Abstract. We have studied proteoglycan secretion using a recently developed system for the preparation of polarized primary cultures of rat uterine epithelial cells. To mimic their native environment better and provide a system for discriminating apical from basolateral compartments, we cultured cells on semipermeable supports impregnated with biomatrix. Keratan sulfate proteoglycans (KSPG) as well as heparan sulfate-containing molecules (HS(PG)) were the major sulfated products synthesized and secreted by these cells. The ability of epithelial cells to secrete KSPG greatly increased in parallel with the development of cell polarity. Furthermore, KSPG secretion occurred preferentially to the apical medium in highly polarized cultures. In contrast, HS(PG) secretion did not increase along with development of polarity, although most HS(PG) (85%) were secreted apically as well. Pulse-chase studies indicated that highly polarized cultures secreted 80-90% of the sulfated macromolecules they synthesized, predominantly to the apical secretory compartment. The half-lives for KSPG and HS(PG) secretion were ~3 and 4 h, respectively.

Parallel studies of cells cultured on tissue culture plastic-coated with biomatrix indicated that neither the state of confluency nor the biomatrix was primarily responsible for inducing the KSPG secretion observed in polarizing cultures. Experiments with uterine strips indicated that the steroid hormone, 17-β-estradiol, markedly stimulated synthesis and secretion of sulfated macromolecules, but had no preferential effect on KSPG production. The ratio of KSPG to HS(PG) secretion from uterine strips was similar to that found in the apical medium of highly polarized cell cultures. Thus, the pattern of proteoglycan secretion observed in polarized cell cultures mimicked that observed for uterine cells, although the preferential increase in KSPG production by polarized cells could not be attributed to an estrogen response. Collectively, these studies describe the major sulfated molecules secreted by rat uterine epithelial cells under varying conditions and provide evidence for a novel influence of cell polarity on the cell's ability to secrete sulfated glycoconjugates.

Epithelial cells line many organs and serve functionally to compartmentalize tissues. To accomplish this task, epithelial cells must differentiate to express different functions at their distinct apical and basolateral cell surface domains. Much interest has been focused on understanding the mechanisms by which epithelial cells sort particular proteins to one or the other individual cell surfaces, thereby contributing to the uniqueness of that plasma membrane (23, 34). Less information is available concerning the production of complex carbohydrates by polarized cells. Given the general role proposed for complex carbohydrates and glycoproteins in cell recognition phenomena (7, 10, 26, 28), it seems likely that the array of glycoconjugates expressed at these cell surfaces is quite different. Clearly, the different interactions of epithelial cells (cell–cell, cell–matrix) are likely to be modulated by these unique arrays of surface components (2, 12). Thus the cell–cell associations of certain epithelial cells could be modulated by uromorulin, a glycoprotein preferentially expressed at the basolateral surfaces of some, but not all, epithelia (37). Similarly, the relationship between an epithelial cell and its basement membrane may be modified by the preferential secretion of extracellular matrix components from the basolateral surface. This seems to be the case for laminin and heparan sulfate proteoglycan secretion in Madin–Darby canine kidney (MDCK) cells (3).

The multiple processes that characterize the relationships between the blastocyst and the uterine endometrium emphasize the necessity for distinct cell–cell and cell–matrix interactions at, respectively, the apical and basolateral surfaces of the uterine epithelial cell. The ability of the apical surface domain of the uterine epithelial (UE) cell to become transiently receptive to blastocyst attachment is regulated by est...
trogen and progesterone (30, 31). Less clear is the control and nature of the relationship between the basal surface of the UE cell and its basal lamina. This interaction appears to decrease reciprocally with the acquisition of blastocyst receptivity by the apical surface (30, 31). Our understanding of these processes is limited, in large part, by our limited information regarding the macromolecules synthesized and secreted by the UE cell. We do not know, for example, whether any of these macromolecules are secreted or expressed preferentially at the apical or the basal cell surfaces. If some are expressed apically, it would be interesting to know whether any of the selectively expressed molecules play a determinative role in the implantation process.

To begin to answer these questions, we used a recently developed culture system for uterine epithelia that allows these cells to attain a polarized state. This approach has permitted us to analyze individually the sulfated glycoconjugates secreted from either the apical or basolateral domains of these cells. Collectively, our results indicate that the state of polarity influences the ability of UE cells to secrete glycoconjugates, particularly at the apical cell surface.

Materials and Methods

Materials

Immature (21-day-old) female Sprague-Dawley rats were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN); tissue culture media and supplements were obtained from either Gibco Laboratories (Grand Island, NY) or Irvine Scientific (Santa Ana, CA). Millicell apparatus were from Millipore Continental Water Systems (Bedford, MA). Tissue culture plates and supplies were obtained from Falcon Labware (Oxnard, CA). Millicell apparati were from Millipore (Bedford, MA). Tissue culture plates and supplies were obtained from Falcon Labware (Oxnard, CA). H213-SO4 (carrier-free) was from ICN Radiochemicals (Irvine, CA); urea and guanidine hydrochloride from Schwarz/Mann (Spring Valley, NY), octylglucoside, Sephadex G-50 (fine), heparinase, chondroitinase ABC, keratanase, heparan sulfate, chondroitin sulfate, keratan sulfate, pronase, 3-{(3-cholamidopropyl) dimethylammonio}propansulfonate (CHAPS), Triton X-100, leupeptin, benzamidine, aprotinin, chymostatin, pepstatin, and 17-β-estradiol were from Sigma Chemical Co. (St. Louis, MO); protein molecular weight calibration standards were from Bethesda Research Laboratories (Gaithersburg, MD); and biomatrix (Matrigel) and NuSerum were from Collaborative Research (Lexington, MA). All chemicals used were reagent grade or better.

Preparation of Polarized Cell Cultures

Epithelial cells were separated from uteri of immature rats using the modified trypsin/pancreatin procedure (24). Homogeneous populations of UE cells were seeded on Millicell HA inserts that had been impregnated with Engelbreth Holm Swarm tumor matrix (Matrigel) and cultured in complete medium (DME/Ham's F12 mixed 1:1, plus 2.5% [vol/vol] heat-inactivated FCS and 2.5% [vol/vol] NuSerum, 15 mM HEPES, and 1% [vol/vol] penicillin-streptomycin), at 37°C in a humidified atmosphere of 95% air/5% CO2. Cells were counted at 24 h and unattached cells removed. Cultures with a plating density of 1,200 cells/mm² typically reached confluence after 48-72 h of culture. The extent of confluency also was monitored by microscopic examination of Mayer's hematoxylin staining of parallel cultures. Under these conditions, these cells maintained their epithelial cell characteristics for at least 22 d, as confirmed by their interaction with specific antibodies raised against simple epithelial cell cytokeratins and against desmoplakins I and II. The development of UE cell polarity was monitored by morphologic examination. Criteria for the establishment of functional polarity included preferential uptake of [35S]methionine from the basal (compared with the apical) surface, the ability to maintain a pH gradient across the UE cell culture, and the restricted basolateral expression of uvomorulin (1la). Filter-cultured UE cells maintained their confluent density (>2,500 cells/mm²) for the duration of the experiments described below. In some cases, UE cells were cultured on tissue culture plates that had been coated with EHS tumor matrix.

Metallic Labeling

In preparation for the study, the complete (serum-containing) medium was removed, the cell layers rinsed with a balanced salt solution, and the medium added. The medium used for metabolic labeling experiments was RPMI 1640 (minus sulfate) supplemented with 3.3 mM MgCl2, 1.2 g/liter NaCO3, 15 mM HEPES (pH 7.2), 2.5 U of penicillin/ml and 2.5 μg of streptomycin sulfate/ml, and including 0.5 mM dH213-SO4. The streptomycin sulfate served as the sole source of nonradioactive sulfate. Routinely, incubations were performed for 9 h in a humidified atmosphere of 95% air/5% CO2 (vol/vol). Isotope incorporation into macromolecules was linear over this time interval for both primary cell cultures and uterine strips. For chase experiments, cells were labeled similarly for 9 to 10.5 h, the cell layers were rinsed several times with prewarmed (37°C) serum-containing, sulfate-replete culture media, and the incubation was continued with the latter (chase) medium.

Uterine strips were prepared from immature rats that had received either no hormone or daily injections of 1 μg of 17-β-estradiol in 0.1 ml of arachis oil for the three preceding days. Uteri were excised and slit longitudinally to expose the epithelial cells lining the uterine lumen. Care was taken to minimize trauma and damage to the tissue, since these experiments were designed to study synthesis of components that would be secreted to the uterine lumen. The explants were labeled metabolically with radioactive sulfate under the same conditions as for metallic labeling of primary cell cultures.

Extraction of Total Glycoconjugates

Macromolecular material in media fractions of the respective apical and basal secretory compartments was obtained by precipitation with ice-cold 10% (wt/vol) TCA/3% (wt/vol) phosphotungstic acid. Precipitates were obtained by centrifugation at 5,000 g for 15 min and the resulting preparation precipitated twice more with 10% (wt/vol) TCA, followed by two precipitations with ice-cold 95% (vol/vol) ethanol. This procedure had precipitated >90% of commercial [1H]heparin in pilot studies, indicating its efficacy at precipitating free glycosaminoglycans in addition to protein-linked glycoconjugates. Cell-associated and Millicell HA filter-associated material was extracted by incubation for 1 h at room temperature with frequent agitation with 4 M guanidine hydrochloride, 1% (wt/vol) CHAPS, 25 mM EDTA (pH 7.0), and a mixture of protease inhibitors (6). Macromolecules were precipitated from this extraction solution using the acid precipitation procedure described above. In some cases, macromolecules were obtained from such extracts by desalting on 0.75 x 22-cm columns of Sephacryl S-50 (fine) eluted with 4 M urea, 0.1% (wt/vol) octylglucoside, 25 mM EDTA (pH 7.0), 20 mM Tris-acetate (pH 7.0), and 0.02% (wt/vol) sodium azide. Material eluting at or near the void volume was used for further analyses. Since there were no significant qualitative or quantitative differences between the results obtained with the desalting or acid precipitation procedures, we routinely used the latter, which was more useful for analyzing a large number of samples.

Liquid Chromatography

Macromolecules were fractionated initially by anion exchange liquid chromatography. The liquid chromatographic system (Beckman Instruments, Inc., Palo Alto, CA) consisted of two model 100A pumps controlled by a model 421A controller. Absorbance was monitored by an online model 163 variable wavelength detector interfaced with a model 427 integrator. Chromatography was performed on a 0.5 x 5-cm column of Mono Q (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with 0.5 M urea, 20 mM Tris-acetate (pH 7.0), 0.01% (wt/vol) octylglucoside, and 0.002% (wt/vol) sodium azide, and developed with a gradient of 0-4 M sodium chloride. The buffer was pumped at room temperature at a flow rate of 1 ml/min with a back pressure of ~400 psi. Fractions were collected every 0.5 min. Recoveries of radioactivity from these analyses ranged between 85 and 95%. Macromolecules also were analyzed by molecular exclusion liquid chromatography on a 1 x 30-cm column of Superose 12 (Pharmacia Fine Chemicals, Uppsala, Sweden). The buffer used was 1 M guanidine hydrochloride, 0.01% (wt/vol) octylglucoside, 20 mM Tris-acetate (pH 7.0), and 0.02% (wt/vol) sodium azide, pumped at a flow rate of 0.7 ml/min with a back pressure of ~330 psi. Fractions were collected every 0.5 min. Recoveries from this procedure exceeded 90%.

Hydrophobic Affinity Chromatography

Keratan sulfate proteoglycans (KSPG) isolated from secretions or filter-
associated material were subjected to hydrophobic affinity chromatography batchwise using octyl-Sepharose as described previously (34). Briefly, the samples (30,000 dpm) were dissolved in 1 ml of 4 M guanidine, 20 mM Tris-acetate, pH 7.0 and incubated with 0.25 ml of octyl-Sepharose resin for 30 min at room temperature with constant rotary mixing. The resin was washed by centrifugation with four changes of 1 ml of this buffer to elute nonbound material. Bound material was eluted by incubation and washed as described above using the same buffer containing 0.1 or 0.5% (vol/vol) Triton X-100. Radioactivity eluted at each step as well as associated with the resin then was determined. Recoveries ranged between 92 and 97%.

Glycoconjugate Digestions

Samples were digested with pronase, chondroitinase ABC, and keratanase as described previously (4). Heparitinase digestions (200 mU/ml) were performed overnight at room temperature in 50 mM Tris-HCl (pH 7.0), 5 mM calcium chloride, 100 mM sodium chloride, 100 μg/ml BSA, and a mixture of protease inhibitors (6). In some cases, samples were reduced before molecular exclusion liquid chromatography by incubation with 50 mM dithiothreitol (DTT) at 90°C for 1 h. Alkaline borohydride hydrolysis was performed in 0.1 M sodium hydroxide/0.25 M sodium borohydride at 37°C for 48 h (27).

Results

Synthesis and Secretion of Sulfated Glycoconjugates

Primary cultures of immature rat UE cells grown on matrix-impregnated, semipermeable supports develop a highly polarized phenotype (11). This culture system allows the individual analysis of components secreted from either the apical or basolateral aspects of the cells. We observed that the pattern of sulfated macromolecules secreted under these conditions changed as the cells developed functional polarity. As is shown in Fig. 1, the most striking change, as cultures matured over the period of days 4–5 through days 10–12 of culture, was the great enhancement in the apical secretion of a class of sulfated components eluting from the anion exchange resin between 1 and 2 M NaCl (A and C). Similar results were obtained using [3H]glucosamine as a precursor (data not shown). Some material with similar ion exchange characteristics was observed in the basal secretions of the highly polarized cell cultures (compare B with D); however, as discussed below, the vast majority of this material was found in the apical medium.

In our routine experimental protocol, we used a medium containing lower than normal sulfate levels to maximize isotope incorporation. Experiments performed in sulfate-replete media gave qualitatively identical patterns of secreted sulfated glycoconjugates (data not shown). Consequently, we concluded that the observed shift in glycoconjugate patterns was not caused by sulfate deficiency in the medium.

In contrast to the results obtained for cells cultured on matrix-impregnated filters, cells cultured on tissue culture plastic coated with the same biomatrix produced the patterns shown in Fig. 1, E and F. Although confluence was achieved at a similar cell density (2,500–3,000 cells/mm²), no qualitative difference in the pattern of secreted sulfated macromolecules was observed with cells cultured on tissue culture plastic as a function of time in culture. The alteration in the pattern of sulfated glycoconjugate production observed for polarizing cell cultures appeared, therefore, to be neither a requisite property of these cells nor attributable to the matrix used to coat the growth surface.

When the isolated macromolecules secreted to the apical media were added to the apical media of matrix-impregnated filters in the absence of a cell layer, we found that these molecules were not "trapped" by the filters and that these components were distributed equally between the apical and basal compartments during the time frame of our metabolic labeling experiments. Consequently, we concluded that the preferential apical distribution observed could not be attributed...
to physical impairment of the release of these components by
the matrix-impregnated filter to the basal media.

It was of interest to determine if the different patterns of
secretion observed under these conditions were the result of
selective sequestering or “trapping” of radiolabeled macromolecules within the cell layer. This possibility was of greater concern for the cells cultured on tissue culture plastic than those cultured on the semipermeable supports. As shown in Table I, a similar percentage of the total macromolecular sulfate was secreted in both cases, that is, ~46%. Furthermore, the patterns of cell-associated material were similar for cells cultured under either condition (see below). Since the cell-associated fraction did not seem to contain an unequal accumulation of sulfated macromolecules, differential sequestering of sulfated macromolecules within the cell layer seemed unlikely to be responsible for the altered composition of the secretory components. The question of trapping of the sulfated macromolecules by the cell-containing filters was addressed further by pulse–chase studies (see below).

Characterization of Secreted Keratan Sulfate Proteoglycans

To characterize the sulfated molecules secreted by epithelial cell cultures, the material eluting from the Mono Q column between 1 and 2 M sodium chloride was pooled for further analysis. As shown in Fig. 2, this material migrated primarily as a single peak of radioactivity on molecular exclusion liquid chromatography with a $K_v$ of 0.35. Compared with protein standards, this material had a hydrodynamic radius similar to that of a 130,000-Mr protein. Similar profiles were obtained for the material isolated from either apical or basal secretions. After exhaustive pronase digestion, this material was shifted to a lower Mr distribution ranging in $K_v$ from 0.41 to 0.85 with a median of 0.63. This observation indicated that the sulfated components were protein linked. After digestion with keratanase, the radioactivity was converted almost completely to a form migrating at or near the fully included column volume. In contrast, neither chondroitinase ABC nor heparitinase digestion released any substantial amount of radioactivity from the intact material. Similar results were obtained for the [3H]glucosamine-labeled material (data not shown). From these observations, we concluded that the sulfated material secreted from the epithelial cells that eluted from the Mono Q column between 1 and 2

Table I. Distribution of $^{35}$SO$_4$-labeled Macromolecules under Different Culture Conditions*

| Culture condition | KSPG | HS(PG) | Other | Percent cell-associated |
|-------------------|------|--------|-------|------------------------|
| Solid supports    | 23 ± 1 | 16 ± 1 | 7 ± 1 | 54 ± 1 |
| Semipermeable filters | | | | |
| Apical            | 41 ± 2 | 1 ± 1 | 1 ± 1 | - |
| Basal             | 1 ± 1 | 2 ± 1 | <1 | - |
| Total             | 42 ± 3 | 3 ± 2 | 1 ± 1 | 55 ± 2 |

* UE cells were prepared and cultured on matrix-impregnated, semipermeable filters for 12–14 d or on matrix-coated tissue culture plates as described in Materials and Methods. These cultures were labeled metabolically with H$_2$[35]SO$_4$, and incorporation into total macromolecules, KSPG, and HS(PG) was determined as described in the text. The data shown indicate the percentage distribution of the total sulfated macromolecules synthesized by these cultures among the indicated fractions. Average ± ranges of values obtained for analyses of at least three separate cultures in each case are presented. Total incorporation into macromolecules for the nonpolarized and polarized cultures was $1.45 \pm 0.05 \times 10^6$ and $2.51 \pm 0.12 \times 10^6$, respectively.
M NaCl consisted primarily of KSPG. Furthermore, it appeared that this was a major glycoconjugate secreted by these cells. The majority, 73% or more, of the secreted macromolecules labeled with \([3H]\)glucosamine comigrated with the \(H_2[35S]O_4\)-labeled components both on anion exchange and molecular exclusion liquid chromatography (data not shown).

To determine whether the KSPG were cross-linked to each other via disulfide bonds, we subjected these proteoglycans to reduction with dithiothreitol. As shown in Fig. 3, this treatment yielded a major peak of radioactivity with similar size characteristics as the untreated control; however, ~10% of the radioactivity was converted to a form that migrated near the fully included column volume. Thus, although most of the KSPG do not seem to be cross-linked by interchain disulfide bonds, it is possible that a small portion of the radioactivity is bound to a very low \(M_r\) fragment linked to the major KSPG by a sulfhydryl group. In other experiments to determine whether the keratan sulfate chains were in N- or O-linkage to the protein core, we found that treatment with alkaline borohydride quantitatively released a broad peak of radioactivity that migrated with a \(K_N\) of ~0.88. In contrast, peptide/N-glycanase, an enzyme capable of releasing a wide variety of N-linked oligosaccharides (13), had no effect on the migration characteristics of this material (data not shown). Collectively, these observations indicated that the keratan sulfate oligosaccharides were in O-linkage to the protein core. Moreover, the observation that the \(M_r\) distribution of the oligosaccharides released by alkaline borohydride treatment was substantially lower than that of the pronase glycopeptides indicated that multiple keratan sulfate chains were closely linked to the same protein cores, making them protease resistant.

Collectively, these observations indicated that the keratan sulfate oligosaccharides were in O-linkage to the protein core. Moreover, the observation that the \(M_r\) distribution of the oligosaccharides released by alkaline borohydride treatment was substantially lower than that of the pronase glycopeptides indicated that multiple keratan sulfate chains were closely linked to the same protein cores, making them protease resistant.

Figure 3. Treatment of keratan sulfate proteoglycans with DTT or alkaline-borohydride. Keratan sulfate proteoglycans were obtained and analyzed by Superose 12 liquid chromatography as described in text. Profiles of eluted radioactivity are shown for intact material (●), material obtained after incubation with 50 mM DTT at 90°C for 1 h (●), and after incubation with 0.1 N sodium hydroxide/0.25 M sodium borohydride at 37°C for 48 h (○). Molecular mass markers are as described for Fig. 2.

Figure 4. Molecular exclusion chromatography of 2.5–4.0 M NaCl eluates from Mono Q. Material eluting from anion exchange liquid chromatography between 2.5 and 4.0 M NaCl was pooled, dialyzed, and analyzed by Superose 12 chromatography as described in text. Profiles are shown of the intact material (●), pronase-digested material (▲), and heparitinase-digested material (○) from either the apical (A) or basal (B) secretions from highly polarized (day 12) filter cultures. At least 80% of the radioactivity was degraded with heparitinase in each case. Molecular mass markers are described for Fig. 2.

Characterization of Secreted Heparan Sulfate-containing Molecules

Radioactivity eluting from the Mono Q column between 2.5 and 4.0 M NaCl was pooled for further analysis as well. As shown in Fig. 4, the intact material secreted to the apical medium migrated on molecular exclusion liquid chromatography as a class of components exhibiting a median \(K_N\) of 0.1. By comparison to protein standards, this material had a hydrodynamic radius similar to that of a 500,000-\(M_r\) protein. After exhaustive pronase digestion, this material was converted to a form migrating as a very broad peak ranging in \(K_N\) from 0.1 to 0.85 with a median of ~0.54. Treatment of the intact material with alkaline borohydride quantitatively released the radioactivity to a molecular mass distribution similar to that observed for the pronase-digested material (data not shown). These observations indicated that the sulfated oligosaccharides were in O-linkage to protein and that the protein-free oligosaccharide chains were only slight-
ly smaller than the pronase glycopeptides. Although this material was resistant to digestion with both chondroitinase ABC and keratanase (data not shown), >75% was digested with heparitinase. Consequently, we concluded that most of the components in these fractions were in the form of heparan sulfate proteoglycans.

Similar analyses were performed on the high-salt eluate obtained from basal secretions of polarized cell cultures (Fig. 4, B). In this case, the intact material migrated as a broad peak of radioactivity with a similar size distribution as the pronase-digested material from the apical secretions. Pronase digestion had little effect on the size distribution of the basal secretion material. Treatment with alkaline borohydride caused only a slight shift to a lower $M_r$ distribution (data not shown); however, heparitinase digested almost all of this material to low $M_r$ products. We concluded that the material in the high-salt eluate from Mono Q in the basal secretions of the epithelial cells was primarily in the form of heparan sulfate linked to little or no protein.

Using these approaches, we estimated the relative distribution of sulfated macromolecules among the cell associated and secreted fractions of epithelial cells grown under polarizing and nonpolarizing conditions. The results are presented in Table I as the percentages of distribution. Regardless of the source, most of the macromolecular radioactivity in the secreted fractions could be accounted for as either keratan sulfate or heparan sulfate proteoglycans. Epithelial cells that achieved confluency on solid supports synthesized and secreted a significantly greater proportion of heparan sulfate-containing molecules (HS[$\text{PG}$]) than their counterparts polarizing on semipermeable filters. Furthermore, these polarized cell cultures primarily secreted KSPG. It was striking that the vast majority of the proteoglycans produced by polarized cell cultures were secreted to the apical medium, although these cells had access to the basal surface. We next analyzed the sulfated macromolecules in the cell- (filter) associated fractions to determine the relationship these molecules bore to the secreted components.

Characterization of Cell-associated, Sulfated Macromolecules

Sulfated macromolecules were extracted from epithelial cells grown on biomatrix-coated tissue culture plastic or matrix-impregnated semipermeable filters. Macromolecules were selected either by acid precipitation or desalting and analyzed by anion exchange liquid chromatography. A profile from one of these analyses is shown in Fig. 5. Similar profiles were obtained from cells cultured under either aforementioned condition. Again, two major peaks were observed that eluted with similar salt concentrations as the secreted KSPG and HS(PG). The material in each peak was pooled as indicated on the figure and each fraction subjected to further analyses.

The material in peak A from Fig. 5 eluted at a similar position on Superose 12 chromatography and exhibited a similar sensitivity to keratanase as secreted KSPG (Fig. 6 A). This material also was sensitive to digestion with pronase or mild alkaline hydrolysis to a similar extent as the secreted KSPG (data not shown). The hydrophobic characteristics of the filter-associated and secreted KSPG were compared as well. As shown in Table II, neither form exhibited significant ability to bind to octyl-Sepharose. Consequently, the secreted
Table II. Hydrophobic Affinity Chromatography of Filter-associated and Secreted KSPG

| Percent Triton X-100 | Filter-associated | Secreted |
|---------------------|-------------------|----------|
| 0                   | 96                | 92       |
| 0.1                 | 1                 | 2        |
| 0.5                 | 2                 | 2        |
| resin               | 1                 | 4        |

* KSPG was isolated by anion exchange liquid chromatography from either filter-associated or secreted material. Approximately 30,000 dpm of each were subjected to hydrophobic affinity chromatography as described in Materials and Methods. The percentage of the total recovered radioactivity eluting with the indicated concentration of Triton X-100 are shown. The total recoveries of input radioactivity ranged between 92 and 97%.

and cell-associated KSPGs exhibited identical characteristics in terms of their ion exchange characteristics, the hydrodynamic radii of the intact molecules or component sulfated oligosaccharides, sensitivity to mild alkaline hydrolysis, pronase, and keratanase as well as hydrophobic character. It was concluded that the cell-associated radioactive material in peak A was largely composed of KSPG similar to the secreted KSPG.

The material in peak B from Fig. 5 was analyzed as well. The intact material eluted in the void volume of Superose 12; however, a large fraction was converted to lower Mr, forms after digestion with either pronase or heparitinase (Fig. 6 B). This material also exhibited a similar sensitivity to mild alkaline hydrolysis as the apically secreted HSPG (data not shown). It was concluded that the cell-associated sulfated components in peak B were largely composed of HS proteoglycans.

To determine if the secreted sulfated macromolecules were derived from the cell-associated forms pulse-chase studies were performed. As shown in Fig. 7 A, loss of KSPG from the filter-associated fraction was accompanied quantitatively by KSPG accumulation in the apical medium compartment. By 6 h of the chase period more than two-thirds of the cell-associated KSPG was released. Chase periods of 24 h resulted in loss of >90% of the 35SO4-labeled micromolecules from the cell layer (data not shown). Similar results were obtained for HS(PG). By 6 h of the chase period ~80% of HS(PG) was lost from the filter-associated fraction and was accompanied by a similar accumulation in the apical and basal media. From these data half-lives for the filter-associated KSPG and HS(PG) were calculated to be 3 and 4 h, respectively. These data strongly suggested that most of the proteoglycans synthesized by highly polarized cells were destined for secretion. Furthermore, it indicated that no more than 20% of the filter-associated proteoglycans were retained or "trapped" within the cells and/or filter within the typical 9-h time frame of our labeling experiments. Consequently, it could be calculated that at least 85% of the sulfated macromolecules synthesized by highly polarized cultures were destined for apical secretion.

Secretion Patterns of Sulfated Macromolecules by Polarized Cell Cultures

Biochemical analyses of the sulfated components released to the apical and basal media of cells grown on matrix-permeated filters revealed the patterns shown in Fig. 5. A dramatic increase in the apical secretion of KSPG was observed as a function of time in culture. Although apical secretion of HS(PG) increased markedly between days 2 and 6 in culture under conditions in which cell numbers did not increase, no further increase in HS(PG) secretion was observed at day 12. This paralleled acquisition of other parameters of functional polarity, for example, development of preferential methionine transport at the basal surface. In contrast, secretion of sulfated macromolecules to the basal media did not change as a function of time in culture. Consequently, these observations indicated not only that these cells developed an increased overall ability to secrete proteoglycans as they became polarized, but that virtually all of this increase was observed in the apical compartment.

Studies of the Secretion of Sulfated Macromolecules by Uterine Strips

It was of interest to determine whether the composition of sulfated macromolecules secreted to the apical media of polarized cell cultures was similar to that of apically, that is, lumenally, secreted sulfated macromolecules in utero. We compared the patterns of sulfated macromolecule secretion from uteri derived from immature rats that had or had not received estrogen treatment. Dutt et al. (4, 5) have demonstrated that this hormone has dramatic effects on glycoprotein production by uteri. We wished, therefore, to determine whether estrogen influenced synthesis and secretion of sulfated glycoconjugate in rat uteri and whether this could account for the changing pattern of glycoconjugate production by the polarized cell cultures.

Preliminary experiments indicated that incorporation of a variety of precursors as well as secretion of newly synthesized components occurred in a time-dependent fashion under these conditions as was observed previously for mouse uterine explants (4, 5). Radioactive components in the medium were presumed primarily to represent apical secretory components; however, since some tissue damage inevitably
occurs, additional components may have been released to the culture media in these experiments.

Anion exchange liquid chromatography indicated that three major charge classes of components were secreted from uterine strips under these conditions (Fig. 6). Two of these eluted with a salt concentration similar to that required for elution of the keratan sulfate and heparan sulfate proteoglycans, but one class eluted with a lower concentration (0.9-1.0 M NaCl). Estrogen preferentially stimulated production of the two charge classes eluting with the higher salt concentrations. Subsequently, molecular exclusion liquid chromatography before and after specific enzymatic digestion (Fig. 7) showed that the material eluting with the lowest salt concentration contained primarily low Mr components migrating with $K_w$ of 0.5 or less. Some of this material was digestible with keratanase (Fig. 7 A). In the media obtained from estrogen-treated rat uteri as much as 80% of this material exhibited the same size distribution and the same sensitivity to keratanase as the KSPG described above (data not shown). This was attributed to increased contamination of this fraction from estrogen-treated uteri by KSPG, which elutes with only a slightly higher salt concentration.

The material eluting from the anion exchange resin with 1.0–2.0 M NaCl had similar characteristics, that is, charge, apparent $M_r$, and sensitivity to keratanase, as the KSPG produced by primary cultures of uterine epithelial cells, and this was so for uterine material exposed or not exposed to estrogen. This was the major charge class of sulfated components released from the estrogen-treated tissue. Consequently, KSPG production appeared to be estrogen responsive, and these proteoglycans seemed to be the major sulfated macromolecules secreted to the uterine lumen under these conditions.

As was found for the primary cell cultures, the sulfated components eluting with 2–4 M NaCl included heparan sulfate–containing molecules. Other components, such as chondroitin and dermatan sulfate–containing molecules were also found in this fraction. Because of this fraction's complexity, it was necessary in every case to digest this material separately with keratanase, chondroitinase ABC, or heparitinase to quantitate its composition accurately. This procedure made it possible to account for 94% or more of the fraction's radioactivity as being in the form of either keratan sulfate–heparan sulfate–, chondroitin sulfate–, or dermatan sulfate–bearing molecules. In all cases, >80% of the radioactivity was in the form of keratan sulfate and heparan sulfate in this Mono Q fraction. Fig. 7 C shows the profile obtained for this fraction from the secretions from estrogen-treated uteri. The fraction contained variable amounts of KSPG, as evidenced by the presence of sulfated components with similar $M_r$ and sensitivity as keratanase, in addition to sulfated components degraded by heparitinase. Less KSPG was in this fraction in the secretions of nonestrogen-treated uterine strips. The KSPG found in this fraction apparently was due to either the production of more negatively charged forms by the uterine strips than the epithelial cells or because the KSPG were incompletely resolved from heparan sulfate–bearing molecules, or both. The fact that 10–20% of the sulfated components in this fraction were digestible with chondroitinase ABC indicated the presence of molecules bearing chondroitin sulfate and/or dermatan sulfate chains (data not shown).

A comparison of the secretion of radiolabeled sulfated macromolecules by estrogenized or nonestrogenized uteri resulted in the data shown Table III. The rate of isotope in-
corporation into both KSPG and HS(PG) increased 12- to 13-fold as a result of estrogen treatment. This increase greatly exceeded the increase in uterine weight observed as a result of estrogen treatment (20, 21). Although the rate of isotope incorporation into both classes of molecules greatly increased as a result of estrogen treatment, the ratio of the two remained quite similar, rising only slightly from 6.6 to 7.3. When this ratio was calculated for apical secretions of highly polarized epithelial cell cultures, the ratio averaged 5.2, whereas that of secretions of epithelial cells cultured on matrix-coated tissue culture plates averaged 1.3. Collectively, these data indicated that estrogen dramatically stimulated radioactive sulfate incorporation into secreted glycoconjugates, although this hormone did not change the relative production of KSPG and HS(PG) by the uterine cells. In fact, the ratio of these two sulfated glycoconjugates was similar in both the apical secretions of highly polarized epithelial cell cultures and in uterine secretions.

Discussion

The various factors reported to affect the phenotype of differentiated cells include hormones (1, 29) and growth factors (22), as well as the composition of the extracellular matrix (14, 17, 19). These factors have been shown to alter dramatically both the morphology and patterns of gene expression of specific cell types. Most epithelial cells exist in a particular three-dimensional configuration in the sense that they not only form close contacts with neighboring cells but must present functionally distinct cell surfaces to differing environments, resulting in cell polarity. Characteristically, the apical plasma membrane of the UE cell lines the lumen of that organ. The basal surface is integrated with the basal lamina, which compartmentalizes these cells from the underlying stroma and its vasculature. We found that the establishment and evolution of polarity markedly alters the extent and direction of UE cell secretion of proteins and glycoproteins, that is, KSPG. These observations emphasize that the

Table III. Effects of Estrogen on Secretion of Sulfated Glycoconjugates

| Source of secretion | dpm x 10^3 per two uterine horns (9 h)* | KSPG | HS(PG) | KSPG/HS(PG) |
|---------------------|----------------------------------------|------|--------|-------------|
| Uterine strips      |                                        |      |        |             |
| No estrogen         | 2.52 ± 0.10                            | 0.38 ± 0.14 | 6.6   |
| Plus estrogen       | 33.4 ± 3.6                             | 4.59 ± 0.49 | 7.3   |
| Primary cultures of epithelial cells† | | | | |
| Semi-permeable filters | –                               | –       | 5.2 ± 1.0 |
| Solid supports      | –                                       | –       | 1.3 ± 0.1 |

* Immature female rats (3 wk old) either were not injected or injected daily for three consecutive days with 1 μg of 17-β-estradiol in 0.1 ml of arachis oil. One day after the last injection, the uteri were excised, slit longitudinally, and metabolically labeled with H_2[35S]SO_4 for 9 h as described in Materials and Methods. As described in the text, secreted macromolecules were obtained by acid precipitation separated initially by anion exchange liquid chromatography, and the amount of radioactivity in the form of KSPG or HS(PG) was determined by the enzymatic digestion protocol. The averages and ranges of quadruplicate determinations are presented.

† UE cells were prepared and cultured on 12-14 d on matrix- (Matrigel) permeated filters or on matrix-coated tissue culture plates as described in the text. These cultures were labeled metabolically with H_2[35S]SO_4 under the same conditions as were used for labeling of the uterine strips. Isotope incorporation into secreted macromolecules was analyzed as described above. The average ratios (± range) of incorporation into KSPG vs. HS(PG) are presented for the values obtained in four separate experiments with the polarized cultures and in two separate experiments with the nonpolarized cultures.
status of cell polarity must be considered among the relevant influences in phenotypic expression of natural epithelial cells. In addition, these data indicate that in certain systems, including UE cells, the patterns of glycoconjugate secretion as well as the spectrum of secreted proteins (11) may be used as an index of cell polarity.

Some reports have described vectorial secretion of glycoconjugates by polarized cells. In this regard, a wealth of information is available on the production of viral glycoproteins by infected cells (for review see reference 23), but relatively little is known about the vectorial secretion of glycoconjugates produced by polarized host cells. Vectorial secretion of the extracellular matrix components, laminin, and heparan sulfate proteoglycans, has been described (3). In this single case, these components were secreted almost exclusively from the basal surface. In contrast, we found that both KSPG and HS(PG) are secreted, in large part, from the apical cell surface of primary cultures of UE maintained under polarizing conditions in vitro and in vivo. Collectively, these observations indicated that proteoglycans can be secreted preferentially from either basal or apical cell surfaces depending on the cell type.

Basally secreted proteoglycans are likely to be deposited in the extracellular matrix where they can function to maintain tissue architecture and participate in interactions with subjacent cells (2, 12). Indeed, localization of proteoglycans in basal lamina has been reported in a number of systems (2, 9, 12). During our typical 9-h incubations, highly polarized cells secreted only 3% of their sulfated macromolecules basally. Pulse-chase studies indicate that 70-90% of the preformed filter-associated components were secreted within this time period. More than 90% of this secretion occurred apically. Consequently, it can be calculated that no more than 6-16% of the total sulfated macromolecules could be sequestered within the cells or filter. Since only ~3% of the sulfated macromolecules are found in the basal media compartment, no more than 9-19% of these molecules could be basally secreted or disposed under these conditions. The remaining 81-91% are secreted apically. Thus, the apical preference for proteoglycan secretion in this system is at least 5- to 10-fold.

The preferential apical orientation of proteoglycan production observed in this study is consistent with our previous observations (36) and those of Morris et al. (25) indicating that HS(PG) were expressed at the apical cell surface of mouse UE (36). Since this epithelial cell surface faces a lumen, the apical proteoglycans are not likely to perform purely structural functions. In contrast to the apical cell surfaces of many other epithelia, that of the UE cell undergoes a regulated interaction with another cell type, the embryonic trophoderm (30, 31). Interestingly, laminin, a heparan sulfate-binding molecule (38), has been found to be expressed at the apical cell surface of the embryonic trophoderm (18). Moreover, HS(PG) seems to be expressed on the trophodermal cell surface and can participate in embryo adhesion events, including interactions with UE cells (8). Finally, we found that specific, high-affinity heparin receptors exist on the cell surface of UE cells (Wilson, Stewart and Carson, manuscript submitted for publication). Consequently, both HS(PG) and molecules capable of binding them characterize the apical surface of the epithelial cells that line the uterus and the embryo. It is possible that HS(PG)-heparan sulfate-binding protein interactions take place between these epithelia at initial stages of embryo-uterine adhesion, as has been suggested for certain other cell adhesion events (8, 10, 16, 17). The relatively rapid turnover rates of the cell-associated proteoglycans observed in these and previous studies of uterine epithelia (25, 36) offer a means for rapid changes in expression of these cell surface components.

The major glycoconjugates secreted apically by the rat UE cells proved to be KSPG. A structurally related class of molecules, lactosaminoglycans, have been found to be cell surface components of mouse UE cells (4). The lactosaminoglycans seem to participate in epithelial cell adhesion events in vitro; whether they perform a similar function in vivo is not clear. We found that a large fraction of the KSPG was secreted, which is inconsistent with a role in epithelial cell-epithelial cell adhesion. The observation that these molecules were secreted apically from polarized cell cultures or from uterine strips suggested that they participate in some function of the uterine lumen. One interesting possibility arises from the fact that sperm must pass through the uterine lumen before fertilization. Shur (33) presented evidence that galactosyltransferase on murine sperm-cell surfaces participates in sperm-egg adhesion. Moreover, lactosaminoglycans have been demonstrated to be excellent substrates for this enzyme and effective inhibitors of sperm binding to zona pellucidae (32). It is possible that the structurally related KSPG interact with sperm and serve to limit or inactivate sperm traversing the uterine lumen. We are currently examining the ability of the secreted KSPG to interact with soluble galactosyltransferase and inhibit sperm-egg binding.

The observation that the synthesis of both keratan sulfate and HSPG was markedly stimulated by estrogen is consistent with other observations of this hormone's strong, stimulatory effect on uterine glycoprotein assembly (6, 24). Since these earlier studies involved analyses of glycoproteins derived from whole uteri, they did not implicate glycoprotein products of a particular uterine cell type as targets of hormone action. We found that estrogen had a profound effect on the synthesis of glycoproteins that are major products of UE cells, the cellular target of other estrogen actions under these conditions (20, 21). It will be particularly interesting to determine to what extent steroid hormone actions influence the patterns of these cells' secretory products. Morris et al. (24) have inferred that estrogen stimulates HSPG turnover in uterine epithelia and may lead to a reduction in its cell surface expression at the time of embryo implantation. The in vitro system we developed should be a useful model for directly studying the effects of steroid hormones on the cell surface components exposed at the apical domain of the uterine epithelium. Determining how this cell surface functions in the highly regulated process of implantation should now be possible.

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References

1. Armelin, M. C. S., and H. A. Armelin. 1983. Glucocorticoid hormone regulation of both cell surface and cytoskeleton related to growth control or rat glioma cells. J. Cell Biol. 96:865.

2. Bernfield, M. R., and S. D. Banerjee. 1978. The basal lamina in epithelial-mesenchymal morphogenetic interactions. In Biology and Chemistry of Basement Membranes. N. A. Kefalides, editor. Academic Press, Inc., New York. 137–148.

3. Caplan, M. J., J. L. Stow, A. P. Newman, J. Madri, H. C. Anderson, M. G. Farquhar, G. E. Palade, and J. D. Jamieson. 1987. Dependence on pH of polarized sorting of secreted proteins. Nature (Lond.). 329: 632–635.

4. Dutt, A., J.-P. Tang, and D. D. Carson. 1987. Lactosaminoglycans are involved in uterine epithelial cell adhesion in vitro. Dev. Biol. 119:27–37.

5. Dutt, A., J.-P. Tang, and D. D. Carson. 1988. Estrogen preferentially stimulates lactosaminoglycan-containing oligosaccharide synthesis in mouse uteri. J. Biol. Chem. 263:2270–2279.

6. Dutt, A., J.-P. Tang, J. K. Welply, and D. D. Carson. 1986. Regulation of N-linked glycoprotein assembly in uteri by steroid hormones. Endocrinology. 118:661–673.

7. Edelman, G. M. 1984. Modulation of cell adhesion during induction, histogenesis and perinatal development of the nervous system. Annu. Rev. Neurosci. 7:339–377.

8. Farach, M. C., J.-P. Tang, G. L. Decker, and D. D. Carson. Heparin/heparan sulfate is involved in attachment and spreading of mouse embryos in vitro. Dev. Biol. 123:401–410.

9. Farquhar, M. G. 1985. The glomerular basement membrane, a selective macromolecular filter. In Cell Biology of the Extracellular Matrix. E. D. Hay, editor. Plenum Publishing Corp., New York. 335–378.

10. Gill, P. J., C. K. Silbert, and J. E. Silbert. 1983. Cell surface heparan sulfate mediates attachment and reattachment of fibroblasts and endothelial cells. Biochemistry. 25:405–410.

11. Glasser, S. R., J. Julian, G. L. Decker, J.-P. Tang, and D. D. Carson. 1988. Development of morphological and functional polarity in primary cultures of immature rat uterine epithelial cells. J. Cell Biol. 107: 2409–2423.

12. Hay, E. D. 1985. Collagen and embryonic development. In Cell Biology of the Extracellular Matrix. E. D. Hay, editor. Plenum Publishing Corp., New York. 379–409.

13. Hirani, S., R. J. Bernasconi, and J. R. Rasmussen. 1987. Use of N-glycanase to remove asparagine-linked oligosaccharides for structural analysis. Anal. Biochem. 162:485–492.

14. Kajava, M. J., and K. Tepperman. 1983. Culturing chick muscle cells on glycosaminoglycan substrates: attachment and differentiation. Dev. Biol. 99:277–286.

15. Lander, A. D., D. K. Fujii, D. Gospodarowicz, and L. F. Reichert. 1982. Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparan sulfate proteoglycan. J. Cell Biol. 94:574–585.

16. Laterra, J., J. E. Silbert, and L. A. Culp. 1983. Cell surface heparan sulfate mediates some adhesive responses to glycosaminoglycan-binding matrices, including fibronectin. J. Cell Biol. 96:112–123.

17. Lee, E. Y.-H., W.-H. Lee, C. S. Kastzel, G. Parry, and M. J. Bissell. 1985. Interaction of mouse mammary epithelial cells with collagen substrate: regulation of casein gene expression and secretion. Proc. Natl. Acad. Sci. USA. 82:1419–1423.

18. Leivo, I., A. Vahteri, R. Timpl, and J. Wartiovaara. 1980. Appearance and distribution of collagens and laminins in the early mouse embryo. Dev. Biol. 76:100–114.

19. Loring, J., B. Gilmelius, and J. A. Weston. 1982. Extracellular matrix materials influence visual neural crest cell differentiation in vitro. Dev. Biol. 90:165–174.

20. Markaverich, B. M., and J. H. Clark. 1979. Two binding sites for estradiol in rat uterine nuclei: relationship to uterotrophic response. Endocrinology. 105:1458–1462.

21. Martucci, C., and J. Fishman. 1977. Direction of estradiol metabolism as a control of its hormonal action: uterotrophic activity of estradiol metabolites. Endocrinology. 101:1709–1715.

22. Massague, J. 1987. The TGF-β family of growth and differentiation factors. Cell. 49:437–438.

23. Matlin, K. S. 1986. The sorting of proteins to the plasma membrane in epithelial cells. J. Cell Biol. 103:2565–2568.

24. McCormack, S. A., and S. R. Glasser. 1980. Differential response of individual uterine cell types from immature rats treated with estradiol. Endocrinology. 106:1634–1649.

25. Morris, M. J., S. W. Potter, and G. Gzda-Bulseco. 1988. Estradiol induces an accumulation of free heparan sulfate glycosaminoglycan chains in uterine epithelium. Endocrinology. 122:542–553.

26. Obrink, B. 1986. Epithelial cell adhesion molecules. Exp. Cell Res. 163: 1–21.

27. Plantes, J.-J., and D. M. Carlson. 1972. Assay for olefinic amino acids: products of the β-elimination reaction in glycoproteins. Methods Enzymol. 27:46–48.

28. Rauvala, H. 1983. Cell surface carbohydrates and cell adhesion. Trends Biochem. Sci. 8:323–325.

29. Rosen, J. M., R. J. Matusik, D. A. Richards, P. Gupta, and J. R. Rogers. 1980. Hormonregulated gene expression in immature rat uteri. Trends Endocrinol. Metabol. 2:339–377.

30. Scharf, S., and A. C. Enders. 1975. Cellular bases of interaction between trophoblast and uterus at implantation. Biol. Reprod. 12:41–65.

31. Sherman, M. L., and D. S. Salomon. 1975. The relationship between the early mouse embryo and its environment. In Developmental Biology of Reproduction. C. L. Markert and J. Papaconstantinou, editors. Academic Press, Inc., New York. 277–309.

32. Shur, B. D. 1984. The receptor function of galactosyltransferase during cellular interactions. Mol. Cell. Biochem. 61:143–158.

33. Shur, B. D., and N. G. Hall. 1982. A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. J. Cell Biol. 95:574–579.

34. Simons, K., and S. P. Fuller. 1985. Cell surface polarity in epithelia. Annu. Rev. Cell Biol. 1:243–288.

35. Deleted in proof.

36. Tang, J.-P., J. Julian, S. R. Glasser, and D. D. Carson. 1987. Heparan sulfate proteoglycan synthesis and metabolism by mouse uterine epithelial cells cultured in vitro. J. Biol. Chem. 262:12832–12842.

37. Vestweber, D., and R. Kemler. 1984. Rabbit antiserum against a purified membrane glycoprotein de, compacts mouse preimplantation embryos and their components. Nature (Lond.). 318:661–673.

38. Woodley, D. T., C. N. Rao, J. R. Hassell, L. A. Liotta, G. R. Martin, and H. K. Kleinman. 1983. Interactions of basement membrane components. Biochim. Biophys. Acta. 761:278–283.