Collagen reduces glycosaminoglycan degradation by cultured mammary epithelial cells: Possible mechanism for basal lamina formation

(proteoglycan/type I collagen/basement membrane/extracellular matrix)

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ABSTRACT   Collagenous substrates are reported to promote the accumulation of extracellular matrix materials by epithelia in culture. Glycosaminoglycan (GAG) metabolism is compared in secondary cultures of mouse mammary epithelial cells maintained on plastic or type I collagen gel substrates. The incorporation of $^{35}SO_4^{2-}$ into GAG during brief labeling indicates no difference between substrates in the rate of GAG synthesis. During prolonged labeling, however, accumulation of [^{35}S]GAG in cultures on collagen exceeds that of cultures on plastic. This increased accumulation is due to a markedly reduced rate of GAG degradation. GAG degradation does not occur in the medium, indicating that degradation is localized to the cells. The cultures on collagen contain a slowly degrading cell-associated [^{35}S]GAG pool and a ruthenium red-stained basal lamina, neither of which is present in cultures on plastic. The cell-associated [^{35}S]GAG in cultures on collagen is, in part, localized to the site of the ultrastructurally identified basal lamina. Formation of the basal lamina, therefore, may result from collagen-mediated reduction in the degradation of GAG-containing molecules.

Collagenous substrates stimulate the incorporation of precursors into glycosaminoglycan (GAG) and collagen by chick embryonic somites (1) and corneal epithelia (2), resulting in enhanced accumulation of extracellular matrix materials. In both instances, the stimulation is considered to be due to increased synthesis of the matrix. In the corneal system, the collagenous substrate is required for formation of a basal lamina, and cell contact with the substrate is required for the increased incorporation (3).

Dissociated epithelial cells cultured on collagenous substrates show properties not found when these cells are cultured on plastic tissue-culture substrates. Primary cultures of adult rat liver epithelial cells grown on floating collagen gels show morphological and functional features characteristic of liver in vivo (4). When cultured under similar conditions in the presence of lactogenic hormones, dissociated mammary epithelial cells from midpregnant mice maintain morphological differentiation (5). On the floating gels, the mammary epithelial cells form a basal lamina, which separates the cells from the underlying collagen, a finding reminiscent of the accumulation of basal laminar material by embryonic corneal epithelium grown on collagenous substrates.

The basal lamina of the postnatal mouse mammary epithelium, like that of several embryonic epithelia (6), is rich in GAG-containing molecules (unpublished data). To investigate the mechanisms whereby collagen influences the accumulation of extracellular materials by epithelial cells, we have examined the effects of a collagen gel substrate on the incorporation of $^{35}SO_4^{2-}$ into GAG by mammary epithelial cells in secondary culture. The results indicate that the collagen substrate stimu-

lates the incorporation of SO_4^{2-} into extracellular matrix materials and promotes the accumulation of a GAG-containing basal lamina. The increased accumulation of newly synthesized GAG is due to a reduced rate of degradation, while the rate of synthesis is unaffected. Formation of the basal lamina on these cells may result, in part, from a collagen-mediated reduction in the degradation of GAG-containing molecules. Preliminary reports of this study were presented in abstract form (7).

MATERIALS AND METHODS

Cells, Culture Media, and Reagents. Namru mouse mammary epithelial cells were provided by contract E-73-2001 N01 within the special virus cancer program, National Institutes of Health, U.S. Public Health Service, through the courtesy of H. S. Smith. This cell line, derived from normal mammary epithelium by selective trypsinization of cultured epithelial fragments, maintains a typical secretory epithelial morphology in vitro and produces benign cystadenomas after injection into isogenic newborn mice (8).

The standard medium was bicarbonate-buffered Dulbecco's modified Eagle's medium (GIBCO), containing 0.45% glucose and supplemented with nonessential amino acids (1X, GIBCO), 10% fetal calf serum (Flow Laboratories, Rockville, MD), 10 μg of bovine insulin per ml (crystalline, Sigma), 100 international units of penicillin per ml, and 100 μg of streptomycin sulfate per ml. Cells were maintained and subcultured in 75-cm² flasks (Falcon) in standard medium with a 1:4 split ratio. Only cells from passages 14–19 that were negative for mycoplasma infection, as assessed by 33258 Hoechst staining (9), were used.

Low sulfate medium was standard medium in which MgCl₂ was used instead of MgSO₄. Labeling medium was low sulfate medium containing 15, 30, or 90 μCi of carrier-free H₂^{35}SO₄ per ml (New England Nuclear). Chondroitin sulfate (B grade) was from Calbiochem; collagenase (type CLSPA) was from Worthington. Thermolysin and 3′,5′-diaminobenzoic acid were from Sigma. Type I collagen was prepared from rat tail tendons (10) and stored at 4°C as a 0.1% solution in 0.1 M acetic acid.

Collagen gels were prepared by pipetting 0.3 ml of the collagen solution, containing a trace of phenol red, into 2-cm² wells (see below) and placing the dish in an NH₃ atmosphere at room temperature until the indicator showed neutralization. The gels were kept overnight at 37°C in a humidified atmosphere, and then washed over several days with several changes of Hank's solution and finally with low sulfate medium.

Labeling and Chase Conditions. For experiments, 4×10^5 cells were plated onto a plastic or collagen gel substrate in a 2-cm² well within a 35-mm tissue culture dish (Falcon). The

Abbreviation: GAG, glycosaminoglycan.

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wells were prepared by gluing the sectioned top portion of a polystyrene tube (no. 2057 Falcon) to the center of the dish. This arrangement allowed 2 cm^2 of cells to be exposed to a larger volume of medium (4.5 ml) to reduce the effects of the gel volume (0.3 ml) during brief incorporation and chase experiments without interfering with gas exchange.

Cells were plated in low sulfate medium. They reached confluent densities within 2 days of culture, and labeling was initiated after 3 days in culture. Based on DNA measurements (11), growth rates after confluence were identical on collagen and plastic substrates. The average increment in DNA per culture was 10.4% between day 3 and day 5 of culture. The amount of DNA in cultures on plastic was 79.4 ± 1.5% (mean ± SEM; n = 9) of that in cultures on collagen, possibly because the surface area for culture on plastic was less than that on the concave gel. From day 3, the cells formed continuous monolayers on both substrates. No cells could be found within the gel. Vital staining, subculturing after prolonged periods of maintenance at confluency, and histological observation suggested equal viability of the cells on either substrate.

For chase experiments, labeling medium was removed, the cells were rinsed three times with 5 ml of serum-free standard medium, and 4.5 ml of standard medium was added. Long-term incorporation experiments were also performed in 2-cm^2 multiwell dishes (Costar) with identical results. When used, 0.9 ml of labeling medium was added to cultures on plastic and 0.6 ml of labeling medium containing 1.5-fold the concentration of $^{35}SO_4^{2-}$ was added to cultures on the 0.3-ml gels, resulting in identical final specific activities.

Harvesting of Materials. After labeling or chasing, the media were aspirated and the cells rinsed twice with 1 ml of Tris-buffered saline (20 mM, pH 7.4; buffer A). Media and rinses were combined and added to 1 ml of buffer A containing 0.06% EDTA. Cells on plastic were collected by scraping in 0.2 ml of buffer A containing 0.02% EDTA (buffer B), followed by two rinses with 0.2 ml of the same buffer, and the suspension was brought to 1.5 ml with standard medium. This fraction was called "cell and substrate" by analogy with the cultures on collagen gels.

Because cells could not be quantitatively separated from the collagen gels, cells cultured on collagen were harvested with the gel and a correction was made for the presence of [^{35}S]GAG in the gel. After the gels were detached from the dish with the tip of a sealed pasteur pipette, 0.1 ml of the exudate resulting from the collapse of the gel was combined with 0.6 ml of buffer B and brought to 1.5 ml with standard medium. The [^{35}S]GAG in the gel interstitial fluid was calculated from its volume and from the label in the exudate. In separate experiments, the volume of the gel interstitial fluid was measured by isotope dilution and was found to be 0.3 ml, a volume equal, within the limits of measurement, to the total gel volume. The remainder of the exudate, the collagen gel, and the cells were combined with 0.6 ml of buffer B and brought to 1.5 ml with standard medium. After correcting for the label in the gel interstitial fluid, this sample represents the cell- and substrate-associated [^{35}S]GAG. Total [^{35}S]GAG in the cultures is defined as the sum of [^{35}S]GAG in the medium, exudate, and cell and substrate fractions.

This indirect method of assessment was validated by showing that culturing cells on gels of different volumes (0.3, 0.4, 0.5, and 0.6 ml), but identical surface area, yielded values for total and for cell- and substrate-associated [^{35}S]GAG that were not significantly different [F (variance ratio) = 0.99; F.05(3,4) = 6.59]. This method was compared directly with an extensive washing procedure (five 1-ml washes on a gyratory shaker), and no significant difference between methods was found [F = 1.83; F.05(1,8) = 5.32].

[^{35}S]GAG Assay. [^{35}S]GAG was assayed by cetyl pyridinium chloride precipitation of samples at 0.06 M sodium ion. Prior to assay, all cell-containing samples were sonicated twice for 20 sec at 0°C. Assay mixtures consisted of 25 mM Na$_2$SO$_4$, 0.05% cetyl pyridinium chloride, 20 μg of chondroitin sulfate, and a 0.05-ml aliquot of sample in a 1-ml volume. Mixtures were incubated at 37°C for 1 hr and the precipitate was collected under reduced pressure on a glass-fiber filter; the filter was washed with a solution containing 0.005% cetyl pyridinium chloride and 25 mM Na$_2$SO$_4$ and, finally, with ice-cold water. After drying, the filters were placed in a toluene scintillant and radioactivity was measured. Predigestion of the samples with thermolysin for 3 hr at 60°C prior to precipitation with cetyl pyridinium chloride did not affect the results, and was thus omitted. Results are expressed as cetyl pyridinium chloride-precipitable $^{35}SO_4^{2-}$ counts per μg of DNA present in the culture. Degradation of GAG is operationally defined as the net loss of precipitable radioactivity from the culture.

Each point represents the mean of duplicate cultures. The deviation from this mean for all points averaged 3%, and the maximum deviation was 8%. All experiments were repeated at least twice.

RESULTS

Incorporation of $^{35}SO_4^{2-}$ into GAG. Confluent cultures on plastic and, after a 60-min lag, cultures on collagen gels incorporated $^{35}SO_4^{2-}$ into GAG at a linear rate during brief labeling (Fig. 1A). The lag in the cultures on the gels is possibly due to a lower initial precursor specific activity in the gel. The rates of incorporation in both culture conditions were nearly identical. When the incorporation rate into GAG associated with the cells and substrate only was examined, again a negligible difference between substrates was observed (Fig. 1B). However, with continued labeling (Fig. 1C), cultures on collagen showed a greater accumulation of [^{35}S[GAG. With regard to cell and substrate [^{35}S]GAG, cells on plastic show a plateau within 24 hr, suggesting that this GAG is in the steady state, while cells on collagen continue to accumulate [^{35}S]GAG.
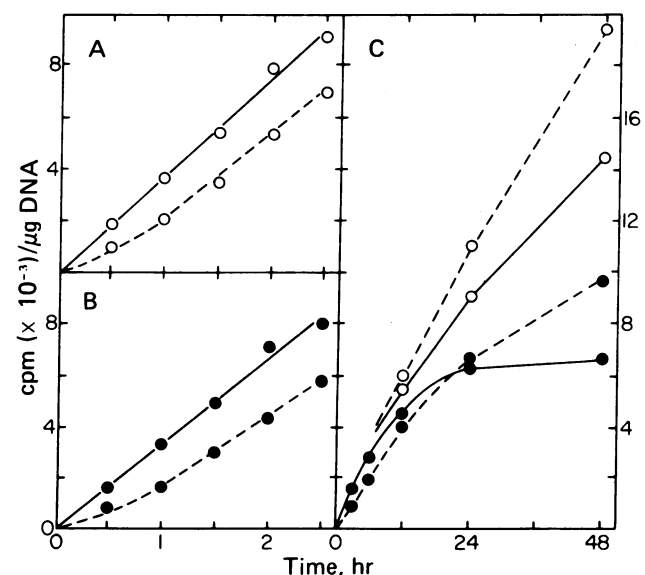

FIG. 1.   Incorporation of $^{35}SO_4^{2-}$ per μg of DNA into cetyl pyridinium chloride-precipitable material (GAG) in cultures on collagen (- - -) and plastic (—) substrates. (*A* and *B*) Initial incorporation; radiosulfate at 90 μCi/ml. (*C*) Prolonged labeling; radiosulfate at 30 μCi/ml; experiment performed in multiwell dishes. ○, Total [^{35}S]GAG accumulating in the cultures; ●, [^{35}S]GAG associated with the cells and substrate only. Each point is the average of duplicate cultures.

The difference between culture types in [^{35}S]GAG accumulation is not due to the presence of collagen itself in the assayed samples because the GAG assays are unaffected by predigestion of gel-containing samples with collagenase or by the addition of fibrillar collagen to samples from cultures on plastic. Nor does the difference result from inadequate removal of labeled materials associated with the plastic. Extraction of the plastic dish with 4 M guanidinium·HCl for 16 hr after harvesting of the cells resulted in recovery of less than 0.2% of the incorporated label.

Degradation and Loss of [^{35}S]GAG during a Chase. Chase experiments were performed after short- and long-term labeling periods. After 1½ hr of labeling (Fig. 2 A and B), at which time less than 10% of the [^{35}S]GAG was in the medium in both culture conditions, the labeling medium was removed and the cultures were rinsed and chased for 48 hr in label-free medium. Initially, the [^{35}S]GAG in cultures on plastic decreased at about a 10-fold greater rate than in cultures on collagen gels (Fig. 2A), indicating a greater rate of net degradation in cultures on plastic. These rates, however, markedly decreased with continued chase. For cultures on plastic, the initial rate of [^{35}S]GAG loss from cell and substrate (Fig. 2B) was similar to the net degradation rate. However, GAG lost from cell and substrate in collagen cultures was only about one-third as rapid as in cultures on plastic, and this rate slowed with continued chase (Fig. 2B).

When the cultures were chased after 48 hr of labeling (Fig. 2 C and D), the rate of net [^{35}S]GAG degradation in both culture types (Fig. 2C) was similar to that observed after brief labeling. Lengthening the labeling period also did not alter the rate of cell and substrate GAG loss in cultures on plastic, but drastically reduced the loss of this GAG in cultures on collagen (Fig. 2D). The collagen gel, therefore, promotes the accumulation of a component associated with the cells and substrate which is very slowly lost. This component seems to account for
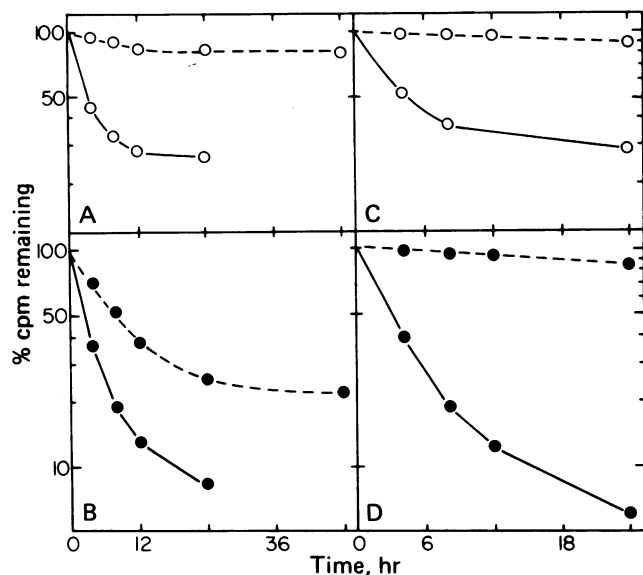

FIG. 2.   Chase of $^{35}SO_4{}^{2-}$ from cetyl pyridinium chloride-precipitable material (GAG) in cultures on collagen (- - -) and plastic (—) substrates. Semilogarithmic plots of the percent of the [^{35}S]GAG remaining against the time of chase. [^{35}S]GAG measured at 0 hr is taken as 100%. (A and B) Chase after 1½ hr of labeling. (C and D) Chase after 48 hr of labeling. ○, Total [^{35}S]GAG in the cultures; ●, cell- and substrate-associated [^{35}S]GAG. Each point is the average of duplicate cultures.

Table 1.   Degradation of exogenous [^{35}S]GAG

Incubations	[^{35}S]GAG remaining	
	cpm	% of control
i. Control	4266	—
ii. Fresh medium	4192	98
iii. Conditioned medium/collagen	4052	95
iv. Conditioned medium/plastic	4066	95
v. Culture/collagen	4262	100
vi. Culture/plastic	4289	101

Equal amounts of [^{35}S]GAG, derived from the medium of cultures on plastic by dialysis at 0°C against precursor and serum-free standard medium, were incubated at 37°C for 7 hr with fresh complete medium (*ii*) or 24-hr conditioned medium from cultures on collagen (*iii*) and plastic (*iv*), and were added in fresh medium to 5-day cultures on collagen (*v*) and plastic (*vi*). Means of duplicate experiments.

the continuing accumulation of cell and substrate GAG in collagen cultures (see Fig. 1C).

Influence of Compartmentalization. The collagen gel represents a separate compartment which is not present in the cultures on plastic. Two different approaches were used to assess whether the presence of this compartment alters the apparent rate of GAG degradation. First, cultures were compared in a configuration that facilitated diffusion of newly synthesized GAG into the medium. Cells were grown directly on Millipore filters or on collagen gels polymerized on Millipore filters, and the filters were floated in the medium. Despite this configuration, net loss of GAG during a chase was unaffected (data not shown). In a second attempt, cells grown on dried collagen gels were compared with cells grown on plastic. The dried gels do not rehydrate and remain as a thin film.* Again, a markedly decreased rate (1/3rd–1/5th) of [^{35}S]GAG loss from the culture and from the cells and substrate were observed in the cultures on collagen (data not shown). These findings indicate that the interstitial compartment in the cultures on collagen gels does not account for the decreased rate of [^{35}S]GAG degradation.

Extracellular Degradation of [^{35}S]GAG. Because GAG is secreted into the medium, the collagen gel and plastic cultures could differ in the extent of extracellular degradation. To evaluate this possibility, we incubated aliquots of [^{35}S]GAG in dialyzed medium from incorporation experiments for 7 hr with conditioned media from the different culture types or directly with the cultures (Table 1). The results show that degradation in the medium is extremely slow and that the exogenous [^{35}S]GAG is not taken up by the cells of either culture type. The virtual lack of GAG degradation in the medium was confirmed by showing that when over 95% of the GAG label was chased from the cells (possible only with cultures on plastic), the total amount of [^{35}S]GAG in the culture remains unchanged for 72 hr. Thus, only cell-associated GAG is degraded in these cultures. The stability of secreted GAG provides an explanation for the deviations from first-order kinetics observed during the chase of total GAG (compare Fig. 2 A and C).

Accumulation of Basal Laminar Material. The chase results indicate that cultures on collagen contain a pool of GAG associated with the cells and substrate that is not detectable in cultures on plastic. Localization of this component was investigated by autoradiography of the cultures (Fig. 3A). Despite the limited resolution of ^{35}S autoradiography, it is clear that there is virtually no label deposited within the gel and that the bulk of label is concentrated over the cells and at the cell–gel interface. Thus, this pool of GAG is localized to the cells.

Ruthenium red staining was performed to assess whether the

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*The volume, measured by isotope dilution, was less than 10 μl.

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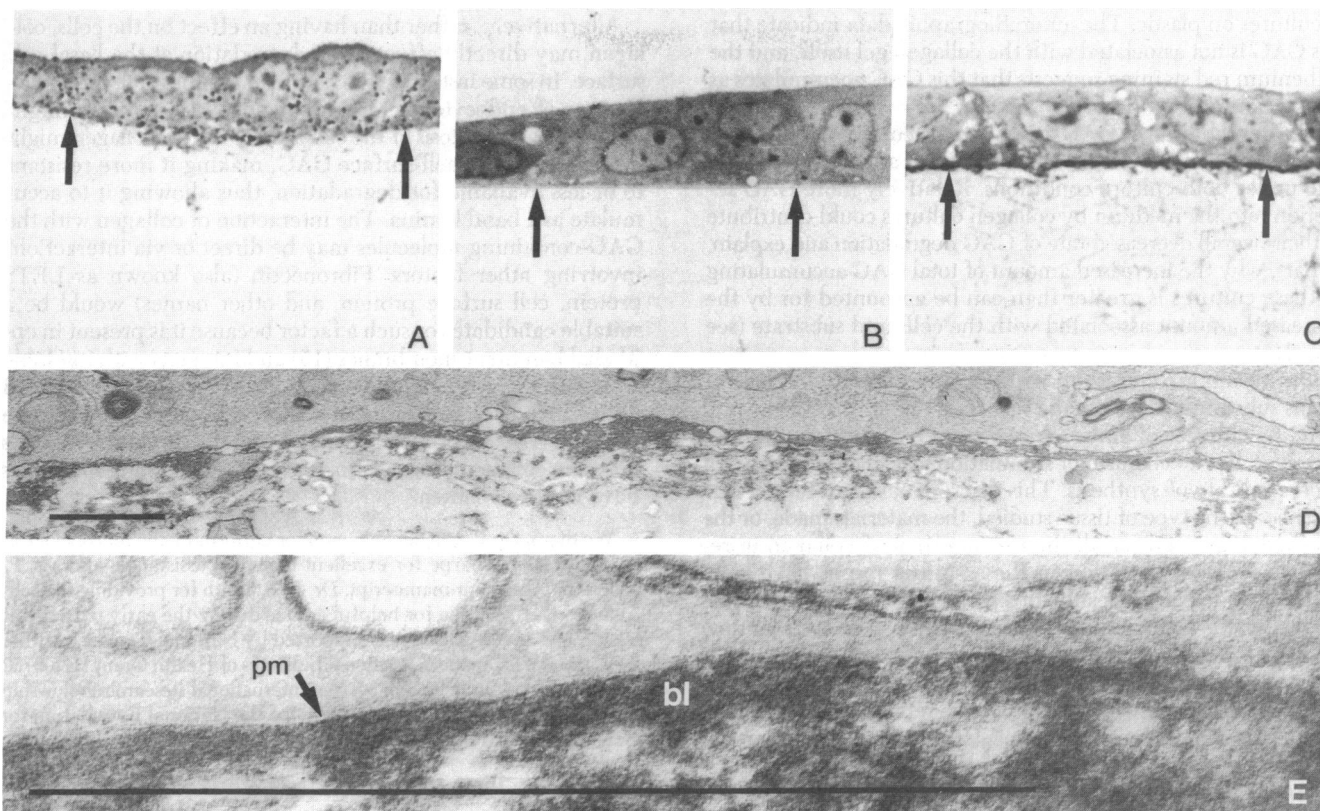*Proc. Natl. Acad. Sci. USA 76 (1979)*   789

FIG. 3. ^{35}S autoradiography and ruthenium red staining of 5-day-old cultures. The cultures were fixed with glutaraldehyde (2.5% in cacodylate buffer, 0.1 M, pH 7.4) and osmium tetroxide (1% in cacodylate buffer) in the presence of ruthenium red according to Luft (12). Tissues were embedded in Epon 8.2 and sectioned perpendicular to the surface of the substrate. (*A*) For autoradiography, 3-day-old cultures of cells on collagen were incubated for an additional 48 hr in the presence of 30 μCi of ^{35}SO$_4^{2-}$ per ml. One-micrometer sections were covered with Ilford L4 emulsion and exposed for 18 days. Label is concentrated over the cells and cell–collagen interface (arrows), whereas there is almost no label over the collagen gel. (×1700.) (*B* and *C*) Basal surface (arrows) of cells grown on a plastic substrate (*B*, ×2300) shows no ruthenium red stain in contrast to cells grown on a collagen gel (*C*, ×1800), where stain is evident. (*D*) Ultrathin section of cells grown on a collagen gel showing ruthenium red-stainable material in a nearly continuous layer at the basal cell surface. Stain is also seen within the gel proximate to the cells. (×15,000; bar = 1 μm.) (*E*) Higher magnification of the basal cell surface of a culture as in *D* showing ruthenium red deposits closely associated with the plasma membrane (pm), resembling the ultrastructural appearance of a basal lamina (bl). (×132,000; bar = 1 μm.)

accumulation of ^{35}SO$_4^{2-}$ near the basal surface of the epithelial sheet was associated with the accumulation of material with characteristics of a GAG-rich epithelial basal lamina. Ruthenium red revealed stainable material at the basal surface of only those cells maintained on collagen gels (compare Fig. 3 *B* and *C*). Electron microscopy of these cultures showed the stained material to be in deposits closely associated with the basal cell surface, approximating the appearance of a GAG-rich epithelial basal lamina (Fig. 3 *D* and *E*).

DISCUSSION

Compared to a number of other substrates, epithelial cells grown on collagen are reported to show increased accumulation of extracellular matrix materials (1, 2). The results presented here demonstrate that, compared to a plastic substrate, the enhanced accumulation of [^{35}S]GAG by mammary epithelial cells cultured on a collagen substrate is due to reduced [^{35}S]GAG degradation. Cultures on collagen contain a slowly degrading cell- and substrate-associated [^{35}S]GAG pool which is not found in cultures on plastic. The cell- and substrate-associated ^{35}SO$_4^{2-}$ is localized to the cells. Label at the cell–gel interface is at the site of an ultrastructurally identified basal lamina, which is also not found in cultures on plastic. Formation of the basal lamina, therefore, may result from a collagen-mediated reduction in the rate of cell-surface GAG degradation.

The similarity of the initial rates of incorporation indicates

that the overall rate of GAG synthesis is unaltered by the presence of the collagen substrate. The linearity of the incorporation reflects a zero-order synthetic rate. The data do not exclude altered rates of synthesis of individual GAG species with the different substrates or qualitative differences in the GAG-containing molecules.

During prolonged labeling, incorporation of ^{35}SO$_4^{2-}$ into cell- and substrate-associated GAG reached a steady-state level for cells on plastic, but cells on collagen continued to accumulate [^{35}S]GAG (see Fig. 1*C*). When synthesis proceeds with zero-order kinetics, the time required to reach a steady-state level of incorporation is a function of only the rate constant of loss or degradation. Thus, the steady-state [^{35}S]GAG level achieved within 24 hr for cells on plastic must reflect a more rapid rate constant of degradation or loss than for cells on collagen.

This expectation was directly confirmed in the chase studies in which the loss of [^{35}S]GAG from the cell and substrate compartment was shown to be substantially greater for cultures on plastic. Comparing the rates at which [^{35}S]GAG is lost after brief labeling with those after prolonged labeling clearly showed the existence of a slowly disappearing pool of cell- and substrate-associated GAG in cultures on collagen. There was no kinetic evidence for a comparable GAG fraction for cells grown on a plastic substrate. Collagen either retards the loss of GAG from the cell and substrate compartment or induces the formation of a slowly degrading GAG species that is not present

in cultures on plastic. The autoradiographic data indicate that this GAG is not associated with the collagen gel itself, and the ruthenium red staining suggests that this GAG accumulates as a basal lamina.

The degradation of GAG appears to be confined to the cells because exogenous GAG was degraded at an extremely slow rate under both culture conditions. Relatively more GAG secretion into the medium by collagen cultures could contribute to their overall decreased rate of GAG degradation and explain, in part, why the increased amount of total GAG accumulating in these cultures is greater than can be accounted for by the increased amount associated with the cells and substrate (see Fig. 1C).

Our results are consistent with the observations that collagenous substrates stimulate the incorporation of precursors into GAG by cultures of embryonic organs (1, 2). In these studies, however, the mechanism of stimulation was suggested to be an increased rate of synthesis. This discrepancy may reflect differences in the type of tissue studied, the materials made, or the methodology used (or all three). An effect of collagen in reducing GAG degradation in these studies might have been difficult to detect, especially for a minor GAG fraction, because the reported rate of loss of tissue-associated [^{35}S]GAG was slow and did not follow first-order kinetics on either collagenous or noncollagenous substrates (1).

The present findings also confirm the reports that collagenous substrates are required for the accumulation of basal laminar material by mammary epithelial cells in primary cultures (5) and by embryonic epithelial organ cultures (2). It should be emphasized, however, that the production of a GAG-rich basal lamina does not always require the presence of exogenous collagen. Embryonic mouse submandibular epithelia, in the absence of any other cell or biological substrate, will synthesize and deposit a virtually complete GAG-rich basal lamina within 2 hr (13). On the other hand, where collagen is required, much longer periods are apparently necessary for the lamina formation (5). This difference implies that a collagen-mediated reduction in the rate of GAG degradation may be necessary for the formation of a basal lamina only where there is low laminar GAG turnover.

In the system studied here, we propose that the reduced degradation of cell-associated GAG by collagen is causally related to extracellular accumulation of GAG into a basal lamina. On plastic substrates, most newly synthesized cell-associated GAG is rapidly degraded. This degradation occurs prior to GAG secretion since extracellular GAG in the medium is quite stable. Collagen substrates may cause newly synthesized GAG to be redistributed, thereby increasing the proportion that is destined for extracellular deposition within the lamina as slowly degrading cell-associated GAG. The collagen substrate may influence the GAG distribution by imposing "polarity" on the cells, by altering the nature of the GAG produced, or by some other mechanism.

Alternatively, rather than having an effect on the cells, collagen may directly affect GAG degradation at the basal cell surface. In some instances, the nature of the substrate influences the rate of endocytosis (14) and, analogously, collagen might reduce the endocytosis of the cell-surface GAG. Collagen might interact with the cell-surface GAG, making it more resistant to or less available for degradation, thus allowing it to accumulate as a basal lamina. The interaction of collagen with the GAG-containing molecules may be direct or via interactions involving other factors. Fibronectin (also known as LETS protein, cell surface protein, and other names) would be a suitable candidate for such a factor because it is present in epithelial basement membranes (15) and interacts with insoluble collagen (16). Although fibronectin has been found associated with GAG (17) and heparin will precipitate cold-insoluble globulin (18), a fibronectin-related protein, direct interactions between collagen, fibronectin, and GAG-containing molecules have not been shown.

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