Glycoconjugate Journal (1996) **13:** 741–747

Cell–cell interactions influence oligosaccharide modifications on mucins and other large glycoproteins

PATRICIA L. STRANAHAN, JENNIFER LAROE‡, RONDA McCOMBS, ALEX GOLDSMITH§, IFFAT RAHIM, MARK OVERLAND and DAVID E. PETTIJOHN*

University of Colorado Cancer Center[1], Department of Biochemistry[2], Biophysics and Genetics[3], University of Colorado Health Sciences[4] Center, Denver, CO 80262, USA

Received 30 August 1995, revised 28 February 1996

Intratumoral phenotypic diversity is well documented with regard to tumor associated carbohydrate antigens (TACA). The factors which control the expression of these cell-surface oligosaccharides on different cells of the same tumor are not understood. We investigated the expression of a panel of mucin associated oligosaccharides in cell lines growing at different surface densities (number of cells per cm^2 of growth flask). Results show that the apparent expression of extended Le^a-Le^x, Le^a and Le^x, sialyl Le^a, Tn and sialyl Tn varies with density of growth by an invasive human squamous cell lung carcinoma cell line (NU6-1), a benign variant (NE-18) and the human lung epithelial cell line BEAS-2B. The results indicate that one of the factors influencing the apparent expression of mucin-associated oligosaccharides is cell-cell interactions.

Keywords: mucins, oligosaccharides, cell-cell interactions cancer, aberrant expression

Abbreviations: Mab, monoclonal antibody; FIT, fluorescein isothiocyanate; TACA, tumor associated carbohydrate antigen

Introduction

The factors controlling the elaboration of complex oligosaccharide sequences on mucins and other large glycoproteins are poorly understood [1–4]. The oligosaccharides of these large glycoproteins are primarily expressed on the cell surface or are part of complexes appearing in the extracellular environment [5–7]. It has often been observed that adjacent cells in normal tissue that are indistinguishable by other criteria can differ in the expression of specific cell surface oligosaccharides. Moreover, nearby cells in the same tumor often vary in their cell surface oligosaccharides detected with specific monoclonal antibodies (Mabs) [8, 9]. Cloned cells derived from such tumors can be selected for homogeneity of oligosaccharide expresssion, but the expression is often unstable, leading rapidly to divergent expression in different cells of the same clone [10]. These observations, while not easily explained, lead one to consider the possibility that environmental triggers acting extracellularly may effect differential responses in genetically identical cells.

Studies in this laboratory have focused on aberrant glycosylation of mucins and other large glycoproteins in squamous cell lung cancer and cancers of the gastrointestinal tract [11–13]. Initially we described expression of a previously unidentified extended Lewis antigen (Le^a-Le^x) in squamous cell lung carcinoma which is recognized by Mab 43-9F. Le^a-Le^x is known to be exclusively associated with glycoproteins in contrast to other Lewis antigens which are glycoproteins/glycolipid carbohydrate moieties [14]. These studies were extended to include the use of additional Mabs to identify expression of other Lewis antigens which have each, individually, been described as tumor associated carbohydrate antigens (TACA) by others [15–19]. Utilization of a panel of

*To whom requests for reprints should be addressed, at Department of Biochemistry, Biophysics and Genetics, Box B188, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262, USA. Tel: +1 303 270 8837; Fax: +1 303 270 3304.
‡Present address: Colorado State University School of Veterinary Medicine, Fort Collins, CO, USA.
§Present address: Department of Internal Medicine, University of Colorado Health Sciences Center, Denver, CO 80262, USA.

biomarkers on patient specimen was initiated in each case in order to increase the probability of concordant aberrant glycosylation which would lead to patterns that might have diagnostic and/or prognostic significance. Further, emerging patterns could alter patient management decisions.

The present study characterizes previously unrecognized cellular diversity of human squamous cell lung carcinoma cells. We demonstrate here that oligosaccharide expression by these variants appears to be regulated by cell-cell interactions. These findings suggest that any experimental strategy for defining prognosis based on cell surface oligosaccharides should take into account the potential capacity for intracellular mosaicism.

Methods

Cell lines

Two previously described clones of the human squamous lung carcinoma line RG-SLC-L11 and the SV40 transformed human lung epithelial cell line BEAS-2B were removed from liquid nitrogen and were allowed to attach to plastic vessels, using RPMI 1640 medium plus 15% fetal bovine serum (clone NE-18 and BEAS-2B) or bovine calf serum supplemented with iron (clone NU6-1) as previously reported [8]. Single cell suspensions were prepared from confluent monolayers by a 5–10 min incubation at room temperature in 0.05% trypsin plus 2 mM EDTA or in 2 mM EDTA alone as previously described [8]. None of the various trypsin or EDTA concentrations or the supplementation with calf serum affected the previously described tumorigenicity [20]. Single cell preparations were prepared for examination by allowing these diluted cells to attach on eight well, teflon coated slides (Cell-Line). These sparse single cells were allowed to grow for 24 h. They were then fixed in 5% phosphate buffered formalin, permeabilized in acetone, at 4 °C for 5 min and stored at 4° in PBS until use. Small colonies were established after single cell plating by allowing growth until an average of 25 cells, each, were counted in 100 colonies. At this point, the eight well slides were washed and treated as the single cell slides. Other cultures were allowed to grow to confluence or post-confluence (2–5 days after confluence). At the appropriate cell density, the slides were washed, fixed and permeabilized as previously discussed [20].

Monoclonal antibodies

Six Mabs specific for different oligosaccharides were used. Anti Le^a-Le^x (43-9F), and anti Le^a (CO-514) were purified from serum-free culture medium of the respective hybridomas as previously described [11, 21]. In some experiments the antibody-containing media was used without purification. Anti-Le^x (P12), anti-Tn (IE3) and anti-sialyl Tn (TKH2) were received as antibody-containing media. Anti sialyl Le^a (Mab 19-9) was received in purified form. The monoclonal antibodies were applied to plated, fixed cells, as previously reported [11, 21].

Immunohistochemistry and evaluation

Each cell specimen was incubated with primary antibody for 1 h in humidity chambers at room temperature, washed, incubated with FITC secondary polyvalent IgG/IgM antibody (goat anti-mouse) and examined using a Leitz Dialux microscope, as previously described [11].

Evaluation of the preparations was on the basis of the intensity of the reaction product (0–4+) and on the percentage of tumor cells which fluoresced (0–100%). With each experiment a known positive carcinoma control as well as a negative control was processed. Also, non-specific reactivity was evaluated by paralleling fixed, permeabilized cells with primary antibody omitted.

Purification of mucins and large glycoproteins

NU6-1 cells were grown to near confluence in plastic flasks in RPMI 1640 media plus 5% bovine calf serum (BCS) supplemented with iron. The medium was removed, the attached cells were washed repeatedly with media lacking BCS, and then grown for 3 days in the serum-free RPMI media containing 2 μCi ml^{-1} [^{3}H] glucosamine (2.0 Ci mmol^{-1}). The spent media was recovered, mixed with identical media from unlabeled cells, centrifuged to eliminate any debris, glycoconjugates precipitated with ammonium sulfate, the precipitate gently solubilized in PBS containing 0.1% Tween + 0.5 mM PMSF, and the glycoconjugates were applied to a Sephacryl S500 column equilibrated with PBS/Tween solution and fractions collected while applying fresh PBS/Tween solution.

Analysis of epitope expression on purified mucins

Utilizing the Bio-rad immunoblot apparatus, 10 μl of each fraction collected off the Sephacryl S500 column was absorbed and washed on appropriately prepared Immubilon, as previously described [11]. The Imobilon was blocked with competing proteins and incubated with Mab 43-9F for 1 h at room temperature as described. After washing three times with PBS the sheet was incubated with ^{125}I labeled IgM secondary antibody, washed, dried and reaction product measured as previously described [11].

Incubation of NE-18 cells (epitope negative) with concentrated media from NU6-1 cells (epitope positive)

The epitope negative cell line NE-18 was grown on eight well teflon coated slides to near confluency in 15% FCS supplemented RPMI media. Mucins and other large glycoproteins were concentrated from previously harvested media of NU6-1 cells by ammonium sulfate precipitation; solubilized, dialysed and added at different concentrations

to the growing NE-18 cells. Growth of cells was allowed to continue for another 24 h. The cells were then stained with oligosaccharides specific Mabs and evaluated as described above.

Results

Three different human cell lines, BEAS-2B, NU6-1 and NE-18, growing as separate cells, in small 25 cell colonies, confluent, or hyperconfluent were stained with six different Mabs specific for different mucin associated oligosaccharides. The term hyperconfluent is used here to denote a distribution of cells resulting from growth in three-dimension so that growing cells pile up on each other. The stained cells on individual eight well slides were evaluated independently by three individuals at different times and the results were scored independently (Table 1).

BEAS-2B cells, which are SV40 transformed normal pulmonary epithelial cells, did not express any of the six mucin associated antigens as single cells. Five of the epitopes continued to be undetectable in small colonies and confluent as well as post-confluent cultures. The sixth antigen, sialylated Le^a, was first detected in cells of small colonies. Fifty per cent of the small colony cells express this epitope in a cytoplasmic membrane bound pattern. When the cultures became confluent 100% of the cells express the epitope. In the hyperconfluent state only 5% of the cells are expressors.

The SLC-11 variant cell line, NE-18, which has been shown to regress both subcutaneously and orthotopically in nude mice, did not express five of the epitopes as single cells or as small colonies (Table 1). With confluence, 5% of the cells express Tn as detected by monoclonal antibody IE3 (Fig. 1). Le^x was expressed by single cells, cells of small colonies as well as confluent and hyperconfluent cells; however, the percentage of cells expressing varied at these different cell densities. The

Table 1. Three variant human lung epithelial cell lines were placed on eight well slides and allowed to plate down and grow to varying cell densities. Expression of each of six mucin associated oligosaccharide epitopes is shown. Variability in expression differed with cell density. Each of the cell lines was plated as single cells, grown into small colonies, grown to logarithmic confluence or allowed to continue past logarithmic phase (hyperconfluence). Oligosaccharide expression by BEAS-2B, NE-18 and NU6-1 cell lines.

	Le^a-Le^x	*Le^a*	*Le^x*	*Sialyl Le^a*	*Tn*	*Sialyl Tn*
BEAS-2B						
Single	–	–	–	–	–	–
Small colonies	–	–	–	4+/50% membrane	–	–
Confluent	–	–	–	4+/100% membrane	–	–
Hyper-confluent	–	–	–	4+/5% cell[a]	–	–
NE-18						
Single	–	–	4+/35% perinuclear	–	–	–
Small colonies	–	–	4+/35% perinuclear	–	–	–
Confluent	–	–	4+/35% perinuclear	–	4+/5% cell	–
Hyper-confluent	–	–	4+/5% perinuclear	–	4+/5% cell	–
NU6-1						
Single	4+/1% cell	4+/1% cell	4+/1% cell	4+/100% cell	–	–
Small colonies	4+/50% cell	4+/25% cell	4+/100% membrane	4+/100% cell	4+/30% cell	4+/30% cell
Confluent	4+/50% plates[b]	4+/35% plates	4+/50% membrane	4+/50% cell	4+/10% cell	–
Hyper-confluent	4+/100% no plates, cell only	4+/75% cell	4+/35% cell	4+/100% cell	–	4+/30% cell

[a]cell, denotes fluorescence throughout; including membrane and cytoplasmic structures.
[b]plates, epitope expression associated with angular material overlying individual cells.
–, denotes lack of epitope expression utilizing Mabs specific for each antigen.

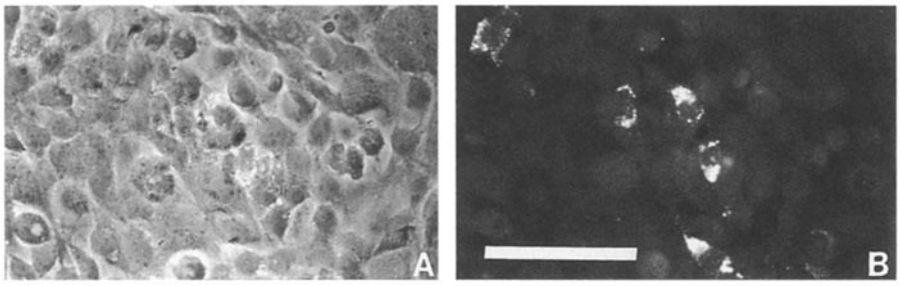

Figure 1. Tn expression by Ne-18 cells grown to confluence. Tn expression was observed by 10% of these cells at confluence. Prior to confluence no Tn expression was apparent utilizing Mab IE3 by single cells or cells in small colonies. (A. Phase microscopy; and B. FITC immunofluorescent microscopy of the same cells.) Bar, 150 μm, × 200.

single cells, cells in small colonies, and confluent cells expressed the epitope in greater concentration than did cells at hyperconfluency (Table 1).

The malignant SLC-11 cell line variant, NU6-1, showed the most variability with cell density (Table 1 and Fig. 2). Single cells expressed four of the epitopes in varying concentrations, but did not express Tn or sialylated Tn. Detection of Le^a-Le^x, Le^a and Le^x on 1 in every 100 cells was observed. One hundred per cent of single cells express sialylated Le^a (Fig. 2H). The subsequent cells of small colonies all expressed these epitopes to a certain degree. The Le^a-Le^x, Le^a, sialylated Tn and Tn oligosaccharides were expressed by only a fraction of the cells in small colonies and the expressing cells were primarily those on the periphery on the colonies (see Fig. 2C,D). By contrast the Le^x and sialylated Le^a oligosaccharides were expressed in 100% of the cells in the small colonies (details not shown). When the colonies grow to the point where cells become confluent, the cells become overlaid with a mucin gel, which, as previously reported [8] is stained by Mabs specific for Le^a-Le^x, Le^a and Le^x in a mosaic pattern which appears in geometric-like plates (Fig. 2E,F). In this case 35–50% of the cells are associated with gels that strongly express the later oligosaccharides. These confluent cells also expressed the sialylated Le^a and sialylated Tn oligosaccharides, but did not preferentially stain the mucin gel and geometric plates were not appreciated. Tn was not expressed by these cells at confluency. When the confluent cells were left to grow and pile upon each other (hyperconfluency), 100% Le^a-Le^x expressivity occurred. However the geometric plate formation is lost (Fig. 2G). In the hyperconfluent state Tn expression is also lost (Table 1).

The observed expression of new oligosaccharide epitopes as single-cells divide to form small colonies is expected to be reliably detected by the monoclonal antibodies employed in this study. However, there is less certainty that the extinction of other previously expressed epitopes would be detected as reliably by the same methods as cells approach confluence. It seems possible that the later oligosaccharide epitopes are actually expressed, but are not available for interaction with antibodies, because of specific adhesion functions in the glycocalyx or with neighboring cells. For this reason we have begun studies of glycoproteins isolated from the different cell types or their media to define the apparent extinction in molecules that have no interactions with cells or glycocalyx. One example of this analysis is shown in Fig. 3.

Soluble mucins and other large glycoproteins from NU6-1 cells were fractionated on a Sephacryl S500 column and analysed for their associated oligosaccharides using immunoblot assays and the Mabs described above. As shown in Fig. 3, glycoconjugates fractionating at positions equivalent to $M_r > 107$ and having properties of mucins [11] are associated with Le^a-Le^x. Other smaller, but also large, glycoconjugates in the M_r range 100 000 to 500 000 are also associated with Le^a-Le^x. Similar analyses using Mabs recognizing the other oligosaccharides also showed that these oligosaccharides are associated with glycoconjugates of comparable size (results not shown). Moreover, a similar analysis of mucins from NE-18 cells (showing extinct expression of Le^a-Le^x) showed no expression of Le^a-Le^x on isolated mucins (results not shown). Thus, at least in this case, there was a good correlation between extinction of an oligosaccharide epitope on confluent cells and in isolated mucins.

The above results show that the expression of different cell-surface oligosaccharides, associated with mucins and other high molecular weight glycoconjugates, varies depending on the growth density of the different types of cells. One cell line, NU6-1 expresses certain oligosaccharides like Le^a-Le^x or Le^a only on rare cells, when the cells grow without neighbors, but the fraction of expressing cells greatly increases when cells grow in colonies or in confluent sheets. However, most of the NU6-1 cells express the sialyl Le^a oligosaccharide when they are growing in single cells, colonies or in confluence. Other cell lines such as BEAS-2B or NE-18 also have characteristic cell-surface oligosaccharides (sialyl Le^a and Le^x respectively) that are expressed differently depending on the state of confluence. We considered the possibility that these varied expressions

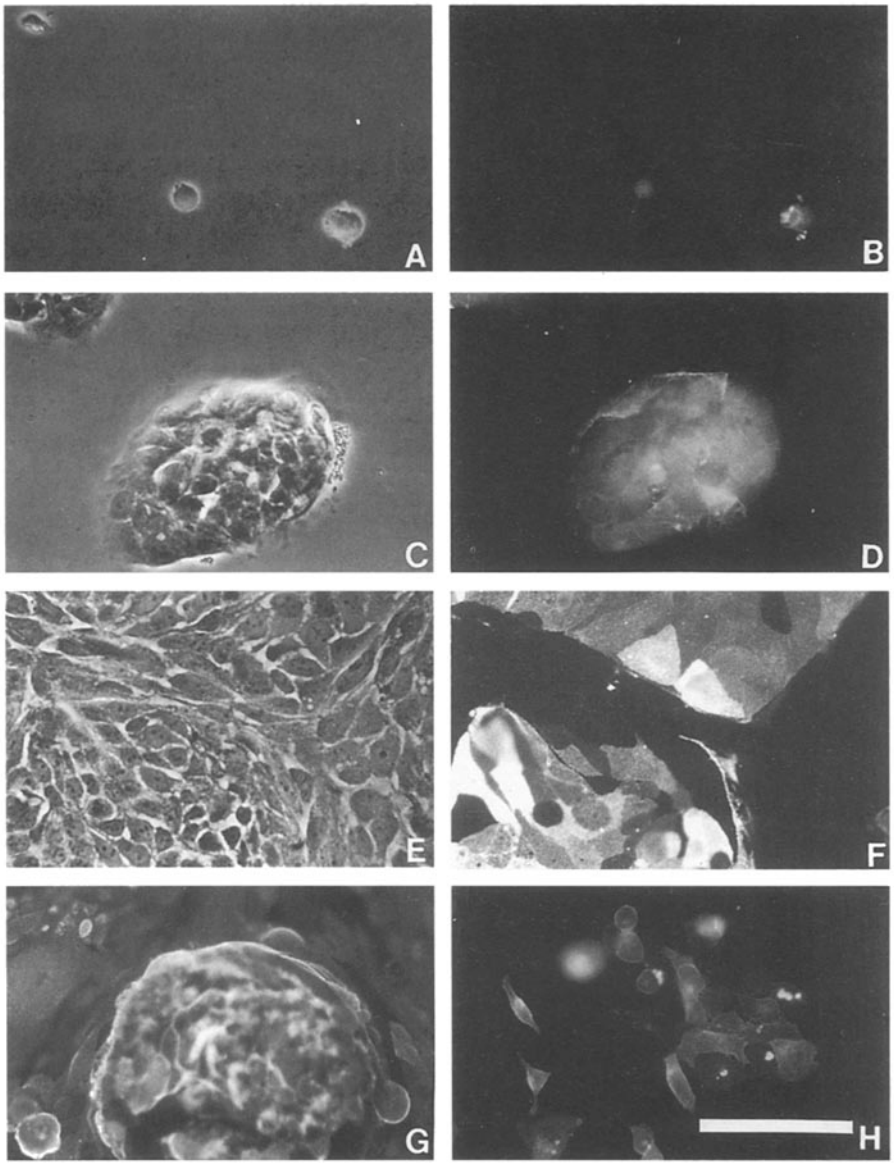

Figure 2. Variation in Le^a-Le^x expression by NU6-1 cells detected by Mab 43-9F. A and B. As single cells only 1 in 100 cells express Le^a-Le^x. A rare field is shown where two expressing cells occur. (A. Phase microscopy; and B. FITC immunofluorescent microscopy of the same cells.) C and D. In small colonies, expression of epitope is by cells at the periphery of each colony. (C. Phase microscopy; and D. FITC immunofluorescent microscopy of the same cells.) E and F. At confluence 30–50% of these cells express Le^a-Le^x and a geometric plate-like gel is apparent. (E. Phase microscopy; and F. FITC immunofluorescent microscopy of the same cells.) G. If these cells are allowed to continue to grow, with adequate new BCS supplemented media, 100% expressivity occurs. FITC immunofluorescent microscopy. H. 100% of single NU6-1 cells express the sialyl Le^a epitope. Of the panel of oligosaccharide epitopes studied sialylated Le^a is the only epitope expressed 100% of the time by single cells. FITC immunofluorescent microscopy. Bar, 150 μm, $\times$ 200.

could be influenced by the methods used to prepare single cell suspensions. For example, the first studies of NU6-1 used trypsin to release confluent cells, which were then plated down as single cells. However, we found that NU6-1 cells prepared by EDTA treatment, or single-cells spontaneously released as they entered mitosis, showed similar decreases in characteristic oligosaccharides, such as Le^a-Le^x when plated as isolated cells (Fig. 1A). Thus the changes in oligosaccharides seem to be characteristic of the cell-cell associations rather than the biochemical treatments that the cells receive.

At this point it is not clear if the observed changes in cell-surface oligosaccharides are attributable to altered regulation in glycosylation (for example via altered expression of glycosyltransferases) or to alterations in the cellular associations of glycoconjugates. An example of the latter would be a selective release into culture media of glycoconjugates carrying specific

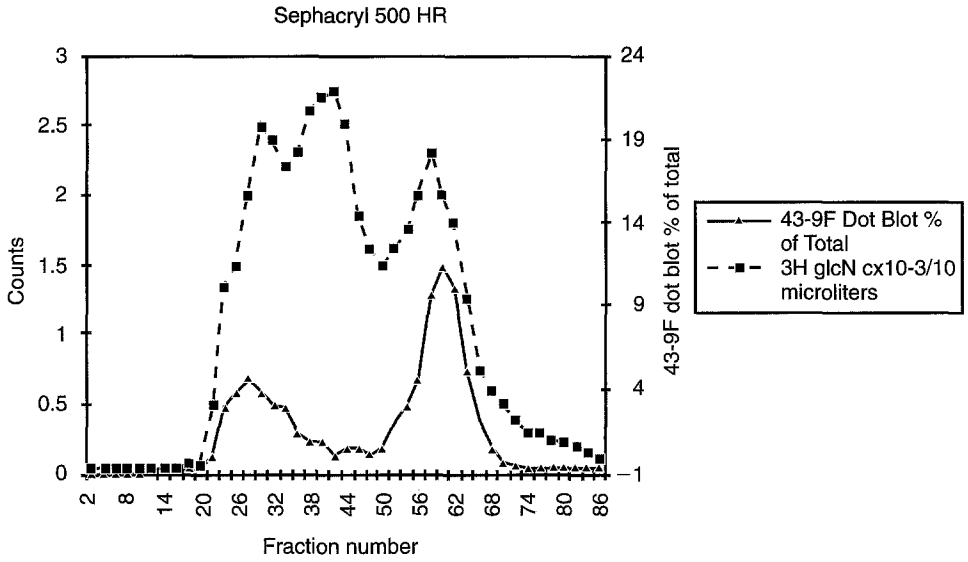

Figure 3. Le^a-Le^x expression by mucins and large glycoconjugates of cell line NU6-1. NU6-1 cells were allowed to grow to near confluence in FCS supplemented RPMI medium. The medium was removed and [^{3}H] glucosamine (10–3/10 μl) added to serum free medium. Growth continued for the next 72 h. The medium was harvested and large glycoconjugates precipitated with ammonium sulfate. These resultant glycoconjugates were applied to a Sephacryl S500 column and fractions collected. Immunoblots of each fraction was performed utilizing Mab 43-9F which identifies the Le^a-Le^x epitope. The oligosaccharide is present in association with the mucin peaks (fractions 18–42) as well as other large glycoconjugates (fractions 43–70).

oligosaccharides when cells grow without contacting neighbors. Alternatively, it also seems possible that mucins and other large glycoconjugates synthesized by one cell could be released and transferred to other neighboring cells and thus present the appearance of more extensive expression when cell densities are increased. These possibilities will be explored in future studies. However, preliminary studies have indicated that the transfer of mucins labeled with specific oligosaccharides does not readily occur from one cell type to another. It was demonstrated that soluble mucins and other large glycoproteins obtained from NU6-1 cells will not associate with the SLC-11 variant clone, NE-18, when the later is grown in media containing mucins decorated with labeled Le^a-Le^x and/or Le^a (data not shown).

Discussion

The present study confirms intracellular oligosaccharide diversity in cell lines (NU6-1 and NE-18) derived from the same tumor which behave very differently when studied in animal tumor models [20]. The results also extend those earlier findings to show that the oligosaccharide diversity on different cells is dependent on the density at which cells grow in tissue culture. Cell density dependent changes in a cell surface oligosaccharide were also observed in the BEAS-2B cell line, derived from normal lung cells, indicating that the phenomena applies to more than just tumor cell lines. It appears as though each cell type is capable of rapidly changing its cell-surface oligosaccharide perhaps either by regulating glycosyltransferase activities, or by modulating the associations of cell membrane glycocalyx quickly in response to associated cells.

It is not clear at this time if the changes in cell surface oligosaccharides that occur, as isolated cells divide and produce neighbors, are directly attributable to the cell-cell interactions that develop, or if the new cell interactions only initiate the changes indirectly through their effects on overall growth. However, it should be noted that the growth conditions, as reflected by cell division rates, do not change appreciably as small colonies develop from single, isolated cells. Yet, in some cases the cell-surface oligosaccharides change during this stage.

The results further suggest that the diversity of cell surface oligosaccharides seen in the developing tumors from these and other tumor cell lines may be at least partly attributable to differences in cell-cell contacts during tumor development. This could be an important consideration as tumor associated oligosaccharides are increasingly used for diagnostic or prognostic indicators.

Acknowledgements

This research was supported in part by Grant P50CA58187 from the US NCI, Grant R25CA49981, ACS-Brooks Trust Account from US NIH and Howard Hughes Medical Institute Grant 71109-500802 to the

University of Colorado-Hughes Undergraduate Biological Sciences Educational Initiative and DOD Grant AIBS 2549.

References

1. Goochee CF, Gromer MJ, Anderson DC, Bahr JB, Rasmussen JR (1992) In: Frontiers in Bioprocessing II (Todd P, Sikdar K, Bier M, eds) pp. 199–240. Washington DC: American Chemical Society.
2. Varki A (1993) *Glycobiology* **3**: 97–130.
3. Schachner M (1990) In: *Morphoregulatory Molecules*, (Edelman GM, Cunningham BA, Thiery JP, eds) pp. 443–68. New York: Wiley.
4. Dwek RA, Edge CJ, Harvey DJ, Wormald MR (1993) *Ann Rev Biochem* **62**: 65–100.
5. Schachter H, Brockhausen I (1989) *Symp Soc Exp Biol* **43**: 1–26.
6. Strous GJ, Dekkar J (1992) *Crit Rev Biochem Mol Biol* **27**: 57–92.
7. Corfield T (1992) *Glycoconjugate J* **9**: 217–321.
8. Stranahan PL, Howard R, Pfenninger O, Cowen ME, Johnston MR, Pettijohn DE (1992) *Cancer Res* **52**: 2923–30.
9. Hakomori S-i (1988) In: *Altered Glycosylation in Tumor Cells* (Reading CL, Hakomor S-i, Marcus DM eds) pp. 207–12. New York: Alan R Liss Inc.
10. Hakomori S-i (1985) *Cancer Res* **45**: 2405–14.
11. Pettijohn DE, Stranahan PL, Due C, Ronne E, Rahbek SH, Olsson L (1987) *Cancer Res* **47**: 1161–69.
12. Engel U, Stranahan P, Hage E, Pettijohn DE, McCombs R, Rahim I, Ollson L (1995) *Proc 86th Ann Meeting, Am Assoc Can Res* **36**: 587.
13. Battifora H, Sorensen HR, Mehta P, Ahn C, Niland J, Hage E, Pettijohn DE, Olsson L (1992) *Cancer* **70**: 1867–72.
14. Stroud MR, Levery SB, Martensson S, Salyan MEK, Clausen H, Hakomori S-i (1994) *Biochemistry* **33**: 10672–80.
15. Sell S (1990) *Human Pathol* **21**: 1003–19.
16. Feizi T (1985) *Nature* **314**: 53–57.
17. Itzkowitz SH (1992) *APMIS* (Suppl 27) **100**: 173–80.
18. Hakomori S-i (1989) *Adv Cancer Res* **52**: 257–331.
19. Irimura T (1994) *Adv Exp Med* **353**: 27–34.
20. Pettijohn DE, Pfenninger O, Brown J, Duke R, Olsson L (1988) *Proc Natl Acad Sci USA* **85**: 802–6.
21. Stranahan PL, Laroe J, Reddy D, Rahim I, Overland M, Pettijohn DE (1994) *Oncol Rep* **1**: 607–11.