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Enhancement of glycosylation of cellular glycoconjugates in the squamous carcinoma cell line MDA886Ln by β-all-*trans* retinoic acid

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Retinoids have been shown to inhibit the growth and modulate the glycosylation of head and neck squamous cell carcinoma (HNSCC) cells including the MDA886Ln cells. To examine the effects of β-all-*trans* retinoic acid (RA) on glycoconjugates in HNSCC MDA886Ln cells, the cells were grown in the absence or presence of 1 μM RA and then labeled with tritiated monosaccharides, extracted and analysed by polyacrylamide gel electrophoresis and fluorography. RA increased markedly the incorporation of [^{3}H]-glucosamine, [^{3}H]-galactose, and [^{3}H]-mannose into numerous cellular glycoconjugates, however, the incorporation of [^{3}H]-fucose and [^{3}H]-leucine was almost unaffected by RA. RA increased the incorporation of glucosamine and galactose but not mannose into high molecular weight (HMW) glycoconjugates of about 220 and 500–600 kDa. To analyse the steady state level of glycoconjugates by lectin blotting, extracts of unlabeled cells were separated by gel electrophoresis and the gels were probed with ^{125}I-labeled wheat germ agglutinin (WGA) and *Maackia amurensis* (MA) agglutinin. Both lectins were found to bind to numerous glycoconjugates including the HMW glycoconjugates, whereas ^{125}I-peanut agglutinin bound only to the HMW glycoconjugates. RA treatment increased the binding of all three lectins to the HMW glycoconjugates. These findings demonstrate that RA enhanced the incorporation of specific monosaccharides into a variety of glycoconjugates and in particular into HMW mucin-like glycoconjugates. This effect of RA may be the result of induction of a more normal differentiation state of the HNSCC cells.

Keywords: retinoids, squamous cell carcinoma, MDA886Ln

Introduction

The exposure of the lining epithelium of the upper aerodigestive tract mucosa to tobacco smoke and environmental carcinogens may lead to the development of cancer [1]. Distinct premalignant lesions, are often clinically recognized and are thought to arise from a process termed field cancerization, i.e. exposure of vast areas of the upper aerodigestive tract to carcinogens [1]. Another process that occurs during carcinogenesis is alterations in glycosylation which result in the expression of both new and altered glycoconjugates [2–4] including mucins [5]. These are thought to modulate cellular processes including growth, differentiation and carcinogenesis itself [2–5]. Several studies have demonstrated changes in glycoconjugates in head and neck cancers [6–11].

Retinoids, vitamin A metabolites and synthetic analogs, have been shown to suppress the carcinogenic process as well as modulate growth, differentiation, and glycosylation [12]. There is a continuing interest in these compounds since retinoic acid has been shown to be active in both preventing progression of premalignant lesions [7] and inhibiting formation of second primary lesions arising during field cancerization [13, 14].

Retinoid-induced alterations in the glycosylation of cell surface glycoconjugates have been demonstrated in various cell types usually in conjunction with altered differentiation [7, 15–26]. Some alterations in glycosylation may result from enhanced expression or activation of glycosyltransferases [9, 17, 18, 22, 23, 26].

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Subtle changes in the glycosylation of cell-surface glycoconjugates have been implicated in the regulation of many processes associated with cell growth, differentiation, and malignant transformation [2–5, 26–28]. The finding that retinoids are able to modulate glycosylation has given rise to the hypothesis that the altered glycosylation of specific cell-surface glycoproteins may be related causally to some of the many effects of the retinoids on cellular growth and differentiation and the expression of the transformed phenotype.

We and others have demonstrated effects of retinoic acid in modulating growth, differentiation, and glycosylation in head and neck squamous carcinoma (HNSCC) cell lines [7, 21, 25]. Although RA treatment is growth inhibitory to HNSCCs [21, 25], mechanistically this inhibition is not just classical cytostasis. In a multicellular tumor spheroid model for HNSCC, growth inhibition was not correlated with altered cell cycle kinetics [29] and in monolayer cells of this spheroid forming cell line (MDA886Ln), a large S-phase fraction is present in growth inhibited-chemosensitive cells.

Previous studies demonstrated alterations in glycosylation in MDA886Ln multicellular tumor spheroids [25]. In the present study, we have extended our analysis of retinoid-induced alterations in glycosylation to MDA886Ln grown in monolayers culture, which enabled us to analyze the effects of retinoids more extensively. We focused on changes in high molecular weight mucin-like material using metabolic labeling with selective sugars and lectin blotting techniques.

Materials and methods

Cells and culture

The human squamous carcinoma cell line MDA886Ln was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 supplemented with 10% fetal calf serum and gentamicin (50 μg ml^{-1}) as described previously [25]. Cells were exposed to β-all-*trans* retinoic acid (RA) (Sigma, St Louis, MO) diluted from a 0.1 mM stock solution in DMSO to a final concentration of 1 μM in the growth medium or to the control vehicle DMSO with medium changed every 48–72 h as described previously [21]. For growth studies, cells were grown in 60 mm diameter tissue culture dishes and treated with RA as above. Cell numbers were obtained by trypsinizing duplicate plates and counting the suspended cells using an electronic particle counter (Coulter Electronics, Hialeah, FL).

Metabolic labeling of cell cultures with tritiated monosaccharides

Cells grown for 8 days in control or RA-containing medium were metabolically labeled for the last 24 h of culture with D-[6-^{3}H]glucosamine (25–40 Ci mmol^{-1}), L-[6-^{3}H]fucose (15–30 Ci mmol^{-1}), D-[6-^{3}H]galactose (10–25 Ci mmol^{-1}), [2-^{3}H]mannose (20–40 Ci mmol^{-1}), and L-[4,5-^{3}H]leucine (40–60 Ci mmol^{-1}) (ICN Radiochemicals, Irvine, CA) all at 10 μCi ml^{-1}.

Cellular solubilization, electrophoretic analysis, and lectin blotting

Radiolabeled cultures were solubilized as described previously [24]. Briefly, cells were washed three times with Ca^{2+} and Mg^{2+}-free phosphate buffered saline, scraped and extracted for 30 min at 4 °C in 0.5% Nonidet P-40 in 10 mM Tris-HCl, pH 7.2, containing 0.25 M sucrose, 0.05 mM $CaCl_2$, and 1 mM phenylmethylsulfonylfluoride. The suspension was centrifuged at 12 000 × **g** for 5 min and the supernatant collected. The protein concentration in the supernatant fraction was determined by a dye-binding assay and adjusted to 10 mg ml^{-1} and mixed 1:2 with a three-fold concentrated sample buffer [15]. Radiolabeled proteins (100 μg in 30 μl per lane) were separated by sodium dodecylsulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) using a 3% stacking and 7% polyacrylamide running gel with a Laemmli (1970) buffer system. Prestained molecular weight protein markers were purchased from Bio-Rad. High molecular weight proteins were examined using a 2.5% stacking and 3% running gel as described by Irimura *et al.* [30]. Briefly, gels were polymerized on Gelbond PAG (FMC Corp., Rockland, ME), which had been attached to one of the glass plates with a two-sided adhesive. Laminin (Telios, La Jolla, CA) was used as a high molecular weight marker. Gels were stained with Coomassie brilliant blue R-250 to visualize proteins and processed for fluorography by impregnation with EN^{3}HANCE (NEN, Boston, MA), dried on filter paper and placed against X-OMAT X-ray film at −70 °C to visualize radiolabeled glycoconjugates. Lectin binding studies used wheat germ agglutinin (WGA), *Maackia amurensis* lectin II (MA) and peanut agglutinin (PNA) (Vector Labs, Burlingame, CA), which were iodinated with ^{125}I by the chloramine-T method. Following SDS-PAGE, gels were probed with 3 × 10^7 cpm ^{125}I-labeled lectin (2 × 10^4 cpm μg^{-1}) as described previously [25]. Following extensive washing, probed gels were dried and placed against X-OMAT X-ray film for autoradiography at −70 °C.

Immunofluorescence

Cells were grown in the absence or presence of 1 μM RA for 6 days. During the last 48 h the cells were subcultured onto glass coverslips. The cells were washed with ice cold PBS and incubated with 50 μg ml^{-1} fluorescein isothiocyanate (FITC)-labeled lectins (Vector Labs) for 1 h at 4 °C. The cells were then washed three times with PBS and fixed with 3.5% paraformaldehyde, mounted in 90%

glycerol, and examined using an epi-fluorescent microscope (Nikon Inc., Garden City, NY).

Results

RA-induced changes in cell growth

When MDA886Ln cells were exposed to 1 μM RA, their growth was inhibited after several days of treatment (Fig. 1). While control cultures continued to increase in cell number, RA-treated cultures reached a plateau at about 7 days of treatment. Thus, growth inhibition continued to increase over time (Fig. 1, inset). Morphologically, control cells reached confluency over the 9 day growth period and exhibited a tightly compact cobblestone-like appearance with evidence of cell piling (Fig. 2A). RA-treated cultures, in contrast, were composed of cells with both a highly compact morphology and with a more spread appearance and they did not pile up (Fig. 2B).

Metabolic labeling of control and RA-treated cultures

Previously, we have determined by metabolic labeling and WGA lectin binding that RA-induced changes in [^{3}H]-glucosamine incorporation into glycoproteins when

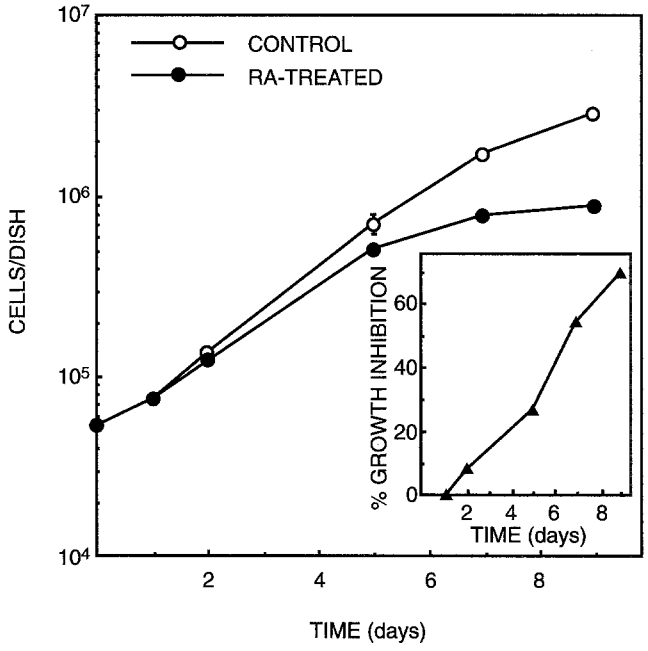

Figure 1. Growth inhibitory effect of RA on MDA886Ln cells in monolayer culture. Cells were grown for 9 days in the absence (control) or presence (RA-treated) of 1 μm RA. At the indicated time points, duplicate dishes were removed from the incubator, the cells were harvested and counted. Inset shows percentage growth inhibition defined as [1–(Nt/Nc)] × 100, where Nt and Nc are the numbers of cells in RA-treated and control cultures, respectively. Errors between plates were ≤12% of the mean.

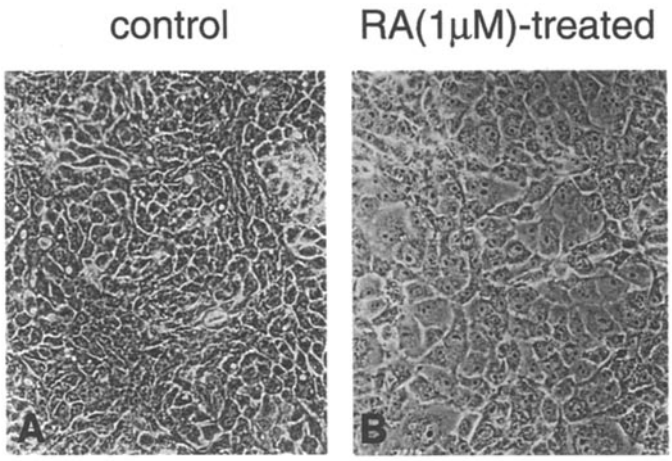

Figure 2. Phase contrast photomicrographs of MDA886Ln cells grown for 8 days in the absence (A, control) or presence of 1 μm RA (B, RA-treated).

MDA886Ln cells were grown in a three-dimensional configuration as a multicellular tumor spheroid [25]. We have extended this observation to monolayer cultures and examined for incorporation of additional sugar moieties using metabolic labeling of 8 day control and RA-treated cultures, which are in a growth-inhibited state (Fig. 1). Figure 3 shows that there is an overall increase in [^{3}H]-

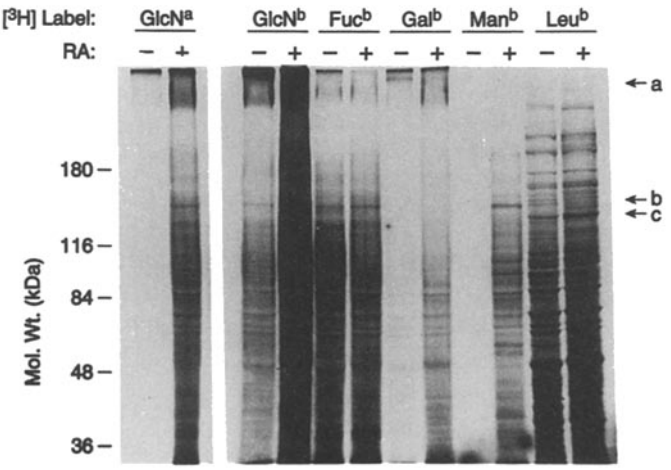

Figure 3. Analysis of glycoconjugates by metabolic labeling. MDA886Ln cells were grown for 8 days in control medium (–) or in medium supplemented with 1 μm RA (+). Cells were treated for the last 24 h with ^{3}H-labeled GlcN (glucosamine), Fuc (fucose), Gal (galactose), Man (mannose) or Leu (leucine), then harvested, extracted, and processed for SDS-PAGE using a 7% polyacrylamide gel. About 100 μg protein were loaded onto each well. Numbers to the left represent the molecular mass in kDa of standard of molecular weight markers. [a]The GlcN-labeled glycoproteins visualized after a short exposure (16 h); [b]The gel was placed against an X-ray film for 5 days. The lettering a, b, and c, on the right represent bands that increase by RA treatment.

glucosamine incorporation in the RA-treated cultures while incorporation of [^{3}H-leucine] is very similar between control and RA-treatment. Although there are some minor differences in leucine labeling (for example, band c, Fig. 3), the comparison indicates that there is an overall RA-induced increase in glycosylation rather than *de novo* protein synthesis. Similarity in protein profile and overall amount per each lane was further confirmed by Coomassie blue staining (data not shown). Additionally, this broad increase in glycosylation was sugar specific being found for glucosamine, galactose, and mannose but not evident with [^{3}H]-fucose (see for example band b in Fig. 3). Although incorporation of [^{3}H]-fucose was similar between control and RA-treated cultures, specific increases or decreases could be found (Fig. 3). Differences between individual sugars in the composition of glycoproteins was also evident. Control cells show a higher level of fucose incorporation than glucosamine, and extremely low levels of galactose and mannose. These low levels are however not due to a loss of specific transferases, since RA induces incorporation of both [^{3}H]-galactose and [^{3}H]-mannose.

Examination of the top of the running gel in Fig. 3 shows the presence of high molecular weight (HMW) glycoconjugates which barely enter the gel (HMW band a in Fig. 3). The apparent lack of incorporation of [^{3}H]-mannose into this material even after RA-treatment suggested that it contains O-linked rather than N-linked oligosaccharide side chains and this combined with its high molecular weight suggested that it is a mucin [5]. To further pursue the nature of these HMW mucin-like glycoproteins, radiolabeled proteins were separated using 3% SDS-PAGE gels (Fig. 4). A significant amount of [^{3}H]-glucosamine-labeled material still remained in the 2.5% stacking gel and an overall increase in glycosylation was still evident. RA-induced increases in glucosamine incorporation were apparent in glycoconjugates of approximately 500–600 and 200–300 kDa, (broad bands a and b, Fig. 4) with the higher molecular weight band also being prominent in control cultures. Additionally, there was a slight increased incorporation of [^{3}H]-fucose and [^{3}H]-galactose but not of [^{3}H]-mannose or [^{3}H]-leucine.

Lectin staining of control and RA-treated cultures

To further characterize the nature of the glycoconjugates in untreated and RA-treated cells, SDS-PAGE gels were probed with ^{125}I-labeled lectins. WGA binds to *N*-acetylglucosamine and to sialic acid in α-2,3 and α-2,6 linkages whereas MA agglutinin binds to sialic acid in α-2,3 linkages. The similarity in the ^{125}I-WGA and ^{125}I-MA patterns suggests that WGA binding to molecules >84 kDa is mostly to sialic acid residues (Fig. 5). The high degree of sialylation of these glycoproteins may be responsible for the lack of PNA staining since sialic acid inhibits PNA binding to most of the molecules recognized

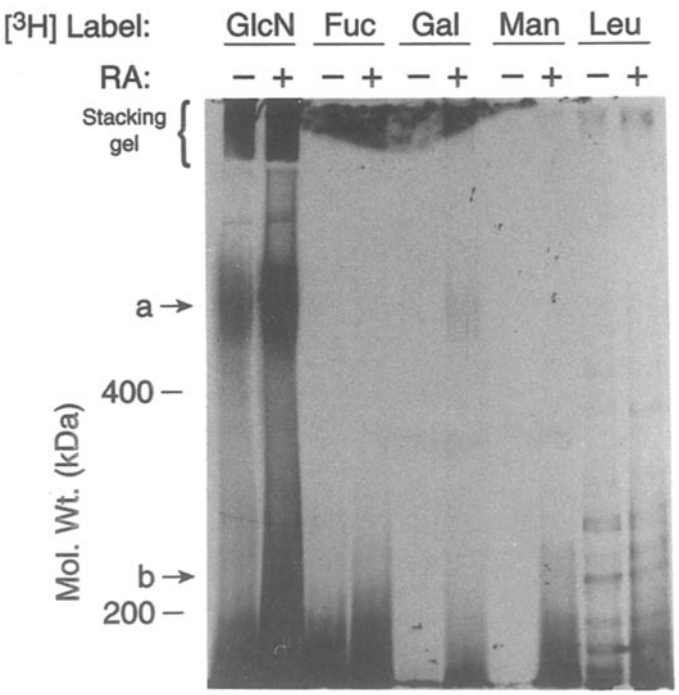

Figure 4. Analysis of high molecular weight glycoconjugates by metabolic labeling. The same samples described in Fig. 3 were separated on a 3% polyacrylamide gel. Laminin was used as the molecular weight marker. The gel was placed against X-ray film at –70 °C for 26 days.

by WGA except for the very high molecular weight ones (band a in Fig. 4). RA treatment caused a decrease in ^{125}I-WGA and ^{125}I-MA binding to glycoproteins b and c (200 and 187 kDa, respectively). The HMW mucin-like glycoproteins on top of the gel stained with all three lectins (band a, Fig. 5). Their intense staining with PNA further supported their mucin-like nature. Further examination of this HMW material in 3% gels demonstrated that there was an increased binding of all three lectins at 500–600 kDa following RA treatment (Fig. 6, band b). Within the 2.5% stacking gel, there was increased binding of WGA and MA to a HMW glycoconjugate(s) (band a, Fig. 6).

Control and RA-treated cells were stained similarly on the cell membrane with FITC-labeled WGA and PNA (Fig. 7).

Discussion

Two methods for analysis of glycoconjugates were utilized in the present study to examine the effects of RA on glycoconjugates in HNSCC MDA886Ln cells: metabolic labeling for *de novo* synthesis and lectin blotting for steady state levels. These analyses have shown that the pattern of both high and low molecular weight glycoconjugates in MDA886Ln cells is complex and that

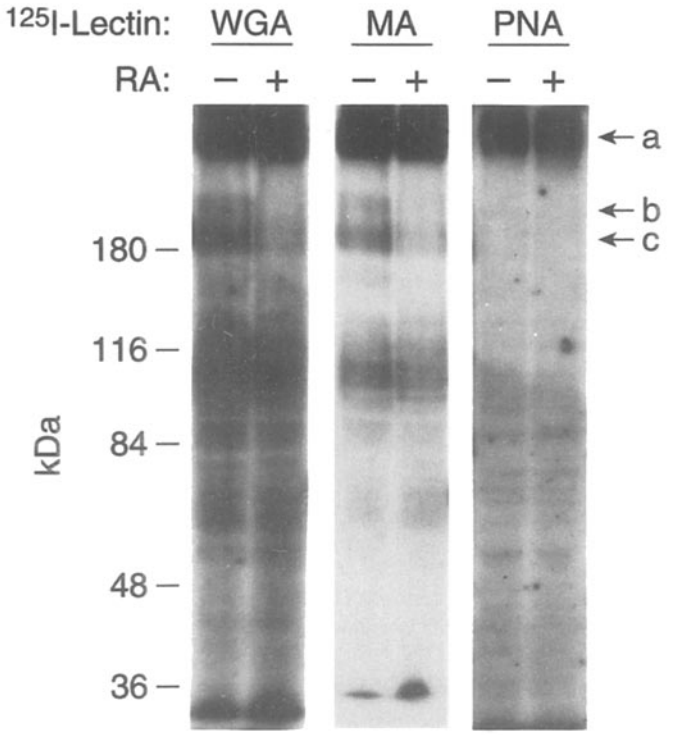

Figure 5. Binding of ^{125}I-labeling WGA, MA, and PNA to MDA886Ln glycoproteins separated in a 7% polyacrylamide gel. Cells were grown in the absence (–) or presence of 1 μm RA (+) for 8 days and extracted and processed for SDS-PAGE. Numbers to the left represent the molecular mass in kDa of standard of molecular weight markers.

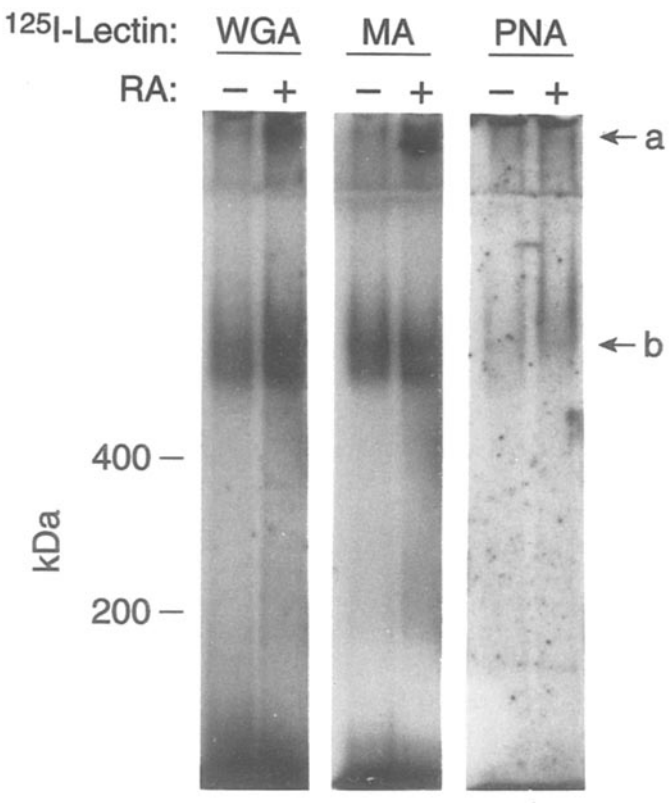

Figure 6. Binding of ^{125}I-labeled lectins to MDA886Ln glycoproteins separated on a 3% polyacrylamide gel. The same samples described in Fig. 5 were separated on a 3% polyacrylamide gel. Laminin was used as the molecular weight marker.

RA-induced alterations in high and low molecular weight glycoconjugates. The steady state level of glycoconjugates as detected by lectin blotting was altered in only a few molecules. In contrast, the metabolic labeling with [^{3}H]-labeled GlcN, Gal, and Man revealed a very marked increase in the glycosylation of numerous glycoconjugates. That this was not a generalized increase in protein synthesis was indicated by the similar labeling of control and RA-treated cells with [^{3}H]-leucine. It is noteworthy that no generalized upregulation of all glycosylation reactions as indicated by the finding that [^{3}H]-fucose incorporation was not enhanced by RA treatment. In this respect the effect of RA on MDA886Ln cells was distinct from RA's effect on F9 embryonal carcinoma cells in which RA increased fucosylation [31].

RA increased [^{3}H]-GlcN incorporation into glycoconjugates with apparent molecular weights greater than 200; 500; and even molecules that failed to enter the 3% gel. These molecules were not labeled by mannose however, they were apparently recognized by WGA, MA, and PNA. These are probably mucin-like molecules because they exhibit characteristics of glycoconjugates containing O-linked oligosaccharide chains [5].

Alterations in glycosaminoglycan synthesis in HNSCC

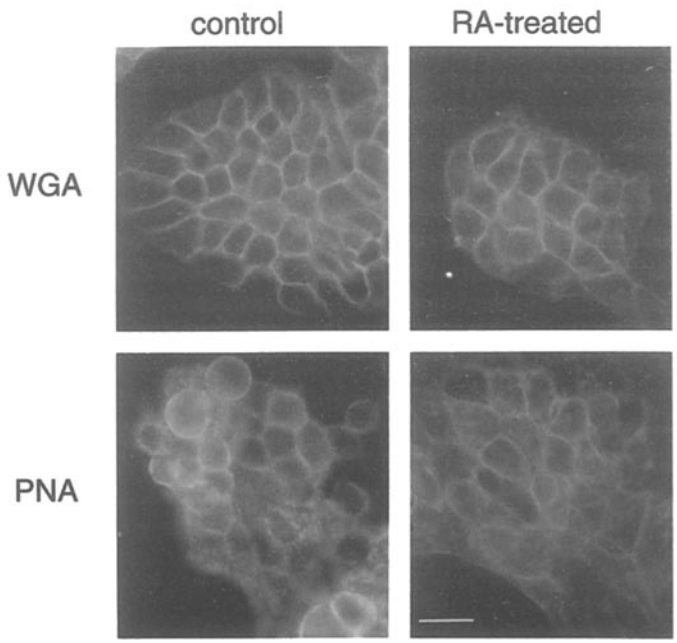

Figure 7. MDA886Ln cells were grown on coverslips in the absence or presence of 1 μm RA for 8 days. They were then stained with FITC-labeled WGA or PNA.

cell lines treated with retinoids have been reported previously by others [7] and by us [21, 25]. The altered glycosylation by RA could be the result of RA-induced changes in glycosyltransferases that have been indentified in diverse cell culture systems [17, 22, 23, 26].

Changes in glycoconjugates also occur during keratinocyte differentiation *in vivo* and *in vitro* [16, 32–34]. Differential lectin binding was shown for various layers of rat oral epithelium [6]. Comparisons between *in vitro* cultures of normal keratinocytes and SCC cell lines have shown both similarities and differences in proteoglycans and glycoproteins [32, 33].

Alterations in glycoconjugates have been shown to occur during development of HNSCCs [10, 11]. Alterations in carbohydrates are associated with abnormalities in proliferation and differentiation, and can be detected in premalignant as well as malignant lesions [8–11]. Our results may represent a restoration of a more normal differentiation state of the HNSCC MDA886Ln cells. Changes in carbohydrate moieties may have clinical significance. For example, Rh factors have been shown to have prognostic values [35] as have antibodies to the A9 antigen [36]. This latter reagent has been shown to recognize the $\alpha_6\beta_4$ integrin [37] and expression of this integrin also correlates with disease progression in the mouse skin carcinogenesis model [38].

Retinoids effect the biology of HNSCC cell lines by modulating basic cellular processes including but not limited to growth, differentiation, sensitivity to chemotherapeutics and glycosylation. The implications of altered glycosylation to these basic biological processes are still unknown and await determination.

Acknowledgements

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