBY CANINE KIDNEY (MDCK) CELLS AFTER TRANSFORMATION BY MURINE SARCOMA VIRUS

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Running title : Glycosylation and progression towards malignancy.

ABSTRACT

The changes in glycosylation of an epithelial cell line (MDCK) before and after progression towards a more malignant phenotype have been studied.

The parental MDCK-3 cells were immortalized after long-term passage in vitro and have shown no tendency for spontaneous acquisition of malignancy-related phenotypes such as tumorigenicity, invasion and metastasis. They conserved morphological and functional characteristics of the epithelial tissue of origin. The ras-MDCK cells acquired the fully malignant phenotype after transformation with a Harvey murine sarcoma virus; they were invasive in vitro and produced invasive and also metastatic tumors after subcutaneous injection into nude mice.

Using immobilized lectins and gel chromatography, before and after liberation of O-linked glycans from the peptide moieties and also after removal of terminal sialic acid, we have found differences in the glycopeptides of both whole cells and cell surface trypsinates from ras-MDCK cultures as compared to the parental MDCK-3 cultures : i) more sialic acid in the N-linked tri- and tetra-antennary structures ; ii) more fucosylation in the N-linked glycopeptides; iii) more bi-antennary N-linked glycopeptides and less O-linked glycans; and iv) a lower molecular weight of the O-linked glycans probably due to a decreased sialylation.

It is concluded that alterations in sialylation and fucosylation of the cell surface exposed glycans accompanied progression of MDCK-3 cells towards a more malignant phenotype.

INTRODUCTION

Alterations of glycans in cell surface exposed N-glycosylpeptides have been correlated with the expression of malignancy-related phenotypes in a range of experimental systems (29). The strategy of such experiments has been to compare cell populations with differences in malignancy-related phenotypes : non-tumorigenic versus tumorigenic (10,13,32); non-invasive versus invasive (4,5); and non-metastatic versus metastatic (9,15,28).

Alterations of the glycans have been mostly found in the terminal sugar residues : sialic acid and fucose. NIH 3T3 cells transfected with DNA from human bladder carcinoma, colon carcinoma or HL60 promyelocytic leukemia cells were tumorigenic in nude mice and showed higher-branched, sialic acid containing glycopeptides (10). Human uroepithelial cells that were tumorigenic in nude mice possessed more highly branched, tri- and tetra-antennary N-acetyllactosaminic glycans than their non-tumorigenic counterparts (13).

Glycopeptides, isolated from the surface of non-invasive cells, eluted behind of those from invasive cells on a gel chromatography column, and this was ascribed to an increased sialylation of the glycopeptides of the latter cells. The invasive phenotype was acquired after mutagenesis, oncogene transfection or epigenetic induction with 1-O-octadecyl-2-O-methylglycero-3-phosphocholine (4,5,20). Decreased fucose incorporation in cell surface carbohydrates was associated with inhibition of invasion of MO_4 cells at reduced temperature and in gap junction defective L929 cells (3,6). Sialylation of particular cell surface carbohydrates was also correlated with the metastatic phenotype of a range of murine tumor cells as reviewed by Schirrmacher et al. (29), of T-cell hybridomas (9) and of mutants from the high-metastatic mouse tumor, MDAY-D_2 (15). Marked inhibition of ^{3}H-fucose incorporation was found in murine tumor cells showing a decrease in metastatic capacity (14,31). By contrast, differences in the metastatic potential of B16 melanoma cell variants did not correlate with differences in total cell surface sialic acid (28).

We have compared the glycopeptides of whole cells and cell surface trypsinates of an epithelial cell line (MDCK) before and after progression towards a more malignant phenotype. The parental MDCK cells conserved morphological and functional characteristics of the renal epithelial tissue of origin; they were immortalized after long-term passage in vitro and have shown little or no tendency for spontaneous acquisition of more malignant phenotypes. After transformation with a Harvey murine sarcoma virus, MDCK cells acquired a more malignant phenotype, since they were tumorigenic, invasive and metastatic in nude mice.

This pair of cell lines provided us with the opportunity to examine whether alterations of glycans were related to early or late steps of malignant progression.

MATERIALS AND METHODS

MDCK cells (obtained from J. Leighton, Department of Pathology, Medical College of Pennsylvania, PA) are Madin-Darby canine kidney cells, coined MDCK-3 to distinguish them from other MDCK variants (2). MDCK-3 cells have repeatedly been shown to be non-invasive in vitro (2,30); they were not tumorigenic after s.c. injection in nude mice (our unpublished results in collaboration with F. Van Roy, Laboratory of Molecular Biology, State University of Ghent, Belgium).

The ras-MDCK cell line (obtained from M.C. Lin, Bethesda, MD through W. Birchmeier, Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Tübingen, FRG) are MDCK cells transformed with Harvey murine sarcoma virus (11); they are invasive in vitro (2) and produced invasive and also metastatic tumors after s.c. injection into nude mice (unpublished results in collaboration with F. Van Roy).

Samples from both cell lines were expanded to obtain three sets of subconfluent monolayers (4 x 150 cm^2/set) on tissue culture plastic substrate (Flow, Irvine, Scotland, cat n° 61-450 B5) at 37°C with Dulbecco's modification of Eagle's medium (DMEM, Flow, cat n° 12-332-54) supplemented with 10% (v/v) fetal bovine serum (Flow, cat n° 29-101-54), 0.05% (w/v) L-glutamine, 250 IU/ml penicillin and 100 µg/ml streptomycin.

Labeling and isolation of glycopeptides

a) Labeling

Cells were labeled metabolically for 48 h with either D-[6-^{3}H]-glucosamine hydrochloride (5 µCi/ml, 30 Ci/mmol) or D-[1-^{14}C]-glucosamine hydrochloride (1 µCi/ml, 54.2 mCi/mmol) (NEN, Boston, MA) and harvested at subconfluent densities.

b) Isolation of the total cell glycopeptides

After extensively washing with phosphate-buffered saline (PBS), the cells were removed from the culture flasks with a policeman and centrifuged in PBS. The cell pellet was fixed with methanol prior to delipidation (27) and exhaustive pronase (grade B, Calbiochem, La Jolla, CA) digestion for 72 h (26) to remove polypeptides. For both cell types, two of the three sets of cultures were used for the analysis of whole cell glycopeptides.

c) Isolation of the cell surface and the cell pellet glycopeptides

In the third set of cultures, cell surface glycopeptides of both cell types were released by trypsin-EDTA (10 min at 37°C; Gibco Europe, cat. n° 043-05300H, Ghent, Belgium) after washing the cell monolayers with Ca^{2+} and Mg^{2+} free salt solution. Trypsinized cells were centrifuged at 150 g for 5 min, fixed with methanol and delipidated. Both the trypsinate and the cell pellet were incubated separately with pronase in order to obtain the glycopeptides.

d) Precipitation of proteoglycans and desalting of glycopeptides

Aliquots of glycopeptides from [^{3}H]-labeled MDCK-3 and from [^{14}C]-labeled ras-MDCK cells, containing equal amounts of radioactivity were mixed. Proteoglycans and nucleic acids were precipitated by cetylpyridiniumchloride (CPC, 0.1 M in 0.1 M Na_2SO_4) in the presence of 0.4 M NaCl (21). After centrifugation and three washings of the CPC-precipitable material, pooled supernatants were concentrated in a rotatory evaporator in vacuo and desalted on a Bio-Gel P_2 column (60 x 1.8 cm; 200-400 mesh; Bio-Rad, Vitry-sur-Seine, France) prior to glycopeptide analysis.

Glycopeptide analysis

a) Affinity chromatography

Affinity chromatography of the glycopeptide mixtures from MDCK-3 and ras-MDCK cells was performed on Concanavalin A (Con A)-Sepharose and on Lens culinaris agglutinin (LCA)-Sepharose at room temperature, in accordance with

Debray et al. (13). Briefly, the desalted glycopeptide mixtures were dissolved in 1 ml buffer (5 mM sodium acetate, 0.1 M NaCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.0 mM $MnCl_2$ and 0.02% NaN_3; adjusted to pH 5.2 with acetic acid) and fractionated on a Con A-Sepharose column (11 x 1.2 cm; Pharmacia, Bois d'Arcy, France, cat. n° 17-0440-01). The eluate was collected in 1.5 ml portions and contained three main fractions with corresponding primary structures of N-linked glycopeptides (24,25) : i) a fraction not retained on the column that was eluted with the Con A-buffer (FNRC), which may consist of tri-, tetra-, bisected biantennary as well as poly-N-acetyl-lactosaminic type structures or O-glycopeptides; ii) a weakly retained fraction that was eluted with Con A-buffer containing 10 mM α-methyl-D-glucopyranoside (FEC_{10mM}), which may consist of bi-antennary and/or immature bisected bi-antennary structures; and iii) : a strongly retained fraction that was eluted with buffer containing 0.5M α-methyl-D-glucopyranoside ($FEC_{0.5M}$), which may consist of oligomannosidic type and/or immature glycopeptides of the N-acetyllactosamine type. A 50 µl sample of each 1.5 ml portion was mixed with 4.0 ml Aqualite liquid scintillation cocktail (J.T. Baker Chemicals, Deventer, The Netherlands) and counted in a Beckman LS-9000 scintillation counter (Beckman Instruments Inc., Fullerton, CA). The radioactivity of the three fractions was calculated as the percentage of the total radioactivity. All portions of each fraction were pooled, concentrated in vacuo and desalted on a Bio-Gel P_2 column (60 x 1.8 cm) equilibrated with deionized water. The desalted Con A-fractions were concentrated in vacuo again, dissolved in 1 ml PBS buffer (pH 7.2) and applied on an LCA-Sepharose column (10 x 1.2 cm; prepared as described by Debray et al. (12) to determine the percentage of α-1,6-fucosylated N-glycosylpeptides interacting with the immobilized lectin (24,25). PBS (5x the column volume) was used as the eluate at a flow rate of 9 ml/h. Portions of 1.5 ml were collected and the retained fractions were eluted with PBS containing 0.15M α-methyl-D-glucopyranoside. As described above for Con A-Sepharose, the radioactivity of each

fraction separated on the LCA-Sepharose column was expressed as the percentage of the original total radioactivity, using counting values of a 50 µl sample of the individual eluate portions.

b) Gel chromatography

The fraction not retained on Con A and not retained on LCA-Sepharose (FNRC/NRL) was chosen for further analysis, because this fraction showed the highest level of radioactivity, and still might contain O-linked glycans and/or poly-N-acetyl-lactosaminic-type structures (24,25). Gel chromatography of the FNRC/NRL was carried out on an Ultrogel ACA 202 column (100 x 1.6 cm; IBF, Villeneuve-la-Garenne, France, cat. n° 48911) at a flow rate of 9 ml/h. Fractions of 1.5 ml were collected and the radioactivity of aliquots was counted as above described. Those fractions containing radioactive label were desalted on Bio-Gel P_2, concentrated in vacuo, treated and finally redissolved in 1 ml elution buffer (0.05 M Tris-HCl, 0.17 M NaCl, 0.02% NaN_3 adjusted to pH 7.4.

In order to liberate the O-linked glycans from remnant peptides, a ß-elimination reaction was used according to Carlson (8). The FNRC/NRL was warmed at 45°C during 16 h in 1 ml NaOH 0.05 M containing 1 M $NaBH_4$. The alkali was neutralized on a 10 ml Dowex-H^+ column (50 W - X_8 : 20-25 mesh; cat. n° 745-6421, Bio-Rad, Richmond, CA). The radiolabeled N-glycosylpeptides and the O-linked glycans were eluted with deionized water (10x the column volume). Traces of borate were evaporated in vacuo after addition of 3 x 10 ml methanol.

Sialic acid was removed by mild acid hydrolisis at 80°C during 60 minutes in 0.05 N trifluoroacetic acid (Janssen Chimica, Beerse, Belgium; cat. n° 13.972.04).

In one experiment the FNRC/NRL of whole cell glycopeptides were subjected to an endo-ß-galactosidase treatment to reveal differences in poly-N-acetyl-lactosaminic type structures. A desalted sample of FNRC/NRL after ß-elimination was dissolved in 500 µl buffer (50 mM sodiumacetate plus 0.02%

NaN_3; pH 5.8) containing 25 mU of endo-β-galactosidase (origin : Bacteroides fragilis lyophilisate; Boehringer, Mannheim, FRG; cat. n° 982954). After 24 h of incubation at 37°C, the mixture was heated for 3 minutes at 100°C and gel chromatographed on Ultrogel ACA 202, as described earlier. The glycans eluting just before phenol red were pooled and chromatographed on Whatmann Chr 3 paper (46 x 57 cm, Whatmann, Maidstone, U.K.; cat. n° 3003917) in ethyl acetate:pyridine:acetic acid:H_2O (5:5:1:3) overnight at room temperature. The paper chromatograms were cut into 1 cm bands. These bands were extracted with 0.5 ml H_2O and the extracts were mixed with 4 ml of Aqualite scintillation cocktail for radioactivity counting; Gal(β1-4)GlcNAc was applied in a separate lane and used as a reference when revealed with aniline oxalate.

RESULTS

a) Affinity chromatography

The results of the affinity chromatography on Con A-Sepharose of the glycopeptides from whole cells, from cell surface trypsinates and from cell pellets are summarized in Table 1. The FNRC, which may contain tri-, tetra-, bisected biantennary structures as well as poly-N-acetyl-lactosaminic type structures or O-glycosylpeptides, was larger in MDCK-3 cells than in ras-MDCK cells, in whole cells, as well as in cell surface trypsinates and cell pellets separately. The FEC_{10mM}, containing essentially bi-antennary structures, was increased in ras-MDCK cells as compared to MDCK-3 cells. The $FEC_{0.5M}$, containing essentially oligomannosidic structures, was lower in cell surface trypsinates than in whole cells and cell pellets.

Fractionation of the different Con A-fractions on LCA-Sepharose are given in Table 2. The sum of the total radioactivity retained on LCA was higher for the ras-MDCK cells than for the MDCK-3 cells. This indicates a higher amount of α-1,6-fucosylated N-glycosylpeptides interacting with the immobilized lectin (24,25) in the ras-MDCK cells.

Table 1 - Fractionation of glycopeptides on Con A-Sepharose : % radioactivity

Fraction	Cell type							
	MDCK-3				ras-MDCK			
	Whole Cells		Cell Surface Trypsinate	Cell Pellet	Whole Cells		Cell Surface Trypsinate	Cell Pellet
FNRC	87.9	84.7	96.4	87.1	75.9	74.9	86.2	77.2
FEC_{10mM}	7.1	3.2	3.0	4.0	15.1	10.4	11.1	12.1
$FEC_{0.5M}$	4.9	12.1	0.6	8.9	9.0	14.6	2.7	10.6

Glycopeptides labeled with D-[6-^{3}H] glucosamine (MDCK-3) or D-[1-^{14}C] glucosamine (ras-MDCK) were applied at equal amounts of radioactivity on a Con A-Sepharose column (11 x 1.2 cm) and eluted with acetate buffer. The numbers represent the percentage of radioactivity found in the 3 fractions : FNRC, fraction not-retained on Con A-Sepharose; FEC_{10mM}, fraction eluted in buffer containing 10 mM α-methyl-D-glucopyranoside; and $FEC_{0.5M}$, fraction eluted with buffer containing 0.5 M α-methyl-D-glucopyranoside. The numbers in the first two columns of each cell type are from experiments in duplo.

Table 2 - Fractionation of glycopeptides from Con A-Sepharose column on LCA-Sepharose : % radioactivity

Fraction	Cell type							
	MDCK-3				ras-MDCK			
	Whole Cells		Cell Surface Trypsinate	Cell Pellet	Whole Cells		Cell Surface Trypsinate	Cell Pellet
FNRC/NRL	85.9	82.1	94.0	84.9	71.0	68.8	80.9	70.6
FNRC/RL	2.1	2.5	2.3	2.2	4.9	6.1	5.2	6.6
$FEC_{10mM/NRL}$	5.0	1.9	1.3	1.9	7.0	4.0	3.5	6.1
$FEC_{10mM/RL}$	2.1	1.4	1.7	2.1	8.1	6.5	7.7	6.1
$FEC_{0.5M/NRL}$	4.9	11.8	0.7	8.8	8.4	11.9	2.0	7.4
$FEC_{0.5M/RL}$	0.0	0.3	0.0	0.1	0.6	2.7	0.7	3.2
Total RL	4.2	4.2	4.0	4.4	13.6	15.3	13.6	15.9

Numbers are percentages of the original total radioactivity found in each group : FNRC (NRL or RL), fraction not retained on Con A-Sepharose and not retained (NRL) or retained (RL) on LCA-Sepharose; FEC_{10mM} (NRL or RL), fraction eluted with buffer containing 10 mM α-methyl-D-glucopyranoside on Con A-Sepharose and not-retained (NRL) or retained (RL) on LCA-Sepharose; FEC 0.5M/NRL or RL : fraction eluted with buffer containing 0.5M α-methylglucopyranoside on Con A-Sepharose and not retained (NRL) or retained (RL) on LCA-Sepharose. The numbers in the first two columns of each cell type are from experiments in duplo.

b) Gel chromatography

Gel chromatography on Ultrogel ACA 202 of the glycopeptide fraction FNRC/NRL from whole cells before and after β-elimination is shown in fig. 1a and 1b. Comparison between fig. 1a and 1b indicated an N-glycosylpeptide region (fraction 35-65) and an O-linked glycan region (fraction 66-100).

Radioactivity in the N-glycosylpeptide region, consisting of tri-, tetra- and bisected biantennary structures, was not different for both cell types : 51,3% for ras-MDCK cells versus 49,2% for MDCK-3 cells. The N-glycosylpeptides of ras-MDCK cells have an apparently higher molecular weight than those from MDCK-3 cells (Fig. 1b). This was largely due to an increased sialylation (Fig. 1b versus 1c).

The amount of O-linked glycans (fractions 66-100) was lower in ras-MDCK cells (17.5%) than in MDCK-3 cells (33.0%). The major peak of O-linked glycans from the ras-MDCK cells appeared in a lower molecular weight fraction (Fig. 1b) and they were less sialylated when compared with those of the MDCK-3 cells (Fig. 1c). As a reference, the tri- and tetrasaccharides O-glycosidically linked to fetuin and also liberated by a β-elimination reaction, were eluted in the fractions 91-100 (data not shown) under experimental conditions comparable with those of figures 1 and 3.

There was no increase of poly-N-acetyl-lactosaminic type structures in the FNRC/NRL of the whole cell glycopeptides as could be concluded from the total radioactivity released by endo-β-galactosidase (5.4% from ras-MDCK cells versus 4.9% from MDCK-3 cells) and from paper chromatograms of these released glycans; their composition did hardly differ between ras-MDCK and MDCK-3 cells (fig. 2).

Gel chromatography of the glycopeptide fraction FNRC/NRL released from the cell surface by trypsinisation (fig. 3) demonstrated profiles before and after β-elimination, and after removal of terminal sialic acid, that were similar to those found for the whole cell glycopeptides (Fig. 1). For ras-MDCK cells a

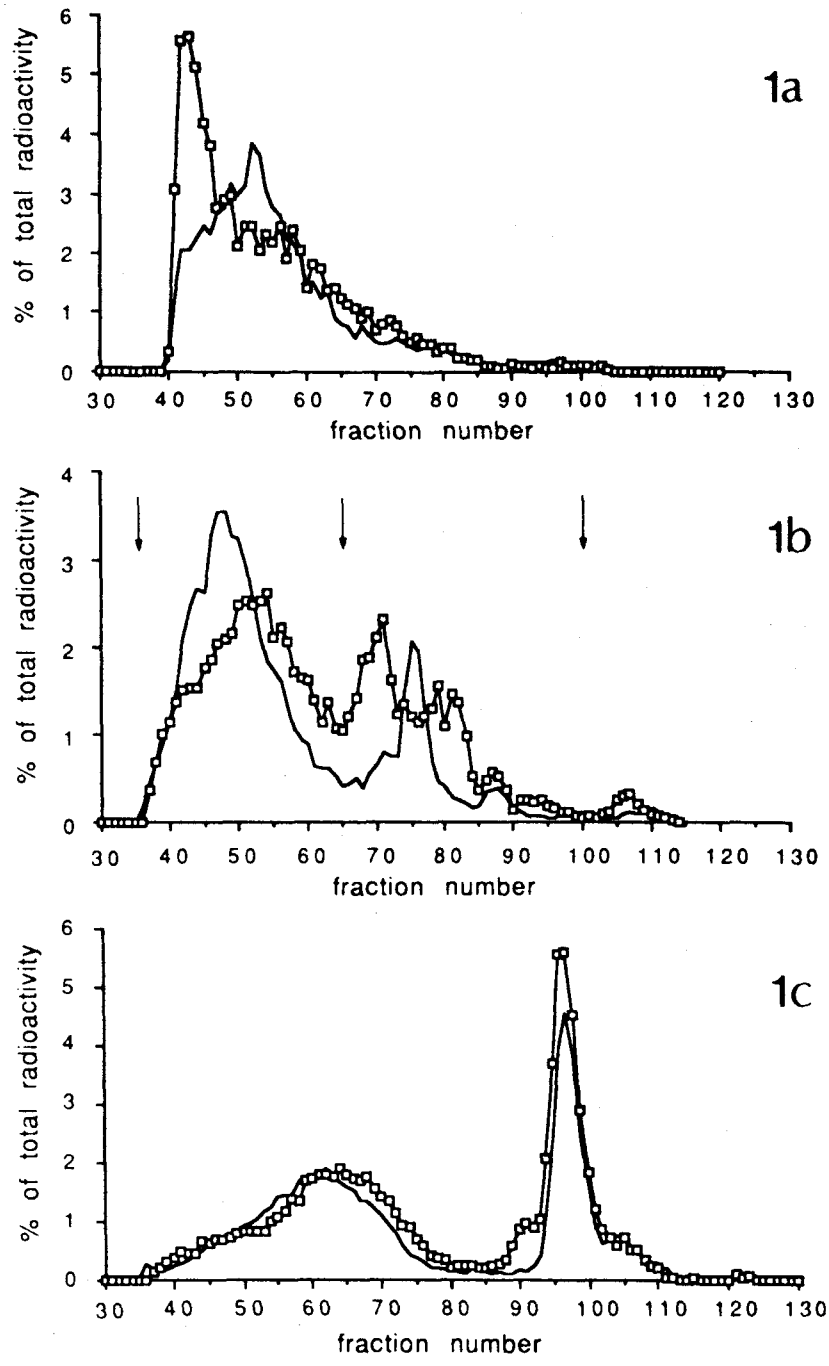

Figure 1 : Gel chromatography profile of the fraction of whole cell glycosylpeptides not-retained on Con A-Sepharose and not retained on LCA-Sepharose. The total cell glycosylpeptides of MDCK-3 cells (□—□: ^{3}H-glucosamine labeled) and of ras-MDCK cells (——— : ^{14}C-glucosamine labeled) were cochromatographed on Ultrogel ACA 202 : i) after pronase digestion (Fig. 1a); ii) after liberation of the O-linked glycans by a β-elimination reaction (Fig. 1b) and iii) after additional removal of terminal sialic acid (Fig. 1c). Arrows indicate a N-linked glycosylpeptide region (Fig. 1b, fractions 35-65) and an O-linked glycan region (Fig. 1b, fractions 66-100). Ordinate : percentage of total the original total radioactivity. Abscissa : fraction number.

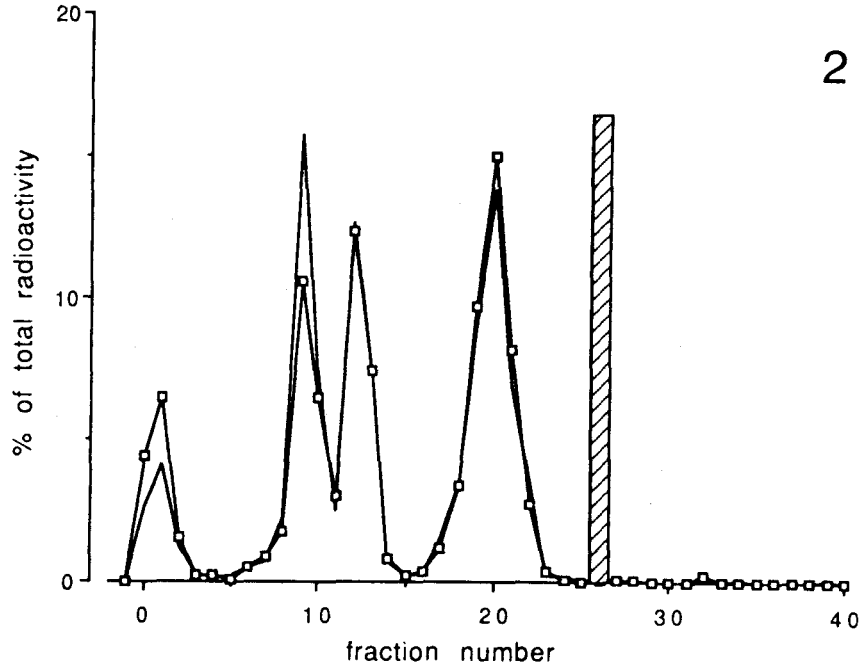

Figure 2 : Distribution profile of the endo-β-galactosidase released glycans from the total cell glycosylpeptides of MDCK-3 cells (□—□) and ras-MDCK (———) cochromatographed on Whatmann Chr3 in ethyl acetate:pyridine:acetic acid:H_2O (5:5:1:3); Gal(β1-4)GlcNAc (▨) migrated in a separate lane as a reference. Ordinate : percentage of total radioactivity applied on the paper. Abscissa : fraction number, representing 1 cm broad paper bands of the chromatogram and starting fraction O 10 cm beneath the site of application.

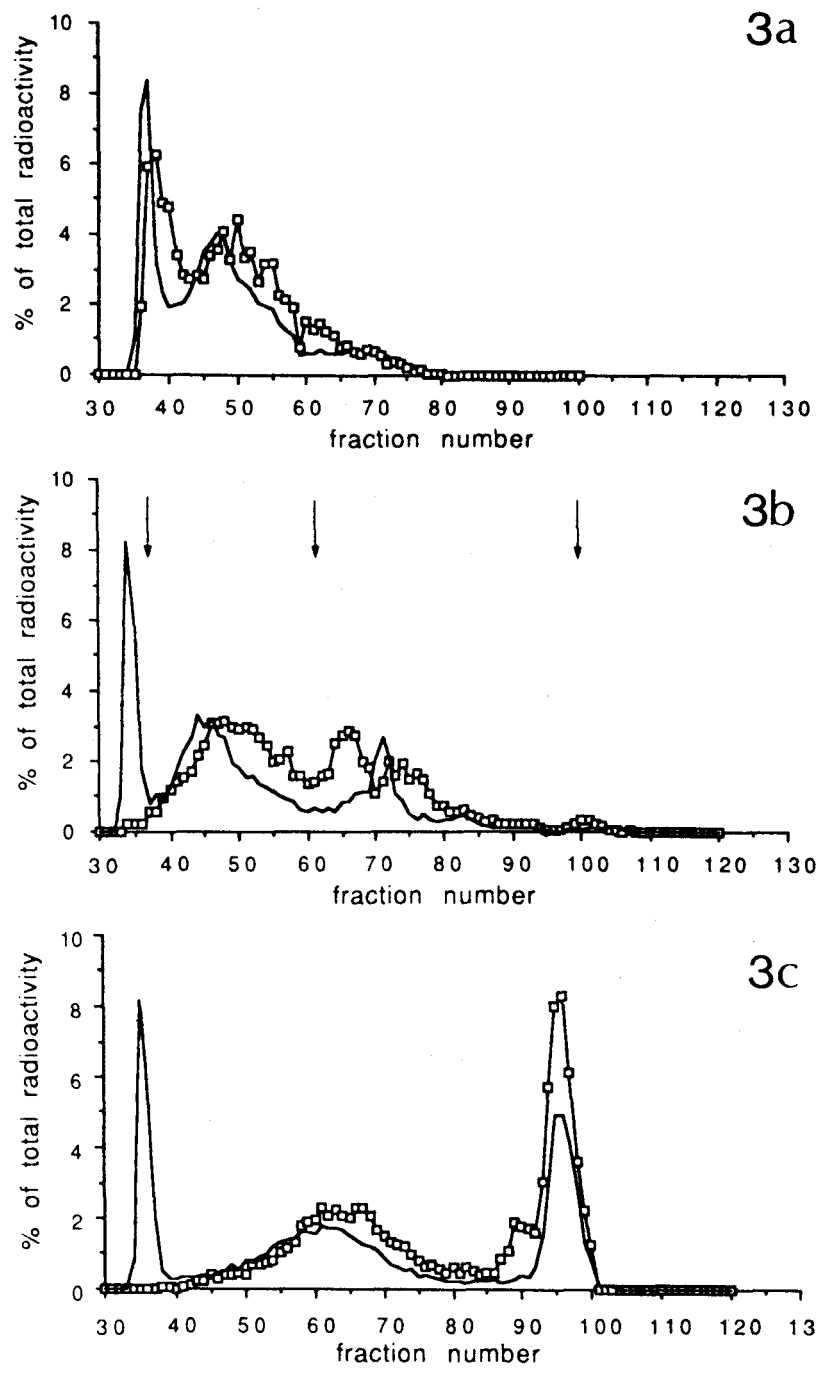

Figure 3 : Gel chromatography profile of the trypsinised cell surface fraction not retained on Con A-Sepharose and not retained on LCA-Sepharose. The cell surface glycosylpeptides of MDCK-3 cells (□—□: ^{3}H-glucosamine labeled) and ras-MDCK cells (———: ^{14}C-glucosamine labeled) were cochromatographed on Ultrogel ACA 202 : i) after pronase digestion (Fig. 3a); ii) after liberation of the O-linked glycans by a β-elimination reaction (Fig. 3b) and iii) after additional removal of terminal sialic acid (Fig. 3c). Ordinate : percentage of the original total radioactivity. Arrows indicate an N-linked glycosylpeptide region (Fig. 3b, fractions 37-62) and an O-linked glycan region (Fig. 3b, fractions 63-100). Abscissa : fraction number.

fraction eluting in the void volume was observed, and this might represent hyaluronic acid, not precipitated by CPC during the preparation of the glycopeptides.

DISCUSSION

Using immobilized lectins and gel chromatography, we have found the following differences in the glycopeptides of ras-MDCK cells as compared to MDCK-3 cells : i) a higher sialic acid content in the N-linked tri- and tetra-antennary structures from the whole cell and from the cell surface glycopeptides (Figs. 1 and 3); ii) a three times higher degree of fucosylation in the N-glycosylpeptides interacting with the immobilized lectin (Table 2); iii) a higher content of bi-antennary N-glycosylpeptides and a lower content of O-linked glycans (Table 1; Fig. 1b and 3b, fractions 65-100); and iv) a lower molecular weight of the O-linked glycans probably due to a decreased sialylation (Figs. 1 and 3, fractions 66-100).

Which alterations in glycan structures would correlate with the more malignant phenotypes ?

Transformation of MDCK-3 cells, to obtain ras-MDCK cells, marks a transition from an immortalized, non-tumorigenic phenotype towards a tumorigenic, invasive and metastatic phenotype. We should, therefore, infer that altered glycans contributed to the acquisition of growth at the site of injection, invasiveness, and growth in distant organs.

Previous experiments in vitro demonstrated that a transition from the non-invasive towards the invasive phenotype, without alteration of growth, was accompanied by an increased sialylation and fucosylation of the N-glycosylpeptides. Temporary induction of the invasive phenotype in constitutively non-invasive baby rat kidney cells with an alkyllysophospholipid correlated with a temporary increase in sialylation of N-glycosylpeptides. Both phenomena were reversible within the same period of time. Pretreatment of precultured embryonic chick heart fragments with the same alkyllysophospholipid resulted in resistance of the heart tissue

to invasion by MO_4 cells, and this was also accompanied by an increase of terminal sialic acid moieties of the N-glycosylpeptides (4,5,7). The role of fucose in invasion was demonstrated by autoradiography in confronting cultures : resumption of MO_4 cell invasion after 10 days of incubation at 28°C was correlated with local recovery of fucose incorporation in the invading MO_4 cells (6). Similarly, fucosyltransferase-deficient L929 cells failed to invade embryonic chick heart fragments in contrast with the corrected L929 X GRSL cells (3).

Fucosylation has also been implicated in the activities of metastatic cells. Adhesion of Lewis lung carcinoma cells to normal lung cells was ascribed to the interaction of an α-fucose-specific lectin of the lung cells with another lectin of the malignant cells. The number of lung colonies was significantly reduced when these carcinoma cells were intravenously injected together with neoglycoproteins bearing α-L-fucose (23).

That a possible role for fucosylation in the expression of malignancy-related phenotypes might not be restricted to experimental systems is illustrated by a number of observations on human tumors. In sera from patients suffering from growing tumors and metastases as compared to healthy individuals, a higher level of fucosylation was found for haptoglobins (35), α-fetoprotein (1) and α-1-antitrypsin (36). The expression of difucosylated carbohydrate antigens marked the transition between hyperplasia, on the one hand, and atypic adenoma and colorectal tumors, on the other hand (16), and novel fucolipids were found to accumulate in human colon adenocarcinoma cells (17).

It is conceivable that an altered sialylation combined with an altered fucosylation results in phenotypic changes of growth and invasion at the same time, as observed in our ras-MDCK cells. These glycan alterations can be expected to affect most, if not all, glycoproteins. Several ligands and their cell-surface receptors implicated in growth and invasion are glycoproteins. Examples of such ligands are : extracellular matrix components (19), growth factors (18,22) and motility factors (33,34). In the case of activating ligands, altered glycosylation of

the receptor might lead to constitutive activation of the receptor. In the case of inactivating ligands, altered glycosylation of the ligand or the receptor or both might interfere with receptor-ligand interaction.

In conclusion, progression of immortalized MDCK cells towards a more malignant phenotype correlates with alterations in sialylation and fucosylation of cell surface exposed glycans.

ACKNOWLEDGMENTS

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CONCLUSION

Nous avons trouvé une augmentation du poids moléculaire apparent des N-glycosylpeptides isolés de la surface de cellules invasives en comparaison avec celui des N-glycosylpeptides de cellules non-invasives. Ceci est essentiellement dû à un degré de sialylation plus élevé, puisque les chromatogrammes coïncident pratiquement après l'élimination de l'acide sialique.

En présence du phospholipide alkylé ET-18-OCH_3, l'invasion des cellules malignes de rein de rat (12 R1C-RK) ou de souris C_3H (MO_4) dans des FCPs est inhibée, tandis que les cellules métastatiques du carcinome de Lewis sont encore invasives. Aucune corrélation entre l'expression du phénotype invasif et les changements des N-glycosylpeptides de la surface cellulaire n'a été trouvée dans ces cas.

Par contre, le prétraitement du tissu-hôte (FCP) par l'ET-18-OCH_3 inhibe l'invasion des cellules MO_4 quand les deux partenaires sont confrontés en absence d'ET-18-OCH_3. Les N-glycosylpeptides de la surface cellulaire des FCP traités à l'ET-18-OCH_3 ont un poids moléculaire apparent plus élevé que les N-glycosylpeptides des FCPs non-traités. Cet effet n'est plus décelable après l'élimination de l'acide sialique. L'expression des "glycannes malins" dans les FCP prétraités à l'ET-18-OCH_3, ainsi que leur résistance à l'invasion par les cellules malignes MO_4 ne sont pas réversibles au cours d'une expérience de 4 jours.

Le prétraitement à l'ET-18-OCH_3 peut induire temporairement le phénotype invasif dans les cellules non-malignes de rein de rat (HSU), dans les fibroblastes murins immortalisés (3T3) et dans les cellules épithéliales de rein de chien (MDCK). Cet effet n'a pas pû être démontré dans les cellules normales et épithéliales de souris, isolées de glandes mammaires (NMuMG) ou dans les cellules non-invasives de sein humain (MCF-7). Une hypersialylation et des changements du type "malin" dans le profil chromatographique des N-glycosylpeptides ont été trouvés pour les cellules HSU et 3T3 traitées par l'ET-18-OCH_3. L'effet de l'ET-18-OCH_3 sur la glycosylation et le phénotype invasif des cellules HSU est réversible après 4 jours. Les changements de la N-glycosylation des cellules MDCK traitées par l'ET-18-

OCH_3 doivent encore être examinés. Nous savons déjà qu'après une transformation maligne à l'aide de l'oncogène ras, les cellules MDCK expriment des glycannes tri- et tetra-antennaires plus sialylés et que le degré de fucosylation en α-1,6 est trois fois plus élevé. Par contre, la quantité des glycannes O-glycosidiquement liés et leur taux de sialylation sont plus élevés dans les cellules MDCK non-transformées par rapport aux cellules transformées.

Nous pouvons donc conclure que les expériences à l'ET-18-OCH_3 nous procurent les outils nécessaires pour élucider la fonction des glycannes dans l'invasion tumorale. Un degré accru de sialylation va de pair avec l'acquisition du phénotype invasif.

CHAPITRE II : L'EFFET DE LA TEMPERATURE SUR L'INVASION ET LA FUCOSYLATION DES CELLULES MO_4 MALIGNES

INTRODUCTION

La température ambiante peut influencer l'invasion tumorale et la formation des métastases (Lucké et Schlumberger, 1949; McKinnell et Tarin, 1984; Zavanella, 1985; De Neve et coll.; ref. 29, 1988). Il a été démontré qu'en réduisant la température, l'invasion des fragments tumoraux du carcinome de Lucké (McKinnell et coll., 1986) et des cellules PNKT-4B (McKinnell et coll., 1988) peut être inhibée in vitro. Nous avons étudié l'invasion des cellules MO_4 dans des fragments de coeur et de poumon d'embryon de poulet afin de comprendre certains aspects de l'invasion tumorale. En même temps, nous avons étudié l'effet de la température sur d'autres activités cellulaires, qui pourraient être impliquées dans l'invasion : i) l'attachement d'agrégats de cellules MO_4 aux FCPs, la première étape de l'invasion (Mareel et coll., 1980); ii) l'assemblage et le désassemblage de microtubules ainsi que la migration directionelle, activités cellulaires nécessaires pour l'invasion (revue par Mareel et De Mets, 1984). Afin d'éclaircir si la croissance est associée à l'invasion, nous avons étudié l'effet de la température sur l'incorporation de la [6-^{3}H] thymidine et aussi sur la croissance de cellules MO_4 comme agrégats isolés et en confrontation avec le tissu cardiaque.

Vu que l'acquisition du phénotype invasif est accompagnée par des changements de glycosylation, nous avons étudié l'incorporation de fucose et de glucosamine à la surface cellulaire et dans les membranes de la cellule entière. Une première expérience avait démontré une inhibition sélective de l'incorporation de fucose à 28°C dans les cellules MO_4; ensuite nous avons testé l'effet de la température sur l'activité de la fucosyltransférase et sur la distribution des précurseurs de la fucosylation. En plus, nous avons examiné l'invasion des cellules L929, qui sont incapables de communiquer entr'elles par manque de fucosylation des glycannes de la surface cellulaire (Smets et coll., 1982).

Ces deux systèmes cellulaires nous offraient l'occasion de tester l'hypothèse qu'une perte du phénotype invasif serait accompagnée par des changements de la fucosylation des glycannes de la surface cellulaire.

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Effect of temperature on invasion of MO_4 mouse fibrosarcoma cells in organ culture

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Invasion by MO_4 mouse fibrosarcoma cells into fragments of embryonic chick heart or lung in organ culture was studied histologically and ultrastructurally at various temperatures between 12 and 40°C. Invasion was absent for at least 7 days at or below temperatures of 29°C. Invasion was invariably observed at or above 30·5°C. Differences in invasion between 29 and 30·5°C could not be ascribed to differences in growth, migration, or microtubule assembly/disassembly of MO_4 cells. Neither could they be explained through differences in the attachment of MO_4 cells to the heart fragments. Possible explanations for the absence of invasion at lower temperature are: altered resistance of the extracellular matrix in heart or lung fragments, and deficient expression of fucosylated glycoproteins at the surface of MO_4 cells. A population of MO_4 cells plated from the parent line and adapted to grow at 28°C ($MO_4$28 cell line) did not differ in invasiveness from the parent MO_4 cells.

We conclude that the temperature dependence of invasion in organ culture might indicate as yet unexplored aspects of the mechanisms of tumour invasion.

Introduction

Temperature has been used as a tool in various ways in experimental oncology. Examples are chemical [10] and viral [13] oncogenesis, homologous and heterologous intercellular contact [9, 29], cell proliferation [14, 36], breakdown of cells and intercellular matrix [16, 37], invasion [15], and metastasis [17].

In an attempt to understand some aspects of tumour invasion we have studied the invasion of MO_4 mouse fibrosarcoma cells into embryonic chick heart and lung fragments in organ culture at temperatures between 12 and 40°C. This assay *in vitro* has been shown to be relevant for at least some aspects of invasion *in vivo* (review in [19]). At the same time, we have examined the effect of temperature on the following MO_4 cell activities which might be implicated in invasion: attachment of MO_4 cell aggregates, which is known to be the first step of invasion in the assay *in vitro* [23]; microtubule assembly/disassembly and directional migration which are known to be necessary for invasion (review in [21]). To further elucidate the question of whether or not growth and invasion were related in MO_4 cells [26] the effect of temperature on growth was examined. Since surface-exposed carbohydrate units of integral

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membrane glycoproteins are involved in various cell–cell and cell–substrate interactions [35], the effect of lowered temperature on the synthesis of fucosyl-glycopeptides and on total protein synthesis was examined.

Material and methods

MO_4 cells

MO_4 cells are virally-transformed C3H mouse fibroblastic cells [2] that are invasive *in vitro* [22] and produce invasive and metastasizing fibrosarcomas in syngeneic mice [28]. They were maintained in culture at 37°C on plastic using Minimum Essential Medium Eagle (modified) with Earle's salts and non-essential amino acids (EMEM, Flow Laboratories Ltd, Irvine, Scotland) supplemented with 10 per cent fetal calf serum, 0·05 per cent (w/v) L-glutamine and 250 I.U/ml penicillin (hereafter called culture medium). MO_4 cell aggregates were prepared by incubation at 37°C of a suspension of MO_4 cells on a gyrotory shaker at 70 r.p.m. as described previously [22]. Aggregates with a diameter of either 0·2 or 0·3 mm were selected under a macroscope (Wild, Heerbrugg, Switzerland) (× 50) after 3 days.

$MO_4$28 cells

$MO_4$28 cells were obtained by plating MO_4 cells at 28°C as follows. Five hundred MO_4 cells were seeded in a 75 cm^2 plastic culture vessel with 25 ml culture medium and incubated at 28°C. After 14 days, an isolated colony was removed with a tissue culture cell scraper, trypsinized, and seeded in a 25 cm^2 plastic vessel. The resulting cell line ($MO_4$28) was maintained as described for the parent MO_4 cell line. $MO_4$28 cells grew more slowly (doubling time in logarithmic phase, 1·7 days) than the parent MO_4 cells (doubling time, 0·65 days) (unpublished results). $MO_4$28 cell aggregates were prepared as described for MO_4 cell aggregates but 7 days of incubation at 28°C were needed to obtain an equivalent amount of aggregates with a diameter of 0·2 mm.

Assays for invasiveness

To test the effect of temperature on invasion we have used confrontations between MO_4 or $MO_4$28 cell aggregates and fragments of 9-day-old embryonic chick heart in organ culture as described earlier [22]. Briefly: the aggregates were brought into contact with precultured heart fragments (diameter, 0·4 mm) on top of a semi-solid agar-agar medium. After attachment of the aggregates to the heart fragments (2 hours to 1 day) the confronting pairs were either further incubated on the semi-solid agar-agar medium or transferred individually into fluid culture medium for further incubation on a gyrotory shaker at 120 r.p.m. Confronting pairs were fixed in Bouin–Hollande's solution after 1 to 7 days for embedding in paraffin and complete serial sectioning into sections 8 μm thick. Consecutive sections were stained with hematoxylin–eosin or with an antiserum against chick heart [25]. For a semiquantitative evaluation of the effect of temperature, the interaction of MO_4 cells with the heart tissue was classified as follows: Grade I, when MO_4 cells were found at the periphery of the outer fibroblastic layers of the precultured heart fragment; Grade II, when MO_4 cells occupied the outer fibroblastic layers; Grade III, when MO_4 cells replaced the cardiac muscle to less than 50 per cent; Grade IV, when MO_4 cells replaced the cardiac muscle to more than 50 per cent. According to Mareel *et al.* [26], Grade III and Grade IV meet the criteria of invasion.

For ultrastructural analysis, cultures were fixed in glutaraldehyde (2·5 per cent in 0·1 M cacodylate buffer, pH 7·4) with postfixation in osmium tetroxide (1·0 per cent

in the same buffer) for embedding in LX-12 resin (Ladd Research Industries Inc., Burlington, Vermont) and sectioning into sections 2 μm thick. Areas were selected for sections 80 nm thick, which were counterstained with uranyl acetate and lead citrate for transmission electron microscopy (JEM-100B, Jeol, Tokyo, Japan).

In two series of experiments fragments of 12-day-old chick embryonic lung (diameter, 0·5 mm) were used instead of heart. Lung fragments were not precultured because this interfered with the attachment of the MO_4 and $MO_4$28 cell aggregates. Confronting pairs were fixed after 1, 3 or 8 days. Consecutive paraffin sections from confrontations between lung fragments and MO_4 cell aggregates were stained with hematoxylin–eosin or in accordance with the diastase–periodic acid Schiff method [18].

Assays for growth

Growth of MO_4 cell populations at different temperatures was studied following three methods.

(1) *Measurement of the volume of confronting pairs of MO_4 cell aggregates and heart fragments*

Confronting pairs incubated in fluid medium as described with the assay for invasiveness were photographed under the macroscope (× 25 or × 50) after 2, 3 or 4, and 7 days. We calculated the volumes following the formula of Attia and Weiss [1] from measurements of the diameters on negatives projected (× 6·3) on tracing paper. We know from previous experiments that growth of confronting pairs is due to proliferation of MO_4 cells [32].

(2) *Measurement of the volume of solitary MO_4 cell aggregates*

Solitary MO_4 and $MO_4$28 cell aggregates (diameter, 0·2 mm) were incubated in 5 ml Erlenmeyer flasks with 1·5 ml culture medium on a gyrotory shaker for 7 days at 120 r.p.m. as described by Storme and Mareel [31]. Volumes [1] were calculated from the diameters of the aggregates measured every 2 days under a macroscope (× 50) equipped with an eyepiece graticule.

(3) *Uptake of ^3H-thymidine by explanted MO_4 cell aggregates*

Individual MO_4 cell aggregates were explanted on coverslips in Leighton tubes [31] and incubated with 1·5 ml culture medium for 2 days at 37°C. Thereafter the cultures were brought to different temperatures for another 2 days. (6-^{3}H)-thymidine (The Radiochemical Centre, Amersham, U.K.; specific activity = 25 Ci/mmol) was added at a radioactive concentration of 0·1 μCi/ml 24 hours before termination of the cultures. After four washings in EMEM at 4°C, cells on the coverslip were solubilized in 1 ml sodium hydroxide (1N, at 37°C for 1 hour). Afterwards, 1 ml perchloric acid (3N) and 100 μl DNA (0·1 per cent w/v in distilled water) were added. After 30 min at 4°C, samples were centrifuged at 2200 *g* for 15 min. ^{3}H-activity was determined in precipitable (DNA) and soluble (pool) material with a Packard Tricarb Liquid Scintillation Spectrometer (Packard Instrument Company, Downer's Grove, Illinois).

Assay for migration

MO_4 and $MO_4$28 cell aggregates were explanted on glass and incubated as described by Storme and Mareel [31]. The diameter of the circular area covered by cells that had migrated from the aggregate was measured with an inverted

microscope (× 40). We know from previous experiments that at 37°C the surface of this area is an index of directional migration, is unrelated to cell proliferation during the first 4 days of culture, and is greatly reduced by microtubule inhibitors (for discussion see [21]).

In some experiments (see Results section) a period of preincubation at 37 or 28°C for 2 days was included and the surface of the area covered by the cells during the period of preincubation was subtracted from the final value. To examine whether migration at 28°C was sensitive to microtubule inhibitors, cultures were treated with the synthetic microtubule inhibitor Nocodazole [5]. Nocodazole was dissolved in dimethylsulfoxide (1 mg/ml) and diluted in culture medium to a final concentration of 1 μg/ml. This concentration is known to inhibit directional migration of MO_4 cells at 37°C [31]. Control cultures were incubated with dimethylsulfoxide (0·1 per cent v/v).

Immunocytochemistry with antiserum against tubulin

To examine the effect of lower temperature on microtubule assembly/disassembly, MO_4 cells trypsinized from stock cultures were seeded at a concentration of 5×10^4 cells/ml on square coverslips in 16 × 55 mm Petri dishes with 3 ml culture medium and incubated at 37°C. After 24 hours, cells were treated with 10 μg Nocodazole/ml for 4 hours at 37°C. Cultures were then washed four times with culture medium at room temperature and further incubated at 28 or at 37°C for 1, 4, and 22 hours. Duplicate cultures were fixed on the coverslip and processed for immunostaining with an antiserum against tubulin (courtesy of J. De Mey and M. De Brabander, Janssen Pharmaceutica, Beerse, Belgium) following the method described by De Mey *et al.* [6]. Control cultures were fixed immediately after treatment with Nocodazole. Qualitative evaluation of the microtubule complex was according to the criteria described earlier [27].

Assay for attachment

Attachment of MO_4 cell aggregates to precultured heart fragments was measured as described earlier [23]. MO_4 cell aggregates and heart fragments were preincubated for 30 to 60 min at the temperature of the experiment, brought together on the bottom of a polystyrene tube, and incubated for 45 min. The rate of attachment was expressed as the number of confronting pairs remaining together per total number tested after sedimentation at 1 *g* in culture medium.

Cell surface carbohydrate studies

For metabolic labelling of cell surface carbohydrates and of proteins, exponentially growing cultures were incubated in the presence of ^{3}H-l. fucose (0·5 μCi/ml) and ^{3}H-leucine (0·5 μCi/ml) respectively. ^{3}H-fucose labelled surface carbohydrates were isolated by mild trypsinization of intact cells and incorporation of ^{3}H-leucine was assessed in the total cold acid-precipitable fraction of trypsinized cells. For surface labelling of membrane sialic acids, cells were exposed to 2 mM sodium periodate (10 min at 0°C) followed by reduction with ^{3}H-borohydride. Surface glycopeptides labelled in their sialic acid residues were subsequently isolated by mild trypsinization. Incorporation of labelled precursor was calculated as bound, non-dialysable radioactivity per 10^6 cells.

Reproducibility

Number of cultures for each type of experiments is given in the Results section. Temperature control showed nominal values ± 0·5°C.

Results

Invasion

MO_4 cells

In a first series of experiments, triplicate confronting pairs of MO_4 cell aggregates and precultured heart fragments were incubated on top of a semi-solid medium at the following temperatures: 12, 15, 18, 21, 23, 25, 28, 32, 35, 37 and 40°C. Cultures fixed after 4 or 7 days showed that invasion was absent (Grade II) at or below 28°C, and present (Grades III and IV) at or above 32°C. In a second series of experiments, confronting pairs were incubated in fluid medium on a gyrotory shaker; the results are summarized in the table. At 28°C (figure 1) and at 29°C invasion was absent since the interaction between MO_4 cells and heart tissue was limited to occupation of the outer fibroblastic layers of the heart fragment by MO_4 cells (Grade II). At 30·5°C, the histology of all cultures met the criteria of invasion since MO_4 cells progressively replaced the cardiac muscle (figure 2). Comparative grading of cultures (see the table) suggested that the rate of invasion was lower at 30·5°C than at 37°C. The total amount of MO_4 cells in cultures fixed after 3 and 7 days appeared to be lower at 28°C than at 37°C. When precultured heart fragments were confronted with two MO_4 cell aggregates (each with a diameter of 0·3 mm) at opposite sides and fixed after 3 and 7 days (total number, 6), invasion was absent as in confronting cultures with one aggregate of 0·2 mm. Histology of sections 2 μm thick from confrontations incubated at 28, 32, 37 and 40°C confirmed the results obtained with sections 8 μm thick. At 32 and 40°C solitary MO_4 cells were found more frequently between the cardiac muscle cells than at 37°C where the occupation was more massive. Ultrastructurally, MO_4 cells inside the heart fragment were identified through the presence of intracisternal A-type viral particles. At or above 32°C, ultrastructural features of invasion were found as described earlier [24, 34]: extensions of MO_4 cells indenting the plasma membrane of cardiac muscle cells or

Semiquantitative analysis of the interaction of MO_4 and MO_4 28 cell aggregates with precultured heart fragments in organ culture at different temperatures.

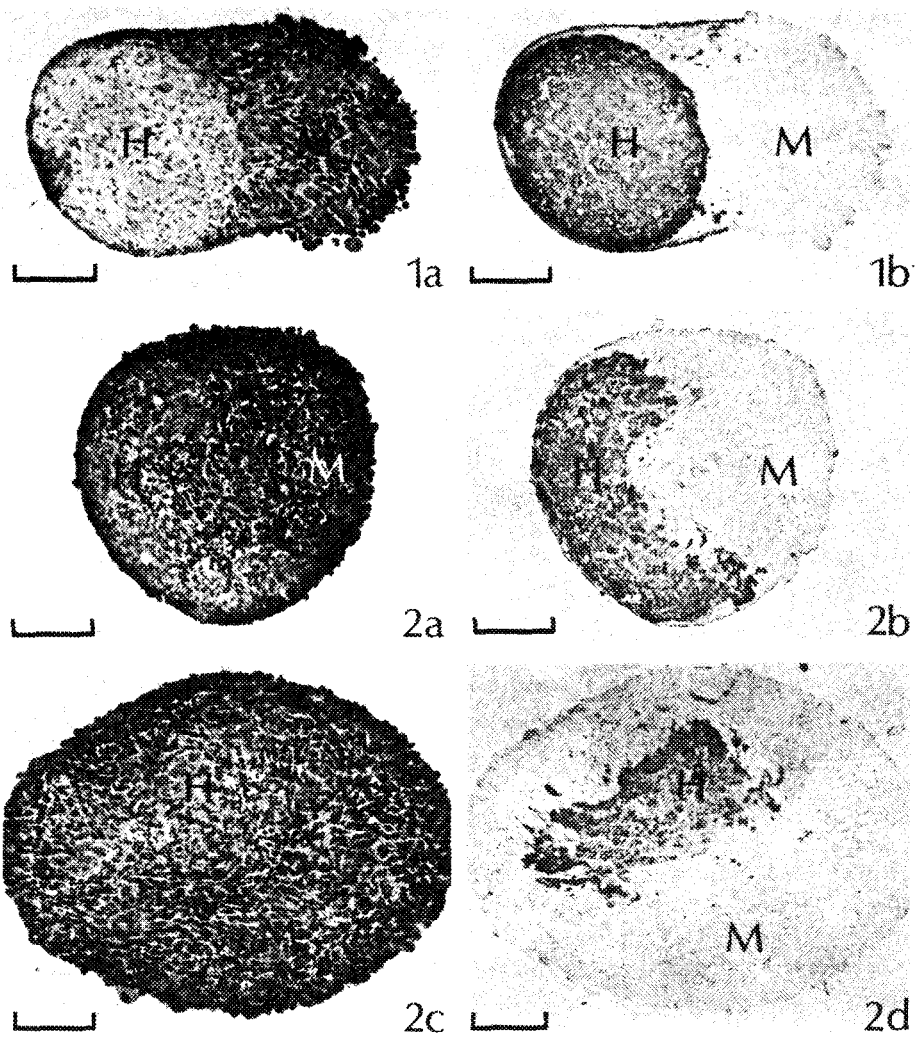
Temperature (°C)	Grading[a] after 2 days	3 days	4 days	7 days
MO_4 cell aggregates[b]				
28	II(2)[c]	II(5)	II(4)	II(12)
29	II(3)	II(6)	II(4)	II(12)
30·5	II(2)	n.d.	III(3)	IV(4)
37	III(2)	n.d.	IV(6)	IV(6)
$MO_4$28 cell aggregates[b]				
28	n.d.	II(4)	n.d.	II(17)
37	n.d.	III(3)	n.d.	IV(3)

n.d., not done.

[a] as described in Materials and Methods; Grades III and IV meet the criteria of invasion.

[b] diameter = 0·2 mm.

[c] number of cultures in parentheses.



Figures 1 and 2. Photomicrographs of sections 8 μm thick from confrontations between MO_4 cell aggregates (M) and precultured heart fragments (H) incubated at 28°C (figure 1) and at 30·5 C (figure 2). Fixation after 4 days (figures 2 (*a*) and (*b*)) and after 7 days (figures 1 (*a*) and (*b*), 2 (*c*) and (*d*)); staining with hematoxylin–eosin (figures 1 (*a*), 2 (*a*) and (*c*) and with an antiserum against chick heart (figures 1 (*b*), 2 (*b*) and (*d*)). Scale bars, 100 μm.

extending between cardiac muscle cells with loss of junctions; and degenerative alterations of cardiac muscle cells (figure 3). At 28°C, MO_4 cells were apposed to the peripheral cardiac muscle cells which had conserved their intercalated discs and desmosomes; fuzzy extracellular material was usually found between the MO_4 cells and the muscle cells (figure 4).

Confrontations between MO_4 cell aggregates and fresh lung fragments incubated at 37 C (total number of cultures, 16) and fixed after 1 to 8 days, indicated progressive occupation of the connective tissue by MO_4 cells followed by degeneration of the epithelium (figures 5 (*a*), (*b*), and 5 (*c*)). At 28°C (number of cultures, 16)

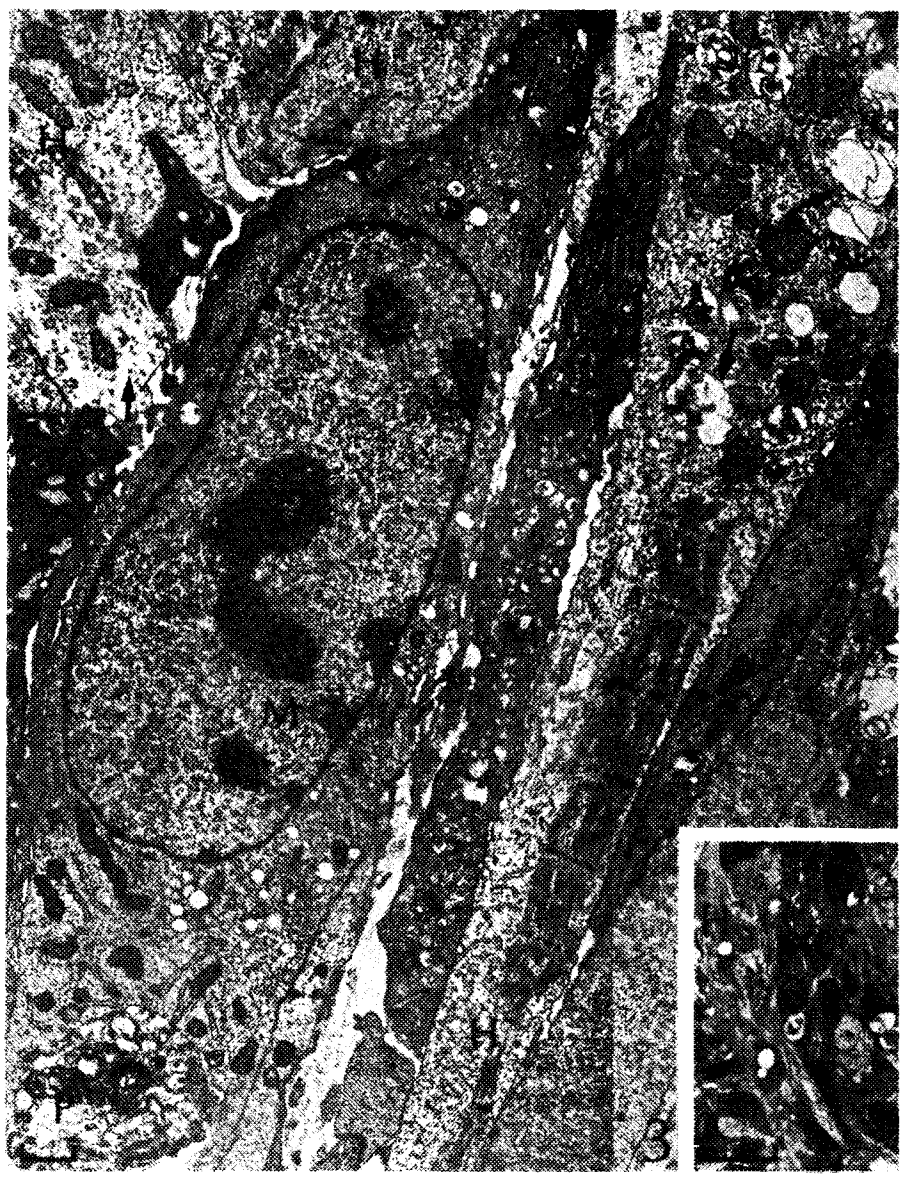


Figure 3. Transmission electron micrograph from a confrontation of an MO_4 cell aggregate with a precultured heart fragment incubated at 32 C and fixed after 4 days. H, cardiac muscle cell; M, MO_4 cell; P, heterophagosome; arrows: degenerative alterations in cardiac muscle cell; asterisk: extracellular material. Scale bar, 1 μm. Inset: photomicrograph of the area from which the ultrathin section was prepared. Scale bar, 20 μm.

Figure 4. Transmission electron micrograph from a confrontation of an MO_4 cell aggregate with a precultured heart fragment incubated at 28 °C and fixed after 4 days. H, cardiac muscle cell; M, MO_4 cell; asterisk; extracellular material. Scale bar, 1 μm. Inset: area from which the ultrathin section was prepared. Scale bar, 20 μm.

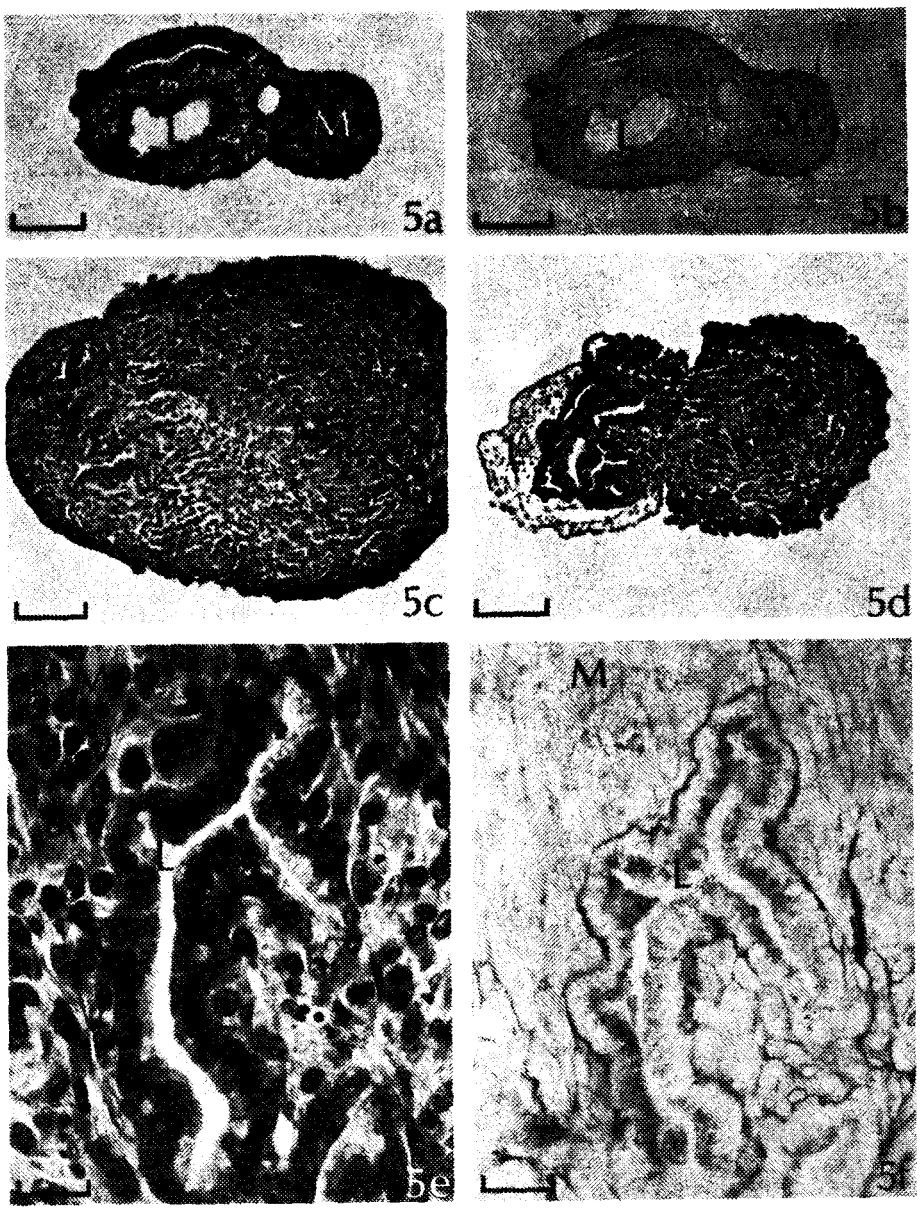


Figure 5. Photomicrographs of sections 8 μm thick from confrontations between MO_4 cell aggregates (M) and lung fragments (L) incubated at 37°C (figures 5 (*a*)–(*c*)) and at 28°C (figures 5 (*d*)–(*f*)). Fixation after 2 hours (figures 5 (*a*) and (*b*)), after 6 days (figures 5 (*c*) and (*d*)) and after 8 days (figures 5 (*e*) and (*f*)). Staining with hematoxylin–eosin (figures 5 (*a*), (*c*), (*d*) and (*e*)) and with diastase-PAS (figures 5 (*b*) and (*f*)). Scale bars, 100 μm (figures 5 (*a*)–(*d*)) and 20 μm (figures 5 (*e*) and (*f*)).

MO_4 cells partly occupied the connective tissue but the epithelium remained intact (figure 5 (*d*)). In cultures fixed after 8 days, bronchi situated at the pole of attachment of the MO_4 cell aggregate showed an intact basal PAS-positive border (figures 5 (*e*) and (*f*)).

$MO_4$28 cells

The histology of confrontations of heart fragments with $MO_4$28 cell aggregates at 28°C did not differ from those with MO_4 cell aggregates at 28°C. When $MO_4$28 cell aggregates were confronted with heart at 37°C, invasion was as obvious as with MO_4 cell aggregates (figure 6).

Growth

MO_4 cells

The growth of confronting pairs of MO_4 aggregates and heart fragments is shown in figure 7A. Growth was obvious in cultures incubated at 29 to 37°C. Growth of solitary aggregates in suspension is shown in figure 8A. Uptake of ^{3}H-thymidine into

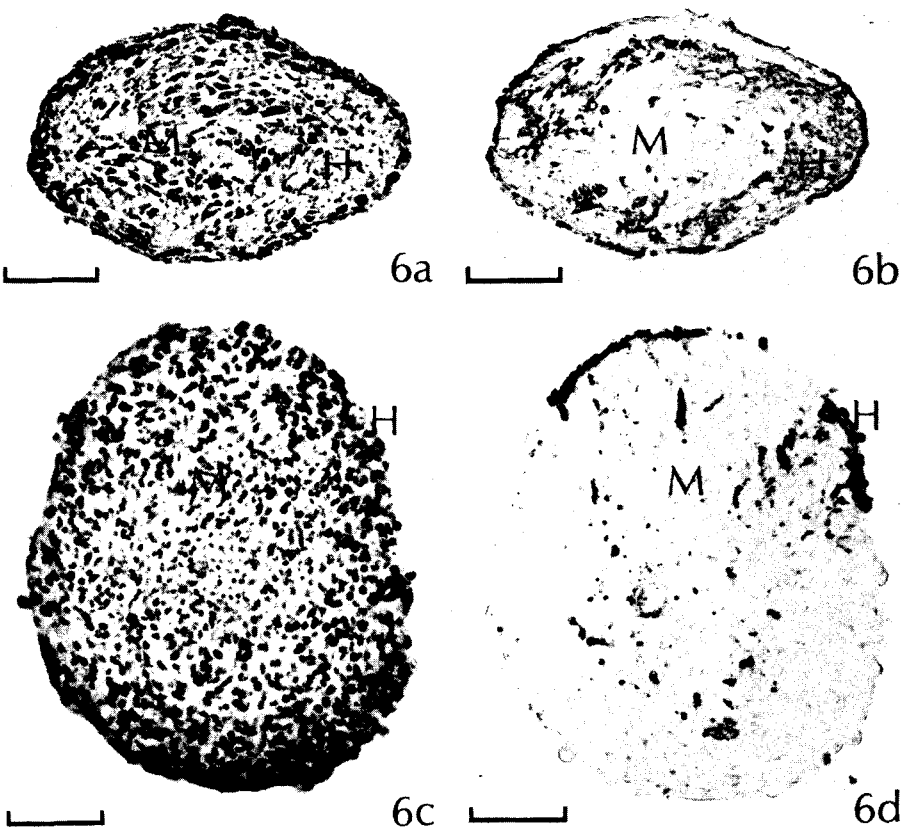


Figure 6. Photomicrographs of sections 8 μm thick from confrontations between $MO_4$28 cell aggregates (M) and precultured heart fragments (H) incubated at 37°C and fixed after 3 days (figures 6 (*a*) and (*b*)) and after 7 days (figures 6 (*c*) and (*d*)). Staining with hematoxylin–eosin (figures 6 (*a*) and (*c*)) and with an antiserum against chick heart (figures 6 (*b*) and *d*)). Scale bar, 100 μm.

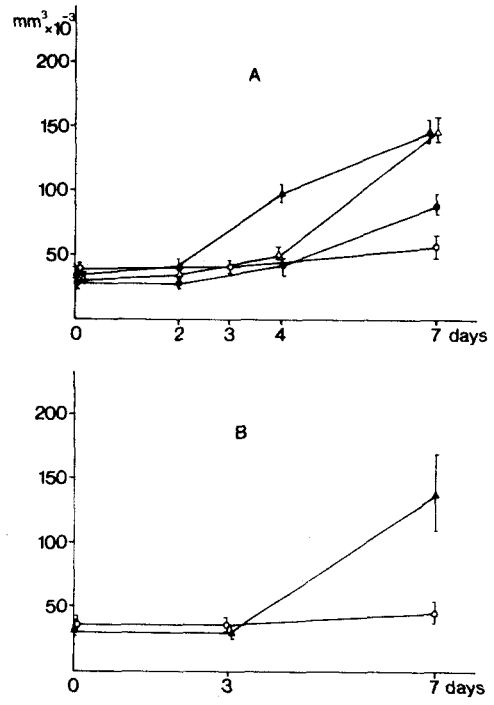


Figure 7. Growth of confronting pairs of MO_4 (A) or $MO_4$28 (B) cell aggregates and precultured heart fragments incubated in fluid medium at 28°C (○——○); 29°C, (●——●); 30·5°C (△——△); and 37°C (▲——▲). Ordinate: volume of confronting pairs; mean ± S.D. from 9 to 35 cultures. Abscissa: time of incubation.

DNA by MO_4 cell aggregates explained on glass is shown in figure 9. The distribution of radioactivity between pool and DNA is approximately the same at temperatures between 28 and 37°C; lowering the temperature below 28°C or increasing it to 40°C affects incorporation of ^{3}H-thymidine into DNA more than into pool (figure 10).

$MO_4$28 cells

The present material did not provide evidence for differences between MO_4 and $MO_4$28 cells (figures 7B and 8B).

Migration

MO_4 cells

Migration of MO_4 cells from an aggregate explanted on glass at temperatures used for our first invasion experiments (cf. *supra*) is shown in figure 11. Migration of MO_4 cells at temperatures that are critical for invasion is shown in figure 12A. Differences in migration between temperatures of 29°C (non-permissive for invasion) and 30·5°C (permissive for invasion) were not considered to be significant. Figure 13 shows that the migration at 28°C is sensitive to the microtubule inhibitor Nocodazole. Further, the area covered by MO_4 cells at 37°C in the presence of

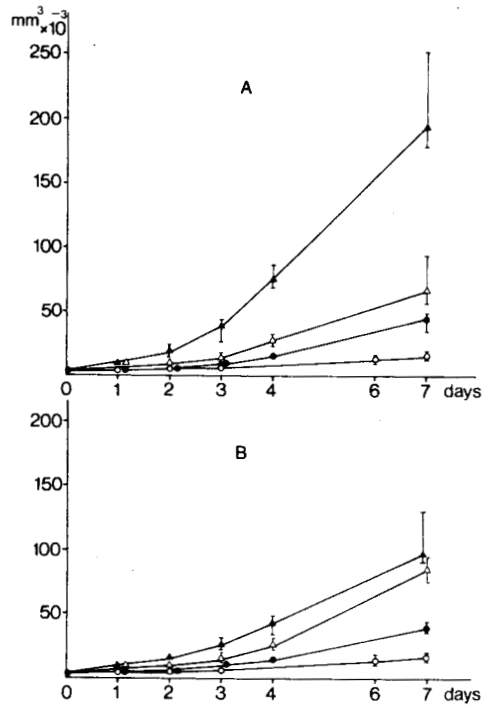


Figure 8. Growth of MO_4 (A) and $MO_4$28 (B) cell aggregates in suspension culture at 28°C (○—○); 29°C (●—●); 30·5°C (△—△); and 37°C (▲—▲). Ordinate: volume of aggregates; median and extreme values from 5 cultures. Abscissa: time of incubation.

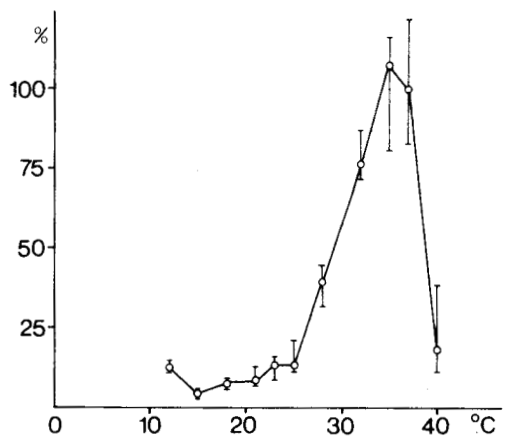


Figure 9. Uptake of ^{3}H-thymidine into perchloric acid precipitable material (DNA) by MO_4 cell aggregates explanted on glass. Ordinate: ^{3}H-activity as a percentage of ^{3}H-activity in cells incubated at 37°C; median and extreme values from 6 to 31 cultures. Abscissa: temperature of incubation.

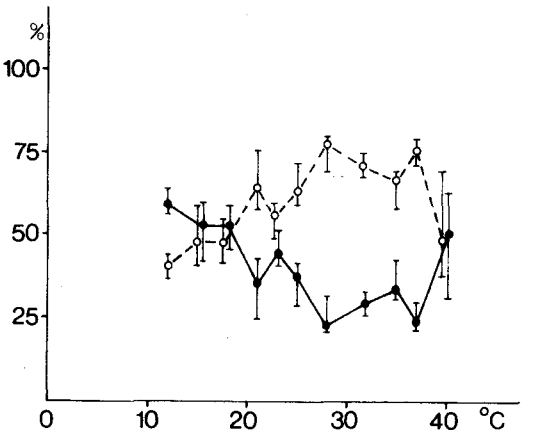


Figure 10. Distribution of ^{3}H-activity between acid soluble (pool) and acid precipitable (DNA) material in cultures shown in figure 9. Ordinate: ^{3}H-activity in pool (●—●) and in DNA (O---O) as a percentage of total activity. Abscissa: temperature of incubation.

Nocodazole is smaller than that at 28°C in the absence of Nocodazole. The polarized shape and radial orientation of cells in the corona was conserved at 28°C and lost when Nocodazole was added.

$MO_4$28 cells

The temperature dependence of the migration of $MO_4$28 cells (figure 12B) was similar to that of MO_4 cells.

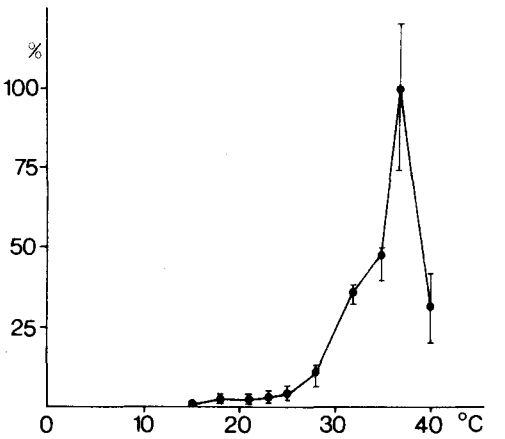


Figure 11. Migration of MO_4 cells from an aggregate explanted on glass. Ordinate: area covered by MO_4 cells (πr^2) after 4 days as a percentage of area covered at 37°C; median and extreme values from five cultures. Abscissa: temperature of incubation. For experiments at or below 21°C cultures were preincubated for 1 day at 37°C (see Materials and methods).

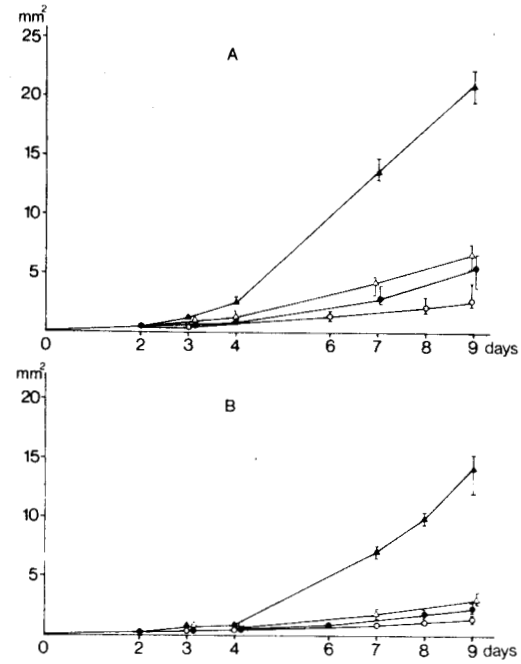


Figure 12. Migration of MO_4 (A) and $MO_4$28 (B) cells from an aggregate explanted on glass at 28°C, (O—O); 29°C, (●—●); 30·5°C, (Δ—Δ); and 37°C, (▲—▲). Explants of MO_4 and $MO_4$28 cell aggregates were pre-incubated for 2 days respectively at 37 and 28°C. Ordinate: area covered by cells (πr^2); median and extreme values from five to nine cultures. Abscissa: time of incubation.

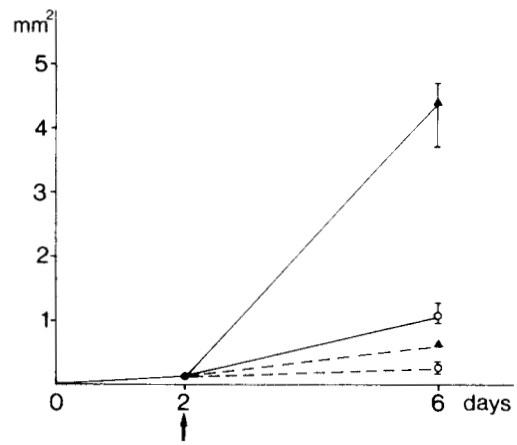


Figure 13. Effect of Nocodazole (dotted lines) on migration of MO_4 cells from an aggregate explanted on glass for 2 days at 37°C and further incubated at 28°C (O—O) or at 37°C (▲—▲). Controls treated with dimethylsulfoxide: full lines. Ordinate: area covered (πr^2) by MO_4 cells; median and extreme values from 10 to 12 cultures. Abscissa: time; arrow indicates addition of Nocodazole.

Immunocytochemistry of microtubules

After treatment with Nocodazole, microtubules were absent in MO_4 cells. After washing and reincubation in fresh culture medium for 1 hour at 28°C or at 37°C, cells contained normal microtubule complexes; no differences were observed between cells reincubated at 28°C as compared to 37°C.

Attachment

The rate of attachment of MO_4 cell aggregates to precultured heart fragments at different temperatures is shown in figure 14. Attachment did not vary within the range of temperatures that were critical for invasion (28–32°C).

Cell surface carbohydrates

MO_4 cells

Incorporation of ^{3}H-fucose and ^{3}H-leucine into MO_4 cells grown for 48 hours at temperatures ranging from 28 to 37°C followed by another 16 hours in the presence of the labelled precursor, is shown in figure 15. The incorporation of ^{3}H-fucose into membrane glycopeptides was almost completely inhibited at or below 34°C. Protein synthesis, monitored by ^{3}H-leucine incorporation, was less affected. Labelling of surface-exposed sialic acids revealed only minor differences between MO_4 cells grown at 37°C or at 28°C. Accordingly, the total amount of surface glycopeptides was not affected by incubation at 28°C.

$MO_4$28 cells

In $MO_4$28 cells, the incorporation of ^{3}H-fucose was also inhibited at 28°C but restored to the level of MO_4 cells when grown at 37°C. These results suggest that both $MO_4$28 cells and MO_4 cells were deficient in the fucosylation of protein-bound carbohydrates at 28°C. A full account of fucosylation and invasiveness will be published separately.

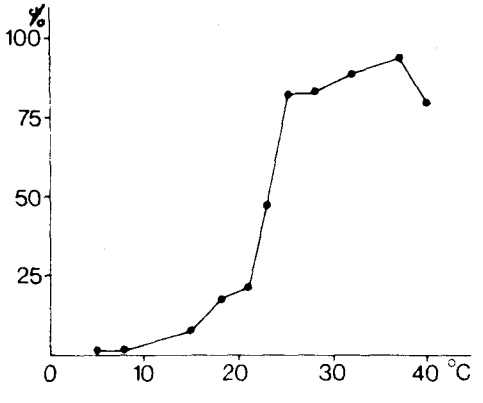


Figure 14. Attachment of MO_4 cell aggregates to precultured heart fragments. Ordinate: percentage of confronting pairs remaining together; values from 20 to 25 pairs. Abscissa: temperature.

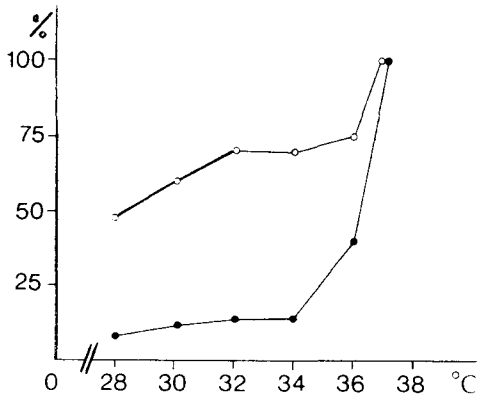


Figure 15. Incorporation of ^{3}H-leucine (O—O) into total cellular protein as compared to incorporation of ^{3}H-fucose (●—●) in surface glycopeptides in MO_4 cells. Ordinate: ^{3}H-activity as a percentage of ^{3}H-activity from cultures at 37°C; mean values from three independent experiments. Abscissa: temperature of incubation.

Discussion

The invasion of MO_4 cells into embryonic chick heart fragments in organ culture was prevented for at least 7 days when the temperature was lowered to 29°C. Histology of confronting pairs fixed after different periods of incubation (see the table) indicated quantitative differences in the rate of invasion between 30·5 and 37°C. Comparison of cultures at 29 and 30·5°C suggested a qualitative difference since invasion did not occur for at least 7 days at 29°C but was invariably found after 4 days at 30·5°C. Occupation of the fibroblastic layers surrounding the heart fragment or of the connective tissue of lung fragments at the pole of attachment of the MO_4 cell aggregate did not meet the criteria of invasion [20, 26]. This phenomenon was also observed when non-malignant cells were confronted with heart [7, 24] or with lung [33] fragments and when inhibitors of invasion were added to the culture medium [27].

Absence of invasion at or below 29°C might be ascribed to changes in the normal tissue, in the MO_4 cells, or in both. The histology of embryonic chick tibia, gonads, and intestine was changed in organ culture when they were incubated at 30°C as compared to 38°C which was optimal for the survival, growth, and differentiation of these organs [3, 4].

Jensen and Therkelsen [11] have found that in adult human skin explants keratinocytes grew optimally at 33°C whereas fibroblasts failed to grow at or below 35°C. It should, therefore, be taken into account that adaptation of the normal tissue may, at least partly, be responsible for absence of invasion at 29°C.

Experiments with $MO_4$28 cells made it unlikely that incubation at temperatures that were non-permissive for invasion led to a selection of a non-invasive subpopulation of cells from the parent MO_4 cell population.

Neither did our experiments provide arguments for accepting that alterations of growth, of microtubule assembly, of migration, or of attachment at non-permissive temperatures, were responsible for absence of invasion. Lack of correlation between growth and invasion of MO_4 cell populations *in vitro* has been discussed previously [26]. The arguments were, that invasion could occur in absence of growth, and that

growth (at 29°C) was not necessarily accompanied by invasion. This lack of correlation is also illustrated by a comparison of inhibition of growth (figure 9) at 28°C (non-permissive for invasion) and at 40°C (permissive for invasion). Immunocytochemical staining with antitubulin of MO_4 cells reassembling microtubules at 28°C after treatment with Nocodazole provided similar images and did not reveal disturbances. In the test-tube the kinetics of microtubule assembly were found to be similar at 28·6 and 30·5°C [12].

The transition temperature of attachment of MO_4 cell aggregates to precultured heart fragments was between 18 and 25°C; the rate of attachment was about the same at 28°C as at 37°C. In our opinion, differences in migration between explants at 29°C as compared to 30·5°C were too small to account for differences in invasiveness. Further, experiments with the microtubule inhibitor Nocodazole suggested that migration at 28°C was directional (for discussion see [21]).

A possible explanation for the absence of invasion at 29°C is failure of breakdown of extracellular matrices. The experiments with lung fragments (figure 5) and ultrastructural data from confrontations of MO_4 cells with heart fragments (figure 4) suggested that extracellular matrix and intercellular junctions between myocytes or between epithelial cells might form at 28°C a barrier to invasion. Data from the literature have shown that the sensitivity to lytic enzymes of defined substrates is temperature dependent. Cleavage of collagen type IV by α-thrombin occurred at 37°C but not at 35°C [16]. Marrow cytolysis by tumour cells in mixed suspension culture could be demonstrated at 37°C but not at 25°C [37].

Another explanation may be found in the deficient fucosylation of surface carbohydrate units of MO_4 cells at temperatures that do not allow invasion. Cell surface carbohydrates have been implicated in various cell–cell and cell–substrate interactions [35]. In mouse L cells deficient fucosylation of surface carbohydrates has been associated with marked variations in adherent and junctional contacts [30].

Although the present experiments do not explain why invasion is arrested by lowering the temperature from 30·5 to 29°C, they show that temperature may be a powerful and rapidly reversible tool for the study of mechanisms of tumour invasion *in vitro*. This tool can also be used *in vivo* under physiological conditions in tumour-bearing ectothermic animals [17] and under experimental conditions in tail tumours [13] of homeothermic animals in which the temperature of the tail varies with the temperature of the environment [8].

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Restored invasion of mouse MO_4 cells into chick heart *in vitro* through mutual conditioning at reduced temperature

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Invasion of malignant mouse MO_4 cells into embryonic chick heart fragments in confronting organ cultures was arrested for 7 days when the temperature of incubation was lowered to 28°C. Afterwards invasion resumed and progression between days 10 and 17 at 28°C was comparable to that between days 0 and 7 at 37°C. This pattern of progression of MO_4 cell invasion at 28°C was unaltered when either MO_4 cells or heart fragments or both were preincubated separately at 28°C for 14 days before confrontation with each other. Invasion at 28°C resumed only when MO_4 cells and heart tissue had been in immediate contact for at least 7 days. Metabolic labelling with [^{3}H]fucose showed a correlation in time between transient suppression of invasion and transient inhibition of incorporation of fucosylation-precursor molecules into glycoproteins by MO_4 cells. The latter activity was far less temperature-sensitive in heart cells. Our observations suggest that metabolic cooperation between invading MO_4 cells and heart tissue is essential for progression of invasion *in vitro*.

Introduction

Environmental temperature may influence tumour invasion and metastasis [11, 18, 32]. It has been shown that invasion *in vitro* of mouse MO_4 cells [15], of frog Lucké renal carcinoma explants [19] and of frog PNKT-4B carcinoma cells [20] can be inhibited by lowering the temperature of incubation. In short-term experiments invasion *in vitro* of MO_4 cells into chick heart showed a transition between 30·5 and 29°C: invasion occurred after 4 days of incubation at 30·5°C, but not after 7 days at 29°C [15]. As a possible explanation, we considered that absence of invasion was due to alterations in the expression of glycopeptides at the surface of MO_4 cells. Alterations related either to fucose uptake [6, 24], to fucosyltransferases [7, 22, 27] or to the structure of fucosylglycopeptides [23, 26, 30] have been implicated in tumorigenicity [5, 26, 31], invasion [3, 16] and metastasis [24].

We have now tested the invasion of MO_4 cells at reduced temperature in long-term experiments to examine the possibility of resumption of invasion and the possible factors involved in it. Therefore, both the confronting MO_4 cells and the host chick heart tissue were adapted to reduced temperature either separately or together.

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Materials and methods

MO_4 and $MO_4$28 cells

MO_4 cells are C3H mouse embryo cells transformed by Kirsten murine sarcoma virus [2]; they are invasive *in vitro* and *in vivo* [12, 21]. MO_4 cells were maintained in culture at 37°C using Eagle's Minimum Essential Medium (modified) with Earle's salts and non-essential amino acids (EMEM, Flow Laboratories, Irvine, Scotland) supplemented with 10 per cent (v/v) foetal bovine serum (Gibco, Paisley, Scotland), 0·05 per cent (w/v) L-glutamine and 250 IU/ml penicillin (hereafter called culture medium).

$MO_4$28 cells were derived from an MO_4 cell culture through colony formation on tissue culture plastic substrate and further maintenance at 28°C as described previously [15]. In the present experiments the cell line was used at passages 8–14.

Assay for invasion and growth

Confrontations of MO_4 or $MO_4$28 cell aggregates and precultured fragments of 9-day-old-embryonic chick heart (PHF) were made as described previously [12] and incubated for 7–21 days at 37 or 28°C. The accuracy of temperature control was ±0·5°C. In these experiments PHF and MO_4 cell aggregates were prepared through incubation for 3 days at 37°C and $MO_4$28 cell aggregates through incubation for 7 days at 28°C. For additional confrontation experiments at 28°C, the two partners prepared as described above were further incubated in suspension culture before confrontation as follows: MO_4 cell aggregates during 14 days at 28°C for confrontation with PHF; MO_4 cell aggregates and PHF during 14 days at 28°C for confrontation with each other; and PHF during 14 days at 28°C for confrontation with either MO_4 or $MO_4$28 cell aggregates. In one experiment fragments dissected from $MO_4$28/PHF confrontations incubated at 28°C for 14 days were reconfronted with fresh PHF. Growth of confronting pairs was evaluated from measurements of the larger (a) and the smaller (b) diameters and calculation of the volume (V) in accordance with $V = 0{\cdot}4ab^2$ [1]. The starting volume of confronting pairs was 0·03 mm^3.

Invasion was scored by at least two independent observers on consecutive serial sections stained with haematoxylin–eosin or with an antiserum against chick heart [13]. These scores describe the following observations: grade I, when MO_4 cells are found at the periphery of the outer fibroblastic layers of the PHF; grade II, when MO_4 cells occupy the outer fibroblastic layers; grade III, when MO_4 cells replace less than half the PHF; and grade IV, when MO_4 cells replace more than half the PHF. According to Mareel *et al.* [17], grades III and IV meet the criteria of invasion. invasion.

Separate assay for growth

Solitary MO_4 and $MO_4$28 cell aggregates were incubated at 28 or 37°C in suspension culture and their volumes (V) were calculated as described for confronting pairs.

Assay for migration

Solitary MO_4 or $MO_4$28 cell aggregates were explanted on glass and the diameter of the circular area covered by cells that had migrated from the aggregate was measured with an inverted microscope (× 40) according to Storme and Mareel [28].

Fucose incorporation and autoradiography

Invasion was assayed as described above and L-[6-^{3}H]fucose (NEN, Albany, Boston; 1 μCi/ml, 86·3 Ci/mmol) was added 16 h before fixation. Consecutive sections were stained with an antiserum against chick heart or processed for autoradiography. Briefly, the sections were dewaxed and coated with a gelatin–chromalumen solution [0·5 and 0·05 per cent (v/v), respectively] at 60°C, dried and overlayered with a fine grain autoradiographic stripping plate (AR 10; Kodak, U.K.). After an exposure time of 14 or 21 days the plates were developed (Kodak D 19), fixed (Agefix) and stained with haematoxylin–eosin. Grains were counted in representative areas and ratios of the number of grains over MO_4 cells versus those over heart tissue were calculated from scores in the same section.

Results

Invasion

Cultures fixed after 7 days showed that invasion of MO_4 or $MO_4$28 cells into PHF was absent at 28°C in all but one culture whereas invasion was observed in all cultures at 37°C (table 1). This is in agreement with our previous results [15]. At 28°C, invasion started after 10 days, leading to complete replacement of the heart tissue in all cultures after 17–21 days of incubation. We have illustrated this progression of invasion by plotting as function of incubation time the percentages of cultures showing invasion (figure 1). This confirmed our impression from histological examination of individual series of experiments that, once invasion had resumed, its progression at 28°C was approximately the same as that at 37°C. Adaptation of MO_4 cell aggregates alone, or of PHF alone, or of both through separate incubation at 28°C for 14 days before confrontation did not alter the course of invasion at 28°C: a delay of invasion was observed for at least 10 days as in all other experiments at 28°C (table 2). We also examined whether or not $MO_4$28 cells, which were expected to be invading on the basis of the results mentioned above (see table 1), would continue to invade when confronted with fresh PHF. Fragments from 14-day-old pairs of $MO_4$28 cells and PHF confronted with fresh PHF at 28°C showed the same progression of invasion as $MO_4$28 cell aggregates confronted with PHF (table 2).

Taken together, these results show that invasion at 28°C resumes only when both MO_4 cell aggregates and PHF are in contact with each other for at least 7 days.

Table 1. Analysis of the interaction of MO_4 and $MO_4$28 cell aggregates with precultured heart fragments (PHF) in organ culture at 37 and 28°C.

Cells	Temperature (°C)	Grading[a] after 7 days	10 days	14 days	17 days	21 days
MO_4	37	IV(6)[b]	N.d.[c]	N.d.	N.d.	N.d.
	28	II(16)	II(10), III(3)	II(3), III(6), IV(5)	IV(8)	IV(6)
$MO_4$28	37	IV(3)	IV(3)	IV(3)	N.d.	IV(2)
	28	II(12), III(1)	II(13), III(1)	II(3), III(10)	IV(4)	IV(7)

[a] As described under Materials and methods; grades III and IV meet the criteria of invasion.
[b] Number of cultures in parentheses.
[c] Not done.

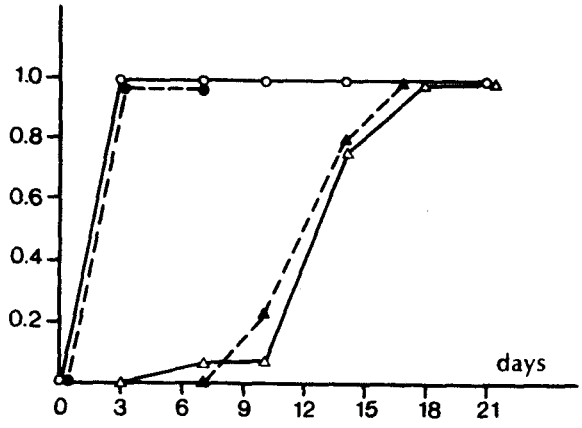

Figure 1. Invasion of MO_4 or $MO_4$28 cells into chick heart in organ culture. Ordinate: fraction of cultures showing invasion; MO_4 cells at 37°C (●--●) and at 28°C (▲--▲) or $MO_4$28 cells at 37°C (○—○) and 28°C (△—△). Abscissa: time of confronting culture.

Table 2. Analysis of the interaction at 28°C of MO_4 and $MO_4$28 cell aggregates with precultured heart fragments (PHF) in organ culture after conditioning of both partners at 28 C for 14 days before confrontation.

		Grading[a] after			
Cells	Conditioning	7 days	10 days	14 days	17 days
MO_4	A[b]	II(4), III(1)[c]	II(1), III(1)	N.d.[d]	N.d.
	B	II(4)	II(2), III(2)	II(1), III(2)	N.d.
	C	II(3), III(1)	II(1), III(2)	III(1), IV(4)	IV(5)
$MO_4$28	C	II(8)	II(8)	II(6), III(3)	II(1), III(3)
	D	II(9)	II(7), III(3)	II(3), III(5)	N.d.

[a] As described under Materials and methods; grades III and IV meet the criteria of invasion.
[b] A, MO_4 cell aggregates cultured at 28°C for 14 days before confrontation with PHF; B, MO_4 cell aggregates and PHF cultured separately at 28°C for 14 days before confrontation; C, PHF cultured at 28°C for 14 days before confrontation with MO_4 or with $MO_4$28 cell aggregates; D, fragments from confrontations incubated at 28°C for 14 days were used instead of $MO_4$28 cell aggregates.
[c] Number of cultures in parentheses.
[d] Not done.

Growth

The growth rate of solitary MO_4 or $MO_4$28 cell aggregates was lower at 28 than at 37°C (figure 2). No alteration of the growth rate of solitary MO_4 or $MO_4$28 cell aggregates was observed between days 7 and 14 at 28°C. For MO_4 cells in confrontation with PHF (figure 3) at 28°C the slope of the curve became steeper between days 10 and 14, suggesting that growth of MO_4 cells was better after conditioning by contact with PHF (compare figures 2 and 3). This was not the case, however, for confronting pairs of $MO_4$28 cell aggregates and PHF at 28°C.

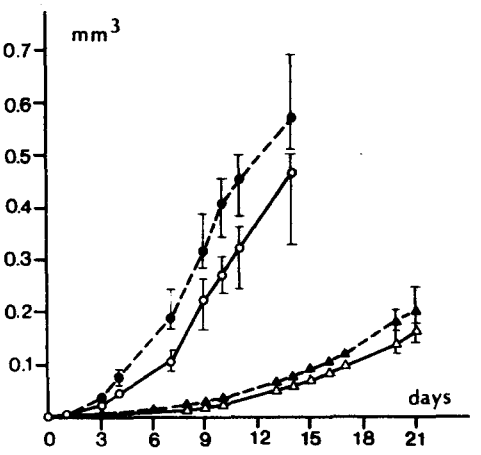

Figure 2. Growth of solitary MO_4 and MO_428 cell aggregates in suspension culture. Ordinate: volume of solitary aggregates of MO_4 cells at 37°C (●--●) and at 28°C (▲--▲) or of MO_428 cells at 37°C (O—O) and at 28°C (Δ—Δ); median and extreme values from at least five cultures. Abscissa: time of incubation.

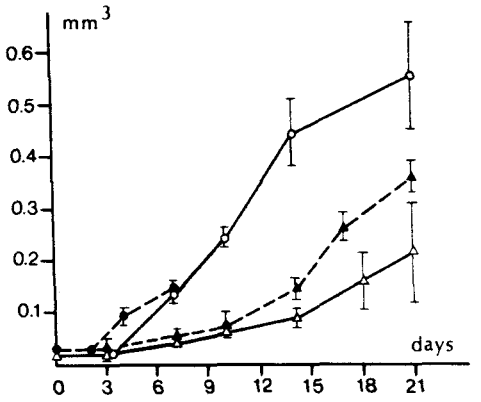

Figure 3. Growth of confronting pairs in organ culture. Ordinate: volume of confronting pairs of PHF with MO_4 cell aggregates at 37°C (●--●) and at 28°C (▲--▲) or with MO_428 cell aggregates at 37°C (O—O) and 28°C (Δ—Δ); median and extreme values from at least five cultures. Abscissa: time of culture.

Migration

Migration of both MO_4 and $MO_4$28 cell aggregates explanted on glass (figure 4) was slower at 28 than at 37°C. No alterations in the slopes of the migration curves were seen between days 7 and 14 of incubation.

Fucose incorporation during invasion

To evaluate interactions between PHF and MO_4 cells, 10 confrontations cultured at 37°C and 20 at 28°C were analysed after metabolic labelling with [^{3}H]fucose.

In confrontations at 37°C, MO_4 cells and heart tissue showed the same incorporation of [^{3}H]fucose at the onset of culture and during the invasion. Counting of grains over MO_4 cells as compared with heart tissue showed ratios between 0·68 and 0·74 in cultures fixed after 1 and 4 days.

In non-invasive (grade II) cultures at 28°C [^{3}H]fucose labelling was lower in the MO_4 cells than in the heart tissue, with a clear transition between both (figure 5). In such cultures the ratios of numbers of grains over MO_4 cells versus heart tissue were 0·15–0·16 after 1 day and 0·17–0·24 after 7 days. Hence lowering the temperature to 28°C decreased the incorporation of [^{3}H]fucose more in the MO_4 cells than in the heart tissue. When MO_4 cells had become invasive (grade III), MO_4 cell labelling in areas containing immunoreactive heart material was higher than in grade II cultures. In grade IV cultures a larger field with heavy MO_4 cell labelling was found in the area of invasion (figure 6). In such cultures MO_4 cells located at the periphery were less heavily labelled than MO_4 cells in the centre. Ratios of grain numbers over MO_4 cells as compared with heart tissue were between 0·34 and 0·39 in the centre and between 0·19 and 0·28 at the periphery. For cultures fixed after 14 days, in which the distinction between central and peripheral areas could no longer be made, a ratio of 0·50–0·57 was found.

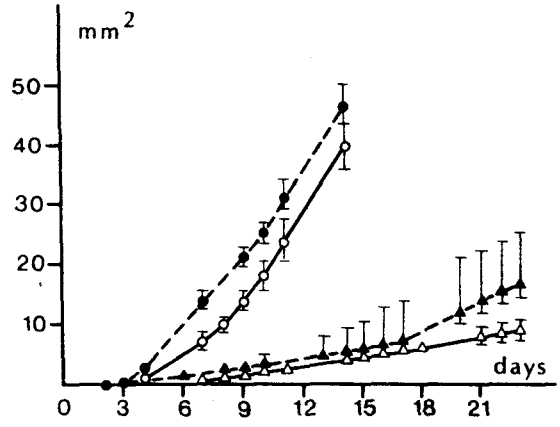

Figure 4. Directional migration of MO_4 and $MO_4$28 cells on glass. Ordinate: area covered by cells migrating from an aggregate explanted on a cover-slip; median and extreme values from at least five cultures; MO_4 cells at 37°C (●--●) and 28°C (▲--▲) or $MO_4$28 cells at 37°C (○—○) and 28°C (△—△). Abscissa: time of culture.

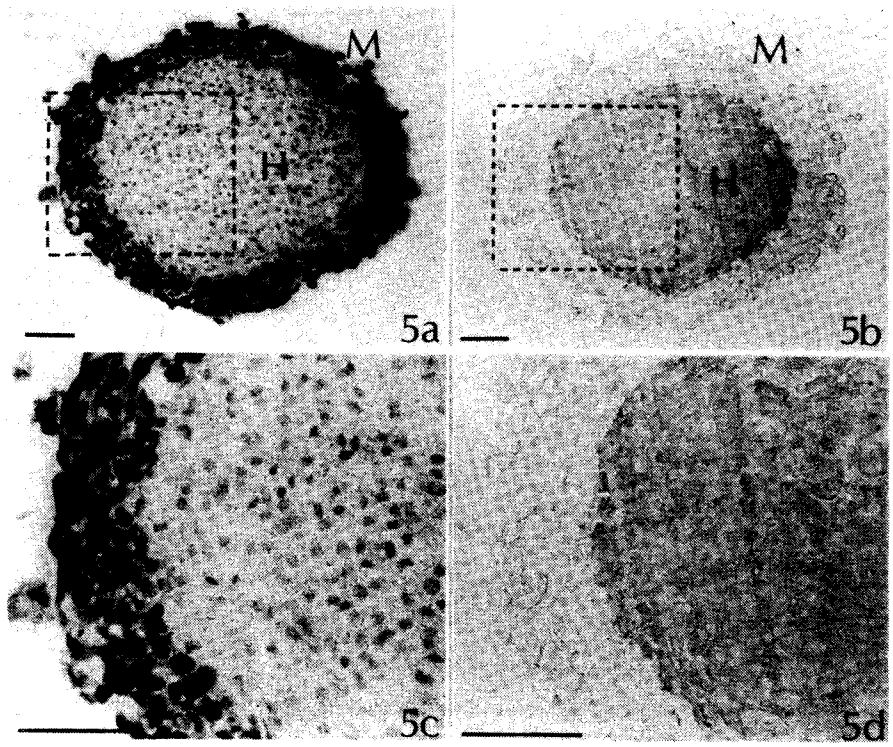

Figure 5. Light micrographs of consecutive sections from confrontations of MO_4 cells (M) with chick heart (H) incubated at 28 °C for 7 days and labelled with [^{3}H]fucose 16 h before fixation. Sections were processed for autoradiography (a) or stained with an antiserum against chick heart (b); (c) and (d) show boxed in areas in detail. Scale bar = 50 μm.

Discussion

These experiments show a correlation in time between transient suppression of invasion and transient inhibition of [^{3}H]fucose incorporation by MO_4 cells in confrontation with PHF at 28 °C.

We interpreted our observations as transient inhibition rather than reduced progression of invasion for the following reasons: (i) grading of invasion (see table 1) in each series of experiments showed a complete absence of invasion during the first 7 days; (ii) the progression of invasion between days 10 and 17 at 28°C was similar to that between days 0 and 7 at 37 °C (see figure 1); and (iii) pooling of all results and plotting the fraction of cultures showing invasion as a function of time (see figure 1 and table 1) indicated a transition from no invasion to maximal invasion between days 7 and 17.

We know from previous experiments that growth of confronting pairs has to be ascribed to proliferation of MO_4 cells [29]. Conditioning of PHF and MO_4 cell aggregates in direct contact at 28 °C led to an increase in the growth rate of MO_4 cells between days 10 and 14 (see figure 3), a phenomenon not observed with solitary MO_4

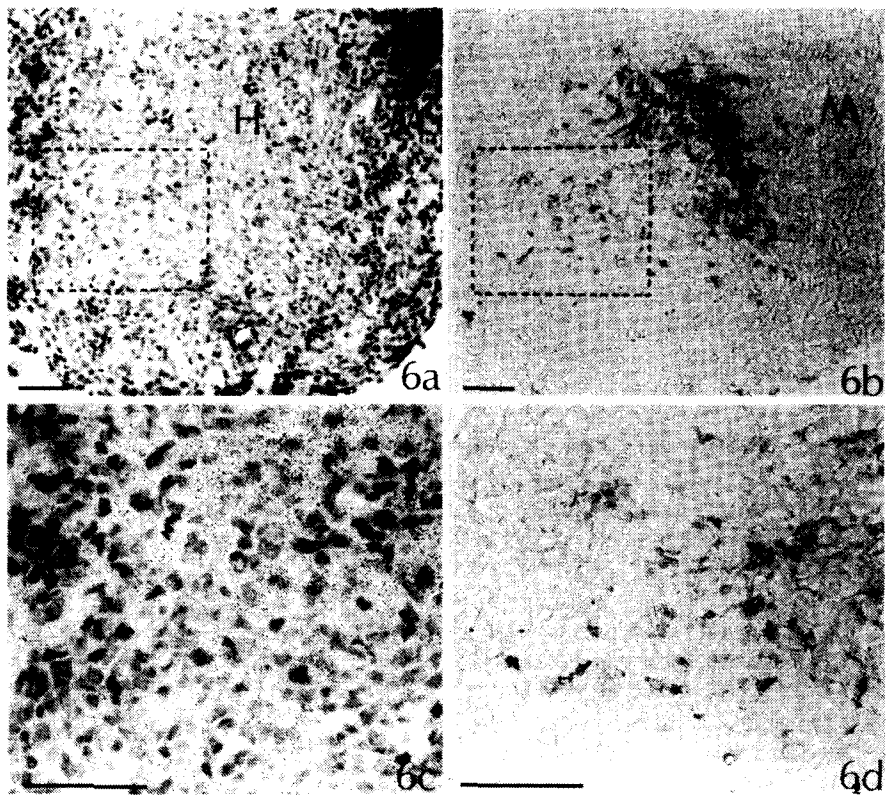

Figure 6. Light micrographs of consecutive sections (a and b) from confrontations of MO_4 cells (M) with chick heart (H) incubated at 28 C for 14 days and labelled with [^{3}H]fucose 16 h before fixation. Sections were processed for autoradiography (a) or stained with an antiserum against chick heart (b); (c) and (d) show boxed in areas in detail. Scale bar = 50 μm.

cell aggregates (see figure 2). It is unlikely, however, that this increase in growth rate was responsible for the start of invasion, for the following reasons: (i) the increased growth rate was observed with MO_4 cells but not with $MO_4$28 cells, whereas progression of invasion was seen for both; (ii) progression of invasion was unchanged when we confronted two MO_4 cell aggregates (diameters = 2 × 0·3 mm), instead of one (diameter = 0·2 mm), with one PHF (data not shown), indicating that the number of cells did not influence invasion; and (iii) we have shown previously that growth and invasion of MO_4 cells *in vitro* are unrelated [14].

Experiments with microtubule inhibitors have demonstrated a role of directional migration in invasion [28]. The course of directional migration of MO_4 cells at 28°C (see figure 4) provided no arguments to explain transient suppression of invasion in the present experiments in terms of transient suppression of directional migration. It should be noted that these observations on solitary MO_4 cell aggregates do not exclude the possibility that contact with PHF might increase the rate of directional migration of MO_4 cells at 28 C.

The possibility that a factor necessary for invasion was produced at a slow rate at 28°C, reaching a critical level after 7–10 days, appears unlikely, because separate preculturing of MO_4 cell aggregates and also PHF at 28°C before confronting them in the invasion assay did not influence the time schedule of invasion. The clear transition previously found in cultures at 30·5°C as compared with 29°C [15] also argues against this possibility.

Confrontations of MO_4 cell aggregates and PHF separately conditioned at 28°C strongly indicated that the onset of invasion was the result of short-range interactions between both partners. That both partners are actively implicated in invasion was also shown by confrontations between cells and tissues from exothermic and endothermic animals. At 28°C, frog tumour cells did invade into frog tissue but not into chick tissue, whereas mouse MO_4 cells invaded into frog tissue (unpublished results, in collaboration with R. G. McKinnell) but not into chick tissue (present data). Here, a possible implication of cell–cell communication is not excluded since metabolic cooperation *in vitro* between cells from distant classes of vertebrates has been described [25].

Histoautoradiographs of confronting cultures labelled with [^{3}H]fucose (see figures 5 and 6) demonstrate that: (i) in contrast to PHF, MO_4 cells non-invading at 28°C have a low incorporation of fucose (see also ref. 15); and (ii) after 14 days of incubation, MO_4 cells at 28°C start to invade and their incorporation of fucose is enhanced. Therefore, we suggest a metabolic conditioning of MO_4 cells at 28°C by the heart tissue, e.g. via junctional cell to cell contacts. Gap junctions between invasive cells and embryonic chick heart muscle have been demonstrated recently [4]. In view of the metabolic conditioning discussed above, the invasion of fragments from 14-day-old confronting cultures presumed to contain invading MO_4 cells (higher fucose incorporation) should have been more pronounced than that of fresh MO_4 cell aggregates (lower fucose incorporation). However, no difference in progression of invasion was observed. This might be explained by the fact that we took fragments from the periphery of confronting pairs in order to avoid necrotic material; such fragments might have consisted of MO_4 cells not taking part in the invasion process. Alternatively, restoration of invasion at 28°C through metabolic conditioning might be a temporary event. The fact that the increased number of grains over MO_4 cells coincided with areas previously consisting of heart tissue and/or still containing immunoreactive heart material might suggest that the labelled glycoproteins were produced by heart cells and not by the MO_4 cells. However, the label was added 16 h before fixation, and at that time only a few or no viable heart cells could be expected in the above-mentioned area. The observation rather indicates that MO_4 cells that had been in contact with viable heart cells had restored their capacity to incorporate fucosylation-presursor molecules into glycoproteins.

Although the lack of incorporation of [^{3}H]fucose indicates an alteration in the fucose metabolism, it should not necessarily be interpreted as a lack of fucosylated glycoprotein synthesis, since two pathways of fucosylation have been described. In the general pathway glucose is converted to GDP–mannose and to GDP–fucose, which is added to the oligosaccharide chain through fucosyltransferase [8, 9]; only in the salvage pathway is exogenous fucose phosphorylated to produce GDP–fucose [10].

In conclusion, our observations showed that the recovery of inhibition of invasion at 28°C was correlated with an increased fucose incorporation by the invading MO_4 cells. These and Bräuner's [4] observations suggest that metabolic

cooperation between invading cells and host cells is essential for the progression of invasion.

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Mémoire 6

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Decreased fucose incorporation in cell surface carbohydrates is associated with inhibition of invasion

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Invasion of malignant MO_4 cells into embryonic chick heart fragments in an organ culture assay was arrested for at least 7 days when the temperature was lowered to 28°C. Prolonged culturing of MO_4 cells at 28°C on tissue culture substrates showed no recuperation of fucose incorporation into cell surface glycopeptides. However, invasion was restored after 10 days of organ culture in confrontation with chick heart tissue at 28°C. A histoautoradiographic study showed that the regained capability to invade was accompanied by an increase in fucose labeling of the MO_4 cells in the invading areas. At 28°C the incorporation of [^{3}H]fucose into total cell protein was drastically reduced, whereas [^{3}H]leucine incorporation as a measure for protein synthesis was less affected. Cell surface glycopeptides, metabolically labeled with either fucose or glucosamine at 28°C, showed a time-dependent decrease in the incorporation of fucose but not of glucosamine and no changes in overall size distribution. Low temperature did not reduce fucosyltransferase activity but the relative accumulation of fucose-1-P suggested inhibited conversion towards GDP-fucose. Moreover, mouse L cells which were incapable of invading chick heart tissue appeared also deficient in fucose incorporation, owing to low levels of fucosyltransferase activity. According to the results, fucosylation of surface carbohydrates may be required for invasive capacity and restored in MO_4 cells invading at 28°C by metabolic cooperation with the host tissue.

Introduction

Malignant transformation has repeatedly been shown to be accompanied by alterations in the structure of fucose-containing, *N*-linked carbohydrate moieties of glycoproteins, exposed at the cell surface [26]. In addition, deficiencies in fucose metabolism [10, 25] and alterations in fucosyltransferase activity [6, 13, 22] have been related to altered tumorigenic and metastatic capacities. Fucose has also been implicated in normal tissue interactions such as proper homing of lymphocytes [14], cell-to-cell communication [27, 28] and embryonic development [9].

Since the acquisition of invasiveness marks the transition from a benign to a malignant condition during tumor progression, we were interested to know whether

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this transition was accompanied by alterations in cell surface glycoproteins. In previous papers [2, 23] it has been shown that induction of invasiveness in several cell lines was indeed associated with characteristic changes in cell surface glycoproteins. Furthermore, treatment of non-invasive cells with an alkyl lysophospholipid induced a similar change in the cell surface phenotype concomitant with invasiveness [2]. Although these observations suggested a positive correlation between the induction of cell surface carbohydrate changes and the acquisition of invasive behaviour, there is little evidence for the opposite, namely that the loss of invasion is accompanied by corresponding structural changes in cell surface carbohydrates, in particular changes involving glycoprotein fucosylation [6, 10, 13, 22, 25].

When malignant mouse MO_4 cells were confronted with fragments of embryonic chick heart in organ culture at 28°C, instead of 37°C, invasion was delayed for at least 7 days [15]. Prolonged culturing at 28°C of MO_4 cells confronted to chick heart tissue restored invasion through mutual conditioning of the two partners [5]. Furthermore, mouse L cells are communication incompetent and deficient in the fucosylation of surface carbohydrates, but correctable for these properties by fusion with competent cells [27, 28]. These two systems offer an excellent opportunity to test the above-described hypothesis, namely that loss of invasiveness may be accompanied by alterations in the fucosylation of carbohydrates.

Here, we report that suppression of invasion is indeed correlated with transient or permanent reduction of fucose incorporation into cell surface glycoproteins, indicating a mechanistic role of these cell components in invasive behavior.

Materials and methods

Cells and virus

MO_4 cells are C_3H mouse cells transformed by Kirsten murine sarcoma virus [1]. These invasive and metastasizing cells are frequently used in studies of the process of invasion [16]. $MO_4$28 cells were derived from MO_4 cells by colony formation at 28°C, and permanently maintained at this temperature [5]. The culture conditions and preparation of cell aggregates at 37 and 28°C were as described earlier [15].

The substrains L929 and A9 of transformed mouse L cells and the somatic cell hybrid A9 × GRSL mouse leukemia were as described [25, 26].

For preparing stocks of the Indiana serotype of *Vesicular stomatitis* virus (VSV; obtained by courtesy of Dr M. Van der Zeist, Utrecht), the virus was cloned twice and propagated in BHK cells [20] at a multiplicity of infection equal to 0·1 PFU/cell.

Preparation and purification of radiolabeled VSV

Monolayers of cells were infected at 20 PFU/cell with stock VSV in 0·1 of the normal volume of medium. After adsorption for 1 h the inoculum was removed and replaced by a regular volume of medium. At 5 h post-infection radiolabeled precursor sugar (L-[5,6-^{3}H]fucose, 60 Ci/mmol, 10 μCi/ml, L-[^{14}C]fucose, 60 mCi/mmol, 5 μCi/ml, D-[6-^{3}H]glucosamine hydrochloride, 30 Ci/mmol, 10 μCi/ml or D-[1-^{14}C]glucosamine hydrochloride, 60 mCi/mmol, 5 μCi/ml) was added. Radiochemicals were purchased from The Radiochemical Centre (Amersham, UK) or New England Nuclear, Boston, MA, USA). Virus was harvested at 16–20 h post-infection and purified by density centrifugation on a linear 20–50 per cent (w/w) sucrose gradient in TEVS buffer (20 mM Tris–HCl, 1 mM

EDTA, 100 mM NaCl and 0·1 per cent serum, pH 7·4) for at least 6 h at 4°C at 25 000 rpm in a Beckman SW 27·1 rotor. Gradients were fractionated and the VSV-containing fractions were subjected to proteolytic digestion [30]. The viral glycopeptides were dialyzed (MW cut-off ca 3500) against deionized water or desalted by Bio-Gel P-2 gel filtration centrifugation and subsequently analyzed by gel filtration on a Bio-Gel P-6 column (200 × 1 cm i.d.), eluted with 0·1 M ammonium acetate (pH 8·0).

Labeling of glycopeptides

Total cellular incorporation of fucose, *N*-acetylmannosamine (sialic acid precursor) and leucine was analyzed as a measure of glycoprotein fucosylation, sialylation and overall protein synthesis, respectively. Semi-confluent cultures were incubated with L-[5,6-^{3}H]fucose (60 Ci/mmol, 1 μCi/ml), *N*-acetyl-D-[6-^{3}H]mannosamine (10 Ci/mmol, 10 μCi/ml) or L-[4,5-^{3}H]leucine (58 Ci/mmol, 0·5 μCi/ml) 16 h before harvest. The cells were washed twice with phosphate-buffered saline (PBS) and scraped off with a rubber policeman in 8 ml of PBS, followed by precipitation in perchloric acid (0·5 N) on nitrocellulose filters (0·2 μm) (Millipore, Bedford, MA, USA). The filters were extracted twice with 5 ml of chloroform–methanol (2 : 1, v/v), dissolved in 10 ml of Picofluor (Packard, Groningen, The Netherlands) and counted in a liquid scintillation counter. Parallel cultures were run for protein determination using Bio-Rad reagents (Bio-Rad Laboratories, Richmond, CA, USA).

Glycopeptides were isolated from cells, metabolically labeled for 16 h at 37 or 28°C with L-[1-^{14}C]fucose (0·5 μCi/ml) or L-[5,6-^{3}H]fucose (1 μCi/ml) and with D-[1-^{14}C]glucosamine hydrochloride (0·5 μCi/ml) or D-[6-^{3}H]glucosamine hydrochloride (1 μCi/ml). Glycopeptides were isolated from the cell surface by trypsinization, further digested with Pronase (Calbiochem, San Diego, CA, USA), dialyzed (MW cut-off 3500) and subsequently analyzed on a 100 × 1 cm i.d. column of Sephadex G-50 (superfine) as described [32]. Co-chromatography of differentially labeled glycopeptides excluded that changes in the elution profiles were due to small variations in resolution of the columns.

Precursor pool analysis

Cells grown at 37 or 28°C were incorporated with either L-[5,6-^{3}H]fucose (5 μCi/ml) or *N*-acetyl-D-[6-^{3}H]mannosamine (10 μCi/ml) for 4 h. After two washes with PBS, cells were harvested by scraping and centrifuged at 3000 rpm for 10 min. An aliquot of the pellet was used for protein determination and the remainder was extracted with chloroform–methanol–water (3 : 2 : 1, v/v/v). The upper phase, containing the radiolabeled precursors, was separated from the protein-containing interphase and the lipid-containing lower phase by repeated washes. Subsequently, the upper phase was evaporated to dryness under a stream of nitrogen, dissolved in the elution buffer (10 mM Bis-Tris, pH 6·4) and analyzed by anion-exchange chromatography. The Mono Q column (PR5) (Pharmacia, Uppsala, Sweden) was eluted with a discontinuous gradient of 0–120 mM NaCl in 10 mM Bis-Tris (pH 6·4). Fractions of 0·3 ml were collected and counted in a liquid scintillation counter. During the whole procedure unlabeled nucleotides were present to prevent degradation of the radiolabeled precursors. Radiolabeled L-[5,6-^{3}H]fucose, L-[1-^{14}C]fucose, GDP-L-[U-^{14}C]fucose (150 mCi/mmol) and [2-^{3}H]AMP (15 Ci/mmol) as well as CMP-*N*-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid (200 mCi/mmol), *N*-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid (200 mCi/mmol), *N*-acetyl-D-[6-^{3}H]man-

nosamine (10 Ci/mmol) and *N*-acetyl-D-[U-^{14}C]mannosamine (200 mCi/mmol) were used as column markers.

Fucosyltransferase and sialyltransferase assay

Fucosyltransferase and sialyltransferase were assayed in a total volume of 150 μl containing 50 mM Tris buffer (pH 5·3), 0·2 per cent (v/v) Trition X-100, 5 mM $MnCl_2$ and 20 μl of cell homogenate (equivalent to 10^6 cells in the same buffer) as enzyme source. For the determination of fucosyltransferase activity this mixture further contained 2 mM GMP, donor GDP-[1-^{14}C]fucose (26·5 μCi/ml, 2 mCi/mmol) and acceptor α-fetuin (200 μg), desialylated and degalactosylated as described [21, 29]. For the determination of sialytransferase activity, CMP-[^{14}C]neuraminic acid (1·8 mCi/mmol) and desialylated α-fetuin were used as donor and acceptor, respectively. After incubation for 2 h, the reaction mixture was precipitated with an equal volume of ice-cold perchloric acid (0·5 N) and the precipitates were collected on filters. After several washes with perchloric acid followed by extraction with chloroform–methanol (2 : 1), the filters were dissolved in 10 ml of Picofluor and counted in a liquid scintillation counter.

Invasion assay

Confrontations of MO_4 or $MO_4$28 cell aggregates and precultured fragments of 9-day-old embryonic chick heart (PHF) were incubated (for 7–21 days) at 37 or at 28°C as described earlier [17]. Because of the incompetence of the L cells to produce useful spheroids, they were tested in the invasion assay as dense suspensions confronted with chick heart fragments.

In some experiments PHF and MO_4 or $MO_4$28 cell aggregates were separately preincubated at 28°C for 14 days and confronted afterwards. Invasion was studied histologically on consecutive sections stained with hematoxylin–eosin or with an antiserum against chick heart [18], and scored with the following classification: grade I and II, when MO_4 cells were found at the periphery of the outer fibroblastic layers of the precultured heart fragment or occupied the outer fibroblastic layers, respectively (denoted as −); grade III, when MO_4 cells replaced the cardiac muscle to less than half (denoted as +); and grade IV, when MO_4 cells replaced the cardiac muscle to more than half (denoted as + +). According to Mareel *et al.* [16], grades III and IV meet the criteria of invasion.

Autoradiography of fucose incorporation

Invasion was assayed as described above in the presence of L-[6-^{3}H]fucose (1 μCi/ml, 16·8 Ci/mmol) for 16 h before fixation. Consecutive sections were processed as described above or subjected to autoradiography. Briefly, the sections were coated with a gelatin–potassium chromic sulfate solution (0·5 and 0·05 per cent, respectively) at 60°C, dried and overlayered with a fine grain autoradiographic stripping plate (AR 10, Kodak, UK). After exposure times of 14–21 days the plates were developed (Kodak D19), fixed (Agofix) and stained with hematoxylin–eosin and grains were counted in representative areas.

Results

Effect of low temperature on cell surface glycopeptides

The effect of culture on tissue culture substrate at 28°C (corresponding to the non-permissive temperature for invasion in organ culture [15]) on the incorporation

of radiolabeled sugars into total cellular proteins and cell surface glycopeptides and on the size distribution of the latter was analyzed in MO_4 and $MO_4$28 cells.

Figure 1 shows the perchloric acid precipitable radioactivity of MO_4 cells grown at 28°C for various days and labeled with [^{3}H]fucose or [^{3}H]leucine for 16 h prior to harvesting. Lowering the temperature from 37 to 28°C drastically reduced the incorporation of fucose to almost 10 per cent of control values, while leucine incorporation was inhibited by maximally 50 per cent. Slightly higher steady-state levels of incorporation were reached after 7 days, which remained unchanged during prolonged culturing at 28°C up to 21 days. Similar incorporations with *N*-acetyl-[^{3}H]mannosamine, being a specific precursor for neuraminic acid residues, revealed that culturing at 28°C had less effect on the incorporation of this sugar compared to fucose, *viz.* a minimum of about 30 per cent of control values at day 3 (data not shown). $MO_4$28 cells, continuously grown at 28°C, differed from MO_4 cells at 28°C specifically in a persistently very low level of fucose incorporation (figure 1, broken line) whereas the levels of leucine (figure 1, between dotted lines) and *N*-acetylmannosamine (data not shown) incorporation by $MO_4$28 cells were similar to those in MO_4 cells at 28°C.

Cell surface glycopeptides, metabolically labeled with fucose or glucosamine, were studied in MO_4 cells grown at 37°C or for various days at 28°C. Co-chromatography of glycopeptides labeled with fucose and normalized for total

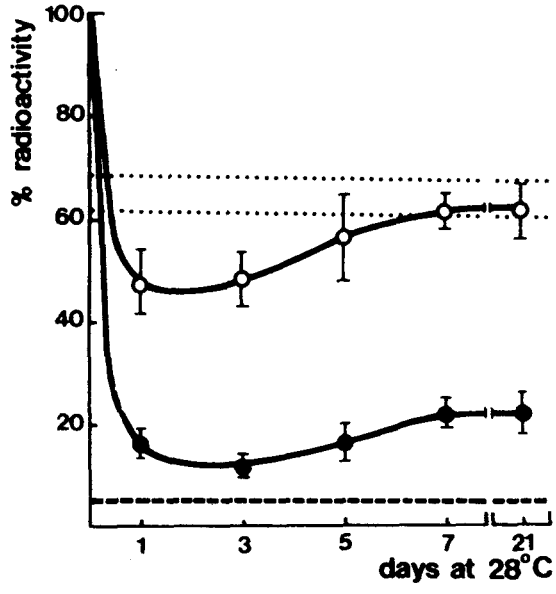

Figure 1. Perchloric acid-precipitable radioactivity in MO_4 cells grown at 28°C for various numbers of days and incubated with [^{3}H]fucose (closed symbols) or [^{3}H]leucine (open symbols) for 16 h before harvest. Perchloric acid-precipitable radioactivity in $MO_4$28 cells continuously grown at 28°C and incubated with [^{3}H]fucose (broken line) or [^{3}H]leucine (between dotted lines) for 16 h before harvest. Ordinate: radioactivity as a percentage of controls at 37°C; mean ± S.E.M. from two triplicate measurements. Abscissa: time of incubation.

cellular protein revealed that fucose incorporation was equally reduced in all size classes and this reduction progressed with the time of culturing at 28°C (figure 2 A). However, a similar analysis using labeled glucosamine (which incorporates in *N*-acetylglucosamine and *N*-acetylneuraminic acid residues) did not show an effect of the lower temperature (figure 2 B). Whereas it appeared almost impossible to label the surface glycopeptides of $MO_4$28 cells with fucose (cf. data in figure 1), the glucosamine-labeled material from $MO_4$28 cells showed a profile very similar to that found with MO_4 cells (figure 2). Figure 3 summarizes the total label incorporation in the eluted glycopeptides (fractions 50–90) at a constant amount of total cell protein. Thus, the combined data indicate that low temperature specifically altered fucose incorporation and not the overall size distribution of cell surface glycopeptides or the incorporation of other sugars.

Fucose metabolism at low temperature

To study the mechanism of the reduction in fucose utilization, fucosyltransferase activity was measured and compared with sialyltransferase activity. The results summarized in table 1 demonstrate that the enzyme activities had not diminished but

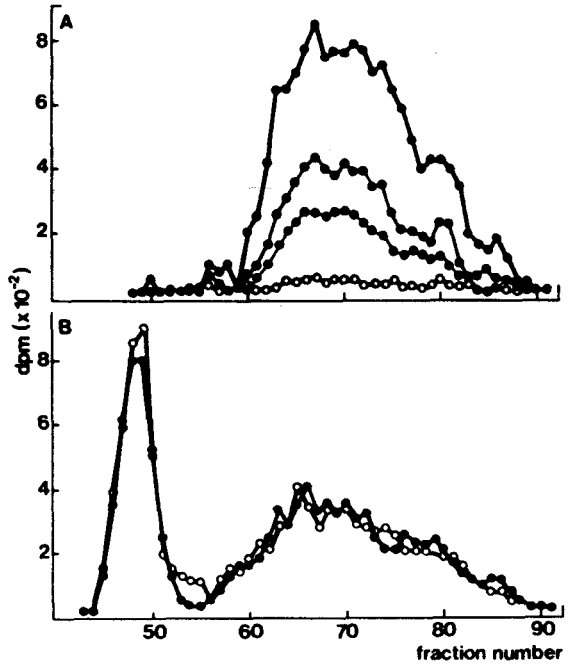

Figure 2. Sephadex G-50 gel filtration profile of glycopeptides derived from MO_4 cells grown at 37°C (closed symbols, thick line), at 28°C for 7 days (closed symbols, intermediate thickness line) or 21 days (closed symbols, thin line) and from $MO_4$28 cells grown at 28°C (open symbols). Glycopeptides were proteolytically derived from cells after metabolic labeling with [^{3}H]fucose (A) or [^{3}H]glucosamine (B) as described under Materials and methods. The size-distribution profiles were corrected for free label, lined up and superimposed by co-chromatography of ^{14}C-labeled material. Ordinate: total dpm/mg cell protein. Abscissa: fraction numbers.

Table 1. Fucosyltransferase and sialyltransferase activity of MO_4 and L cells.

Cells	Temperature (°C)	Fucosyltransferase activity	Sialyltransferase activity
MO_4	37	7·5 ± 0·6[a]	75·2 ± 8·5
MO_4	28[b]	13·3 ± 1·8	62·5 ± 9·3
$MO_4$28	28	11·6 ± 1·5	66·1 ± 6·1
L cells	37	1·6 ± 0·6	56·6 ± 7·1
L × GRSL[c]	37	16·1 ± 2·0	73·3 ± 9·8

The enzyme activities were measured in crude extracts of cells grown at 37 or at 28°C, using asialoagalactofetuin or asialofetuin as acceptor. Specific conditions as described under Materials and methods.

[a] Mean values from three experiments ± S.E.M., expressed as dpm × 10^{-3}/mg cellular protein/h.

[b] Cultured for 3 days at 28°C.

[c] Somatic hybrid of mouse L cells and mouse leukemic GRSL cells.

rather increased in cells grown at 28°C. In contrast, the reported reduction of fucose incorporation in mouse L cells (substrains L929 and A9) [27, 28] may well be ascribed to a reduction in fucosyltransferase activity as this was only 10 per cent of the activity found in the corrected somatic cell hybrid of the L cells (shown for strain A9, table 1) and also much lower than in MO_4 cells. The sialyltransferase activities in MO_4 cells grown at 37 or 28°C were not significantly different and comparable to those found in L cells.

To analyze further the effect of temperature on fucose metabolism, the conversion of fucose into the activated precursor GDP-fucose *via* fucose-1-phosphate was measured. The [^{3}H]fucose-containing precursor pools were isolated from MO_4 cells cultured for various days at 28°C and separated in free fucose, fucose-1-P and GDP-fucose by high-performance liquid chromatography (HPLC) on an ion-exchange column. The amount of radioactivity in the precursor pools relative to total cellular protein and the partition over the individual pools is summarized in table 2. The overall amount of precursors had declined by more than

Table 2. Partition of label over fucose precursors.

Days at 28°C	Per cent of total radioactivity[a] F[b]	F-1-P	GDP-F	Total precursor pool (dpm/mg protein)
MO_4 cells				
0	53·1	11·1	35·8	152000
	50·7	11·7	37·6	190000
4	22·3	39·7	38·0	65190
	24·7	40·0	35·3	68370
12	25·6	38·2	36·2	32300
15	20·6	43·2	36·2	43750
$MO_4$28				
continuous	26·2	26·5	47·3	27880

[a] [^{3}H] Fucose-containing precursors were separated by HPLC on an ion-exchange column.

[b] F, fucose.

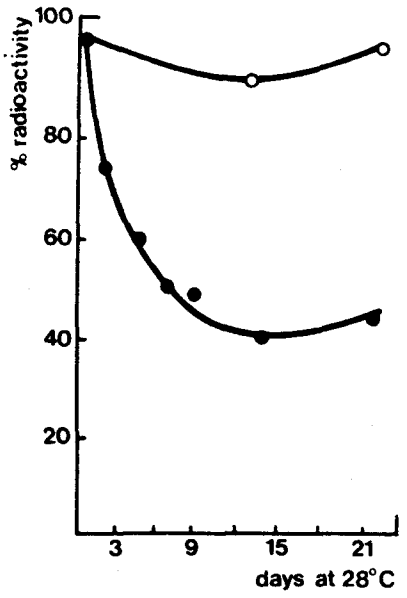

Figure 3. Total radioactivity incorporated in glycopeptides [^{3}H]fucose (closed symbols) or [^{3}H]glucosamine (open symbols), eluted from gel filtration columns as illustrated in figure 2. Ordinate: radioactivity as a percentage of controls at 37°C. Abscissa: time of incubation.

50 per cent after 4 days of culture at 28°C, reaching a minimum of about 20 per cent after 12 days, and was even lower in $MO_4$28 cells. Moreover, the partition of label over the three precursors was drastically changed. The relative amount of fucose-1-P had increased approximately 4-fold, whereas the amount of free fucose had decreased about 2-fold. The distribution of the fucose-containing precursors in $MO_4$28 cells was different from MO_4 cells at 28°C, indicating a different metabolic defect in these cells which may account for their persistently low incorporation of exogenous fucose (cf. figure 1). When *N*-acetylmannosamine was used instead of fucose, such redistribution over the various precursor pools (i.e. *N*-acetylmannosamine, *N*-acetylmannosamine-6-P, *N*-acetylneuraminic acid-9-P, *N*-acetylneuraminic acid and CMP-*N*-acetylneuraminic acid) was not observed (data not shown).

To establish whether the reduction in fucosyltransferase activity in L cells was the only lesion in carbohydrate biosynthesis, they were monitored for their capacity of proper protein-bound carbohydrate biosynthesis by lytic infection with VSV. VSV infecting L cells (strain A9) contained a normal set of glucosamine-labeled carbohydrate units, terminating in 0 to maximally 3 sialic acids residues, respectively (figure 4; cf. ref. 12). Unlike the control BHK cells, however, the carbohydrates synthesized in infected L cells did not detectably incorporate fucose. The small shift in elution of the various peaks of glucosamine-labeled VSV/L glycopeptides compared with fucose-labeled VSV/BHK glycopeptides corresponded with the difference in molecular weight due to presence or absence of fucose residues (unpublished results).

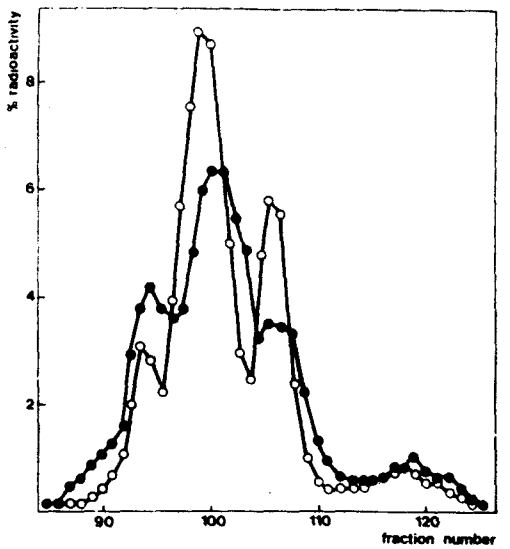

Figure 4. Bio-Gel P-6 gel filtration of glycopeptides from VSV grown in L cells as compared with BHK cells. Glycopeptides from glucosamine-labeled VSV/L and fucose-labeled VSV/BHK were mixed and analyzed co-chromatographically. Closed symbols, [^{3}H]glucosamine VSV/L glycopeptides; open symbols, [^{14}C]fucose VSV/BHK glycopeptides. The peaks (fractions 92–96, 98–104, 105–110 and 115–124) represent glycopeptides containing 3–0 sialic acid residues, respectively [12]. Ordinate: radioactivity as a percentage of total eluted radioactivity. Abscissa: fraction numbers.

Fucosylation and invasion

Previously, it was shown that the specific inhibition of fucose incorporation in cell surface carbohydrates on MO_4 cells was concomitant with an arrest of invasion into PHF at 28°C [15]. Invasion of MO_4 cells at 28°C was restored to normal progression after prolonged culturing at 28°C, probably through mutual conditioning [5]. Fucose incorporation of chick heart target tissue was, however, much less affected by lowering the temperature to 28°C, i.e. a reduction of about 25 per cent versus 80 per cent in MO_4 cells, as evident from histo-autoradiographic analysis (data not shown; cf. ref. 5). In view of the presumed role of fucose in invasion, the possibility that fucose incorporation of invading cells was concomitantly restored was therefore investigated. As described in a previous paper [5], the invasion analysis was repeated in the presence of radiolabeled fucose during the last 16 h of confrontation. After fixation and serial sectioning, consecutive sections were processed for autoradiographic analysis or stained with an antiserum against chick heart. The invading MO_4 cells were indeed more heavily labeled than peripherally located MO_4 cells, suggesting mutual conditioning [5] and the possibility of a prominent role for fucose.

To study further the corollary that fucosylation of surface glycoproteins is required for invasiveness, fucosyltransferase deficient L cells (L929 and A9 substrains) were tested for their ability to invade into chick heart tissue (table 3). In the confronting cultures the L cells were actively growing and completely surrounded the chick heart fragments (figure 5). However, the cells failed to invade

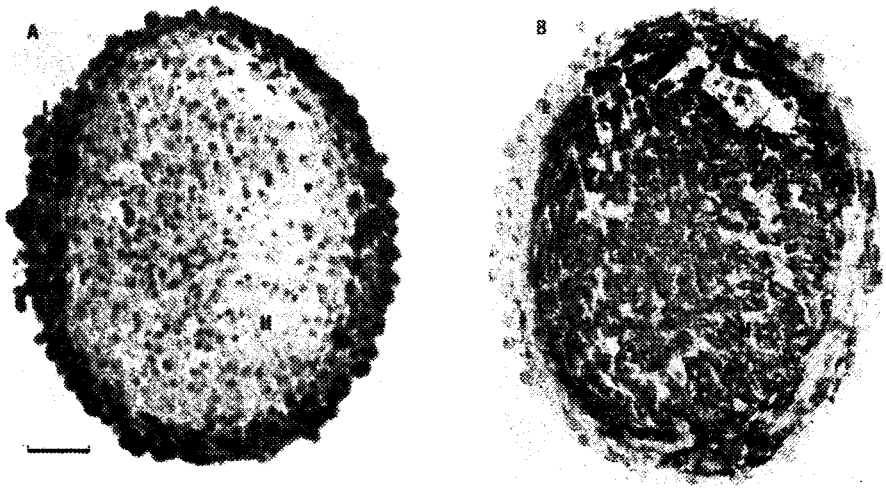

Figure 5. Photomicrographs of 8-μm thick sections of confrontations between L929 cells (L) and precultured heart fragments (H), fixed after 7 days and stained with hematoxylin–eosin (A) or with an antiserum against chick heart (B). Scale bar: 50 μm.

Table 3. Analysis of the interaction of L cells with precultured heart fragments in organ culture.

	Grading after		
Cell	Day 2	Day 4	Day 7
L929	−(3)[a]	−(3)	−(1), ±(1)
A9	−(3)	−(3)	−(5)
A9 × GRSL[b]	−(4)	−(2), ±(2)	±(4)

[a] Number of cultures in parentheses.
[b] ±: Some single cell infiltration (up to 20 per section).

and destroy the target tissue. On the other hand, the fucosyltransferase corrected AGR hybrid appeared also unable massively to invade the chick heart tissue, but a few cells appeared able to infiltrate the heart tissue.

Discussion

This study concerned the effect of reduced fucose incorporation into glycoproteins on the capacity to invade normal tissue. Lowering the temperature to 28°C reduced the incorporation of [^{3}H]fucose into surface glycopeptides of malignant MO_4 cells (figures 2 and 3), whereas $MO_4$28 cells, permanently grown at 28°C, were totally inhibited in this respect (figure 1). This temperature-dependent inhibition was fairly specific for fucose since the incorporation of glucosamine and *N*-acetylmannosamine was less affected and almost paralleled the overall inhibition of protein synthesis as monitored by leucine incorporation.

Fucosyltransferase activities of MO_4 and MO_428 cells grown at 28°C were slightly increased rather than decreased relative to the activities in cells grown at 37°C (table 1). No effect of low temperature was found for the sialytransferase activities. Analysis of the label distribution in the precursor pools of MO_4 cells showed a temperature-dependent reduction, but with a relative accumulation in the fucose-1-P pool (table 2). Apparently, the reduced [^{3}H]fucose utilization at lower temperature is the result of inhibited conversion of fucose-1-P into GDP-fucose and not of effects on glycosyltransferases. The fact that fucose is the only sugar which utilizes phosphorylated guanosine for its activation for protein glycosylation may provide for a clue to explain the relative specific effect of low temperature on the incorporation of this sugar. A similar difference in fucose utilization has been observed in high and low metastatic lymphoma variants: low metastatic cells accumulated fucose-1-P without incorporation of fucose into protein-bound carbohydrates [20]. Nevertheless, a more extensive kinetic study of the fucose utilization is needed for final clues.

A previously reported deficiency in utilization of [^{3}H]fucose in transformed mouse L cells [27, 28] could now be ascribed to a drastic reduction in fucosyltransferase activity to values of about 10 per cent of that found in AGR cells, i.e. cells corrected by somatic hybridization with mouse leukemia cells (table 1). In agreement with Braüner [4], the L cells were unable to invade normal chick heart tissue (figure 5). Apparently, reduced fucose incorporation either by low temperature (MO_4 cells) or by a genetic deficiency in fucosyltransferase (L cells) is involved in arresting the invasive capacity of the cells.

Prolonged incubation of MO_4 cells confronted with chick heart tissue at 28°C showed a resumption of invasion after 10 days [5]. The MO_4 cells which actively invaded chick heart tissue in these long-term confrontations regained the capacity to incorporate [^{3}H]fucose, as measured in a histo-autoradiographic analysis. This restoration of fucose incorporation was specifically observed in the invading areas and not in the periphery of the confrontations nor in MO_4 aggregates grown separately at 28°C. Similarly, preculturing at 28°C of MO_4 cells did not shorten the delay in invasion. Apparently, metabolic cooperation between MO_4 cells and the host tissue at 28°C corrected the deficiency in fucose utilization and is required for resumption of the invasive capability of MO_4 cells which they possessed before being put at 28°C. In contrast, the fucosyltransferase-deficient mouse L cells remained incapable of invasion even after 21 days (data not shown). This is conceivable since gap-junctional communication does not allow transport of large and membrane-bound enzymes, i.e. fucosyltransferase as opposed to low molecular weight precursors involved in the inhibition of fucose incorporation in MO_4 cells at 28°C. Contrary to expectations, only a few cells of the fucosyltransferase-corrected hybrids of L cells had regained invasive capacity (table 3). This may not necessarily invalidate the suggested correlation between glycoprotein fucosylation and invasiveness. The non-segregating mouse–mouse hybrids represent a mixed phenotype of L fibroblasts and GRSL leukemic cells which may be incompetent in tissue interaction required for invasion. Moreover, the marginal invasion may also be due to mechanical obstruction since the hybrids were 2–3 times larger than either individual cells. Comparable size-dependent minimal invasion was previously suggested for the myeloid leukemia cell lines K652 and HL-60 in contrast with leukemic blasts, freshly obtained from the peripheral blood of a patient with acute lymphocytic leukemia, which massively invaded the test tissue [23]. This question

could be resolved by transfection of the fucosyltransferase gene into small-sized L cells.

The possibility that the resumption of invasion at low temperature in MO_4 cells was due to mutual conditionings other than communication-dependent restoration of fucose incorporation cannot be ruled out. However, many studies [3, 7, 11, 19, 20, 24] have indicated a crucial role for surface carbohydrates in invasion and metastasis. It is conceivable that the absence of fairly specific, terminal fucose residue will have profound implications for the functioning of these surface molecules, as already described for metastasis [25] and normal tissue interactions [9, 14, 31].

Acknowledgements

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CONCLUSION

L'invasion des cellules MO_4 dans des fragments de coeur et de poumon d'embryon de poulet est inhibée à 28°C pendant au moins 7 jours. Ce phénomène n'est pas dû aux différences de croissance, de migration directionelle ou d'assemblage et de désassemblage des microtubules des cellules MO_4. Les différences d'attachement des cellules MO_4 aux FCPs ne peuvent non plus expliquer l'inhibition à 28°C. A cette température l'incorporation de fucose dans la totalité des protéines cellulaires est fortement réduite, tandis que l'incorporation de leucine, comme mesure de la synthèse des protéines, avait beaucoup moins changé. L'analyse des N-glycosylpeptides de la surface cellulaire par chromatographie sur gel démontre qu'il n'y a pas de changements de taille après marquage à la glucosamine. La dimunition de la température n'affecte pas la fucosyltransférase, mais bien les précurseurs fucosylés. De plus, une accumulation de fucose-1-P suggère une inhibition de la conversion en GDP-fucose. Ceci est moins clair pour la lignée MO_4 clonée et maintenue à 28°C, que pour les cellules MO_4 conditionées temporairement à 28°C. De plus les cellules L929, qui sont incapable d'envahir le tissu cardiaque, sont déficientes pour l'incorporation de fucose par une activité trop faible de la fucosyltransférase. Par contre, les cellules hybrides L-GRSL, corrigées pour leur manque d'activité de fucosyltransférase, sont capables d'envahir le FCP.

Des expériences, allant jusqu'à 21 jours d'incubation, ne démontrent pas un redémarrage de l'incorporation de fucose dans les N-glycosylpeptides de la surface cellulaire MO_4, cultivé en mono-couche.

Pourtant l'invasion des cellules MO_4, incubées ou clonées et en contact avec le FCP à 28°C, est restorée après 10 jours d'incubation. La progression de l'invasion entre 10 et 17 jours d'incubation à 28°C est comparable à celle observée entre 0 et 7 jours à 37°C. Dans les deux cas il faut 7 jours pour remplacer complètement un FCP, ayant un diamètre de 0.4 mm au début du test. Le déroulement de l'invasion change à peine quand les cellules MO_4 ou le tissu cardiaque ou les deux sont préincubées séparément pendant 14 jours à 28°C avant leur confrontation. Il a été

démontré par autoradiographie que là, ou l'invasion des cellules MO_4 démarre à 28°C, il y a une augmentation de l'incorporation métabolique du fucose dans les cellules MO_4. Nos observations suggèrent donc qu'une collaboration métabolique entre les cellules MO_4 et le tissu cardiaque est nécessaire pour la progression de l'invasion.

CHAPITRE III : LES EFFETS DES INHIBITEURS DE LA GLYCOSYLATION SUR L'INVASION DES CELLULES MO_4 ET L'EXPRESSION DES N-GLYCOSYLPEPTIDES DE LA SURFACE CELLULAIRE

INTRODUCTION

L'invasion marque la différence entre une tumeur bénigne et une tumeur maligne, cette dernière étant localement agressive et potentiellement métastatique (Mareel, 1980). L'adhésion entre la cellule et le substrat, la sécrétion de facteurs lytiques, et la migration directionelle sont des activités cellulaires impliquées dans l'invasion (Bruyneel et Mareel, 1981; Mareel et De Mets, 1984; Sträuli et Haemmerli, 1984; Liotta, 1986). Pourtant, aucune de ces activités cellulaires n'est spécifique pour des cellules malignes invasives. Nous avons suggéré que le contrôle de ces activités pourrait se situer au niveau de la transcription ou de la translation et dépenderait de la transmission de signaux endogènes ou exogènes (Bracke et coll., 1986; Mareel et Van Roy, 1986). Les glycoconjugués exposés à la surface cellulaire sont parmi les premiers candidats de la perception de ces signaux et peut-être aussi de la transduction de ces signaux vers l'intérieur de la cellule. Cela explique notre intérêt dans le rôle des glycoconjugués dans l'invasion tumorale. Des changements dans l'expression de glycannes à la surface cellulaire ont été liés aux différents aspects de l'invasion et de la métastase (Schirrmacher et coll., 1982; Yogeeswaran, 1983; Smets et Van Beek, 1984; Dennis et Laferte, 1987; Mareel et De Mets, 1989).

Les inhibiteurs de la glycosylation interviennent dans les différentes étapes du cycle de dolicholphosphate ou dans le processus de l'élagage ou de l'élongation des chaînes glycanniques (Elbein, 1987).

Les inhibiteurs de glucosidases ou de mannosidases peuvent provoquer une accumulation de certains types de chaînes glycanniques. Nous avons utilisé ces substances

dans le but d'étudier si l'intégrité des chaînes glycanniques à la surface cellulaire serait requise pour l'invasion des cellules MO_4.

L'invasion des cellules MO_4 dans les FCPs a été démontrée en culture organotypique (Mareel et coll., 1979). La spécificité du test et son importance pour certains aspects de l'invasion in vivo ont été discutés (Mareel, 1982). Les inhibiteurs de la glycosylation tel que la tunicamycine, la 2-désoxy-D-glucose, la β-hydroxynorvaline, la monensine, la 1-désoxynojirimycine, la N-méthyl-1-désoxynojirimycine, la castanospermine, la 2,5-dihydroxyméthyl-3,4-dihydroxypyrrolidine, la 1-désoxymannojirimycine et la swainsonine ont été utilisées dans le test d'invasion. En plus, leurs effets biochimiques ont été étudiés par chromatographie sur gel des glycopeptides N-liés des cellules MO_4 et des cellules cardiaques après marquage métabolique par le [6-^{3}H] fucose ou par le [2-^{3}H] mannose (Gilfix et Sanwal, 1984).

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Effect of inhibitors of glycosylation and carbohydrate processing on invasion of malignant mouse MO_4 cells in organ culture†

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Inhibitors of glycosylation and carbohydrate processing were used to investigate the role of carbohydrates exposed at the cell surface in invasion. Malignant mouse MO_4 cells were confronted with embryonic chick heart in organ culture, an assay shown to be relevant for a number of aspects of invasion *in vivo*. Tunicamycin (1·0 μg/ml), 2-deoxy-D-glucose (100 mM), β-OH-norvaline (1·0 mM), and Monensin (0·1 μg/ml) reversibly inhibited the invasion of MO_4 cells. At these concentrations the drugs also inhibited the growth of MO_4 cells. 1-Deoxynojirimycin (10 mM), swainsonine (0·4 μg ml), and Marcellomycin (0·1 μg ml) permitted invasion. Marcellomycin also reversibly inhibited the growth of MO_4 cells. These results show that drugs known to interfere with the glycosylation or processing of carbohydrate chains of glycoproteins in different ways have different effects on the invasion of MO_4 cells *in vitro*.

Introduction

Carbohydrates exposed at the cell surface might be implicated in tumour invasion [35, 37]. Differences in invasive capacity between T cell lymphoma variants have been explained on the basis of differences in the expression of cell surface carbohydrates [36], and the absence of invasion of MO_4 cells at temperatures lower than 29° has been shown to coincide with a decrease of fucose incorporation into cell surface carbohydrates [22]. In baby rat kidney cells transfected with Adenovirus 12 gene sequences, differences in surface carbohydrates were correlated with differences in invasiveness (J. Bolscher, personal communication). A correlation between cell surface carbohydrates and activities related to invasion has been described also with normal cells: mature granulocytes transiently express altered carbohydrates during egression from the bone marrow [42]. The amount of cell surface sialic acid influences the ability of T-derived lymphocytes to leave the thymus, to circulate, or to home in other lymphoid tissues [9, 31]. The trophoblast undergoes temporal changes in lectin binding prior to implantation into the uterine wall [6]. In all these cases a causal relationship between invasion, egression, or implantation and surface changes is difficult to establish.

Recently, drugs that interfere at different levels with the glycosylation of glycoproteins and with the processing of their carbohydrate moieties became

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available. It appeared to us that comparative experiments with a series of such drugs might allow us to test the hypothesis that distinct alterations of cell surface carbohydrates could alter the invasive behaviour of tumour cells. We have, therefore, examined the effect of tunicamycin, 2-deoxy-D-glucose, β-OH-norvaline, monensin, 1-deoxynojirimycin, swainsonine, and Marcellomycin on the invasion of MO_4 mouse cells using an organ culture assay that proved to be useful for the study of anti-invasive agents [4, 24, 39].

Materials and methods

MO_4 cells

MO_4 cells are C3H mouse embryo cells transformed by murine sarcoma virus [3]. They were maintained on tissue culture plastic substrate in Minimum Essential Medium Eagle (modified) with Earle's salts and non-essential amino acids (EMEM, Flow Laboratories Ltd, Irvine, Scotland) supplemented with 10 per cent (v/v) foetal bovine serum, 0·05 per cent (w/v) L-glutamine, and 250 I.U. penicillin/ml (hereafter called culture medium). MO_4 cells are invasive *in vitro* [25] and metastatic *in vivo* [26] and these characteristics have been demonstrated repeatedly afterwards.

Drugs

Tunicamycin (TM, NSC 177382, provided by the Natural Products Branch, Division of Cancer Treatment, NCI, Bethesda, U.S.A.), 2-deoxy-D-glucose (DOG, Merck, Darmstadt, F.R. Germany), and β-OH-norvaline (HNV, Sigma, St Louis, Missouri, U.S.A.) were dissolved in culture medium at concentrations of 0·1 to 10 μg/ml, 1 to 100 mM, and 0·01 to 10 mM respectively (table 1). Monensin (MON, Calbiochem-Behring, La Jolla, California, U.S.A.) was dissolved in ethanol (1 mg/ml) and further diluted in culture medium to concentrations between 0·01 and 10 μg/ml. 1-Deoxynojirimycin (dNM, provided by D. Schmidt and E. Truscheit, Bayer-A.G., Wupperthal, F.R. Germany) and swainsonine (SW, provided by P. Dorling, School of Veterinary Studies, Murdoch University, Murdoch, Western Australia) were dissolved in culture medium to concentrations of 5 and 10 mM and 0·1 to 0·4 μg/ml respectively. Concentrations were chosen on the basis of known ranges of activity. The relatively small ranges of concentration used with dNM and SW were due to restricted availability. Marcellomycin (MCM) tartrate (obtained from Bristol Laboratories, Syracuse, New York, U.S.A. through M. Rozencweig) was dissolved in Ringer's solution (100 μg/ml) and further diluted in culture medium to concentrations between 0·003 and 0·1 μg/ml. Controls with solvents alone were included in all experiments.

Assay for invasiveness

The assay for invasiveness *in vitro* was performed as described previously [25]. Briefly, MO_4 cell aggregates with a diameter of 0·2 mm were confronted on top of a semi-solid agar-agar medium with 9-day-old embryonic chick heart fragments (diameter = 0·4 mm), and incubated for about 2 h. After attachment to each other, individual confronting pairs were transferred into fluid culture medium for further incubation on a Gyrotory shaker at 120 rev min^{-1}. Drugs were added to the fluid culture medium; they did, therefore, not influence the initial attachment between MO_4 cell aggregates and chick heart fragments. After 4 days of incubation on a Gyrotory shaker, part of the cultures were washed with fresh culture medium and further incubated for 4 days without drug. After incubation for 4 days or 4 + 4 days,

triplicate confronting pairs were fixed and processed for paraffin sections. Consecutive sections were stained with haematoxylin–eosin and with an antiserum against chick heart [23]. For a semiquantitative evaluation of invasion we used the grading published by Bracke *et al.* [4]: Grades III and IV indicate increasing degrees of invasion, Grade II indicates complete inhibition of invasion.

Assay for growth

We know from previous experiments [39] that growth of confronting pairs has to be ascribed to proliferation of MO_4 cells. Confronting pairs were photographed under a Macroscope (Wild, Heerbrugg, Switzerland, × 25 or × 50). After 4 and 4 + 4 days, volumes were calculated according to the formula of Attia and Weiss [1] from measurements of the larger and the smaller diameter on negatives projected (× 6·3) on tracing paper.

Assay for toxicity on heart tissue

To examine the toxicity of drugs on the heart tissue, chick heart fragments (diameter = 0·4 mm) were cultured as described for confronting pairs but without MO_4 cells. After 4 days incubation part of the heart fragments ($n = 3$) were fixed for histology. The others ($n = 6$) were washed in Moscona's solution and transferred into Nuclon Delta SI 24-well multidishes (Nunc, Roskilde, Denmark) with 500 µl EDTA (0·03 per cent w/v in Moscona's solution). After 10 min at room temperature EDTA was replaced by 200 µl trypsin (0·05 per cent w/v in Moscona's solution) plus EDTA (0·02 per cent w/v). After incubation at 37° for 20 min 1 ml culture medium was added to each well and 6 h later half of the medium was replaced. All cultures were followed with an inverted microscope throughout the 3 weeks. The number of cultures showing proliferation of heart cells over the total number of cultures was used as an index of viability.

Results

Invasion

Invasion of MO_4 cells into the chick heart tissue was obvious (Grades III and IV) in all cultures without the drug. Inhibition of invasion (Grade II) was observed with TM, DOG, HNV, and MON at concentrations indicated in table 1. In these cultures (figures 1–3), MO_4 cells were found around the heart tissue, except for TM where MO_4 cells remained at the pole of attachment (figure 1). With DOG at concentrations of 1 mM (in 1 out of 3 cultures) and 5 to 20 mM (in all cultures) only a few MO_4 cells were found inside the heart tissue (figure 2). Although these cultures were scored as grade III according to the criteria used [4] they were clearly different from all cultures without drug. After treatment with TM, DOG, HNV and MON at anti-invasive concentrations the histology of both the heart tissue and the MO_4 populations differed from untreated cultures through vacuolization of the cytoplasm and absence of postmetaphase figures. dNM, SW, and MCM (figure 4) permitted invasion. In confronting pairs treated with MCM at concentrations of 0·03 and 0·1 µg/ml postmetaphase figures were absent.

Growth

Examples of growth curves are shown in figure 5. Growth of confronting pairs was inhibited in cultures with TM, DOG, HNV, and MON at anti-invasive concentrations and with MCM (table 1). dNM and SW had no effect on growth.

Table 1. Semiquantitative evaluation of invasion and growth of MO_4 cells confronted with precultured chick heart fragments in organ culture.

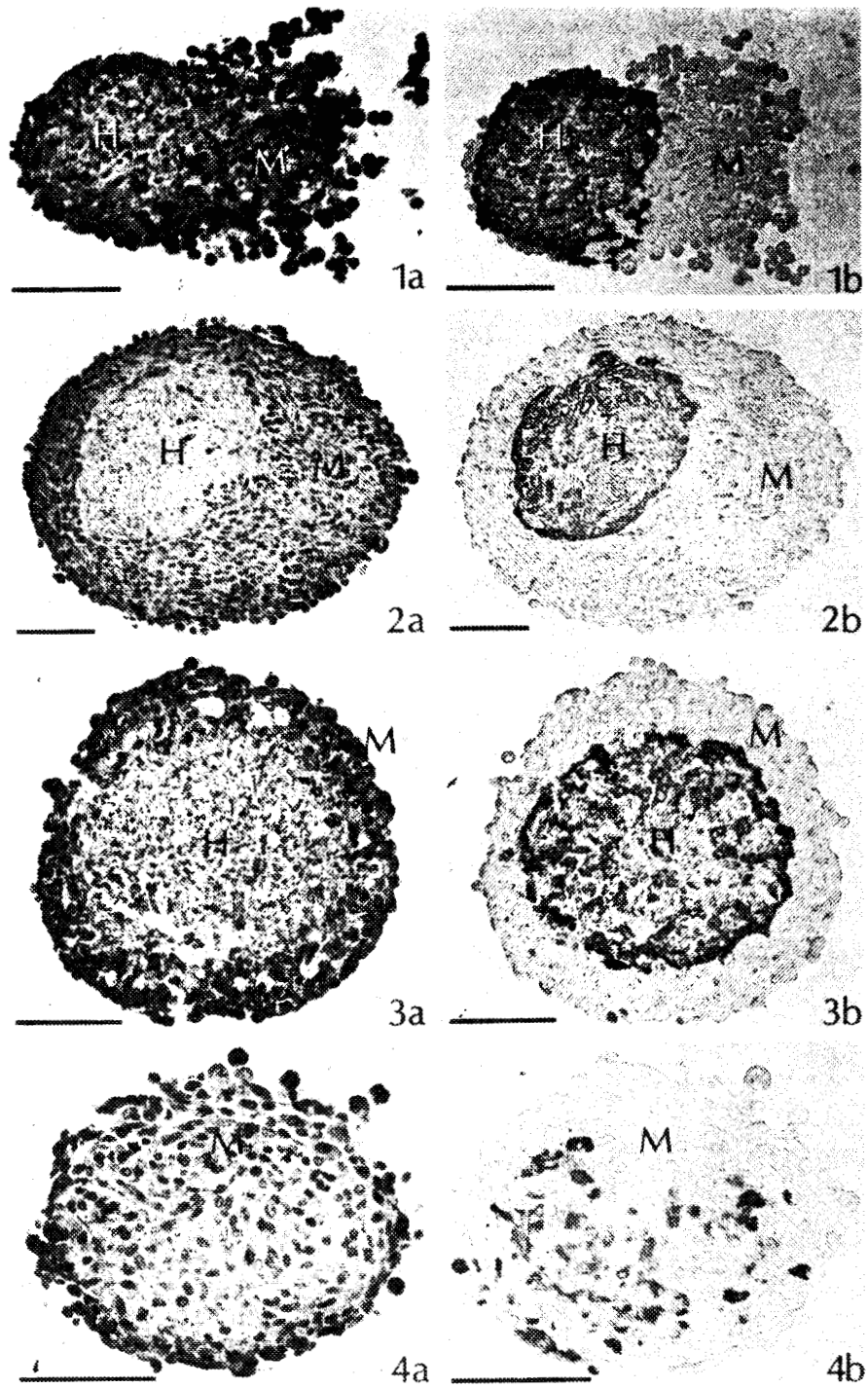
Drug	Concentration	Grading[a] of invasion after 4 days	Grading[a] of invasion after 4+4 days[b]	Type of growth curve[c]
Tunicamycin (TM)	0·1 μg ml	IV	IV	A
	0·3 μg ml	III	ND	ND
	1·0 μg ml	II	III, IV	C
	10 μg ml	II	ND	ND
2-Deoxy-D-glucose (DOG)	1 mM	III, IV	IV	A
	5 mM	III	IV	B
	10 mM	III	IV	B
	20 mM	III	IV	B
	100 mM	II	III, IV	C
β-OH-Norvaline (HNV)	0·01 mM	IV	IV	A
	0·1 mM	IV	IV	A
	1·0 mM	II, III	IV	B
	10 mM	II	II	C
Monensin (MON)	0·01 μg ml	III, IV	IV	A
	0·1 μg ml	II	IV	B
	1·0 μg ml	II	IV	B
	10 μg ml	N	N	C
1-Deoxynojirimycin (dNM)	5 mM	IV	IV	A
	10 mM	III, IV	IV	A
Swainsonine (SW)	0·1 μg ml	IV	IV	A
	0·2 μg ml	IV	IV	A
	0·4 μg ml	IV	IV	A
Marcellomycin (MCM)	0·003 μg ml	IV	IV	A
	0·01 μg ml	III, IV	IV	A
	0·03 μg ml	IV	IV	B
	0·1 μg ml	IV	IV	B
None[d]		III, IV	IV	A

[a] Extreme values.
[b] 4 days with drug, washing, and 4 days without drug.
[c] For examples see figure 5.
[d] Included in each experiment.
N, necrosis (due to toxicity); ND, not done.

Absence of postmetaphase figures in non-growing confronting pairs suggested that inhibition of MO_4 cell proliferation was responsible for absence of growth. In all confronting pairs without inhibition of growth central necrosis was observed after 4+4 days.

Reversibility

Reversibility of inhibition of invasion (figure 6) and of growth (table 1) was observed in most cultures. The slope of type B curves (figure 5) suggested that



Figures 1–4. Light micrographs of histological sections from confrontations between an MO_4 cell aggregate (M) and a chick heart fragment (H) cultured for 4 days with 1 μg tunicamycin/ml (figure 1), 1 mM 2-deoxy-D-glucose (figure 2), 0·1 μg monensin/ml (figure 3), or 0·1 μg Marcellomycin/ml (figure 4). Staining with haematoxylin–eosin figure 1–4 (*a*) and with an antiserum against chick heart figures 1–4 (*b*). Scale bar: 100 μm.

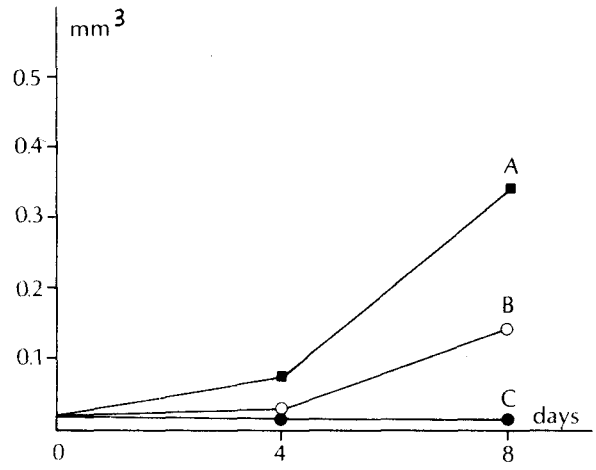


Figure 5. Examples of growth curves. Ordinate: volume of confronting pairs; median values from 3 to 6 cultures. Abscissa: time of culture; drugs were removed at day 4 and cultures were continued for another 4 days (day 4+4). ■——■, untreated, type A curve; O——O, with 10 mM 2-deoxy-D-glucose, type B curve; ●——●, with 10 mM β-OH-norvaline, type C curve.

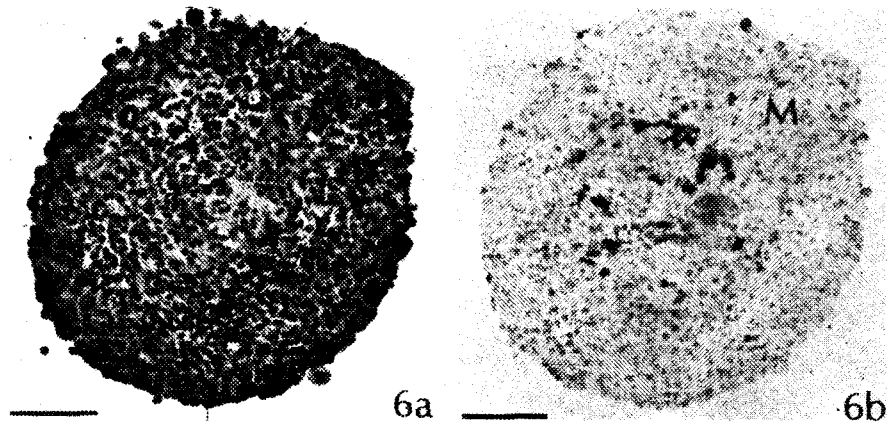


Figure 6. Light micrographs of histological sections from a confrontation between an MO_4 cell aggregate (M) and a chick heart fragment cultured for 4 days with 0·1 μg monensin ml followed by washing and further incubation for 4 days without drug. Staining with haematoxylin–eosin (*a*) and with an antiserum against chick heart (*b*). Scale bar: 100 μm.

recovery from growth inhibition was not due to a minor resistant subpopulation of MO_4 cells. With 100 mM DOG and with 1·0 μg TM/ml inhibition of invasion was reversible but recovery from growth inhibition was not obvious within 4 days after removal of the drug.

Toxicity on heart tissue

Histology of heart fragments cultured without MO_4 cells showed the same effects of treatment as described for confronting pairs: vacuolization of the cytoplasm. With

Table 2. Growth on tissue culture plastic substrate of cells from heart fragments pretreated during 4 days in organ culture.

Pretreatment		
Drug	Concentration	Growth[a]
Tunicamycin (TM)	0·1 μg/ml	4/6
	1·0 μg/ml	1/6
2-Deoxy-D-glucose (DOG)	100 mM	5/6
β-OH-Norvaline (HNV)	0·01 mM	5/6
	0·1 mM	5/6
	1·0 mM	4/4
Monensin (MON)	0·01 μg/ml	4/6
	0·1 μg/ml	4/4
	1·0 μg/ml	5/5
Swainsonine (SW)	0·1 μg/ml	3/4
	0·2 μg/ml	4/4
	0·4 μg/ml	2/3
Marcellomycin (MCM)	0·003 μg/ml	4/6
	0·01 μg/ml	5/6
	0·03 μg/ml	5/6
	0·1 μg/ml	0/4
None		17/22

[a] Number of explants showing growth over total number of explants.

the exception of TM at 1·0 μg/ml and MCM at 0·1 μg/ml, treated heart fragments produced viable cultures after explantation on tissue culture plastic substrate in the absence of the drug (table 2).

Discussion

The present paper shows that drugs known to interfere with the glycosylation or the processing of carbohydrate chains of glycoproteins in different ways have different effects on the invasion of MO_4 cells in organ culture. TM, DOG, HNV and MON did interfere with invasion whereas dNM, SW and MCM permitted invasion.

TM, DOG and probably also MCM inhibit lipid-dependent N-linked glycosylation [27, 29, 36, 40]. HNV also completely prevents N-linked glycosylation, but only when the glycosylation site is Asn-X-Thr [15, 32]. Indeed, glycosylation cannot occur when threonine is replaced by its analogue HNV.

MON is a sodium ionophore that interferes with several metabolic processes occurring in the Golgi cisternae, including the processing of N-linked and O-linked sugars of glycoproteins and the synthesis of glycolipids [12, 18, 19, 30]. dNM is a glucosidase inhibitor that prevents trimming of the glucose residues of high-mannose sugars. The resulting N-linked carbohydrates are thus of the high-mannose type [5, 33].

SW is an inhibitor of mannosidase II [10, 41]. Cells treated with SW usually produce hybrid N-linked sugars [13].

Our results clearly show that the integrity of N-linked carbohydrates of glycoproteins is not required for invasion. We have indeed shown [14, 34, unpublished results] that cells grown in the presence of SW of dNM have altered cell-surface glycoproteins. Our results obtained with MON, TUN and DOG suggest that some degree of N-linked glycosylation may be required for invasion. Since MCM does not prevent invasion, we assume that it inhibited glycosylation only partially. Alternatively, the inhibition observed with MON, TM, DOG and HNV on invasion might not result exclusively from the effect of these compounds on glycosylation. We have concluded from previous experiments that fucosylation of cell surface oligosaccharides is important for invasion [22]. This opinion is supported by the findings that TM inhibits fucosylation [7, 28] whereas SW allows fucosylation [13].

The present results and data from the literature showed that the anti-invasive drugs TM, DOG, HNV and MON affect a number of cellular activities which might or might not be related to their anti-invasive effect. All four drugs inhibited the proliferation of MO_4 cells. An antiproliferative activity was demonstrated with TM on lymphoblastoid cells [11, 28] and on B16 melanoma cells [16], and with MON on rat fibrosarcoma cells [12]. We have shown that growth and invasion are unrelated activities of MO_4 cells [21] and this was confirmed by recent experiments [4, 38]. Experiments in the presence of MCM (after 4 days) and of 100 mM DOG (after 4 + 4 days) shown in table 1 provide other examples of invasion without MO_4 cell proliferation. It is, therefore, unlikely that the anti-invasive effect of TM, DOG, HNV and MON has to be ascribed to inhibition of MO_4 cell proliferation.

TM, DOG, HNV and MON resemble microtubule inhibitors in as much as they inhibit both proliferation and invasion at the same concentration [24]. It is not likely that these four drugs affect microtubules since multimicronucleated MO_4 cells, which are typical after treatment with microtubule inhibitors [8] were not observed in the present experiments. It is, however, not excluded that both types of drugs have common effects. MON and colchicine were synergistic in producing randomization of Golgi complexes throughout the cytoplasm [20]. Bennett and Haddad [2] showed retention of [^{3}H]fucose in Golgi complexes of the ciliary processes of the eye by colchicine as well as by vinblastine. In chicken embryo fibroblasts vinblastine affected transport of glycoproteins similarly to MON [17]. We have ascribed the anti-invasive effect of microtubule inhibitors to inhibition of directional migration [24]. We are actually examining the effect of the drugs used in the present experiments on the migration of MO_4 cells.

We conclude that the inhibitors of glycoprotein synthesis and processing TM, DOG, HNV and MON represent a new class of anti-invasive agents. They are interesting for the analysis of the role of cell surface oligosaccharides in invasion particularly because the inhibitors of glycoprotein processing SW, dNM and MCM are permissive for invasion. Some of the latter drugs may affect specific cellular functions without evident cytotoxicity [14, 34].

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Mémoire 8

EFFECT OF GLYCOSYLATION INHIBITORS ON N-LINKED CELL SURFACE GLYCOPEPTIDES AND ON INVASION OF MALIGNANT MOUSE MO_4 CELLS IN VITRO

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Key words : glycosylation, invasion, MO_4 cells, organ culture.

SUMMARY

Cell surface glycan chains are believed to play a role in tumour invasion and metastasis. Yet, we have previously shown that the inhibitors of N-linked glycan processing swainsonine (SW) and 1-deoxynojirimycin (dNM) did not prevent invasion of chick heart fragments by MO_4 murine fibrosarcoma cells in organ culture. We now present biochemical evidence that these and other inhibitors of processing were indeed effective in remodelling cell surface glycans. After metabolic labeling with tritiated mannose or fucose, glycopeptides were obtained by pronase treatment of material released from cell surfaces by trypsin. Glycopeptides were separated by Biogel P-10 chromatography. With all drugs tested, there was a shift towards lower molecular weight of the glycan chains. There were, however, major quantitative differences between the different drugs and also, for monensin (MON; 0.1 µg/ml), between fucose-labeled and mannose-labeled chains. The shift in apparent molecular weight affected mainly fucose-labeled peptides after treatment of MO_4 cells with SW (0.4 µg/ml). The shift induced by dNM (10 mM) + SW (0.4 µg/ml) in both fucosylated and mannosylated chains was much larger than that induced by SW given alone. 1-Deoxymannojirimycin (dMM; 1 mM) had major effects on both mannose and fucose-labeled structures and so did N-methyl-1-deoxynojirimycin (MdNM; 2 mM) and castanospermine (CS; 100 µg/ml). With the latter drugs, incorporation of fucose in complex-type glycopeptides was dramatically reduced. The effect of SW on fucose-labeled glycopeptides of embryonic chick heart was similar to that observed on MO_4 cells. After removal of sialic acid, control and SW-treated glycopeptides from both MO_4 and embryonic chick heart cells had similar gel chromatographic profiles, suggesting that a decrease in cell surface sialic acid accounts to a large extent for the difference between glycans from control and SW-treated cells. Additional biological experiments were done with dMM (1 mM), MdNM (2 mM), CS (100 µg/ml), 2,5-dihydroxymethyl 3,4-dihydroxypyrrolidine (DMDP; 250 µg/ml) and SW (0.4 µg/ml) + dNM (10 mM). All these compounds or combinations failed to inhibit invasion. The observation that

inhibitors of N-linked glycan processing did not interfere with invasion although they clearly modified the glycosylation of cell surface proteins indicated that the integrity of cell surface glycans might not be a prerequisite for invasion in vitro.

INTRODUCTION

Changes in cell surface glycosylation have been correlated with malignant transformation on one hand and with tumour progression towards invasiveness and metastatic capability on the other hand. The biochemical evidence rested on structural analyses of glycans and glycosylating enzymes (Smets and Van Beek, 1984; Dennis et al., 1987; Bolscher, 1988a, Rademacher et al., 1988). Along the same line, biological studies have relied mainly on the use of glycosylation-defective mutant cell lines and of inhibitors of protein glycosylation (Irimura et al., 1981; Humphries et al., 1986a and 1986b; Dennis, 1986; Finne et al., 1989). Using the latter approach, Irimura et al. (1981) showed that melanoma cells precultured in the presence of tunicamycin (TM) formed less pulmonary nodules than control cells after i.v. injection into syngeneic mice. Humphries et al. (1986a and 1986b) have made a similar observation with swainsonine (SW) or castanospermine (CS).

Using an assay in vitro, we have shown that inhibitors of N-linked glycosylation such as TM inhibit invasion of mouse malignant MO_4 cells. In contrast, swainsonine (SW) and 1-deoxynojirimycin (dNM) had no effect on the invasion of these cells (Mareel et al., 1985). Yet, both compounds have been described to interfere with the processing of N-linked glycans and to prevent the synthesis of malignancy-related carbohydrate structures (Elbein, 1987; Mareel and De Mets, 1989).

Since it could be argued that the drugs were not active in our cell system, we undertook the biochemical characterization of the cell-surface glycans synthesized in the presence of these and several other inhibitors of glycan-processing. The latter included N-methyl-1-deoxynojirimycin (MdNM), castanospermine (CS), 2,5-dihydroxymethyl 3,4-dihydroxypyrrolidine (DMDP), 1-deoxymannojirimycin (dMM), and dNM + SW, all of which also failed to inhibit invasion in vitro (this paper). With

all inhibitors tested, the pattern of newly synthesized cell-surface glycans was clearly modified. Taken together, our data indicated that major changes in cell surface carbohydrates were compatible with invasion in vitro.

MATERIALS AND METHODS

MO4 cells

MO_4 cells (obtained from A. Billiau, KUL, Leuven, Belgium through M. De Brabander, Janssen Life Sciences, Beerse, Belgium) are C3H mouse cells transformed by Kirsten murine sarcoma virus (Billiau et al., 1973); they were cultured in Eagle's modified Minimal Essential Medium with Earle's salts and non-essential amino acids (EMEM, Flow Laboratories Ltd, Irvine, Scotland) supplemented with 10% (v/v) fetal bovine serum, 0.05% (w/v) L-glutamine, 250 IU penicillin per ml and 100 µg streptomycin per ml (hereafter called culture medium). MO_4 cells were invasive in vitro (Mareel et al., 1979) and produced invasive and metastasizing sarcoma-like tumours in syngeneic mice (Meyvisch and Mareel, 1982).

Drugs

MON (Calbiochem-Behring, La Jolla, CA) was dissolved in ethanol (1 mg per ml) and further diluted in culture medium to a final concentration of 0.1 µg per ml; control cultures with solvent alone are included in these experiments. The other drugs were directly dissolved in culture medium at the given concentrations : TM (NSC 177382, provided by the Natural Products Branch, Division of Cancer Treatment, NCI, Bethesda, MD) at 1 µg per ml; 2-deoxy-D-glucose (dGlc) (Merck, Darmstadt, FRG) at 5 and 20 mM; dNM (a gift from E. Trusheit and D. Schmidt, Bayer-AG, Wuppertal, FRG) at 10 mM; MdNM (a gift from R. Schwarz, The Rockefeller University, NY, and U. Klein, Institut für Virologie, Universität Giessen, Giessen, FRG) at 2 mM; CS (a gift from L. Fellows, Royal Botanic Gardens, Kew, UK) at 100 and 400 µg/ml; DMDP (a gift from L. Fellows) at 250 µg/ml; dMM (a gift from E. Trusheit and D. Schmidt) at 1 mM; SW (a gift from Dr. P. Dorling, School of Veterinary Studies, Murdoch University, Western Australia) at 0.4 and 3.0 µg/ml.

Concentrations were chosen according to published data (reviewed by Mareel and De Mets, 1989) and to personal communications from the donators.

Isolation and analysis of cell surface glycopeptides

MO_4 cells were seeded at an initial density of 1.5×10^5 cells per 75 cm^2 plastic tissue culture flasks (Flow, Irvine, Scotland, cat n° 61-450B5).

Chick embryo heart (CEH) cells were prepared from 9-day old embryos. After dissection, the ventricles were fragmented, rinsed in a Ca^{2+}- and Mg^{2+}-free salt solution containing 0.04% EDTA (w/v) and trypsinised for 15 minutes at 37°C. Fetal bovine serum was added to neutralize the trypsin, and after centrifugation the pellet was resuspended in culture medium to obtain 0.7×10^6 cells per 20 ml.

MO_4 or CEH cells were incubated on tissue culture plastic at 37°C during 4 days; the medium was refreshed after 2 days and/or not supplemented with one of the drugs. Cells treated or not were metabolically labeled with either L-[6-^{3}H] fucose (1 µCi/ml; 30 Ci/mmol) or D-[2-^{3}H] mannose (1 µCi/ml; 19 Ci/mmol) during the last 20 h of incubation (radiochemicals were obtained from New England Nuclear, Boston, MA, or from the Radiochemical Center, Amersham, UK).

Cell surface glycopeptides were isolated in accordance with the technique of Gilfix and Sanwal (1984). Briefly : cells were washed with phosphate-buffered saline (PBS) and trypsinized for 10 minutes at 37°C. After centrifugation the supernatant (called trypsinate) was exhaustively digested with pronase (from Streptomyces griseus, cat. nr 6174-01, Koch & Light, Genzyme Ltd, Suffolk, UK), and loaded on a 90 x 1.6 cm Bio-Gel P10, 200-400 mesh (Bio-Rad, Richmond, CA, cat. n° 150-150) column, with dextran blue and phenol red added to each sample in order to determine the exclusion and inner volumes of the column; fractions of 1.5 ml were collected. Radioactivity was measured in the digested supernatant and in the cell pellet. Percentages of total radioactivity in the supernatant and radioactivity per mg protein of the cell pellet, as measured by the method of Lowry (1951), were calculated.

Terminal sialic acids were removed from glycopeptides by incubation at 80°C in 0.1 M H_2SO_4 during 90 minutes. To liberate O-linked glycans from remnant peptides a reductive β-elimination reaction was done according to Carlson et al. (1968). Extraction of glycolipids was performed according to Oliver and Hemming (1975).

These methods permitted to analyse : i) the effect of the different glycosylation inhibitors on the apparent molecular weight of radiolabeled N-linked cell surface glycopeptides; ii) the amount of radioactivity released by trypsin; iii) the change of radiolabeled sugar incorporation per mg protein in cultures on solid tissue culture substrate.

Assay for invasion

The assay for invasion in vitro has been described previously (Mareel et al., 1979). Spheroids of MO_4 cells (diameter = 0.2 mm) were allowed to attach to precultured embryonic chick heart fragments (PHF, diameter = 0.4 mm), which consisted of a core of myoblasts surrounded by a capsule of fibroblast-like cells, on top of a semi-solid agar medium for at least 2 h. Culture medium, supplemented or not with drugs, was then added and individual confronting pairs were transferred into a 5 ml Erlenmeyer flask containing 1.5 ml of the same medium. They were further incubated for 4 days at 37°C on a Gyrotory^R shaker (New Brunswick Scientific Co, New Brunswick, NJ) at 120 rpm, fixed in Bouin Hollande's solution, embedded in paraffin, and completely sectioned into 8-µm thick sections. Consecutive sections were stained with haematoxylin-eosin or with an antiserum against chick heart (Mareel et al., 1981). Histological examination of invasion was done by at least two observers, based on an evaluation of occupation and degeneration of the PHF. The score was as follows : Grade I, when MO_4 cells were at the periphery of the outer fibroblast-like cells of the PHF; Grade II, when MO_4 cells had occupied only the outer fibroblast-like cell layers, but not the myoblasts; Grades III and IV, when MO_4 cells had occupied respectively less or more than half of the

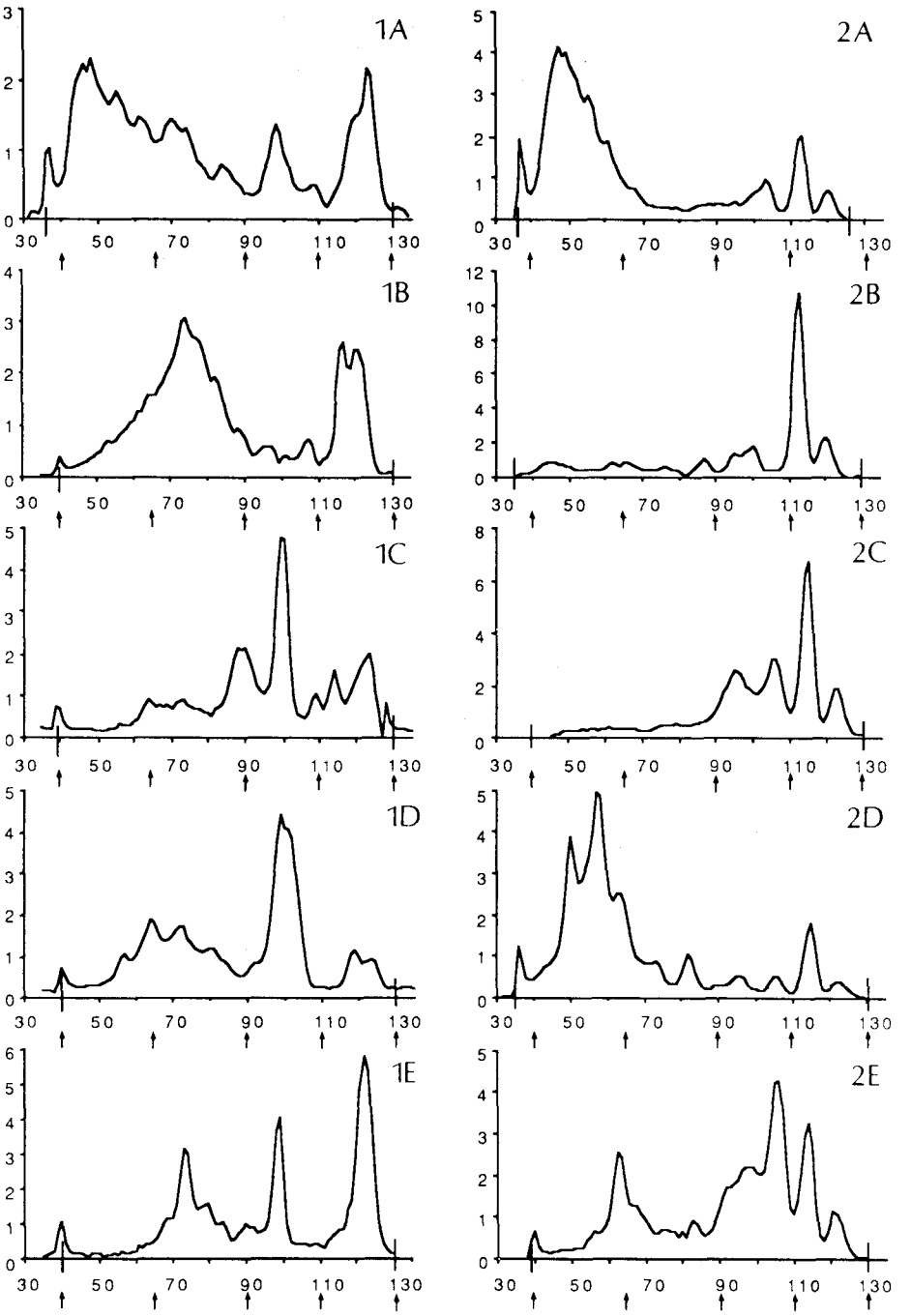
PHF. According to Bracke et al. (1984), Grades III and IV meet the criteria of invasion.

RESULTS

Cell surface glycopeptides

The radioactivity found in the trypsinates of MO_4 or of CEH cells varied between 19% and 35% of the total cellular radioactivity. The total radioactivity/mg cellular protein of most treated cultures varied within 20% of that of the control cells, showing a substantial overall incorporation of fucose and of mannose. Exceptions were : CS (400 µg/ml) doubled the incorporation of mannose when compared with untreated cells; MON (0.1 µg/ml) reduced the incorporation of fucose to about half the value of control cells whereas no quantitative differences were found after mannose labeling.

The gel chromatography patterns of the pronase-digested glycopeptides from the radiolabeled MO_4 (Figs. 1 to 3) and CEH cell surfaces (Fig. 4) could be interpreted as follows (Warren et al., 1978) : fractions 35 to 40, eluting with or near to dextran blue, corresponded to proteoglycan-derived material; fractions 41 to 65 contained the complex type N-linked glycans; fractions 66 to 90 represented the hybrid, oligomannosidic and immature N-linked glycan structures; finally, fractions 91 to 110 and fractions 111 to 130 eluting close to phenol red would contain respectively small precursors and free fucose or mannose. This interpretation was supported by preliminary results from applications of the different groups to different lectins (data not shown). The distribution of the radioactivity found for fucose-labeled glycopeptides of untreated MO_4 cell surfaces (fig. 2A) was different from that of the corresponding mannose labeled sample (fig. 1A), indicating that fucose was preferentially incorporated in larger oligosaccharides, while mannose was distributed over all types of structures. Even after extensive washing, precursors or free label were found.



Figures 1 and 2 : Gel chromatography profiles of MO_4 cell surface glycopeptides on Bio-Gel P10 : MO_4 cells are grown at 37°C in culture medium (Fig. 1A and 2A), supplemented with monensin (0.1 µg/ml; Fig. 1B and 2B), 1-deoxymannojirimycin (1 mM; fig. 1C and 2C), swainsonine (0.4 µg/ml; Fig. 1D and 2D); swainsonine + 1-deoxynojirimycin (0.4 µg/ml + 10 mM; Fig. 1E and 2E); for 48 h, and metabolically labeled with D-[2-^{3}H] mannose (fig. 1) or L-[6-^{3}H] fucose (fig. 2) during the last 20 h of incubation. Ordinate : percentage of total radioactivity. Abscissa : fraction number; vertical bars in the abscissa indicate the elution maxima of dextran blue (left) and of phenol red (right); each experiment was repeated at least once, with similar results (compare for example fig. 2A with fig. 3A, and fig. 2D with fig. 3C). Arrows delineate zones containing, from left to right, the complex type glycopeptides (fractions 41-65); the hybrid, oligomannosidic or immature N-linked glycopeptides (fractions 65-90); the small precursors (fractions 91-110) and free label (fractions 111-130).

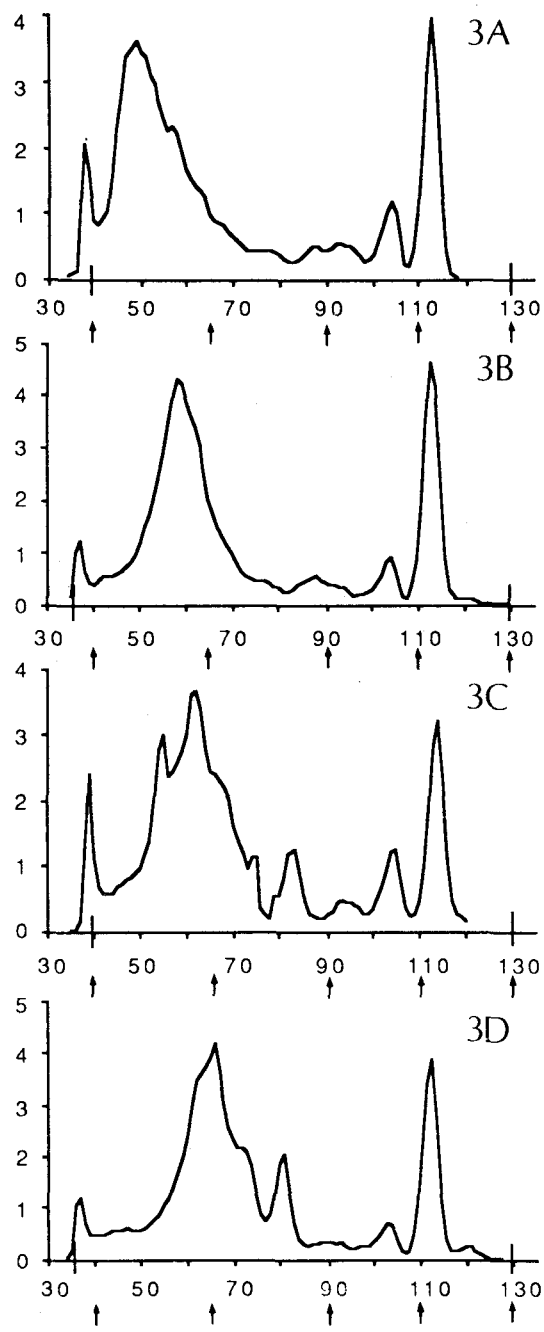


Figure 3. Gel chromatography profiles of MO_4 cell surface glycopeptides on Bio-Gel P10. MO_4 cells were untreated (3A and 3B) or treated with swainsonine (0.4 µg/ml) (3C and 3D) and labeled with L-[6-^{3}H] fucose; trypsinates were acid treated (3B and 3D) or not (3A and 3C). Ordinate : percentage of total radioactivity. Abscissa : fraction number; vertical bars in the abscissa indicate the elution maxima of dextran blue (left) and of phenol red (right). Arrows are as for Figs. 1 and 2.

The following controls have been performed : i) the gel chromatography profile of untreated MO_4 cell surface glycopeptides was influenced neither by extraction of glycolipids nor by a reductive β-elimination reaction (the latter procedure liberates O-linked glycans and destroys remnants of the peptide moieties, if still present); ii) glycosidase contamination of the pronase by α-D-glucosidase, α-D-mannosidase, α-D-galactosidase, α-L-fucosidase, β-D-glucuronidase, β-D-galactosidase and N-acetyl-β-D-hexosaminidase could not be detected in a solution of 2 mg/ml pronase (J. Kint, personal communication).

The effects of drug treatments on gel chromatography profiles of trypsinates from cultures radiolabeled with fucose and/or mannose were illustrated in Figs. 1 to 4.

A general feature of all treatments was the decrease of labeled complex type N-linked glycans or a shift towards a lower molecular weight with an increase in small precursors and/or free fucose and mannose.

Since mannose was present in the core of all N-linked glycans, labeling with ^{3}H-mannose provided a convenient approach to investigate the effect of inhibitors. In the absence of any treatment (Fig. 1A), a large fraction of the label was recovered in complex, and in hybrid plus oligomannosidic type structures. Small precursors and free label were also found. The labeling of complex type glycans with ^{3}H-mannose was dramatically reduced with all inhibitors tested (Fig. 1), in particular with dMM (Fig. 1C), when SW and dNM were used together (Fig. 1E), and with MdNM (2 mM) and CS (100 and 400 µg/ml) (data not shown). There was a compensatory increase of hybrid and oligomannosidic type structures after treatment with MON (Fig. 1B), and with MdNM and CS (data not shown). Small precursors accumulated in cells treated with SW (Fig. 1D), with dMM (Fig. 1C), with MdNM and with CS (data not shown).

Labeling of MO_4 cells with fucose nearly exclusively occurred in complex type glycopeptides (Fig. 2A). After treatment with SW, labeled fucosyl glycopeptides were smaller than in control cells (Fig. 2D). This was even more the case after

treatment with SW plus dNM (Fig. 2E) and with TM (profile not shown). Labeling of complex type fucosyl glycopeptides was very low after treatment with MON or dMM (Fig. 2B, 2C). At 5 mM, dGlc produced gel profiles (not shown) similar to these obtained after SW treatment (see fig. 2D), while 20 mM dGlc gave rise to a profile (not shown) comparable to that of dMM (see fig. 2C).

Mild acid treatment of trypsinates (removing terminal sialic acids, caused a shift of the profile towards lower molecular weights in fucose-labeled MO_4 cells (Fig. 3). Such a shift was observed in fucose-labeled MO_4 cells that were untreated (compare figs. 3A and 3B) or treated with SW (compare figs. 3C and 3D). Gel chromatography profiles from MON-treated MO_4 cells labeled with mannose or with fucose (see figs. 1B and 2B) remained unaltered after removal of sialic acids (profiles not shown).

To examine whether inhibitors of glycoprotein processing would affect also the N-linked surface glycosylation of the heart cells, CEH cultures were treated with 0.4 µg SW/ml. The gel chromatography profile of fucosylated glycopeptides of untreated CEH cells shows a broader distribution of apparent molecular weights (fig. 4A) as compared with that of untreated MO_4 cells (see fig. 2A). As observed for MO_4 cells (see fig. 2D), SW caused a shift of CEH fucosylated complex type glycans towards lower molecular weight fractions (fig. 4B). As for MO_4 cells, elimination of terminal sialic acids by acid treatment shifted the gel chromatography profile of control and SW-treated CEH cells to a region of lower molecular weights (fig. 4C and 4D).

Invasion

Previous experiments have demonstrated that TM, dGlc and MON inhibited invasion of MO_4 cells in vitro in a reversible way whereas dNM and SW permitted invasion (Mareel et al., 1985). Here, we have extended these observations to the effects of other glucosidase and mannosidase inhibitors on invasion. Treatment with SW, dNM or a combination of SW plus dNM in a matched experiment did not

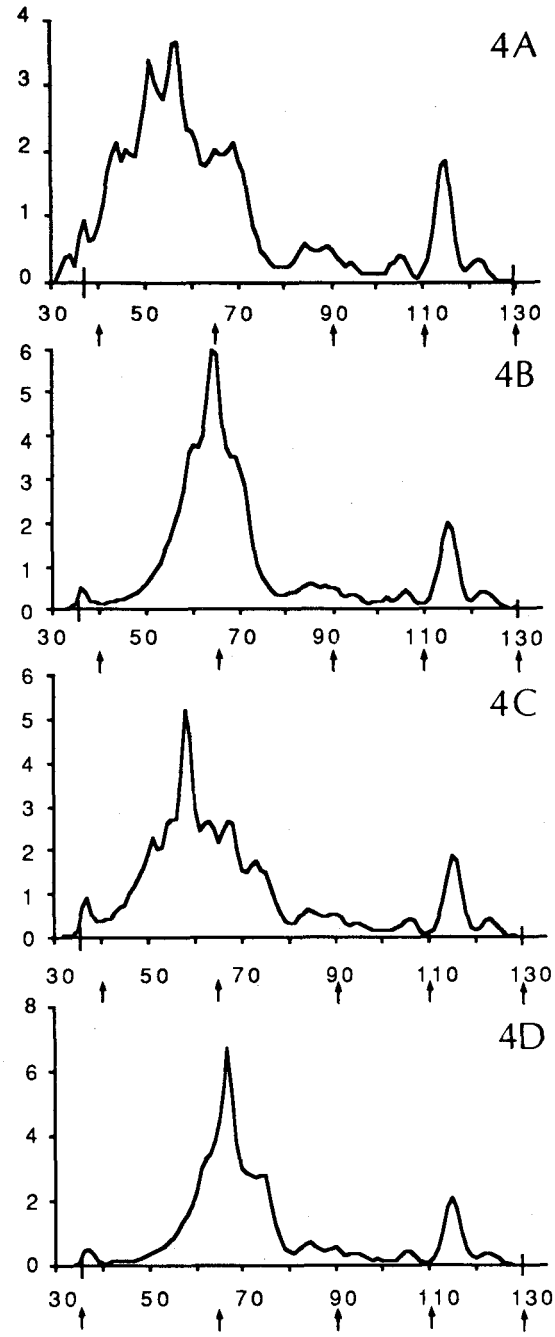


Figure 4. Gel chromatography profiles of the cell surface glycopeptides of chick embryo heart cells (CEH) on Bio-Gel P10. CEH cells were grown on tissue culture plastic in culture medium alone (fig. 4A and 4C) or in presence of 0.4 µg/ml swainsonine (fig. 4B and 4D) and labeled with L-[6-^{3}H] fucose. Sialic acid was removed from trypsinates of untreated (fig. 4C) or swainsonine-treated (fig. 4D) CEH cells. Ordinate : percentage of total radioactivity. Abscissa : fraction number; vertical bars in the abscissa indicate the elution maxima of dextran blue (left) and of phenol red (right). Arrows are as for Figs. 1 and 2.

inhibit invasion of MO_4 cells into the PHF (table 1). The other trimming inhibitors dMM (fig. 5), MdNM, CS, DMDP and dNM + SW did also permit invasion (table 1).

DISCUSSION

Acquisition of the invasive phenotype by cultured cell populations after genetic or epigenetic manipulation has been correlated with a shift of cell surface-exposed N-linked glycopeptides towards a higher molecular weight (Collard et al., 1986; Bolscher et al., 1986; Bolscher et al., 1988a and 1988b; Bruyneel et al., 1989b). Here, we were wondering whether drug-mediated reduction of the complexity of these glycopeptides would interfere with the expression of the invasive phenotype by MO_4 cells in vitro. Our results indicated that a glycopeptide profile "normalized" by treatment with inhibitors of Golgi glucosidases or mannosidases was compatible with the expression of the invasive phenotype, at least in vitro.

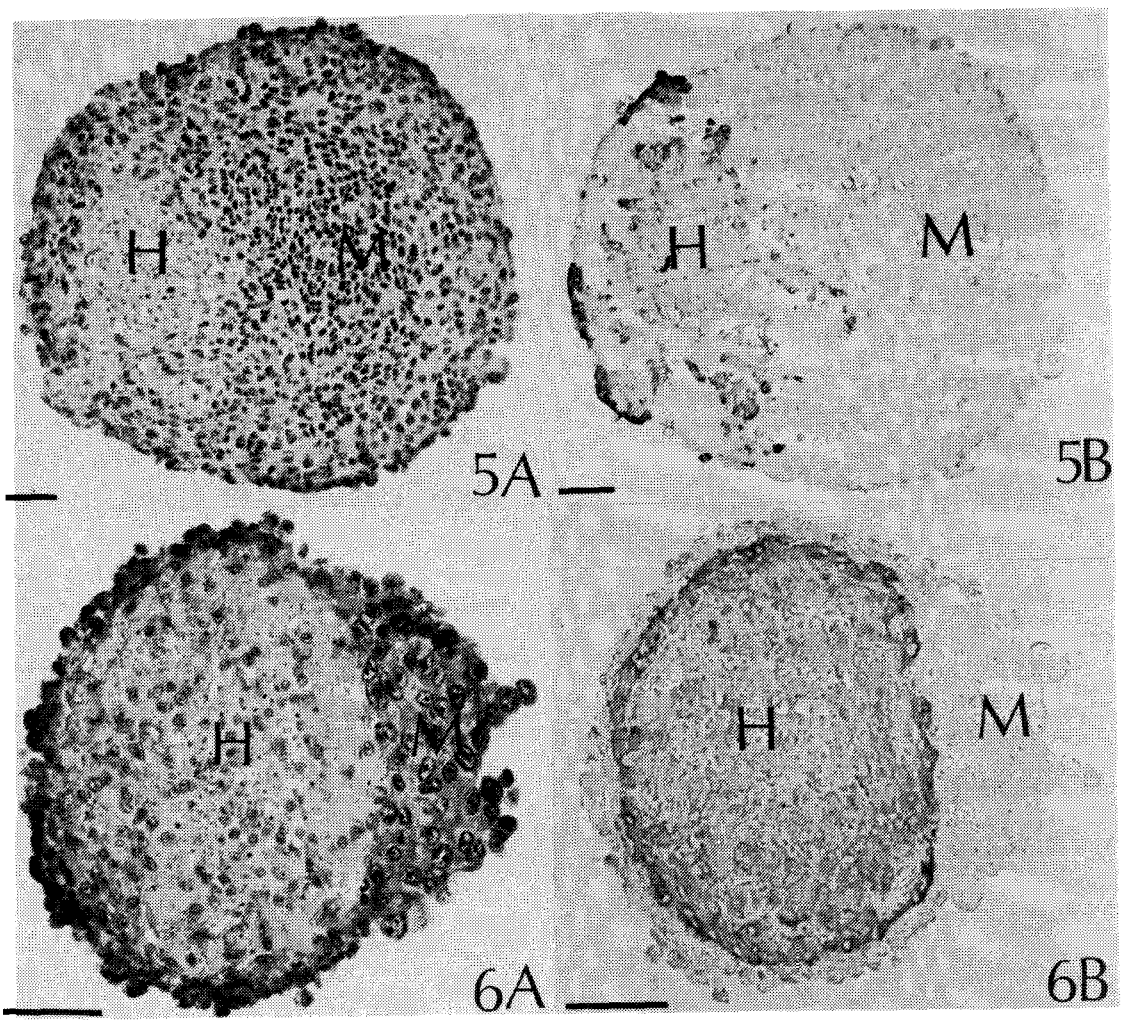
Although glucosidase and mannosidase inhibitors have been extensively studied for their effects on a variety of cellular functions (reviewed by Elbein, 1987; Rademacher et al., 1988; Mareel and De Mets, 1989), they rarely have been used in assays for invasion. CS and SW were reported to reduce the lung colony forming capability of mouse B16 melanoma cells (Irimura et al., 1981; Humphries et al., 1986a and 1986b; Dennis, 1986). This effect might be ascribed to reduced invasiveness of the treated cells, or to interference with other cellular functions implicated in lung colony formation.

Gel chromatography profiles, as performed in the present study, were not meant to reveal the nature of the molecular alterations responsible for shifts towards lower molecular weights. Comparison between profiles from SW-treated MO_4 cells and profiles from untreated cells with removal of terminal sialic acids (Figs. 3D and 3B) suggested a reduced sialylation due to SW-treatment. On the other hand, analysis on Con A-Sepharose columns has shown that in untreated cells tri-and tetra-antennary structures (85%) prevailed over bi-antennary structures (15%), whereas after treatment with SW the former (14%) were inferior to the latter (86%) (unpublished results).

Table 1 : Effect of inhibitors of glycoprotein processing on invasion of MO_4 cells into embryonic chick heart fragments in vitro.

Drug	Concentration	Grading[a] of invasion after 4 days			
		I	II	III	IV
1-deoxynojirimycin*	10 mM	0	0	0	4[b]
N-methyl-1-deoxynojirimycin	2 mM	0	0	2	3
Castanospermine	100 µg/ml	0	0	2	3
2,5 dihydroxymethyl-3,4 dihydroxypyrrolidine	250 µg/ml	0	0	0	4
1-deoxymannojirimycin	1 mM	0	1	2	1
Swainsonine*	0.4 µg/ml	0	0	0	4
Swainsonine + 1-deoxynojirimycin*	0.4 µg/ml + 10 mM	0	0	1	3
None*		0	0	2	9

[a]as described in Material and Methods : Grades III and IV meet the criteria of invasion. For comparison : All confronting cultures treated with monensin (0.1 µg/ml), 2-deoxy-D-glucose (100 mM), or tunicamycin (1 µg/ml) were scored as Grade II (Mareel et al., 1985).
[b]number of cultures examined histologically.
*matched experiments.



Figures 5 and 6. Photomicrographs of consecutive sections from confronting cultures between MO_4 cell aggregates (M) and embryonic chick heart fragments (H) cultured for 4 days in presence of 1 mM 1-deoxymannojirimycin (fig. 5); inhibition of invasion by 100 mM 2-deoxy-D-glucose as also published previously (Mareel et al., 1985) is shown for comparison (fig. 6); staining with haematoxylin-eosin (fig. 5A and 6A) or with an antiserum against chick heart (fig. 5B and 6B). Scale bars : 50 µm.

Dramatic reduction in fucosylation of N-linked glycopeptides was observed after treatment of MO_4 cells with the antiinvasive compounds TM (not shown) and MON (fig. 2B), but also with dMM (fig. 2C) which permitted invasion. Inhibition of fucosylation has been related to the lack of invasion by MO_4 cells in vitro at 28°C (Bruyneel et al., 1989a), and to reduced metastatic capability of Eb as compared to ESb cells (Schwartz et al., 1984) or of Wa4 as compared to B16-F1 melanoma cells (Finne et al., 1982 and 1989). In the present experiments, dMM-treated MO_4 cells were an example of low fucosylation together with invasion. Thus, there was no simple correlation between the glycans expressed during invasion and during metastasis.

The antiinvasive agents (TM, dGlc and HNV), which interfered with the dolichol-P cycle in the endoplasmic reticulum, also inhibited growth (Mareel et al., 1985). Concentrations that differentially affected growth and invasion, as described for other agents (Mareel et al., 1982) were not found with these inhibitors of glycosylation, making them less appropriate for the study of invasion mechanisms.

Taken together, our present experiments indicated that the integrity of N-linked surface glycosylation was not necessary for invasion in vitro. A number of considerations, however, may question this statement. i) The present gel chromatography profiles from metabolically labeled cells demonstrated a modified glycosylation pattern between 28 and 48 h after administration of the drugs. Extrapolating to the invasion assay, we could state that MO_4 cells had fully processed glycoproteins at the moment of initial contact with the PHF, and that this was sufficient for invasion to occur. ii) Glycosylation inhibitors affected both MO_4 cells and CEH cells, in contrast with the aforementioned experiments in vivo where only the tumor cells were treated (Irimura et al., 1981; Humphries et al., 1986a and 1986b; Dennis, 1986). For 1-O-octadecyl-2-O-methylglycero-3-phosphocholine, an alkyllysophospholipid affecting N-glycosylation, it was shown that the antithetic effects on invasion critically depended upon treatment of either one or both of the confronting partners (Schallier et al., 1988; Bolscher et al., 1988a; Bruyneel et al.,

1989b). iii) MO_4 cells and CEH cells were treated separately to obtain glycopeptides for chromatography, whereas in the invasion assay both partners were treated together. Since metabolic collaboration between MO_4 cells and heart tissue has been suggested from other experiments (Bruyneel et al., 1989a), it was not excluded that cell surface glycosylation in confronting organ culture responded to the glucosidase and mannosidase inhibitors in a different way as compared to separate cell cultures. iv) It could be that modification of glycosylation by the drugs, that permitted invasion, was never complete. Metabolic labeling allowed only the characterization of newly synthesized glycans and glycoproteins with a low turnover, having unaltered glycans at their cell surface, could remain present during the invasion assay. Therefore, it might be that the molecules critical for invasion remained normally glycosylated.

Our present data confirmed previous observations indicating that inhibitors of N-glycan processing do not prevent invasion and that this was the case for concentrations of inhibitor that do alter glycosylation. Therefore, we supposed that the integrity of N-glycan chains was not a prerequisite for invasion in vitro.

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CONCLUSION

Nous avons démontré que les inhibiteurs de la biosynthèse des glycannes N-glycosidiquement liés tels que la tunicamycine (1 µg/ml), la 2-désoxy-D-glucose (100 mM), la ß-hydroxynorvaline (1 mM) et un inhibiteur des activités golgiennes, notamment du transport des glycoprotéines, la monensine (0.1 µg/ml) inhibent l'invasion des cellules MO_4. Les inhibiteurs de glucosidases ou de mannosidases, qui permettent l'invasion, sont : la 1-désoxynojirimycine (dNM : 10 mM); la N-méthyl-1-désoxynojirimycine (2 mM), la castanospermine (100 µg/ml); la 2,5-dihydroxyméthyl-3,4 dihydroxypyrrolidine (250 µg/ml); la 1-désoxymannojirimycine (1 mM); la swainsonine (SW : 0.4 µg/ml) ou la dNM (10 mM) + SW (0.4 µg/ml). Du point de vue biochimique, il paraît utile d'analyser l'effet des substances qui permettent l'invasion aussi bien que l'effet des substances qui inhibent l'invasion. En résumé, l'invasion ne s'observe plus après inhibition du cycle de dolichol. Un certain degré de glycosylation semble donc requis. Par contre, toutes les substances qui empêchent la conversion d'oligosaccharides du type oligomannosidique en formes hybrides ou complexes sont compatibles avec l'invasion.

Une conclusion possible est que la glycosylation complexe des glycannes N-glycosidiquement liés de la surface cellulaire n'est pas nécessaire pour l'invasion des cellules MO_4 *in vitro*. Néanmoins, il faut considérer que les modifications biochimiques présentées se situent entre 28 et 48 hrs après l'addition de l'inhibiteur. Cela veut dire qu'au départ du test d'invasion les cellules MO_4 sont normalement glycosylées et un ralentissement de l'invasion peut échapper à l'analyse histologique après 4 jours de confrontation. Un prétraitement de l'un ou des deux partenaires avant la confrontation est donc à envisager. En plus, des expériences avec un lysophospholipide alkylé, ET-18-OCH_3, ont démontré un effet sur la N-glycosylation et une absence de l'invasion quand le tissu d'hôte ou les cellules à confronter sont prétraitées (Schallier et coll., 1988; Bolscher et coll., 1988; Bruyneel et coll., 1989).

Une autre explication serait que la N-glycosylation en confrontation soit différente de celle des deux partenaires traités séparément avec les inhibiteurs de la glycosylation. Une collaboration entre les cellules MO_4 et le tissu cardiaque n'est pas exclue, comme il a été démontré en confrontation à 28°C par autoradiographie (Bruyneel et coll., Clin. Exp. Metastasis, 1989) et surtout si on tient compte des considérations préalablement faites.

Finalement, l'analyse des N-glycosylpeptides est faite sur les glycoprotéines provenant de toute la surface cellulaire et, d'après les chromatogrammes sur gel, l'inhibition n'est jamais complète. Ceci pourrait impliquer que certaines glycoprotéines, qui sont critiques pour l'invasion, sont quand même normalement glycosylées. Il pourrait s'agir d'une glycoprotéine particulière, ou d'un nombre limité de glycoprotéines ou encore de toutes les glycoprotéines d'une zone restreinte de la surface cellulaire.

CONCLUSION GENERALE

Le phénomène de prendre possession des tissus environnants avec la tenacité d'une crabe qui tient sa proie est nommé l'invasion; elle est une caractéristique des tumeurs malignes. Les métastases, qui sont le résultat de cette invasion, peuvent être distinguées qualitativement de leur tissu d'origine et d'une tumeur bénigne par leur contribution dans l'évolution maligne du cancer.

A partir d'une série d'observations, tel que la locomotion et l'inhibition de la locomotion par contact, faites sur des populations de cellules invasives et non-invasives in vivo et in vitro, il parait que la locomotion n'est pas une caractéristique spécifique pour des cellules invasives. Le manque de respect pour les frontières tissulaires et la locomotion des cellules pénétrantes dépenderait du contact direct entre les cellules invasives et le tissu d'hôte. Les mécanismes moléculaires de ces interactions sont encore mal connus. Nous suggérons que les glycannes N-glycosidiquement liés de la surface cellulaire seraient parmi les premiers candidats pour la réception et éventuellement pour la transduction vers l'intérieur de la cellule de ces signaux qui controleraient le phénotype invasif.

Notre étude a démontré que la complexité des glycannes N-glycosidiquement liés de la surface des cellules invasives est augmentée par rapport à celle des cellules non-invasives. Ceci est le cas pour : i) les cellules malignes R1C, dérivées de rein de rat et transfectées avec l'ADN du virus Adéno 12 (0-16%), en comparaison avec les cellules non-malignes HSU du même origine, mais transfectées avec l'ADN du virus Adéno 12 (0-7,2%); ii) les cellules malignes RB14-T, dérivées de cerveau de rat, traité à l'éthylnitroso-urée en comparaison avec les cellules RB-22 de cerveau de rat non traité; iii) les cellules malignes MO_4, dérivées de souris C3H, transformées avec le virus du sarcome de Kirsten, en comparaison avec les cellules non-malignes MOH_{22}, dérivées de souris C3H non-traitée; iv) les cellules malignes T13, qui sont des cellules transformées par l'oncogène ras de l'ADN du carcinome de vessie, en comparaison avec les cellules non-malignes NIH/3T3 de souris; v) les

cellules malignes MDCK-ras, dérivées de rein de chien Madin-Darby et transformées avec l'oncogène ras, en comparaison avec les cellules MDCK-3, qui sont des cellules immortalisées et non-transformées du même origine. Cette augmentation de la complexité des glycannes N-glycosidiquement liés est essentiellement due à une augmentation du taux de l'acide sialique et du fucose (Mémoires 1 et 3, Chapitre I).

La 1-O-octadécyl-2-O-méthylglycéro-3-phosphocholine (ET-18-OCH_3) augmente la complexité des glycannes N-glycosidiquement liés et induit le phénotype invasif dans des cellules initialement non-invasives, tel que les cellules HSU, NIH/3T3 et MDCK. Le prétraitement par l'ET-18-OCH_3 n'a pas d'effet sur l'absence d'invasion dans les cellules NMuMG et MCF-7. Pour la lignée cellulaire HSU, les deux phénotypes malins sont inductibles par et réversibles dans le même délai après le prétraitement par l'ET-18-OCH_3 (Mémoires 1 et 2, Chapitre I).

Le prétraitement par l'ET-18-OCH_3 du FCP induit une résistance dans le tissu cardiaque vis à vis de l'invasion des cellules malignes MO_4 et R1C. Par chromatographie sur gel, il a été démontré que la complexité des glycannes N-glycosidiquement liés des fibroblastes et myoblastes en monocouche est augmentée après prétraitement par l'ET-18-OCH_3. Cette différence en glycannes N-glycosidiquement liés est essentiellement due à une augmentation du taux de l'acide sialique. L'induction par l'ET-18-OCH_3 de la résistance du FCP contre l'invasion et l'augmentation du taux de l'acide sialique est irréversible pour au moins 4 jours (Mémoire 2, Chapitre I).

En réduisant la température à 28°C, il est possible d'arrêter l'invasion des cellules malignes MO_4 en confrontation avec des FCPs pendant au moins 10 jours. A cette température, l'incorporation du [^{3}H]-fucose dans les glycoprotéines des cellules MO_4 est fortement réduite, tandis que l'incorporation de la [^{3}H]-leucine, comme mesure de la synthèse protéique, est beaucoup moins affectée. La chromatographie sur gel démontre une diminution de l'incorporation du fucose dans les N-glycosylpeptides complexes qui dépend du temps d'incubation des cellules MO_4 à

28°C. Par contre, l'incorporation de la glucosamine par mg de protéines n'est pas affectée et le poids moléculaire apparent des N-glycosylpeptides, marquées à la glucosamine, de cellules MO_4 parentales à 37°C est comparable à celui des N-glycosylpeptides de cellules MO_4 clonées et maintenues à 28°C.

La réduction de la température n'influence pas significativement l'activité de la fucosyltransférase, mais bien les précurseurs de la fucosylation. De plus, une accumulation de fucose-1-P suggère une inhibition de la conversion en GDP-fucose dans les cellules MO_4 parentales, cultivées à 28°C. Pour les expériences durant jusqu'à 21 jours, l'invasion des cellules MO_4 à 28°C redémarre après 14 jours d'incubation. La progression de l'invasion entre 10 et 17 jours d'incubation à 28°C est comparable à celle à 37°C entre 0 et 7 jours. Cette transition de l'invasion à 28°C ne peut pas être attribuée à des différences de croissance, de migration directionnelle ou de l'assemblage et désassemblage des microtubules. Une étude autoradiographique a démontré que l'invasion des cellules MO_4 clonées et maintenues à 28°C démarre dans ces zones ou l'incorporation du fucose est augmentée. Par le fait que l'incorporation du fucose est beaucoup moins affectée dans le tissu cardiaque, nous suggérons une collaboration métabolique entre le FCP et les cellules MO_4 clonées et maintenues à 28°C (Mémoires 4, 5 et 6; Chapitre II).

Les inhibiteurs de la glycosylation, interférant avec le cycle du Dolichol-P, tel que la tunicamycine, la ß-hydroxynorvaline et le 2-désoxy-D-glucose, inhibent la glycosylation et l'invasion des cellules MO_4. La monensine, un inhibiteur du transport des glycoprotéines, inhibe la fucosylation et la sialylation des glycannes N-glycosidiquement liés en même temps que l'invasion. Les inhibiteurs du processus de maturation, tel que la 1-désoxynojirimycine (dNM), la N-méthyl-1-désoxynojirimycine, la 2,5-dihydroxyméthyl-3,4-dihydroxypyrrolidine, la castanospermine, la 1-désoxymannojirimycine ou la swainsonine (seul ou ensemble avec la dNM) n'inhibent pas l'invasion des cellules MO_4. Pourtant, pour certaines de ces substances leur effet sur la glycosylation est aussi important que celui des substances qui inhibent l'invasion. Une conclusion possible serait alors que la

complexité des glycannes N-glycosidiquement liés n'est pas requise au début de l'invasion des cellules MO_4. Néanmoins, il faut envisager que ces substances ont aussi un effet sur la glycosylation du tissu cardiaque. Il pourrait bien que l'invasion dépend de modifications discrètes des glycannes N-glycosidiquement liés présentes sur certaines glycoprotéines du FCP ou des cellules MO_4. Vu que l'inhibition de la glycosylation n'est jamais complète, la glycosylation de certaines glycoprotéines pourrait être normale en confrontation. De plus, la glycosylation est normale au début du test d'invasion, puisque ni les cellules MO_4, ni le tissu cardiaque ont été prétraitées. Un prétraitement d'un ou des deux partenaires est donc à envisager (Mémoires 7 et 8, Chapitre III).

En conclusion, une hypothèse simple qui peut expliquer la nature complexe et la dynamique de l'invasion tumorale n'est pas encore possible. Pourtant, ce travail a démontré que l'acide sialique et le fucose des glycannes N-glycosidiquement liés de la surface de cellules invasives peuvent jouer un rôle-clef dans l'invasion tumorale. Nous situons ce rôle dans l'attachement et le détachement pendant la locomotion des cellules malignes. Les changements dynamiques de ces molécules pourraient s'accomplir par l'action de glycosyltransférases ou de glycosidases in situ, influençant ainsi la conformation spatiale des glycannes N-glycosidiquement liés et les interactions des cellules malignes avec le tissu-hôte.

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Les références incluses dans l'introduction ou dans les conclusions d'un chapitre, et dans les articles se retrouvent à la fin des articles. Ces références ne sont pas reprises ici. La présente liste se rapporte donc exclusivement à l'introduction générale.

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RESUME

L'invasion marque la différence entre une tumeur bénigne et une tumeur maligne; elle peut être testée in vitro en cultures organotypiques.

La complexité des glycannes N-glycosidiquement liés de la surface de cellules invasives est augmentée par rapport à celle des cellules non-invasives : ces glycannes contiennent davantage de fucose et d'acide sialique. Le phénotype invasif a été conféré à des cellules non-transformées d'une part par une transfection à l'aide d'oncogènes, d'autre part par un prétraitement par le 1-O-octadécyl-2-O-méthylglycéro-3-phosphocholine.

En réduisant la température des cultures organotypiques de 37° à 28°C, il a été possible de lever les propriétés invasives de cellules de fibrosarcome murin MO_4 pendant dix jours. A 28°C l'incorporation de fucose dans les protéines de cellules MO_4 est diminuée bien plus fort que l'incorporation de leucine. Après deux semaines à 28°C, l'invasion démarre, et l'autoradiographie démontre précisément une incorporation accrue de fucose dans les cellules MO_4 aux sites d'invasion. Une collaboration métabolique entre les cellules MO_4 et le tissu-hôte semble plausible.

Les inhibiteurs de la glycosylation qui, comme la tunicamycine, interfèrent avec le cycle du dolichol, préviennent l'invasion par les cellules MO_4. Par contre, les inhibiteurs de la maturation des glycannes N-glycosidiquement liés, qui réduisent la conversion de structures oligomannosidiques en formes hybrides ou complexes n'affectent en rien l'invasion. Un certain degré de glycosylation est probablement requis au début du test, ce qui n'exclut pas que la glycosylation des cellules MO_4 peut devenir plus complexe en confrontation avec le tissu-hôte.

Cette étude a démontré que l'acide sialique et le fucose des glycannes N-glycosidiquement liés de la surface de cellules invasives peuvent jouer un rôle-clef dans l'invasion.