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Repression of the Lewis Fucosyl Transferase by Retinoic Acid Increases Apical Sialosyl Lewis^a Secretion in Colorectal Carcinoma Cultures

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Abstract The rate of polarised secretion of sialosyl Lewis^a(19-9) molecular species (SiaLeams) by SW1116 colorectal carcinoma cells is stimulated at least ninefold by the presence of 3 μM retinoic acid (RA). In order to investigate the intracellular origins of this augmentation, carcinoma cell membranes, membrane subfractions, and media were studied to determine alterations in sialosyl Lewis^a levels, oligosaccharide composition, and core structures accompanying the capacity to increase export of this epitope. We observed a nine- to twentyfold increase in sialosyl Lewis^a epitope levels in a light membrane subfraction from RA-treated cells. Antigenic molecules of $<200{,}000$ Mr on acrylamide gradient gels were concentrated in two doublets in the apparent Mr range 106,000–152,000 on Western blots. Carbohydrate analyses of oligosaccharides from SiaLeams of membrane subfractions and apical media indicated much higher fucose/mannose, fucose/sialic, fucose/sialosyl Lewis^a, fucose/total CHO, and (^{3}H) fucose incorporation in control samples than RA samples. Western blots of samples from membrane subfractions and media indicated that, in contrast to the effect of RA on the sialosyl Lewis^a epitope, RA treatment did not augment cysteine-rich, PDTRP, blood group H-2, blood group A, and EGF receptor-like region epitopes in the media. In addition, Northern blots using the Lewis fucosyl transferase (FTIII) cDNA showed a dramatic diminution of mRNA encoding FTIII but apparently unaltered levels of sialyl transferase (ST4) mRNA. Since subterminal fucosylation of lactosyl termini blocks terminal sialylation, we conclude that one mechanism of sialosyl Lewis^a induction in this culture system is the lower expression of the Lewis fucosyl transferase mRNA. Therefore less subterminal fucosylation of GlcNAc permits the prior sialylation of terminal Galβ1-3 moieties at oligosaccharide termini destined for export from the Golgi.

Key words: carcinoma, glycoproteins, secretion, retinoic acid, epithelial cell biology

Several colorectal carcinoma cell lines have been found to secrete mucin or mucin-like glycoproteins—for example, HT-29 [Devine et al., 1991], T-84 [Roumagnac and Laboisse, 1987; McCool et al., 1990], and the human derived SW1116 [Yedgar et al., 1992; Liepkalns and Icard-Liepkalns, 1993]. The SW1116 cell line has proven to be particularly useful in our previous studies because the cells are relatively large, adhere well to plastic and membrane surfaces, and are enriched in carbohydrate epitopes such as sialosyl Lewis^a (S-Le^a), and Lewis^a [Liepkalns et al., 1985, 1988]. The sialosyl-Le^a epitope has the reported structure NeuNAcα2-3Galβ1-3(Fucα1-4)GlcNac- [Magnani et al., 1982], and the monoclonal antibody against this structure (Mab 19-9) has been used to screen for the presence of gastrointestinal tumors [Steinberg, 1990].

This same epitope has been shown to be present on mucin-like glycoproteins [Yedgar et al., 1992; Klug et al., 1988], mucins from cystic fibrosis patients [Roberts et al., 1986], and colorectal carcinoma cell gangliosides [Magnani et al., 1982]. One mucin-like protein from SW1116-conditioned media has been purified (apparent Mr 210,000). This glycoprotein has some significant differences in composition from gel forming mucins from other sources, although it still apparently carries the sialosyl Lewis^a epitope [Klug et al., 1988]. Thus, the sialosyl Lewis^a monosialoganglioside carries one epitope, while O-glyco-

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sylated carbohydrate rich (mucin type) glycoproteins can carry the epitope in superfluity at termini of oliogosaccharide chains [Carraway and Hull, 1989].

Our original interest was to develop a membrane filter growth system for colorectal carcinoma cells in synthetic media in an effort to provide conditions more conducive to differentiation and polarization. The effects of various agents could then be studied on the polarised secretion of sialosyl Lewis^a–carrying substances by cells surrounded by media.

We subsequently reported a dramatic rise in the secretion of sialosyl Lewis^a of about an order of magnitude (per 24 h) after a 10 day treatment of cultures with 2.6 μM retinoic acid (RA). This increase in secretion of the epitope into apical media was accompanied by a 40% decrease in cell division rate and a 40% increase in protein per cell in these RA fed cells [Liepkalns and Icard-Liepkalns, 1993].

The purpose of the current study is to determine the intracellular origins of this massive increase in secretion of the epitope. We fractionated and subfractioned SW1116 cells and purified sialosyl Lewis^a molecular species from intracellular membranes and from media. We followed antigenicity by ELISA (solid-phase bound 19-9 antibody) and Western blotting and analyzed sugars and the transferases responsible for the biosynthesis of the epitope by direct assay and molecular biological techniques.

Our immunochemistry and isotope incorporation studies indicated that a light membrane fraction from the cells contained >100,000–200,000 Mr sialosyl Lewis^a carrying glycoproteins which increased in specific antigenicity nine- to twentyfold in cells fed RA. Western blots with an array of monoclonal antibodies, sugar analyses, and Northern blots demonstrated that one mechanism of RA induction of sialosyl Lewis^a secretion is through elaboration of oligosaccharide termini by diminution of Lewis fucosyl transferase (FTIII) messenger RNA, lowering the specific activity of FTIII in Golgi compartments and permitting sialylation of terminal Galβ moieties by an α2-3 sialyl transferase, since the ST-4 sialyl transferase level of expression remains, by and large, unaltered by RA treatment in these cells.

Our results portend functional significance in view of recent reports on a role for sialosyl Lewis^a in carcinoma cell adhesion [Majuri et al., 1992; Varki, 1994].

MATERIALS AND METHODS

Cell Culture on Membrane Supports and Preparation of Retinoate-Treated Cells

SW1116 human colorectal carcinoma cells were originally supplied by Dr. Hilary Koprowski of the Wistar Institute, and their growth characteristics and antigenic properties have been published previously [Liepkalns and Icard-Liepkalns, 1993; Liepkalns et al., 1985].

Cells were seeded on Millipore (Bedford, MA) HA membranes in 7% fetal calf serum (mycoplasma and virus screened) at 200,000/cm^2 in DME/F12 medium fortified with glutamine, Pen-Strep, and gentamycin (Gibco, Grand Island, NY). After 24 h all media were changed to synthetic (UG) substitute in DME/F12 plus or minus 3 μM RA. Our conversion (to convert cells to high secretors) period was consistently five medium changes in 10 days. Previous experiments had indicated that 1.5 μM RA for 10 days or 2.6 mM for 3 days was sufficient to alter epitope secretion. Twenty-four hours after the last medium change we could start a time course for secretion of sialosyl Lewis^a or add isotope.

Sialosyl Lewis^a assays, protein determinations, and cell counts were performed as described previously [Liepkalns and Icard-Liepkalns, 1993].

After washing the cell surfaces (apical and basal) two times with Hanks BSS (Gibco) containing 1 mM PMSF, the surfaces were again washed with an isolation buffer (300 mM sucrose, 5 mM imidazole, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml phenanthroline, 1 mM benzamidine, 1 mM PMSF, and 50 μg/ml NaN3, pH 7.1) [Mason and Jacobson, 1985]. In isotope incorporation studies control and RA-converted cells were prepared as described above, but 1 μCi/ml of (^{3}H) fucose 17 Ci/mmol (NEN, Boston, MA) was added in DME/F12 medium 24 h after the last medium change to the interior and exterior of the filter growth surfaces for 24–48 h. Cell surfaces were washed three times and placed in fresh medium for postincubation studies and/or fractionation. Control experiments showed that the effect of retinoate on secretion was not significantly abated during its 48 h absence (after the 10 day treatment).

For fractionation, apical and basal surfaces were washed with Hanks BSS (Gibco) containing 1 mM PMSF (Sigma, St. Louis, MO) and then with isolation buffer. The cells were then harvested by gently scraping the membranes

under 1 ml of isolation buffer. The cell suspension was then submitted to a Potter homogenizer (P. Braun) equipped with a teflon piston 20 times, 5 seconds each, maintaining temperatures at or near 4°C.

This homogenate was then centrifuged 100*g* for 10 min in a Sorvall centrifuge, and the supernatant from that centrifugation was filtered through a 10 micron nylon mesh. The cell diameter of SW1116 averages much more than 10 microns (approximately 30–100 microns depending on the cell density, rate, and stage of division). The filtrate was centrifuged at 6,000*g* for 10 min to obtain a "6K pellet." The supernatant from this preparation was centrifuged at 48,000*g* for 60 min to obtain a "48K pellet." Both pellets could be subfractionated on discontinuous sucrose gradients consisting of 0.8 ml each of sample resuspended in isolation buffer, on 0.8 ml of 0.8 M sucrose, 1.1 M sucrose, 1.25 M sucrose, 1.5 M sucrose, and 2.0 M sucrose, all in imidazole, pH 7.1. Gradients were subjected to 260,000*g* in a SW 50.1 swinging bucket rotor by a Beckman L8-60 M centrifuge for 2 h. Subfractions of 400 μl each were collected for assay of protein (BCA method; Pierce Biochemicals, Rockford, IL) and sialosyl Lewis^a by ELISA (CIS-BIO, Saclay, France; a license of Centocor, Malvern, PA), Western blotting techniques, and purification of sialosyl Lewis^a carrying molecular species (SiaLeams) by binding to solid phase 19-9 antibody in ELISA assay tubes (CIS-BIO).

Sialyl Transferase and Fucosyl Transferase Assays for the Galβ1-3GlcNAc Acceptor

Sialyl transferase assays and fucosyl transferase assays on the 48K membrane preparations from control and RA cells were performed by procedures modified from those published previously [Liepkalns et al., 1988; Jolif and Liepkalns, 1988]. Control and RA enzymatic activities were optimized for pH, detergent (taurocholate), and were saturating for Galβ1-3 GlcNAc acceptor and the nucleotide donors, CMP-NeuNAc (sp. act 11.4 μCi/mmol, diluted from 15 Ci/mmol; NEN) and GDP-fucose (10 μCi/mmol, diluted from 7.4 Ci/mmol). Instead of paper and florisil chromatography we isolated the products by column chromatography from Dowex 1 columns. Sialylated carbohydrates were eluted with 5 mM phosphate buffer from Dowex phosphate columns, and fucosylated carbohydrate products were eluted with H_2O from Dowex chloride columns. Activities shown here are in linearity for time and saturating for the substrates with boiled enzyme and "minus acceptor" values subtracted.

Binding of SiaLeams to Solid Phase Antibody, Washing, and Recovery

Aliquots of sucrose gradient subfractions or apical or basal media were adjusted to total volumes of 200–300 μl with phosphate buffered saline (plus PMSF, pepstatin, and NaN3, pH 7.2) (PBS) were added to assay tubes containing solid phase 19-9 antibody for immunobinding. We incubated these preparations either 8 h at ambient temperature or 16 h at 4°C. The washing procedures to remove bound substances were the following:

1. for nonspecific water soluble amphipathic (0.5 ml each) 2× PBS, 1× 0.3% Tween in PBS, followed by 3× PBS, pH 7.2, wash (A)];
2. for specifically bound antigens, wash (A) followed by 0.1 M triethyl amine (1 h at ambient temperature) followed by 0.1 M glycine, 50–200 μl each (pooled extracts were analysed);
3. for removal of lipids and gangliosides, wash (A) followed by 1.0 mL of chloroform/methanol/H_2O, 4/8/3, vortex, followed by 200 μl of the same, vortex [Kaufman et al., 1984; Fredman and Svennerholm, 1980], pool organic extracts [wash (LX)];
4. for removal of ester-bound radioactivity, wash (A), then wash (LX) and then 1.5 M neutral NH_2OH 200 μl (30°C, 4 h) or 0.15 M KOH in anhydrous CH_3OH, 600 μl (37°C, 1 h) [Slomiany et al., 1984; Laemmli, 1970; Liepkalns et al., 1993].

Separation and Detection of SiaLeams by Gradient SDS-PAGE, Western Blotting; Testing for the Presence of Sialosyl Lewis^a, Mucin, and Mucin-Like Core Structures, H-2 Antigen, Blood Group A, and EGF Receptor-Like Regions

Fractions, subfractions, media, and purified antigens were preincubated prior to gel analysis on a SDS-PAGE on 5–12% gradient, not at 100°C [McIlhinney et al., 1985], but rather at much milder conditions, which permit retention of oxyester and thioester bonds: 37°C in loading buffer (SDS 2%, mercaptoethanol 5%, glycerol 10% in 0.6 mM tris, pH 6.8) for 10 min. After SDS-PAGE the gel was blotted onto an immobilon PVDF nylon transfer membrane (Millipore) and reacted with monoclonal 19-9 Mab (1:1,000) (CIS-BIO), F4 Mab (1:1,000), 29.1 Mab (1:

1,500), anti-M1 (straight hybridoma supernatant), Muse11 Mab (Hybridoma sup.) or anti H-2 Mab (Hybridoma sup.) for 12 h and then with conjugated antimouse IgG, peroxidase-linked species-specific whole antibody (from sheep) (Amersham Life Sciences, Buckinghamshire, UK) (1:5,000), 30 min. The immunological reactions took place in 0.15 M NaCl, 0.1% Tween 20, 5% skim milk, 10 mM Tris-HCl, pH 7.5, and were visualized with "ECL" reagents from Amersham by auto radiography (Amersham MP film).

In some cases to determine protein-bound radioactivity instead of Western blotting, the gel lanes were dried, cut into 1 cm^2 pieces, which were then incubated with 300 μl of 0.4 M KOH in methanol overnight at 37°C and counted in biodegradable emulsifying scintillation fluid (Zinsser analytical).

Northern Blot Analyses

Northern blotting was performed by standard procedure, using a hybond N^+ membrane from Amersham, except that total RNA was extracted with RNazol (Bioprobe, Paris, France). Three different cDNAs from the plasmids pCDM7(α1,3/1,4) (kindly provided by Dr. John B. Lowe) [Kukowska-Latallo et al., 1990], ST3N-1 (kindly provided by Dr. James C. Paulson) [Wen et al., 1992], and pUC119-ST4 (kindly provided by Dr. Tatsunari Nishi) [Sasaki et al., 1993] were labeled with (α-^{32}P)dCTP using a random priming kit (Amersham) to a specific activity of 2×10^9 dpm/μg and used as probes at 4×10^6 disintegrations per minute per blot. A 28s oligonucleotide, CCACCAAGATCTGCACCTGCGGCGGCTCCA, was labeled with (γ-^{32}P)dATP using T4 polynucleotide kinase and hybridized to the same blots in order to control for RNA levels from control and RA-treated cells. The hybridized membranes were washed twice in 2× SSC (0.3 M NaCl, 30 mM NaCitrate, pH 7.0) containing 2% SDS at 65°C and submitted to autoradiography for 16 h [Icard-Liepkalns et al., 1992].

Immunofluorescence and Light Microscopy

Cells for immunofluorescence were cultured identically to those for immunochemical studies. After conversion of some cultures by the RA treatment described above, media were removed and incubated 1 h with 3% paraformaldehyde in Hanks BSS. The filters were further incubated in 1% paraformaldehyde, 0.1% Triton in Hanks BSS and washed 3× with PBS, pH 7.2. We then incubated with $NaBH_4$ (1 mg/ml) for 15 min to diminish aberrant fluorescence and washed with PBS three times and then two times with 1% human serum albumin in PBS. At this point we cut the filters out with a scalpel and set the filters cell side up back into sterile wells which had been coated with parafilm to deter evaporation. Each filter piece then received 400 μl (sufficient to cover) of anti-α-tubulin (1:500; Sigma Immunochemicals) or 19-9 antibody for incubation at 4°C. The next day the filters were washed six times with PBS and two times with 1% human serum albumin in PBS. We then deposited 400 μl of sheep antimouse fluorescent IgG (Diagnostics-Pasteur, Paris, France) (1:125) in Hanks BSS and incubated for 2 h. After three washings with Hanks BSS we prepared semipermanent slides by cutting 2 mm^2 pieces and sealing them in coverslips, cell side up, with acrylic nail polish. Control slides were prepared by incubating with 1% BSA instead of the primary antibody [Sambuy and Rodriguez-Boulan, 1988].

Observations and photography of cultures fluorescent or simply fixed were made with an Olympus Vanox Universal research microscope fully equipped with vertical fluorescence illuminator, phase contrast attachments, and fully automated exposure control unit.

Immunocytochemistry and Electron Microscopy

Cells, control and RA treated, were prepared on membrane inserts as described above and then fixed with 3% paraformaldehyde and then with 0.5% gluteraldehyde for 3 h. The membranes were then rinsed and dehydrated through 70% ethanol and then infiltrated and embedded in LR White resin which was polymerized at 50°C. Thin sections were then rinsed thoroughly in PBS and soaked in PBS 0.01 M glycine and then 1% ovalbumin in PBS and glycine-PBS again. Sections were incubated with drops of 2 μg/ml mouse 19-9 Mab IGg (CIS-Bio) for 2 h and rabbit antimouse IgG 5 μg/ml for 2 h and then with protein A-gold (Pasteur) 50 μg/ml for 2 h. After thorough washing with distilled water the sections were stained in uranylacetate and examined in a JEOL EX transmission electron microscope [Roth et al., 1978].

Analyses of Purified Sialosyl Lewis^a Antigenic Compounds

Analysis of sugars of purified antigens recovered from 19-9 Mab solid phase binding was

performed by Dr. Chris Starr and Dr. Chuck Hague of the Glyko Corporation (Novato, CA).

RESULTS

We determined the time course of sialosyl Lewis[a] secretion under our culture conditions over a 24 h time period into apical and basal compartments (Fig. 1). Soluble sialosyl Lewis[a] (2,000*g*, 5 min) was assayed by an ELISA technique which entails formation of antigen-antibody complexes to solid phase 19-9 antibody and then the binding of a second antibody conjugated for enzymatic color development (see Materials and Methods). Thus, sialosyl Lewis[a]–carrying molecules with at least two binding epitopes (designated SiaLeams) are required for colorimetric detection, and monosialogangliosides do not qualify. Results of the time-course experiment indicated up to ninefold greater secretion of epitopes in RA converted cultures into the apical media after 6 h and as much as twentyfold greater accumulation after 3 h incubations (not shown). Furthermore, secretion was partially polarized with a twofold greater rate of secretion into apical than basal media per unit volume per milligram of cells.

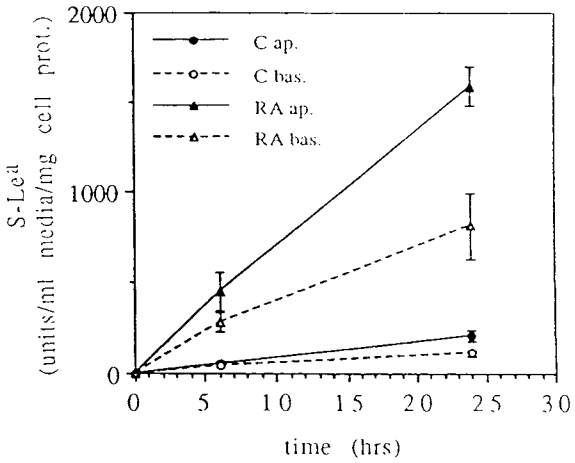

Fig. 1. Time course of the secretion of sialosyl Lewis[a] antigen into apical and basal culture media of SW1116 human colorectal carcinoma cells. Cells were seeded at $2 \times 10^5/cm^2$ on membrane filters in 7% FCS on day 0. On day 1 all media were changed to synthetic growth support media (see Materials and Methods), 1.5 ml in the interior (apical side) of the supports and 2.0 ml in the exterior of the supports. To RA cells was added retinoic acid in EtOH (1 μl/ml) to a final concentration of 3 μM. To C cells only the same volume of EtOH was added. Media were changed thence in the same fashion every other day until five media changes were completed. All cell surfaces were then washed three times with control media, and all wells received control media for 24 h postincubation. Aliquots of media were collected for analysis and assay for secretion of the antigen at 0, 6 and 24 h time points from the interior and exterior chambers of the filter supports. ap, apical; bas, basal; C, control media; RA, media from cells treated with retinoate. Means of three determinations each ± standard error.

Immunochemical Detection of Sialosyl Lewis[a] Epitopes in Subcellular Fractions, Subfractions, and Compounds Recovered From Immunobinding

As an initial approach to possible sources of secreted SiaLeams we looked at the levels of sialosyl Lewis[a] antigenic sites in 6K and 48K membranes by Western blotting. Membrane-bound glycoproteins carrying sialosyl Lewis[a] and penetrating acrylamide gels were at least twentyfold higher in 48K pellets (per unit protein) by densitometry in RA-converted cells (Fig. 2, lane 7) than in the comparable fraction from control cells (Fig. 2, lane 5). This fraction was also particularly sensitive to Brefeldin A (4.5 μg/ml, 24 h) (Fig. 2, lanes 7, 8).

Gradient-purified subfractions from these cells were compared as well (Fig. 3, lanes 1, 2). Membrane-bound epitopes which were carried on SiaLeams in the Mr range just over 100,000 to 170,000 were three- to ninefold more highly expressed, particularly in two doublets, one at apparent Mr 106,000–116,000 and another at 140,000–152,000.

These subfractions were used as a source of SiaLeams for binding to solid phase 19-9 antibody, which were released with 0.1 M triethylamine and then submitted to SDS-PAGE separation and Western blotting (Fig. 3, lanes 3, 4). The SiaLeams recovered in this way, from RA-converted cells, expressed epitopes much more evidently in the range Mr > 100,000 to > 210,000 than those recovered from control cells. Binding to solid phase 19-9 Mab was very strong even after an extensive washing procedure, and we were unable to accomplish complete recovery (50–80%) of SiaLeams with 0.1 M triethylamine; this method proved to be nonetheless useful for some comparisons between retinoate and control samples.

When apical media SiaLeams from 18 h postincubation were subjected to SDS-PAGE and Western blotting, we obtained the characteristic doublet at just over 150,000, which was an order of magnitude more highly detectable in RA media than in control media (Fig. 3, lanes 5, 6).

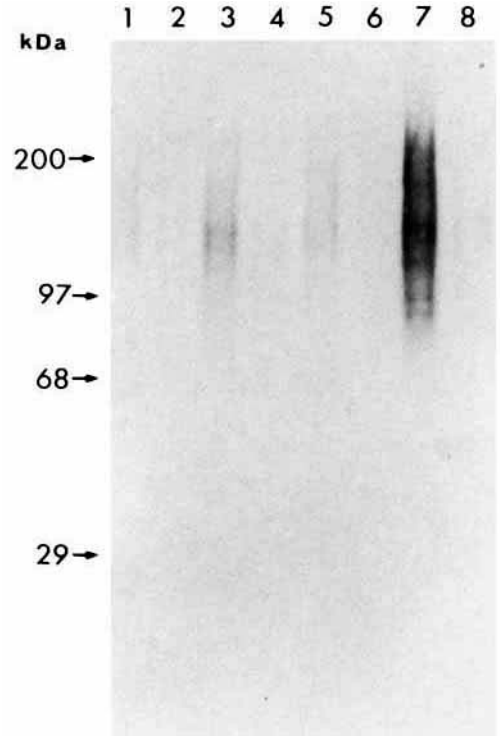

Fig. 2. Western blots of 5–12% gradient SDS-PAGE of subcellular fractions from SW1116 colorectal carcinoma cells which had been cultured in the presence and absence of retinoic acid (RA). Samples of gels were blotted on to Immobilon PVDF transfer membrane and incubated with 19-9 Mab and then with rabbit antimouse IGg peroxidase linked whole antibody as described in Materials and Methods. **Lanes 1–4:** 20 μg of protein from 6K pellets. **Lanes 5–8:** 10 μg of protein from 48K pellets. Lanes 1,2,5,6 from control cell fractionation; lanes 3,4,7,8 from RA-treated cells; lanes 2,4,6,8 contained samples from cells also treated with 4.5 μg/ml Brefeldin A.

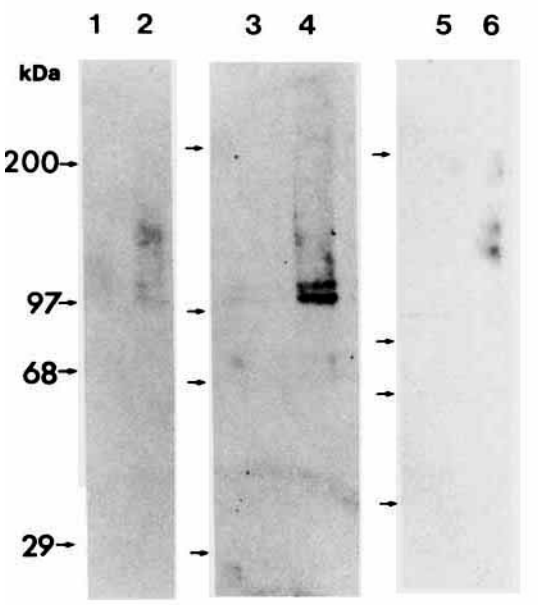

Fig. 3. Western blots of 5–12% SDS-PAGE gels of samples from subfractions of SW1116 colorectal carcinoma cells. **Lanes 1,2:** Light membranes (0.8–1.1 M pooled) of sucrose gradients subfractionation of the 48K pellets. **Lanes 3,4:** Antigenic glycoproteins recovered from the light membrane subfractions by affinity binding, washing, and 0.1 M triethylamine extraction (see Materials and Methods). **Lanes 5,6:** Samples from apical media (triethylamine extract). The samples in lanes 1,3,5 came from control cultures, and the samples in lanes 2,4,6 (from equivalent amounts of membrane or cell protein) came from RA-treated cultures.

Increased Expression of Core Structures vs. Glycosylation at Oligosaccharide Termini as the Basis of RA-Induced Alteration of Sialosyl Lewis^a Secretion; Control Experiments Using Other Monoclonal Antibodies

With the aid of monoclonal antibodies of known specificity, we looked at peptide and core structures of mucin-like and EGF receptor-like compounds present in these cells. Samples from control and RA-converted apical media were subjected to Western blotting using as primary antibodies the Muse-11 antibody against PDTRP-GalNAc-Gal regions, the 19-OLE/12-4 LE antibody against H type 2 epitopes (Fig. 4), and the 29.1 and F4 antibodies against EGF receptor-like epitopes (Fig. 5). We also tested for the presence of cysteine-rich regions on Western blots with M1 antibody (not shown). We observed a decrease in H-2 of compounds migrating at just over 100,000 Mr but not at the band just under 200,000 Mr (Fig. 4, lanes 1, 2). There was no apparent effect on Muse-11 (Muc-1) epitopes (Fig. 4, lanes 3, 4), and there was no major increase in M1 epitope secretion by RA treatment (not shown). Thus, our results from these experiments indicated no apparently major increases in the expression of these peptidic regions or H-2 epitope in the apical media as the result of RA treatment. This is in contrast to the order of magnitude increases in sialosyl-Lewis^a epitope appearance in the media (Fig. 1) and in intracellular membrane fractions and subfractions (Fig. 2).

The results of the studies with monoclonal antibodies to EGF receptor-like regions is shown in Figure 5. The F4 antibody is specific for peptidic regions, and the 29.1 antibody recognizes the blood group A epitope. RA conversion of the SW1116 cell cultures did not result in any obvious stimulation in secretion of blood group A epitopes or EGF receptor-like peptide regions

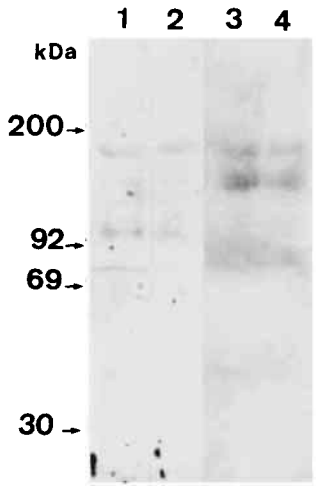

Fig. 4. The presence of H-2 (lanes 1,2) and Muse-11 (lanes 3,4) antigen-carrying glycoproteins in the apical media (24 h postincubated) of colorectal carcinoma cells cultured on membrane inserts. Media samples from control and retinoate-treated cells were applied to SDS-PAGE and Western-blotted onto Immobilon PVDF transfer membrane for exposure to antibodies and visualisation as described in Materials and Methods. **Lanes 1,3:** Apical media from control cultures. **Lanes 2,4:** Apical media from retinoate-treated cultures.

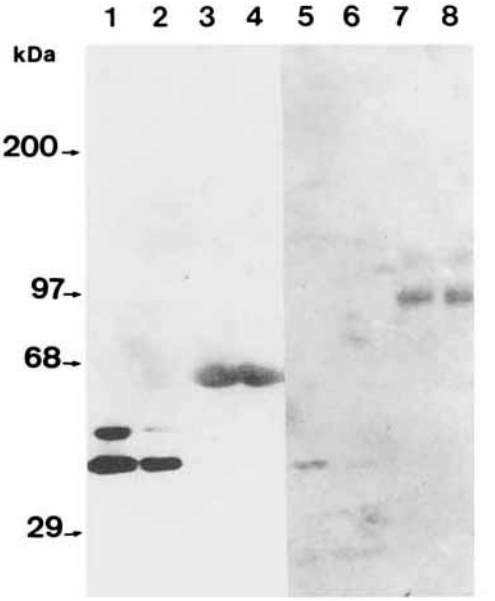

Fig. 5. The presence EGF receptor-like regions in SW1116 colorectal carcinoma cell membranes and apical media as determined by Western blotting. Aliquots from membrane subfractions from 48K pellets and apical media were subjected to SDS-PAGE and Western blotting as described in Materials and Methods. **Lanes 1–4:** F4 antibody against peptidic regions of EGF receptor was used as the primary antibody. **Lanes 5–8:** Antibody 29.1 against carbohydrate (blood group A) epitopes was used. Lanes 1,2,5,6: Membrane subfractions were applied. Lanes 3,4,7,8: Apical media were applied. Lanes 1,3,5,7 had samples from control cultures; lanes 2,4,6,8 had samples from retinoate-treated cells.

(Fig. 5, lanes 3,4,7,8). On the other hand, the expression of intracellular blood group A–carrying molecular species was clearly much greater at Mr 50,000 (Fig. 5, lanes 5,6) and of EGF receptor-like peptide regions at 60,000 (Fig. 5, lanes 1,2) in samples from control cells, indicating that RA treatment, which also restrains cell division, results in diminished intracellular expression of these epitopes. This is in marked contrast to the effect of RA conversion on the secretion and intracellular expression of the sialosyl-Lewis^a epitope (Figs. 1, 2).

Carbohydrate Analyses, Isotope Incorporation, and Glycosyl Transferase Specific Activities

We also submitted purified SiaLeams to carbohydrate analyses (Table I). The major and most consistent finding of this part of the study was that fucose mass levels were always higher in control samples from cells or apical media regardless of the manner in which we expressed the relationship of this sugar to other sugars or to the epitope. Fucose levels were also higher with respect to total carbohydrate in purified antigenic compounds from control cell membranes (60 pmoles/nmole CHO) than in purified antigenic compounds from RA-treated cells (20 pmoles/nmole CHO). Furthermore, radioactivity incorporation from (^{3}H) fucose into SiaLeams was ninefold greater in control cells than in RA-converted cells per unit sialosyl Lewis^a epitope (Table I). We have presented "total sialic" in this table, but we found that the immunobound compounds also contained N-glycol as well as N-acetyl neuraminyl residues in their oligosaccharides.

We also tested for sialyl transferase and fucosyl transferase specific activities to the Galβ1-3GlcNAc acceptor in 48 k membranes (Table 1). We determined initial rates under optimal conditions by methods published previously [Liepkalns et al., 1988; Holmes and Levery, 1989] (see Materials and Methods). The results from these in vitro assays consistently indicated that RA treatment did not generate an elevation of these glycosyltransferase specific activities in SW1116 colorectal carcinoma cells. On the contrary, after optimization of control and RA membrane-derived enzymatic activities, fucosyl transferase specific activities appeared to be about twofold greater in control cell membranes than in membranes from RA-treated cells, consistent

TABLE I. Fucose and Sialosyl Residues Recovered From Glycoproteins (Oligosaccharides) of SW1116 Colorectal Carcinoma Cell Membranes and Apical Media, Recovered From Immunobinding to Solid Phase 19-9 Antibody (Control and RA Treated [10 Days, 3 μM])

	Cell membranes[a]		Media	
	Control	Retinoate-fed	Control	Retinoate-fed
n moles Fuc/n moles man	0.25	0.12	0.25	0.18
p moles Fuc/nmoles sialic	130	50	69	42
p moles Fuc/Unit sialosyl-Le^a	2.5	0.5	2.2	0.3
f moles [^{3}H] Fuc/Unit sialosyl-Le^a	6.3	0.7	0.7	0.2
n moles sialic/n moles man	2.0	3.4	3.4	4.1
p moles sialic/Units sialosy-Le^a	19.5	9.5	31.8	5.9
Fucosyl transferase[b] (n = 2) (β-DGal1-3GlcNAc acceptor)	4,901/5,400	2,717/2,902	not determined	
Sialyl transferase[b] (n = 2) (β-DGal1-3GlcNAc acceptor)	461/468	317/302	not determined	

[a]48 K membrane subfractions (see Materials and Methods and Fig. 3).
[b](^{3}H) dpm/h/100 μg membrane protein.

with the (^{3}H) fucose incorporation data (Table I).

Determination of Glycosyl Transferase mRNA Expression by Northern Blot Analysis

The level of mRNA expression for three glycosyl transferases was determined in control and RA-treated cells (Fig. 6) by Northern blots probed with cDNA from plasmids provided by laboratories which have cloned the Lewis fucosyl transferase (LeFT) or FucTIII [Kukowska-Latallo et al., 1990] and two species of α2-3 sialyl transferases, a mammalian liver sialyl transferase (ST-3) [Wen et al., 1992] and a human melanoma sialyl transferase (ST-4) [Sasaki et al., 1993]. The results shown in Figure 6 indicate that the Lewis fucosyl transferase (LeFT) messenger RNA levels are significantly lowered in cells treated with retinoic acid (Fig. 6, lanes 1, 2) but that the level of ST-4 remains high in both control and retinoate-treated cells (Fig. 6, lanes 3, 4). Control experiments using the same blot showed that this alteration was not due to differences in RNA extraction or transfer during the experiment, since we observed a signal of similar intensity with the 28S probe in control and RA-treated cells (Fig. 6, lanes 5, 6). We were unable to obtain informative levels of ST-3 sialyl transferase mRNA expression in these colorectal carcinoma cells.

Light Microscopy, Immunofluorescence, and Immunocytochemistry Studies

Light microscopy studies showed that RA-converted cells had developed a different mor-

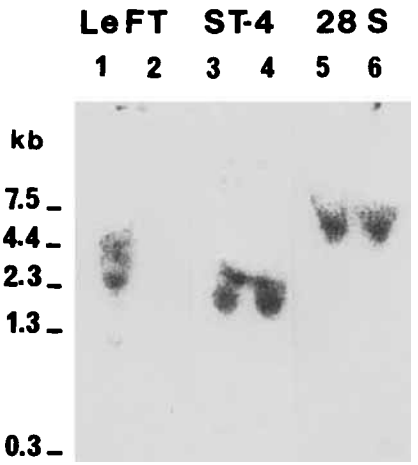

Fig. 6. Northern blot analysis of RNA samples from SW1116 colorectal carcinoma cells. **Lanes 1,3,5:** Pooled RNA from control cells. **Lanes 2,4,6:** Pooled RNA from retinoate treated cells which were blotted and then probed with ^{32}P-labeled α(1-3/1-4)fucosyl transferase cDNA. Lanes 1,2: LeFT (Lewis fucosyl transferase). Lanes 3 and 4: An α2-3 sialyl transferase cDNA (sialyl transferase cloned from human melanoma [ST-4]). Lanes 5,6: ^{32}P-labeled 28s oligonucleotide which was labeled with γ-^{32}P dATP and hybridized on the same blot.

phology than control cells on these membrane inserts particularly with regard to the location of vacuoles toward the lumen or apical surface (not shown). In RA-converted cells these vacuoles are predominantly at the apical surface, while in control cells most of these vacuoles are distributed away from the apical surface.

Independent immunocytochemistry experiments revealed the localization of antigenic molecular species (resistant to ethanol extraction) at a high density along the apical plasmalemma

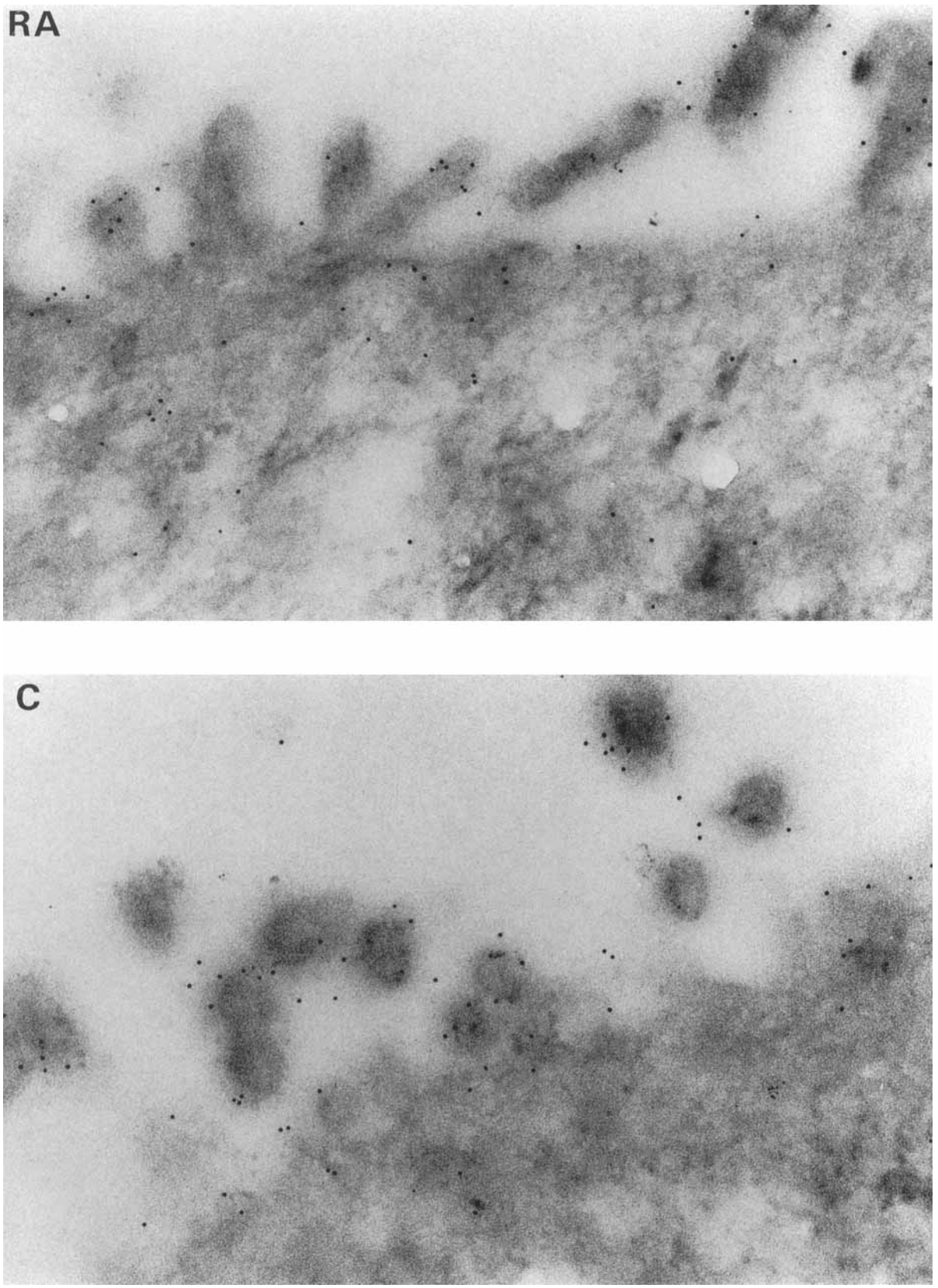

Fig. 7. Electron microscopy immunocytochemistry of SW1116 colorectal carcinoma cells cultured on HA membrane inserts in the presence (RA) and absence (C) of 3 μM retinoic acid. Apical surfaces are shown. Colorectal carcinoma cells were grown on filters for 10 days and then washed and fixed with 3% paraformaldehyde, dehydrated through 70% ethanol, and infiltrated and embedded in LR White resin. Thin sections were incubated with mouse monoclonal IgG against sialosyl Lewis^a (19-9 Mab), rabbit antiserum against mouse IgG, and finally with protein A-gold. Cellular localization of the antigen occurred at a high density along the apical plasmalemma including its microvillous extensions. ×145,000.

including its microvillous extensions in both control and RA-treated cells (Fig. 7).

Finally, anti-α-tubulin staining by immunofluorescence showed that, even after the 10 day RA exposure period, SW1116 cells were healthy to the extent that they were capable of forming mitotic figures (not shown).

DISCUSSION

This study yielded a number of significant and novel findings relevant to the functions of secretion, glycosylation, and adhesion in carcinoma cells. Our results also pertain to experimental epithelial cell biology since our cell culture system provided conditions particularly favorable to differentiation and polarized secretion of glycoproteins (i.e., growth on membrane inserts permitting complete access to synthetic media and to retinoic acid).

To our knowledge, we demonstrate here one of the highest in vitro inductions and intracellular accumulations of an oligosaccharide carcinoma-associated epitope, accompanied by specific, relative increases in polarized secretion of sialosyl Lewis^a. We have also demonstrated here the first finding of a mRNA repression of a fucosyl transferase (FTIII) by retinoic acid. This finding is significant in that sialyl transferases and fucosyl transferases terminate glycosylation sequences in glycoprotein oligosaccharides. Our results have suggested to us some conclusions and proposals regarding the mechanisms of the induction of the secretion of sialosyl Lewis^a by RA.

Migration of highly sialylated, highly glycosylated molecules is governed by the extent of sialylation as well as molecular mass on SDS gels [Litvinov and Hilkens, 1993]. Nevertheless, under these experimental conditions, in these human colorectal carcinoma cells, we detected a family of nongel-forming, highly sialylated molecular species carrying the sialylated Lewis^a epitope which penetrated the acrylamide gradient gel, and these molecular species appear on Western blots to be under 200,000 Mr. The antigenic compounds are membrane-bound (or enclosed) intracellularly but are released in a soluble form at the apical surface, detectable within 3 h (Figs. 1, 2). A postnuclear 6,000–48,000*g* fraction and its gradient subfractions were particularly enriched in these compounds, whose buildup was sensitive to Brefeldin A treatment; this suggests that these subfractions may contain vesicles of Golgi origin headed for the apical surface (Figs. 1–3). The presence of ethanol-insoluble sialosyl Lewis^a epitopes was localised to the outer plasma membrane of apical microvilli, suggesting that this is the compartment from which these compounds are released to the medium (Fig. 7).

A central question relevant to the mechanisms of RA-induced secretion of sialosyl Lewis^a is whether, on the one hand, the biosynthesis of peptidic core sequences is elevated, thereby providing more peptide chains for the support of oligosaccharide chains, or, on the other hand, glycosylation at oligosaccharide termini is affected, permitting the adduction of more epitopes. The mechanism we propose for the augmentation in the secretion of the sialosyl Lewis^a epitopes generated by RA in colorectal carcinoma cells is the following: the primary postnuclear cause of higher sialosyl Lewis^a levels in RA-treated colorectal carcinoma cells is the negative regulation of the Lewis fucosyl transferase (FTIII) (Fig. 6). Alpha 1-4 fucosylation of the subterminal GlcNAc moiety blocks sialylation of the terminal galactosyl moiety of lactosyl acceptors [Hansson and Zopf, 1985]. If the level of FTIII enzyme and its α1-4 fucosyl transferase specific activity is lowered in the Golgi as a consequence of lower amounts of messenger, this leaves more Galβ 1-3GlcNAc moieties available for sialylation. Fucosylation of the subterminal GlcNAc moiety of the sialylated lactosyl still occurs because sialylated (neo)lactosyl moieties have K_m in the range of the lactosyl moiety and can act as competitive inhibitors of lactosyl fucosylation [Holmes and Levery, 1989]; this is in the case of cocompartmentalization of FTIII with the α2-3 sialyl transferase. Another way in which fucosylation of the sialylated lactosyl moiety could be ensured is by the presence of a sufficiently nonhomologous fucosyl transferase (i.e., under separate genetic regulation) which recognizes the sialylated lactosyl moiety with a low enough Km.

We have suggested ST-4 as the α2-3 sialyl transferase in these cells in the pathway to terminal sialosyl Lewis^a because we were unable to detect significant levels of ST-3 messenger RNA (Fig. 6); we do not, however, exclude the presence of ST-3 species in these cells. The ST-4 enzyme, which was cloned from a human cancerous tissue, has only about 15% homology in its amino acid sequence with the ST-3 enzyme, which was cloned from rat liver. Nevertheless, this homology appears in the catalytic site and

may account for their similar acceptor specificity [Wen et al., 1992; Sasaki et al., 1993]. Furthermore, ST-4 sialylates lactosyl as well as neolactosyl substrates in vitro [Sasaki et al., 1993]. Thus, the sialylation of Galβ 1-3 or Galβ 1-4 moieties may not be exclusively reserved for one enzyme species or the other but may instead be determined by the presentation and compartmentalization of acceptors with Galβ termini—whichever α2-3 sialyl transferases may be expressed and active in the Golgi.

Carbohydrate analyses were consistent with the Northern blot results: there were higher levels of fucosyl moieties in mass as well as incorporation of fucose radioactivity in antigenic molecules of control cells (Table I). Part of this accumulation of fucosyl groups may be due to more Lewis fucosyl transferase enzyme in control cells via gene expression (Table I; Fig. 6), although α1-4 fucosylation of subterminal GlcNAc as well as α1-2 fucosylation of terminal Galβ 1-3/4 moieties would block sialosyl Lewis^a biosynthesis.

The antigens defined by M1 appear to be restricted to precancerous and cancerous colonic tissue of epithelial origin [Bara et al., 1988, 1991; Bara and Decaens, 1991]. The Muse-11 monoclonal antibody reacts with an epitope that contains the PDTRP sequence with -GalNac-Gal, O-linked to the threonine residue [Bara et al., 1993] (Fig. 4). This is a very common sequence in episialin and other epithelial highly sialylated secreted glycoproteins. Sequences which contain PDTRP map to chromosome 1 (21 or 22q region), and sequences which contain the M1 antigenic regions map to chromosome 11 (15p region) [Ligtenberg et al., 1990; Swallow et al., 1987].

Further control experiments with commercially available F4 monoclonal antibody to EGF receptor-like protein regions were performed in order to determine if a general augmentation in membrane-bound proteins had occurred in the presence of RA or if RA caused shedding of membrane spanning proteins relative to controls (Fig. 5). We conclude from these experiments and from the immunofluorescence experiments with anti-α-tubulin (see Results) that RA does not cause a relative increase of antigens in culture media through cell death or shedding of membrane sheets. It was, however, interesting that control cells specifically expressed a truncated EGF receptor-like region which was almost absent in RA-treated cells at this Mr (Fig. 5, lanes 1,2,5,6). One possible explanation is suggested by reports that some oncogene products (V-erbB) have sequence homology with the EGF receptor [Ullrich et al., 1984] and human epidermal growth factor receptor cDNA is homologous to a variety of RNAs from carcinoma A431 [Xu et al., 1984].

Taken together, our control studies with M1, Muse 11, EGF receptor-like (Mab F4) monoclonal antibodies which recognize peptidic core regions, and H type 2 and blood group A (Mab 29.1) monoclonal antibodies which recognize fucosyl glycosidic linkages (Figs. 4, 5) support our conclusion that there is a certain degree of specificity to RA-induced molecular alterations in SW1116 cells directed at oligosaccharide termini and therefore sialosyl Lewis^a at oligosaccharide termini. As form ever follows function, this most certainly has functional sequelae at the apical surface where these compounds are bound and released (Figs. 1, 7). Our results should be taken in context with those of other laboratories which demonstrate that adhesion proteins such as E-selectin may mediate binding through the participation of sialosyl Lewis^a termini [Varki, 1994]. One function of the antigenic compounds we report here, termed Sialeams, which we have observed by SDS-PAGE Western blots, immunofluorescence, and immunocytochemistry may be to carry sialosyl Lewis^a to the apical surface and cell junctions where they serve as ligands. Indeed, a functional mucin-like glycoprotein ligand for P-selectin which is a homodimer of 220 kDa has been cloned recently [Sako et al., 1993]. Thus, the adhesion properties of carcinoma cells may be altered by retinoic acid.

Effects on glycosylation pathways by RA have been reported in teratocarcinoma cells recently by other laboratories: a 3.5-fold increase in UDP-Gal:β-D-Gal α1,3-galactosyl transferase and two- to fourfold increases in β1-3 and β1-4 galactosyl transferases [Cummings and Mattox, 1988; Heffernan et al., 1993] specific activities compared to controls. The presence of RA in F9 teratocarcinoma cell cultures also reduced the levels of the fucosylated stage specific antigen (SSEA-1) which is structurally analogous to Lewis^a (neolacto series). Our study is the first to demonstrate by quantitative analysis of sugars, glycosyltransferase assays, Western blotting techniques, and Northern blotting techniques the negative regulation of a Lewis fucosyl transferase(FTIII) coupled with much higher secretion of sialylated epitopes.

Since our carbohydrate analyses indicated a prominent presence of N-glycolneuraminyl groups and other workers have reported these residues in high molecular weight O-linked mucin carbohydrate, we cannot rule out a role for the CMP-Neu5Ac hydroxylase [Shaw and Schauer, 1988]. It is conceivable that higher CMP-Neu5Ac hydroxylase activity in control cells could also lower NeuNAc incorporation required for biosynthesis of the sialosyl Lewis^a epitope and for 19-9 Mab recognition.

In summary, we have analysed glycosidic and peptidic domains of secreted sialosyl Lewis^a–carrying molecular species as well as mRNAs for glycosyl transferases in the sialosyl Lewis^a pathway. Our results demonstrate that RA-induced secretion of sialosyl Lewis^a in colorectal carcinoma cells is due to the diminution in the level of mRNAs encoding for the Lewis fucosyl transferase. Our results also suggest that the functional properties of colorectal carcinoma cells could be altered by RA treatment through a relative enrichment in sialosyl Lewis^a ligands participating in adhesion functions at the cell surface and at intercell junctions.

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