

## Type I Collagen Reduces the Degradation of Basal Lamina Proteoglycan by Mammary Epithelial Cells

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**ABSTRACT** When mouse mammary epithelial cells are cultured on a plastic substratum, no basal lamina forms. When cultured on a type I collagen gel, the rate of glycosaminoglycan (GAG) synthesis is unchanged, but the rate of GAG degradation is markedly reduced and a GAG-rich, basal lamina-like structure accumulates. This effect of collagen was investigated by comparing the culture distribution, nature, and metabolic stability of the  $^{35}\text{S}$ -GAG-containing molecules produced by cells on plastic and collagen. During 48 h of labeling with  $^{35}\text{SO}_4$ , cultures on collagen accumulate 1.4-fold more  $^{35}\text{S}$ -GAG per microgram of DNA. In these cultures, most of the extracellular  $^{35}\text{S}$ -GAG is immobilized with the lamina and collagen gel, whereas in cultures on plastic all extracellular  $^{35}\text{S}$ -GAG is soluble. On both substrata, the cells produce several heparan sulfate-rich  $^{35}\text{S}$ -proteoglycan fractions that are distinct by Sepharose CL-4B chromatography. The culture types contain similar amounts of each fraction, except that collagen cultures contain nearly four times more of a fraction that is found largely bound to the lamina and collagen gel. During a chase this proteoglycan fraction is stable in cultures on collagen, but is extensively degraded in cultures on plastic. Thus, collagen-induced formation of a basal lamina correlates with reduced degradation and enhanced accumulation of a specific heparan sulfate-rich proteoglycan fraction. Immobilization and stabilization of basal lamina proteoglycan(s) by interstitial collagen may be a physiological mechanism of basal lamina maintenance and assembly.

A basal lamina, a uniformly thick extracellular matrix, is formed by parenchymal tissues wherever they contact connective tissues (24). The lamina contains specific varieties of collagens (see reference 13 for review), glycoproteins (8, 16, 23), glycosaminoglycans, and proteoglycans (1, 2, 9–11). The process by which these components are assembled into a basal lamina is unclear, but there are implications that interstitial collagen may be involved. For example, type I collagen stimulates normal epithelial cells to accumulate extracellular materials (18) and contact with a type I collagen substratum allows several epithelia to form a basal lamina in vitro (6, 7, 17, 19). Similarly, when cultured on a type I collagen gel, cells of a mouse mammary epithelial line deposit GAG at the cell-gel interface where they accumulate ultrastructurally identifiable basal lamina materials. In contrast, when these cells are cultured on plastic, despite a nearly identical rate of GAG synthesis, no lamina and GAG accumulate apparently because of a nearly sixfold greater rate of GAG degradation (3).

The mechanism by which collagen reduces the rate of GAG degradation and promotes accumulation of lamina materials is unclear. The collagen could alter the nature of the materials synthesized, affect their secretion, stabilize secreted proteogly-

can to degradation, or act in some other way. To investigate these possibilities, we have compared the culture distribution, nature, and metabolic stability of the  $^{35}\text{S}$ -proteoglycans produced by mouse mammary epithelial cells on a plastic or type I collagen gel substratum. The results indicate that similar proteoglycans are produced, but they are distributed differently in the culture, apparently because of binding to collagen, and that collagen reduces the degradation of a specific heparan sulfate-rich fraction that has the characteristics of a basal lamina proteoglycan. A preliminary report of this study was presented in abstract form (4).

### MATERIALS AND METHODS

#### *Cells, Culture, Labeling, and Chase Conditions*

The medium for plating, growing, and labeling of the cells was as reported previously (3). Cells from low passage (13–20) cultures of the NMuMG cell line (20) were plated at half confluent density ( $10^5$  cells/cm<sup>2</sup>) on a 2-cm<sup>2</sup> plastic or collagen gel substratum. The cells were confluent within 2 d and were labeled for 48 h on day 3 by substituting fresh medium containing 100  $\mu\text{Ci}/\text{ml}$   $\text{H}_2^{35}\text{SO}_4$  (carrier free; New England Nuclear, Boston Mass.) for the original medium. This labeling period ensured that the  $^{35}\text{S}$ -GAG associated with monolayers on plastic was in a steady state (3). Cultures were chased by substituting label-free medium

with a normal  $\text{SO}_4^{2-}$  content for the labeling medium. The chase was prolonged (48 h) to minimize the contribution from labeled proteoglycan fractions that were formed early during the chase.

## Buffers

HBSS was Hanks' balanced salt solution, pH 7.4. EDTA buffer was  $\text{Ca}^{++}$ -,  $\text{Mg}^{++}$ -free HBSS containing 0.02%  $\text{Na}_4\text{-EDTA}$ , pH 7.4. Detergent buffer was HBSS containing 0.5% Triton X-100 (B-grade; Calbiochem-Behring Corp., San Diego, Calif.), 100 mM 6-aminohexanoic acid (Eastman Kodak), 5 mM benzamidine-HCl (Aldrich Chemical Co., Inc., Milwaukee, Wis.), 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Calbiochem), pH 7.4. Extracting buffer consisted of 4 M guanidine-HCl (ultrapure; Schwarz-Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), 100 mM 6-aminohexanoic acid, 5 mM benzamidine-HCl, 0.5 mM PMSF, 10 mM EDTA, and 50 mM sodium acetate, pH 5.8.

## Harvesting and Definition of Culture Fractions

After removal of the labeling medium, the monolayers grown on plastic were quickly rinsed with HBSS and this rinse was pooled with the medium. The cells were then removed by incubation in EDTA buffer for 10 min at 37°C, or by scraping in HBSS with the aid of a rubber policeman. The resulting cell suspension was spun at 200 g for 5 min and the supernate (wash) and pellet (cells) were collected separately. Substratum-associated material was material remaining on the plastic dish after removal of the cells as described above, or, after their removal by exposure to ice-cold detergent buffer.

For cultures on collagen the medium was collected as described above. The monolayers and supporting gels were then lifted from the dish with a spatula and washed gently in HBSS by repetitive centrifugation at 200 g with several changes of buffer. These supernates, designated as wash, included the medium from the interstices of the gel. The pellet represented the cell and substratum-associated materials. Cells were obtained by gently agitating the pellet in EDTA buffer for 10 min at 37°C, physically removing the gel from the tube, and centrifuging at 200 g for 5 min. This EDTA supernate contained <2% of the cell and substratum-associated label and it was not further analyzed.

A cell-free substratum preparation that retained the basal lamina was obtained by extensively rinsing and agitating the pellet in ice-cold detergent buffer. The absence of cells and retention of the lamina was confirmed by transmission electron microscopy. Cell-free substratum material was also obtained by treating the pellet with purified protease-free clostridial collagenase (30  $\mu\text{g/gel}$ ) in HBSS containing 0.1% bovine serum albumin (3  $\times$  crystalline; Sigma Chemical Co., St. Louis, Mo.). Incubation for 15 min at 37°C resulted in complete solubilization of the gel and its associated materials.

## $^{35}\text{SO}_4$ Proteoglycan Extraction

Fluid culture samples were made 4 M in guanidinium ion by adding an equal volume of 2  $\times$  extracting buffer. Cells or substratum samples were kept overnight at 4°C in extracting buffer (0.75–1 ml/original  $\text{cm}^2$ ). Each sample was spun at 25,000 g at 4°C for 30 min and stored at –20°C. The supernates contained >95% of the total radioactivity in the samples except for the cell samples (~85%).

## Chromatography

The extracts were thawed, centrifuged at 25,000 g for 30 min without loss of label, and passed through a 10-ml (minimum 3  $\times$  sample volume) Sephadex G-25 column to remove any free  $^{35}\text{SO}_4$ . Labeled materials from the G-25 void volume were applied to a Sepharose CL-4B column (0.8  $\times$  100 cm) and eluted at 4°C into 1-ml fractions. All chromatography was performed in extracting buffer. Aliquots of each fraction were diluted with 0.5-ml of 70% ethanol and, after the addition of 10 ml of Aqasol (New England Nuclear), were counted at an efficiency of 86–91%. Recovery from the Sephadex and Sepharose columns exceeded 85%.  $^3\text{H}$ -glucosamine was included in each run to mark the  $V_i$ .  $K_{av}$  values given in the text are means (and range) of separate experiments.

## GAG Analyses

Total  $^{35}\text{S}$ -GAG in the culture compartments was determined as described previously (3). Proteoglycans in pooled eluates were precipitated at –20°C by the addition of 3 vol of 95% ethanol/1.3% K-acetate in the presence of 20  $\mu\text{g}$  of carrier chondroitin-6-sulfate (Calbiochem). The  $^{35}\text{S}$ -GAG types were identified as described previously (9) by cetylpyridinium-Cl precipitation at low and high salt concentrations after chondroitinase ABC digestion.

## RESULTS

After 5 d of culture, the mammary epithelial cells on collagen have accumulated a layer of GAG-rich basal lamina materials, whereas those on plastic have not (3). Monolayers on plastic reach a steady-state level of  $^{35}\text{SO}_4$  incorporation after 24 h of labeling, whereas  $^{35}\text{S}$ -GAG continues to accumulate in monolayers on collagen (3).

### Distribution of $^{35}\text{S}$ -GAG

By 48 h of labeling, cultures on collagen contained 1.40 ( $\pm 0.05$ , mean and SD;  $n = 6$ ) times more  $^{35}\text{S}$ -GAG per microgram of DNA than cultures on plastic and distributed the  $^{35}\text{S}$ -GAG differently (Table I). In cultures on plastic, half of the  $^{35}\text{S}$ -GAG was in the medium. In the other half associated with the monolayer, 60% was with the cells; the rest was in the cell wash, representing soluble materials accumulating between and under the cells. Only minute amounts of  $^{35}\text{S}$ -GAG were found on the plastic substratum, whether the cells were harvested by EDTA treatment, scraped off with a rubber policeman, or extracted with detergent buffer, and whether the plastic was extracted with hot SDS, 0.1 N NaOH, or 4 M GuHCl. In contrast, in cultures on collagen, the bulk of  $^{35}\text{S}$ -GAG was associated with the collagen gel substratum and basal lamina; only 10% was in the medium. The cells and cell wash, which included the medium present in the interstices of the gel, together contained a similar proportion of  $^{35}\text{S}$ -GAG (~50%) as in cultures on plastic.

The proportion of  $^{35}\text{S}$ -GAG associated with the collagen gel and lamina was the same (~45%) whether the cells were removed with EDTA or extracted with detergent buffer (procedures that leave the lamina intact), or, when the lamina and collagen were solubilized by treating intact monolayers with collagenase. Collagenase treatment released only 0.5% of the label from cells grown on plastic. These results indicate that the major difference in  $^{35}\text{S}$ -GAG distribution between the cultures is in the extracellular materials, which are soluble in cultures on plastic but largely immobilized with the substratum in cultures on collagen.

### Nature of $^{35}\text{S}$ -GAG in the Culture Compartments

The materials in the various compartments (Table I) were analyzed by chromatography on Sepharose CL-4B in 4 M GuHCl and protease inhibitors to determine whether the compartments contained different  $^{35}\text{S}$ -GAG containing molecules.

TABLE I  
Distribution of  $^{35}\text{S}$ -GAG in Cultures Labeled for 48 h

| Culture compartment | Percent of total $^{35}\text{S}$ -GAG |                 |
|---------------------|---------------------------------------|-----------------|
|                     | Plastic                               | Collagen        |
| Cells               | 30.2 $\pm$ 5.6                        | 15.0 $\pm$ 2.7  |
| Cell wash           | 20.3 $\pm$ 2.4                        | 31.7 $\pm$ 3.9* |
| Substratum          | <1                                    | 43.7 $\pm$ 1.9  |
| Medium              | 49.2 $\pm$ 2.4                        | 9.42 $\pm$ 2.7  |

$^{35}\text{S}$ -GAG is defined as counts precipitable by cetylpyridinium chloride. The numbers represent the mean and standard deviation of three separate experiments for plastic and four for collagen, each experiment involving two cultures.

\* Contains the medium present in the interstices of the collagen gel.

## Cultures on Plastic

The  $^{35}\text{S}$  in the cells from cultures on plastic resolved into three approximately equal fractions; a peak in the excluded volume ( $V_0$ ), at  $K_{av} = 0.327$  (0.010) and  $0.872$  (0.020) (mean (range) for  $n = 3$ ; Fig. 1A). Digestion of the cell extract with thermolysin caused the label to elute as a broad peak at  $K_{av} \cong 0.55$  and a more narrow one at  $K_{av} \cong 0.87$  (Fig. 1A). Similar results were obtained with cells harvested by scraping.

The label in the EDTA wash of the cells eluted as a single asymmetric peak with  $K_{av} = 0.342$  (0.029) (Fig. 1B;  $n = 3$ ). To assess the asymmetry, materials of apparently larger size ( $K_{av} = 0.15$ – $0.25$ ) were rechromatographed. These eluted at  $K_{av} \cong 0.18$  as a symmetric well-defined peak (Fig. 2). When digested with thermolysin, the label in the entire cell wash eluted as a single broad peak with  $K_{av} \cong 0.55$  (not shown). The  $^{35}\text{S}$  in the medium, the largest compartment, was more heterogeneous, eluting as a double peak, with a shoulder near  $K_{av} \cong 0.2$  and maxima at  $K_{av} = 0.399$  (0.019) and  $0.548$  (0.037) (Fig. 1C;  $n = 3$ ). Again, protease treatment of the media fractions yielded a single broad peak eluting near  $K_{av} \cong 0.55$  (not shown).

## Cultures on Collagen

The  $^{35}\text{S}$  in the cells from cultures on collagen displayed peaks in the void volume and at  $K_{av} = 0.332$  (0.034) and  $0.875$  (0.025), duplicating almost exactly the profile of cells grown on plastic (Fig. 1D;  $n = 5$ ). Protease treatment of the cellular

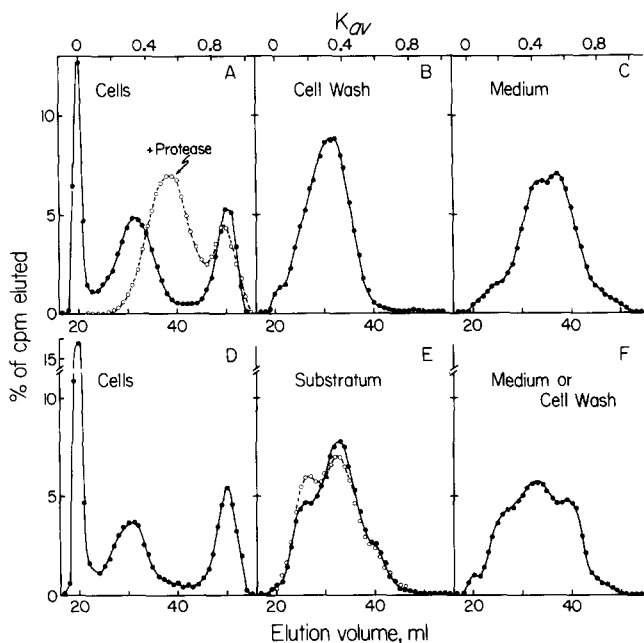


FIGURE 1 (A–C) Elution profiles of  $^{35}\text{SO}_4^{2-}$ -labeled materials in cultures grown on plastic substrata labeled for 48 h. (A) Materials present in EDTA-dissociated cells (●); same material digested with thermolysin (○). (B) Materials present in the EDTA buffer used to dissociate the monolayers. (C) Materials present in the culture medium. (D–F) Elution profile of  $^{35}\text{SO}_4^{2-}$ -labeled materials in cultures grown on collagen substrata labeled for 48 h. (D) Materials present in EDTA-dissociated cells. (E) Materials released from the cells by collagenase treatment (●); materials remaining on the collagen substratum after complete removal of the cells with Triton X-100 (○). (F) Materials present in wash of the collagen gel. Sepharose CL-4B eluted at  $4^\circ\text{C}$  in 4 M guanidinium chloride (pH 5.8) containing protease inhibitors, as described in Materials and Methods. Ordinate: % of the total cpm eluted. Recovery was  $>85\%$  of the  $\sim 100,000$  cpm loaded for each profile.

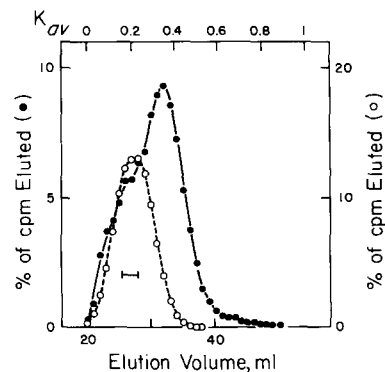


FIGURE 2 Characterization of the  $^{35}\text{SO}_4^{2-}$ -labeled material present in the EDTA wash of cells grown on plastic. Sepharose CL-4B chromatography as in legend to Fig. 1. The materials in the cell wash (●) that eluted in the  $K_{av} = 0.2$  region of the column were pooled (indicated by the bar) and 30,000 cpm were rechromatographed over the same column (○). Note the difference in scale for the ordinates.

fractions also yielded results similar to those obtained for cells on plastic (not shown).

The label remaining with the basal lamina and collagen gel after removal of the cells with detergent buffer eluted as a double peak with maxima at  $K_{av} = 0.188$  (0.015) and  $0.362$  (0.045) and a shoulder near  $K_{av} = 0.55$  (Fig. 1E;  $n = 3$ ). After protease digestion, these materials also eluted in a single broad peak with  $K_{av} \cong 0.558$ – $0.562$  (not shown). The chromatograph of the label solubilized by collagenase treatment of intact monolayers, which removed the lamina and collagen gel, was similar to that of the detergent insoluble materials: a double peak with maxima near  $K_{av} \cong 0.2$  and  $0.35$  (Fig. 1E).

The material in the cell wash eluted as a broad peak with a maximum at  $K_{av} = 0.389$  (0.033) and shoulders near  $K_{av} = 0.199$  (0.04) and  $0.577$  (0.015) (Fig. 1F;  $n = 3$ ). This profile was identical to that shown by the small amount of  $^{35}\text{S}$  material accumulating in the medium.

## Comparison of Cultures

These results indicate that, regardless of substratum, the cells produce similar types of several chromatographically distinct  $^{35}\text{S}$ -labeled fractions; those eluting near  $K_{av} = 0, 0.2,$  and  $0.3$ – $0.4$  (subsequently referred to as  $V_0, V_{0.2},$  and  $V_{0.35}$  fractions) are in proteoglycans containing similar-sized GAG chains, whereas those eluting near  $K_{av} = 0.55$  and  $0.87$  ( $V_{0.55}$  and  $V_{0.87}$ ) appear to be free GAG chains. The fractions are in different compartments of the cultures: the  $V_0$  proteoglycans and the  $V_{0.87}$  GAG fractions are found only in the cells, whereas the  $V_{0.2}$  proteoglycan and the  $V_{0.55}$  GAG fractions are only extracellular. The  $V_{0.35}$  proteoglycan fraction is mostly extracellular (wash, media) but is also found within the cells. Slight differences in the  $K_{av}$  values were obtained for the  $V_{0.35}$  fractions derived from the different culture compartments (cf. above) but whether these differences are significant remains to be established.

The labeled GAG compositions of the fractions are also similar; the proteoglycans and the  $V_{0.87}$  GAG fraction have 80–85% of their label in heparan sulfate and the remainder in chondroitin sulfate, whereas the  $V_{0.55}$  GAG fractions have a larger proportion of  $^{35}\text{S}$ -chondroitin sulfate (Table II). These values may underestimate the proportion of heparan sulfate because it generally has a lower sulfate content than chondroitin sulfate.

To compare the relative amounts of each fraction in the cultures, the amount of  $^{35}\text{S}$  per microgram of DNA in each fraction was determined from the various profiles of both cultures (cf. Fig. 1). This amount of label was then expressed relative to the amount of  $^{35}\text{S}$  in the  $V_0$  fraction from cultures on plastic (Fig. 3). This comparison reveals that the cultures contain the same relative amount of label in the  $V_0$  and  $V_{0.87}$  fractions, but that the collagen cultures contain slightly (<20%) more in the  $V_{0.35}$  and  $V_{0.55}$  fractions and greater than four times more in the  $V_{0.2}$  fraction. In cultures on collagen 60% of this extracellular proteoglycan is immobilized on the substratum, whereas in cultures on plastic it remains soluble, with roughly equal amounts present in the wash and media fractions. Thus, the increased  $^{35}\text{S}$ -GAG in the cultures on collagen is attributable primarily to greater accumulation of the  $V_{0.2}$  proteoglycan fraction.

### Proteoglycan Stability during a Chase

To determine whether the greater accumulation of the  $V_{0.2}$  proteoglycan fraction could result from its reduced degradation, we assessed the stability of the proteoglycans during a

chase after prolonged (48-h) labeling. After labeling on collagen, the cell and substratum-associated  $^{35}\text{S}$  material eluted as a  $V_0$  peak, a large  $V_{0.35}$  peak ( $K_{av} = 0.360$ ; [0.032]) with a shoulder near  $K_{av} \cong 0.2$ , and a small  $V_{0.87}$  peak ( $K_{av} = 0.870$ ; [0.047]) (Fig. 4A;  $n = 3$ ). After a 48-h chase, the label remaining in the cells and substratum ( $69.2 \pm 2.6\%$  [mean  $\pm$  SD;  $n = 3$ ] of the original amount) eluted as a small  $V_0$  peak and distinct  $V_{0.2}$  ( $K_{av} = 0.189$  [0.020]) and  $V_{0.35}$  ( $K_{av} = 0.359$  [0.047]) peaks, (Fig. 4A;  $n = 3$ ). The chase profile was very similar to that seen for the substratum material immediately after labeling (cf. Fig. 1E). Indeed, after the chase, 96% of the cell and collagen gel label was Triton X-100 insoluble and, when the cells were removed with EDTA, virtually all of their label eluted in the void volume (not shown). The materials in the cell wash were also stable; after the chase,  $72.4 \pm 9.6\%$  (mean  $\pm$  SD;  $n = 3$ ) of the original label remained and its elution profile was virtually unchanged (Fig. 5;  $n = 2$ ). Macromolecular label lost to the medium during the chase was <10% of the original label. Thus, on collagen, the cell associated fractions ( $V_0$ ,  $V_{0.35}$ ,  $V_{0.87}$ ) chase rapidly, whereas the extracellular ( $V_{0.2}$ ,  $V_{0.35}$ ) proteoglycans chase very slowly.

After 48 h of labeling on plastic, the  $^{35}\text{S}$  in the monolayer eluted as a  $V_0$  peak, a large and slightly asymmetric  $V_{0.35}$  peak ( $K_{av} = 0.350$  [0.042]), and a small  $V_{0.87}$  peak ( $K_{av} = 0.872$  [0.057]) (Fig. 4B;  $n = 3$ ). After a 24-h chase, only  $11 \pm 3.1\%$  (mean  $\pm$  SD;  $n = 4$ ) of the original label remained with the monolayer. These materials eluted as a  $V_0$ , a symmetrical  $V_{0.35}$ , and a very small  $V_{0.87}$  peak (Fig. 3B;  $n = 2$ ). Macromolecular label lost to the medium during the chase ( $30 \pm 4.5\%$  [mean  $\pm$  SD;  $n = 4$ ] of the original label) eluted as a symmetrical peak with  $K_{av} = 0.350$ – $0.393$  (Fig. 4B;  $n = 2$ ). The rest of the original label comigrated with free  $\text{SO}_4$  on the Sephadex G25 column that was routinely run before the Sepharose 4B chromatography (not shown). Importantly, the ~8–10% of the original label in the  $V_{0.2}$  fraction could not be detected after the 24-h chase; this extracellular proteoglycan appeared to chase as rapidly as the proteoglycans in the cells. Thus, reduced degradation of the  $V_{0.2}$  proteoglycan fraction can account for its enhanced accumulation in cultures on collagen.

TABLE II  
Characterization of the Sepharose CL-4B  $^{35}\text{SO}_4$ -labeled Peaks

| Fraction | Origin* | $^{35}\text{SO}_4$ -Heparan sulfate† (% of total $^{35}\text{S}$ -GAG per peak) |                             |
|----------|---------|---|-----------------------------|
|          |         | Plastic   | Collagen                    |
| $K_{av}$ |         |   |                             |
| 0        | C       | 81.5 $\pm$ 0.9  | 85.9 $\pm$ 2.5              |
| 0.2      | EC      | 84.9 $\pm$ 1.9  | 84.2 $\pm$ 1.4 <sup>b</sup> |
| 0.35     | C       | 85.4 $\pm$ 1.8  | 86.7 $\pm$ 1.1              |
|          | EC      | 82.6 $\pm$ 1.9 <sup>a</sup>   | 83.5 $\pm$ 2.1 <sup>b</sup> |
| 0.55     | EC      | 57.5 $\pm$ 1.8  | 55.5 $\pm$ 1.5              |
| 0.85     | C       | 84.5 $\pm$ 1.3  | 87.3 $\pm$ 3.6              |

Average and range of two separate experiments on plastic, means and standard deviations of three experiments on collagen: a, medium samples; b, substratum samples.

\* C, cellular; EC, extracellular.

† Total  $^{35}\text{S}$ -GAG is defined as the  $^{35}\text{SO}_4$  precipitable with cetylpyridinium-Cl at 0.05 M  $\text{Na}^+$ . Heparan sulfate is the  $^{35}\text{S}$ -GAG that is precipitable at 0.6 M  $\text{Na}^+$  after digestion with chondroitinase ABC (9, 21). The heparan sulfate nature of this GAG was confirmed in selected samples ( $V_{0.2}$ ) by determining its sensitivity to nitrous acid.

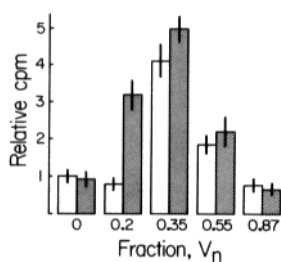


FIGURE 3 Accumulation of the  $^{35}\text{S}$ -labeled fractions in cultures on collagen and plastic substrata. White columns, plastic; stippled columns, collagen. Data derived from the separate chromatograms in Fig. 1. For each profile the percent of the total cpm eluted in a particular peak was multiplied by the amount of label in that culture compartment. Values from corresponding peaks in the different profiles were added and the sum was expressed as a ratio to the amount of  $^{35}\text{S}$  present in the  $V_0$  peak derived from cultures on plastic.  $V_0 = K_{av} 0$ – $0.10$ ;  $V_{0.2} = K_{av} 0.11$ – $0.25$ ;  $V_{0.35} = K_{av} 0.26$ – $0.45$ ;  $V_{0.55} = K_{av} 0.46$ – $0.70$ ;  $V_{0.87} = K_{av} 0.71$ – $1.0$ . More than 90% of the  $^{35}\text{S}$  was in GAG, except for the  $V_0$  fractions where, in both instances, only 50–60% of the label was precipitable by ethanol or CPC.

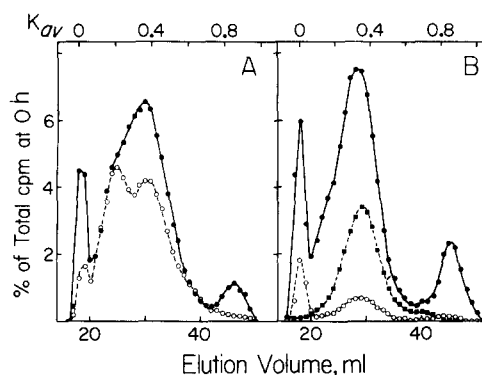


FIGURE 4 Effect of a chase on the  $^{35}\text{SO}_4$ -labeled materials in cultures on collagen (A) and plastic (B). (A) Material present in the cells and on the substratum in cultures on collagen labeled for 48 h, before (●) and after (○) a 48-h chase. Total label associated with the cells and substratum at the start of the chase defined as 100%. (B) Material present in intact monolayers of cultures on plastic labeled for 48 h before (●) and after (○) a 24-h chase, and the material recovered in the 24-h chase medium (■). Total label present in the monolayer at the start of the chase defined as 100%. Sepharose CL-4B chromatography as in legend to Fig. 1. Total cpm loaded: 80,000–100,000 cpm for each run, except for the extract from chased monolayers grown on plastic (60,000 cpm).

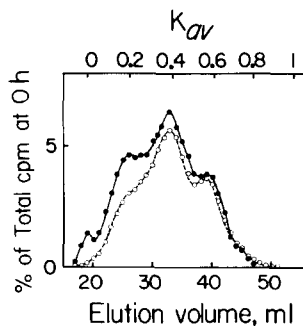


FIGURE 5  $^{35}\text{S}$ -labeled materials in the cell wash of cultures on collagen. Chromatography as in Fig. 1. (●) 0-h chase; (○) 48-h chase. The total cpm present in the wash fraction at 0 h of chase are taken as 100%.

## DISCUSSION

The mouse mammary epithelial cells used in this study form a continuous basal lamina when grown in athymic nude mice (5). These cells fail to form a lamina when cultured on plastic but will form a basal lamina-like layer when cultured on a type I collagen gel (3). We show here that regardless of the substratum, the cells produce similar types of several heparan sulfate-rich proteoglycan fractions and that formation of the lamina on collagen is accompanied by a substantial increase in a specific proteoglycan fraction. Enhanced accumulation of this fraction on collagen is attributable, at least in part, to a reduction in its rate of degradation. The collagen may be providing a substratum that allows physiological mechanisms of basal lamina assembly and maintenance to proceed *in vitro*.

The proteoglycan fraction ( $V_{0.2}$ ) that selectively accumulates on collagen gels may be a novel basal lamina proteoglycan. In these cultures, ruthenium red staining localizes polyanionic materials to the lamina and autoradiography shows that the  $^{35}\text{S}$  label is confined to the cells and the cell-gel interface (3). The  $V_{0.2}$  fraction is present only extracellularly, is found in largest amounts physically associated with the lamina and collagen gel and is the only fraction to increase significantly during formation of the lamina. A proteoglycan fraction of nearly identical Sepharose 4B elution characteristics and GAG composition has been isolated from a basement membrane-producing tumor (10). The  $V_{0.2}$  fraction differs, however, in elution characteristics from a proteoglycan of similar composition isolated from the glomerular basement membrane (12).

In cultures on collagen, the heparan sulfate-rich proteoglycans are largely in an insoluble matrix whereas the analogous fractions are soluble in cultures on plastic. This insolubility is attributable to the collagen gel, but whether the proteoglycans bind directly to the collagen or to some intervening component(s) remains to be established. This proteoglycan deposition would be consistent with known glycosaminoglycan-collagen interactions (15). Additionally, laminin, a constituent of basal laminae, can bind heparinlike polysaccharides (22), as can fibronectin (14, 25). These proteins could immobilize the heparan sulfate proteoglycan in the lamina on the preformed collagen gel substratum.

The degradation rate of the  $V_{0.2}$  proteoglycan fraction is markedly reduced in collagen cultures, apparently because of its binding and interaction with the collagen substratum. On the other hand, most of the proteoglycans eluting near  $K_{av} = 0.35$  also bind to the collagen gel (Fig. 1E), but this fraction doesn't accumulate (Fig. 3), suggesting that reduced degrada-

tion upon interaction with collagen may be limited to the  $V_{0.2}$  proteoglycan fraction. Thus, despite their similar GAG composition and chain size, the proteoglycan fractions represent functionally distinct entities. Analogous interactions could also reduce the degradation rate of several other lamina components, ultimately resulting in the appearance of the ultrastructurally identified lamina.

Although the collagen substratum causes the basal lamina proteoglycan to accumulate, this effect is not analogous to "differentiation" of the cells as suggested in prior studies (18, 19). Rather, the collagen selectively stabilizes a proteoglycan fraction that is also produced in the absence of exogenous collagen. This *in vitro* effect of the collagen gel may have physiological significance. A collagen substratum may not be required for basal lamina accumulation when the lamina is rapidly turning over (2). Under such circumstances, the production rate may be high and adequate enough, even in the absence of any stabilizing factor, to allow a lamina to form. This may be the case during early embryonic development where the lamina must turn over rapidly to accommodate growth and changes in tissue shape. On the other hand, when lamina turnover is slow, for instance in mature organisms, exogenous collagen may be needed to stabilize the lamina components to degradation, thus maintaining the integrity of the lamina.

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## REFERENCES

- Bernfield, M. R., S. D. Banerjee, and R. H. Cohn. 1972. Dependence of salivary epithelial morphology and branching morphogenesis upon acid mucopolysaccharide-protein (proteoglycan) at the epithelial surface. *J. Cell Biol.* 52:674-689.
- Cohn, R. M., S. D. Banerjee, and M. R. Bernfield. 1977. Basal lamina of embryonic salivary epithelia. Nature of glycosaminoglycan and organization of extracellular materials. *J. Cell Biol.* 73:464-478.
- David, G., and M. R. Bernfield. 1979. Collagen reduces glycosaminoglycan degradation by cultured mammary epithelial cells: possible mechanism for basal lamina formation. *Proc. Natl. Acad. Sci. U. S. A.* 76:786-790.
- David, G., and M. R. Bernfield. 1980. Basal lamina formation involves enhancement by collagen of proteoglycan processing. *Eur. J. Cell Biol.* 22:426a (Abstr.).
- David, G., B. van der Schueren, and M. R. Bernfield. Basal lamina formation by normal and transformed mouse mammary epithelial cells duplicated *in vitro*. *J. Natl. Cancer Inst.* In press.
- Emerman, J. T., S. J. Burwen, and D. R. Pitelka. 1979. Substrate properties influencing ultrastructural differentiation of mammary epithelial cells in culture. *Tissue Cell.* 11:109-119.
- Emerman, J. T., and D. R. Pitelka. 1977. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In Vitro (Rockville)*. 13:316-328.
- Foidart, J. M., E. W. Bere, M. Yaar, S. I. Rennard, M. Gullino, G. R. Martin, and S. Katz. 1980. Distribution and immunoelectron microscopic localization of laminin, a noncollagenous basement membrane glycoprotein. *Lab. Invest.* 42:336-342.
- Gordon, J., and M. R. Bernfield. 1980. The basal lamina of the postnatal mammary epithelium contains glycosaminoglycans in a precise ultrastructural organization. *Dev. Biol.* 74:118-135.
- Hassell, J. R., P. G. Robey, H. J. Barrach, J. Wilczek, S. I. Rennard, and G. R. Martin. 1980. Isolation of a heparan-sulfate-containing proteoglycan from basement membrane. *Proc. Natl. Acad. Sci. U. S. A.* 77:4494-4498.
- Kanwar, Y., and M. G. Farquhar. 1979. Presence of heparan sulfate in the glomerular basement membrane. *Proc. Natl. Acad. Sci. U. S. A.* 76:1303-1307.
- Kanwar, Y., M. Lemkin, V. Hascall, and M. Farquhar. 1980. Structure of newly synthesized heparan sulfate-proteoglycan of glomerular basement membrane in kidney organ culture. *J. Cell Biol.* 87(2, Pt. 2):121a (Abstr.).
- Kefalides, N. A. 1978. Chemistry and metabolism of basement membranes. Composition and structure. *In Biology and Chemistry of Basement Membranes*. N. A. Kefalides, editor. Academic Press, Inc., N. Y. 215-228.
- Lattera, J., R. Ansbacher, and L. A. Culp. 1980. Glycosaminoglycans that bind cold-insoluble globulin in cell-substratum adhesion sites of murine fibroblasts. *Proc. Natl.*

*Acad. Sci. U. S. A.* 77:6662-6666.

15. Lindahl, U., and M. Hook. 1978. Glycosaminoglycans and their binding to biological macromolecules. *Annu. Rev. Biochem.* 47:385-417.
16. Linder, E., A. Vaheri, A. Ruoslahti, and J. Wartiovaara. 1975. Distribution of fibroblast surface antigen in the developing chick embryo. *J. Exp. Med.* 142:41-49.
17. Mann, P. R., and H. Constable. 1977. Induction of basal lamina formation in epidermal cell cultures *in vitro*. *Brit. J. Dermatol.* 96:421-426.
18. Meier, S., and E. D. Hay. 1974. Control of corneal differentiation by extracellular materials. Collagen as a promoter and stabilizer of epithelial stroma production. *Dev. Biol.* 38:249-270.
19. Meier, S., and E. D. Hay. 1975. Simulation of corneal differentiation by interaction between cell surface and extracellular matrix. I. Morphogenetic analysis of transfilter "induction." *J. Cell Biol.* 66:275-291.
20. Owens, R. B., N. S. Smith, and A. J. Hackett. 1974. Epithelial cell cultures from normal glandular tissue of mice. *J. Natl. Cancer Inst.* 53:261-269.
21. Rodén, L., J. R. Baker, A. Cifonelli, and M. B. Mathews. 1972. Isolation and characterization of connective tissue polysaccharides. *Methods Enzymol.* 28(B):73-140.
22. Sakashita, S., E. Engvall, and E. Ruoslahti. 1980. Basement membrane glycoprotein laminin binds to heparin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 116:243-246.
23. Timpl, R., M. Rohde, P. Gehron Robey, S. I. Rennard, J. M. Foidart, and G. R. Martin. 1979. Laminin—A glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933-9937.
24. Vracko, R. 1978. Anatomy of basal lamina scaffold and its role in maintenance of tissue structure. *In* Biology and Chemistry of Basement Membranes. N. A. Kefalides, editor. Academic Press, Inc., N. Y. 165-176.
25. Yamada, K. M., D. W. Kennedy, K. Kimada, and R. M. Pratt. 1980. Characterization of fibronectin interactions with glycosaminoglycans and identification of active proteolytic fragments. *J. Biol. Chem.* 255:6055-6063.