# Effects of Heparan Sulfate Removal on Attachment and Reattachment of Fibroblasts and Endothelial Cells†

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ABSTRACT: Human skin fibroblasts and calf aorta endothelial cells were grown as tissue culture monolayers in the presence of [$^{35}$S]sulfate in order to label the glycosaminoglycan portions of proteoglycans for investigation of their role in cell attachment. The [$^{35}$S]glycosaminoglycans were then selectively removed from the cell monolayers by the addition of various glycosaminoglycan-degrading enzymes. As previously described, in contrast to trypsin treatment none of these enzymes removed any cells from the culture plates. Incubation with a preparation from *Flavobacterium heparinum* left only small stubs of [$^{35}$S]glycosaminoglycans on the cell monolayers, indicating that all the cell-surface proteoheparan [$^{35}$S]sulfate and proteochondroitin [$^{35}$S]sulfate was accessible to this enzyme preparation. The treatment did not change the amount or time of incubation with trypsin necessary for release of the cells from the monolayers. Thus, cell attachment was not weakened by removal of heparan sulfate or chondroitin sulfate. In contrast, neither fibroblasts nor endothelial cells in suspension would reattach in the presence of the *F. heparinum* preparation while reattachment occurred readily in the presence of chondroitin ABC lyase. This provides evidence that heparan sulfate, but not chondroitin sulfate, is involved in the process of cell attachment even though neither is necessary for maintaining attachment.

Heparan sulfate proteoglycan is a major component of the extracellular matrix of tissues and is a cell-surface component of many if not all cells (Kraemer, 1971; Buonassisi, 1973; Kleinman et al., 1975; Glimelius et al., 1978; Middendorf et al., 1980; Vogel & Kendall, 1980; Kjellen et al., 1981; Simionescu et al., 1981; Shimada et al., 1981; Heifetz & Allen, 1982; Oohira et al., 1983; Rapraeger & Bernfield, 1983). Much of the data concerning production of this substance has developed from work with cells in culture. Moreover, use of cultured cells has indicated a variety of possible functions for this cell-surface proteoglycan. Cell-surface heparan sulfate may be related to the binding of various substances such as platelet factor 4 (Laterra et al., 1983), fibronectin (Yamada et al., 1980; Laterra et al., 1983; Rollins et al., 1983), and lipoprotein lipase (Shimada et al., 1981; Ching et al., 1981). In addition, it may be related to cell permeability (Simionescu et al., 1981) and migration (Kramer et al., 1982). There is evidence that heparan sulfate appears at attachment sites of cells in monolayer culture (Rollins et al., 1979, 1983), is particularly enriched while cells are in the process of attaching (Rollins et al., 1983), and may be involved in fibronectin-mediated cell spreading during the attachment process (Harrison & Culp, 1983). Addition of various glycosaminoglycans to media of cell suspensions has been shown to inhibit attachment of cells, suggesting a competition with cell-surface glycosaminoglycans which in turn implies a role for these substances in attachment (Schubert & LaCorbiere, 1980). Thus, heparan sulfate may be important in the initial attachment of cells to a culture plate, but the evidence for this function has been indirect.

We previously described the use of a preparation from *Flavobacterium heparinum* which removed 85–90% of the sulfated glycosaminoglycans including heparan sulfate from cell layers of cultured human skin fibroblasts (Gill et al., 1981). The cells remained attached following this treatment. In contrast to the removal of [$^{35}$S]sulfate-labeled material, when skin fibroblasts in culture were grown in the presence of [$^{3}$H]leucine, [$^{3}$H]proline, or [$^{3}$H]fucose, there appeared to be no net removal of labeled material by the *F. heparinum* preparation. Thus, the *F. heparinum* preparation did not appear to have significant protease activity nor did it appear to affect glycoprotein. Following the removal of the sulfated glycosaminoglycans by the *F. heparinum* preparation, there was no change in the appearance of the cells in culture nor was there any change in subsequent glycosaminoglycan formation by these cells. This implied that cell-surface heparan sulfate and other glycosaminoglycans might not be necessary in order to maintain attachment of fibroblasts. However, we did not exclude the possibility that attachment could have involved the remaining 10–15% of the surface glycosaminoglycan. We also examined the effects of the *F. heparinum* preparation on monolayers of bovine pulmonary artery endothelial cells (Shimada et al., 1981). These, too, remained attached following the enzymatic removal of 85–90% of the [$^{35}$S]glycosaminoglycans.

We have now repeated the use of the *F. heparinum* preparation for removal of sulfated glycosaminoglycans from human skin fibroblasts and from bovine aorta endothelial cells. We have examined the 10–15% of glycosaminoglycan remaining in both fibroblast and endothelial cell monolayers subsequent to treatment with this preparation. We have also used the *F. heparinum* preparation and fractions enriched by gel filtration together with fibroblast and endothelial cell suspensions to determine whether the removal of heparan sulfate would have an effect on subsequent reattachment. In contrast to the results with cells that were already attached,

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removal of heparan sulfate from suspended cells coincided with the elimination of cell reattachment.

## Experimental Procedures

Sodium [$^{35}$S]sulfate (0.8 Ci/mmol) and [*methyl*-1,2-$^{3}$H]-thymidine (102 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Minimum essential medium (MEM), RPMI 1640, trypsin–ethylenediaminetetraacetic acid (EDTA), L-glutamine, and gentamycin were purchased from Irvine Scientific, Santa Ana, CA. Fetal calf serum (Hyclone) was purchased from Sterile Systems, Logan, UT. Chondroitin ABC lyase, chondroitin AC lyase, chondroitin 4-sulfate, and heparitinase (heparanase) were obtained from Miles Laboratories, Naperville, IL. Trypsin was purchased from Worthington Biochemical Corp., Freehold, NJ. Heparin was purchased from Calbiochem-Behring, La Jolla, CA. Sepharose CL-6B, Sephadex G-25, and Sephadex G-50 were purchased from Pharmacia, Piscataway, NJ. The *Flavobacterium heparinum* preparation was obtained as previously described (Gill et al., 1981).

Human skin fibroblasts were obtained from our liquid nitrogen storage bank where cell suspensions from early-passage monolayers were kept frozen in complete medium containing 10% dimethyl sulfoxide. These cells had been initially obtained from skin biopsies of normal 20–30-year-old males and had the characteristic appearance of cultured fibroblasts. Bovine aorta endothelial cells were also obtained from our liquid nitrogen bank, where they were similarly stored in suspension at early cell passages. Cells initially had been derived from calf aorta obtained from the slaughterhouse. Endothelial cell cultures had been initiated by standard techniques after collagenase treatment of the aortas (D'Amore & Shepro, 1977). These cells had the characteristic appearance of endothelial cells in culture, were contact-inhibited in monolayer culture, and were positive for factor VIII antigen (Macarak et al., 1977) and negative for mycoplasma (Bioassay Systems, Woburn, MA).

*Cell Culture and Reattachment.* Culture procedures were, in general, similar to those we had used previously (Gill et al., 1981; Shimada et al., 1981). After removal of cell suspensions from liquid nitrogen storage, the fibroblasts were seeded into 25 cm$^2$ flasks (Corning, Corning, NY) containing 5 mL of minimal essential medium (MEM), 10% fetal calf serum, 2 mM glutamine, and 50 $\mu$g/mL gentamycin and incubated at 37 °C in 5% $CO_2$–humidified air. Confluent cell monolayers were lifted with 0.1% trypsin and passed 1–2 times before use. Endothelial cells were plated in the same fashion except that RPMI 1640 media containing 18% fetal calf serum was used. The endothelial cell monolayers were lifted with 0.05% trypsin–0.5 mM EDTA and passed 1–2 times before use.

For experimental studies, both the fibroblasts and endothelial cells were plated at a density of (3–5) $\times$ 10$^5$ cells in 5 mL of their respective complete medium as described above. After 2–4 days, the incubation medium for the fibroblasts was replaced with 5 mL of sulfate-free MEM, 2 mM glutamine, and 10% fetal calf serum, but without gentamycin. [$^{35}$S]Sulfate (125 $\mu$Ci) and $Na_2SO_4$ were added to a final concentration of 0.4 mM, and cell monolayers were incubated for 24 h at 37 °C. Labeling with [$^{3}$H]thymidine was achieved by incubating monolayers for 72 h in 2 $\times$ 10$^{-5}$ mM [$^{3}$H]-thymidine (10 $\mu$Ci). Endothelial cells were labeled in the same fashion and with the same medium used for fibroblasts, involving a change from the RPMI 1640 medium and a change from 18% to 10% fetal calf serum.

For cell reattachment studies, the medium was removed from near-confluent monolayers of [$^{35}$S]sulfate- and [$^{3}$H]-thymidine-labeled cells. The cells were then rinsed with phosphate-buffered saline (PBS: 138 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, and 1.47 mM $KH_2PO_4$) that was $CaCl_2$ free and $MgSO_4$ free and lifted with 0.5 mM ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Culp, 1974). The suspended cells were centrifuged and washed in the PBS buffer. Aliquots of 10$^5$ cells were added to each well of a six-well cluster dish (Costar, Cambridge, MA) containing 1 mL of PBS with 0.68 mM $CaCl_2$ and 0.4 mM $MgSO_4$ or 1 mL of this buffer plus glycosaminoglycan-degrading enzymes. On occasion, 0.1% bovine serum albumin was added to the cluster dishes. Attachment of cells was monitored by [$^{3}$H]thymidine, and glycosaminoglycan lyase activity was monitored by release of [$^{35}$S]sulfate-labeled products. The cluster dishes were also visually monitored for cell attachment and cell spreading.

*Use of Glycosaminoglycan-Degrading Enzymes.* These procedures were similar to those we had used previously (Gill et al., 1981). $^{35}$S-Labeled cell monolayers were washed repeatedly with iced PBS containing $CaCl_2$ and $MgSO_4$ to remove free [$^{35}$S]sulfate. Glycosaminoglycan-degrading enzymes in 1.0 mL of this PBS were added to the cell layers, which were then generally incubated for 40 min at 37 °C. Following this, the enzyme solutions, containing some degraded [$^{35}$S]-glycosaminoglycan products and some residual [$^{35}$S]sulfate, were removed and the cell monolayers rinsed several times with the iced PBS. The cell layers were generally incubated with 0.25% trypsin for 15 min at 37 °C in order to obtain the remaining cell-layer [$^{35}$S]glycosaminoglycans or [$^{35}$S]glycosaminoglycan stubs. The flasks were then treated with 0.5 mL of 0.5 M NaOH to release any remaining radioactivity, which was never more than a few percent of the total. The cells, which were quantitatively released during the trypsin treatment, were collected by centrifugation and analyzed for DNA (Kapscinski & Shoezybes, 1977) or [$^{3}$H]thymidine. The trypsinate was saved for analysis of the $^{35}$S-labeled material. Glycosaminoglycan-degrading enzymes were used on cell suspensions in the same fashion as they were used on monolayers.

Enzyme activity was measured by the appearance of $A_{232}$ after incubation with appropriate substrates. Units of activity were expressed as micromoles of unsaturated double bond formed from substrates per hour. The *F. heparinum* preparation had varying activities depending upon the glycosaminoglycan substrate. Relative to its activity on heparin, its activity on heparan sulfate was 50%, chondroitin 6-sulfate 36%, chondroitin 4-sulfate 16%, dermatan sulfate 14%, and hyaluronic acid 9%. A preparation containing 1 unit of heparin-degrading activity had a protease activity measured with L-$N^{\alpha}$-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) (Erlanger et al., 1966) equivalent to a trypsin solution of less than 0.00002%. A 2-h incubation with this same *F. heparinum* preparation had no discernible effect on [$^{14}$C]fibronectin (produced by culture of the bovine endothelial cells with [$^{14}$C]proline) as determined by sodium dodecyl sulfate (SDS) gel electrophoresis.

The *F. heparinum* preparation was also chromatographed on a 1 $\times$ 70 cm column of Sepharose CL-6B at 4 °C. Fractions were analyzed for the presence of glycosaminoglycan lyase activity, the ability to remove [$^{35}$S]sulfate from intact cells, and the inhibition of cell attachment (measured by [$^{3}$H]thymidine attachment to a culture well).

*Characterization of $^{35}$S-Labeled Material.* Trypsinates from untreated and enzyme-treated monolayers were incubated overnight at room temperature in 0.5 M NaOH and 1 M

Table I: Production of Proteoglycans

| | [$^{35}$S]sulfate incorporated | | % of total glycosaminoglycan[a] | | |
|---|---|---|---|---|---|
| | cpm/$10^6$ cells | nmol/$10^6$ cells | DS | CS | HS |
| fibroblasts | | | | | |
| media | $4.1 \times 10^5$ | 4.3 | 28 | 61 | 11 |
| cell layer | $0.7 \times 10^5$ | 0.7 | 8 | 46 | 46 |
| endothelial cells | | | | | |
| media | $4.8 \times 10^5$ | 5.0 | 0 | 74 | 26 |
| cell layer | $2.0 \times 10^5$ | 2.1 | 0 | 35 | 65 |

[a] Abbreviations: DS, dermatan sulfate; CS, chondroitin sulfate; HS, heparan sulfate.

$NaBH_4$, neutralized with HCl, and chromatographed on Sepharose CL-6B columns using PBS containing $CaCl_2$ and $MgSO_4$. Fractions containing $^{35}$S-labeled material were pooled, concentrated, and dialyzed. Samples were characterized by incubation with glycosaminoglycan-degrading enzymes as previously described (Gill et al., 1981) followed by chromatography on a column of either Sephadex G-50 or Sephadex G-25 to determine susceptibility to the enzymes. Radioactivity was measured in a liquid scintillation spectrometer with Aquassure (New England Nuclear, Boston, MA) as the "scintillation cocktail".

## Results

Production of [$^{35}$S]proteoglycans by monolayers of human skin fibroblasts and bovine aorta endothelial cells during a 24-h incubation with [$^{35}$S]sulfate is shown in Table I. Total cell-layer [$^{35}$S]proteoglycan was measured as [$^{35}$S]sulfate in trypsin-releasable [$^{35}$S]glycosaminoglycan. The media from the endothelial cell cultures contained approximately the same amount of proteoglycan as the media from fibroblasts. However, dermatan sulfate was not detected in the media from the endothelial cells, and the amount of heparan sulfate was greater relative to chondroitin sulfate. The endothelial cells had 3 times as much cell-layer proteoglycan as the fibroblasts, and heparan sulfate was an even larger portion of the total (65%).

We have previously shown that the *F. heparinum* preparation removed 85–90% of human skin fibroblast monolayer $^{35}$S-labeled material while trypsin removed most of the remainder (Gill et al., 1981). The 1–3% left behind after trypsin treatment (removable with 0.5 M NaOH) yielded a mixture of $^{35}$S-oligosaccharides. Since cells remained attached following this initial treatment, we questioned whether this might be due to a small amount of intact [$^{35}$S]glycosaminoglycan remaining as part of cell-surface proteoglycan in an area of attachment protected from the enzyme. Alternatively, if the remaining proteoglycan contained only small stubs of [$^{35}$S]-glycosaminoglycan, involvement of heparan sulfate in attachment would be less likely, since this would indicate that no proteoglycan remained completely intact. Furthermore, this would indicate that the enzyme could reach all proteoglycans including those that might be near cell attachment sites. In order to examine this, we used trypsin to remove the $^{35}$S-labeled material that remained after prior treatment of monolayers with either chondroitin ABC lyase or the *F. heparinum* preparation. Following the trypsin treatment, the $^{35}$S-labeled material was incubated overnight in 0.5 M NaOH and 1 M NaOH and 1 M $NaBH_4$. Aliquots were then chromatographed on Sepharose CL-6B in order to demonstrate the size of the remaining $^{35}$S-labeled material. Results are shown in Figure 1.

The [$^{35}$S]glycosaminoglycan removed by trypsin from PBS-treated (control) fibroblasts or endothelial cells (Figure 1A) was heterogeneous in size, with a peak at a $K_{av}$ of 0.3. There was also a trace of material near the $V_t$. The [$^{35}$S]-glycosaminoglycan from the endothelial cells had an additional component with a $K_{av}$ of approximately 0.6–0.9. Use of chondroitin ABC lyase alone (Figure 1B) left a large peak of heparan [$^{35}$S]sulfate plus a lesser amount of smaller material. In all cases, the $^{35}$S-labeled material appearing near the $V_t$ (fractions 49–52) of the Sepharose CL-6B column also appeared near the $V_t$ upon chromatography with Sephadex G-25. These fractions were not examined further. Following *F. heparinum* treatment of fibroblast monolayers and endothelial cell monolayers (Figure 1C), only 10–15% of the original $^{35}$S-labeled material remained to be removed by trypsin. With both cell types, this material was much smaller in size than the [$^{35}$S]glycosaminoglycan found in the trypsinate of control cells, appearing with a $K_{av}$ of approximately 0.8–0.9 (fractions 40–48) and near the $V_t$. Fractions 40–48 from Figure 1C subsequently appeared in the void volume when chromatographed on a column of Sephadex G-25. Reincubation with the *F. heparinum* preparation resulted in degradation, indicating that this material represented small stubs of [$^{35}$S]-glycosaminoglycans or $^{35}$S-oligosaccharides that had not been degraded completely by the *F. heparinum* preparation while the cells were in the monolayer. When purified heparanase (from Miles and from F. Linker, Salt Lake City, UT) was used in conjunction with chondroitin ABC lyase (not shown), removal of $^{35}$S-labeled material was not as extensive. The remaining stubs were considerably larger than those remaining after use of the *F. heparinum* preparation, indicating that the purified heparanase lacked some heparan sulfate degrading enzymes contained in the *F. heparinum* preparation.

The [$^{35}$S]glycosaminoglycans isolated after trypsinization of untreated cells were identified by susceptibility to chondroitin ABC lyase and chondroitin AC lyase (Table I). This was compared with the susceptibilities of the [$^{35}$S]glycosaminoglycans to glycosaminoglycan-degrading enzymes while on the cell monolayers (Figure 1). The comparison indicated

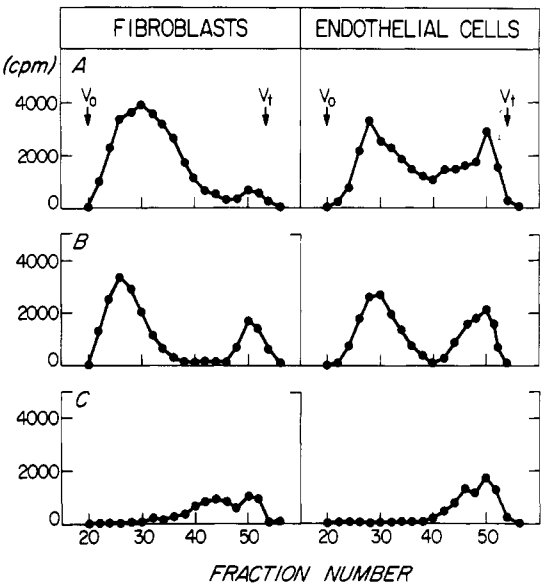


FIGURE 1: Sepharose CL-6B chromatograms of [$^{35}$S]glycosaminoglycan remaining after treatment of cell layers by glycosaminoglycan-degrading enzymes. Trypsinates from [$^{35}$S]sulfate-labeled fibroblasts and endothelial cells were chromatographed on Sepharose CL-6B (1 × 70 cm) with PBS as eluant. Fractions of 1 mL were collected. Prior to treatment with trypsin, the cell monolayers had been incubated for 40 min with (A) PBS, (B) 15 units of chondroitin ABC lyase, or (C) 1.6 units of *F. heparinum* preparation. Units of activity are expressed as micromoles of substrate degraded per hour.

Table II: Release of Cells by Use of Varying Amounts of Trypsin after Pretreatment with the *F. heparinum* Preparation[a]

| trypsin concn (%) | trypsin incubn time (min) needed for detachment of: fibroblasts pretreated with PBS | fibroblasts pretreated with *F. heparinum* | endothelial cells pretreated with PBS | endothelial cells pretreated with *F. heparinum* |
|---|---|---|---|---|
| 0.125 | 8 | 8 | 5 | 8 |
| 0.063 | 10 | 10 | 8 | 10 |
| 0.031 | 13 | 14 | 12 | 11 |
| 0.013 | 26 | 25 | 25 | 30 |
| 0.006 | 53 | 54 | 41 | 40 |
| 0.003 | nd[b] | nd[b] | nd[b] | nd[b] |

[a] PBS or 1 unit of the *F. heparinum* preparation was added to washed cell monolayers, and incubation at 37 °C was continued for 40 min. The *F. heparinum* preparation was removed, and varying concentrations of trypsin were added. Time for cell detachment was then monitored for 1 h. [b] nd, no detachment.

that essentially all of the cell-layer [$^{35}$S]glycosaminoglycans were at least partially accessible to the enzymes.

It was clear from our previous work that removal of heparan sulfate did not dislodge cells from monolayers. However, we considered the possibility that prior treatment with *F. heparinum* enzymes might loosen the attachment so that less trypsin would subsequently be required for lifting these cells. Therefore, we compared control (PBS) cells with the *F. heparinum* preparation treated cells to observe the time interval for complete lifting using varying amounts of trypsin. Results are shown in Table II and indicated that prior removal of the glycosaminoglycans by pretreatment with the *F. heparinum* preparation had no effect on loosening the attachment of either fibroblasts or endothelial cells. In all cases, 0.006% trypsin was necessary to observe any detachment. Furthermore, cells readily attached in 0.004% trypsin. Prior incubation with the *F. heparinum* preparation also had no effect on the relative ease of lifting cells with EGTA (not shown).

We then considered the possibility that removal of glycosaminoglycans from suspended cells might affect their capacity to reattach, even though this removal did not affect attachment once it had taken place. Therefore, endothelial cells that had been prelabeled with [$^{3}$H]thymidine and [$^{35}$S]sulfate were lifted and suspended by EGTA (or by trypsin treatment) and examined for their reattachment in the presence of glycosaminoglycan-degrading enzymes. In addition to the use of the *F. heparinum* preparation, we utilized fractions after chromatography of *F. heparinum* on a Sepharose CL-6B column and correlated the activity of attachment measured by [$^{3}$H]thymidine-labeled cells with the degradation of standard heparan sulfate and the release of [$^{35}$S]sulfate-labeled products. These results are shown in Figure 2. Heparan sulfate degrading activity was found in fractions 38–46 with a maximum at fraction 42. The protein content of these fractions was estimated by $A_{280}$ as approximately 10% of the total protein of the crude sonicate. There was also some particle-bound heparan sulfate degrading activity in fractions 22 and 23. This activity could only be elicited by repeated shaking of the suspended particles with the substrate. The fractions that contained the heparan sulfate degrading activity contained the [$^{35}$S]sulfate-releasing activity and were the only fractions that inhibited cell attachment as measured by the percent attachment of [$^{3}$H]thymidine-labeled cells. Furthermore, since 1 unit of the *F. heparinum* preparation had a protease activity (measured by BAPNA) less than that of 0.00002% trypsin, and attachment of our cells could take place in 0.004% trypsin, this indicated that the small amount of protease activity of the *F. heparinum* preparation was at least 2 orders of magnitude less than would be expected to have any effect on attachment. The purified heparanase (plus chondroitin ABC lyase), which was not as effective in removal of [$^{35}$S]glycosaminoglycan as the *F. heparinum* preparation, did not prevent attachment. The presence of 0.1% bovine serum albumin did not counteract the effects of the *F. heparinum* preparation in prevention of attachment.

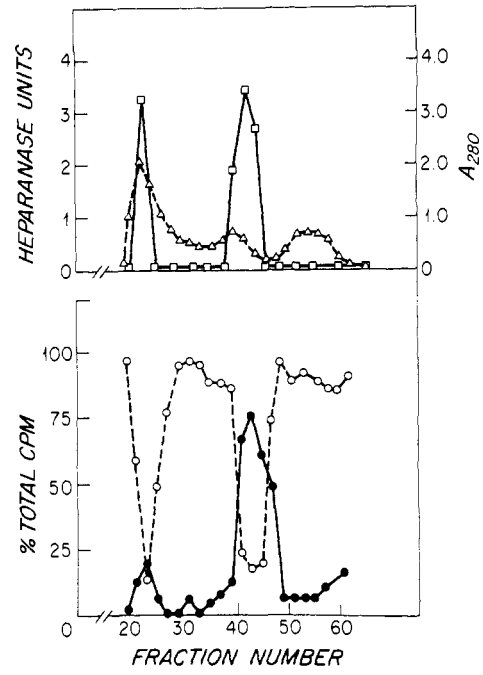


FIGURE 2: Activities of *F. heparinum* preparation fractions after chromatography on Sepharose CL-6B. *F. Heparinum* preparation (40 units) was chromatographed on a 1 × 70 cm column of Sepharose CL-6B at 4 °C. PBS containing $MgSO_4$ and $CaCl_2$, pH 7.0, was used as eluant, and fractions of 0.9 mL were obtained. The upper panel shows heparanase units (□) and protein content (△) for each fraction analyzed. The lower panel shows net release of [$^{35}$S]sulfate (●) and attachment of [$^{3}$H]thymidine-labeled cells (○) after 0.4-mL aliquots were incubated with $10^5$ suspended [$^{35}$S]sulfate- or [$^{3}$H]thymidine-labeled cells.

Reattachment and subsequent spreading were also monitored by a microscope as shown in Figure 3, which provided a means of examining degrees of attachment. Results of these experiments are shown in Figure 4. The presence of small amounts of the *F. heparinum* preparation effectively prevented reattachment, while large amounts of chondroitin ABC lyase had no effect. Thus, the removal of chondroitin sulfate, dermatan sulfate, and hyaluronic acid by chondroitin ABC lyase had no effect on cell reattachment, while removal of all but short stubs of heparan sulfate by *F. heparinum* enzymes coincided with the elimination of reattachment. When the *F. heparinum* preparation was removed from the suspended cells by centrifugation and washing with PBS, effective reattachment resumed.

The amount of the *F. heparinum* preparation necessary to eliminate reattachment of suspended cells was also compared with the amount needed to remove [$^{35}$S]glycosaminoglycans from monolayers or suspended cells. Results (Figure 5) indicated a good correlation. Thus, the amount of preparation required for maximal removal of [$^{35}$S]glycosaminoglycan from cells was the amount necessary to abolish reattachment of suspended cells, while lesser amounts had partial effects. These results were consistent with those of Figure 2.

## Discussion

When cultures of skin fibroblasts or endothelial cells are grown in the presence of [$^{35}$S]sulfate, $^{35}$S-labeled proteoglycans

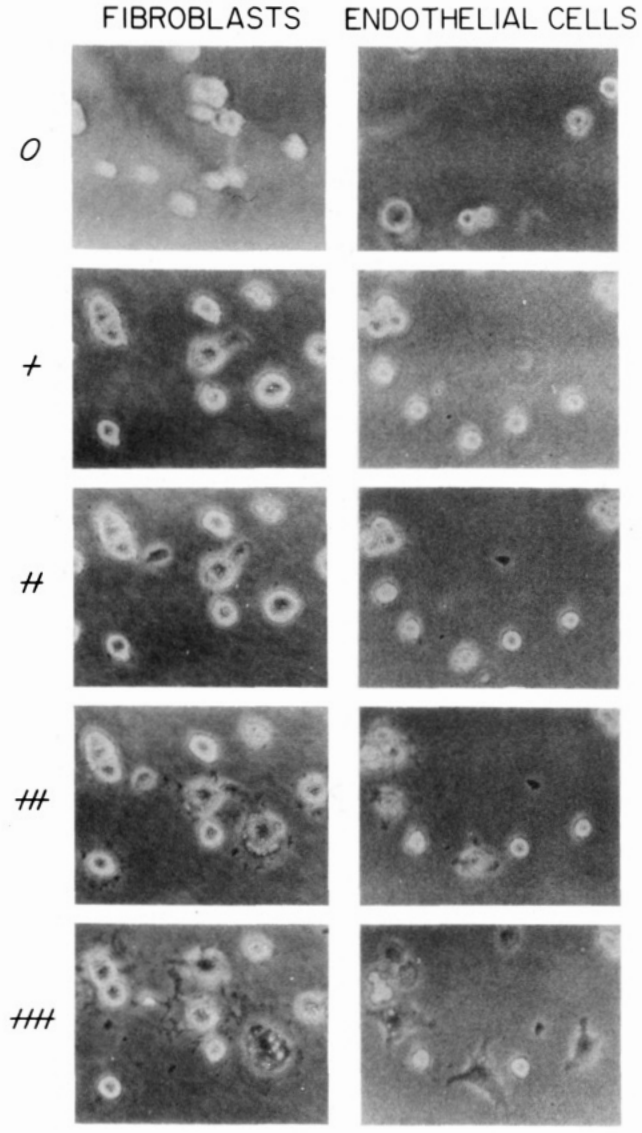


FIGURE 3: Visual assessment of reattachment. (0) All cells floating in media; (+) most cells attached but vibrate upon motion of media; (++) all cells attached, no vibration; (+++) some cells spreading; (++++) most cells spreading.

are found both in the cell media and in the monolayers. Furthermore, the [$^{35}$S]proteoglycans account for essentially all of the cell-layer $^{35}$S and essentially all the $^{35}$S-labeled macromolecules that subsequently appear in the culture medium. The intracellular pool of free [$^{35}$S]sulfate is negligible. Thus, any removal of $^{35}$S from well-washed cell layers corresponds to the removal of $^{35}$S-labeled glycosaminoglycan or $^{35}$S-labeled proteoglycan.

Culture medium from the bovine endothelial cells was found to contain approximately the same amount of [$^{35}$S]proteoglycan as culture medium from fibroblasts (Table I). When washed monolayers of human skin fibroblasts or bovine endothelial cells were treated with sufficient trypsin, cells were quantitatively lifted from the plates, accompanied by solubilization of approximately 90–95% of the cell-layer $^{35}$S. All this material could be recovered as [$^{35}$S]glycosaminoglycan. When the cell-layer-derived [$^{35}$S]glycosaminoglycan was examined, endothelial cells were found to produce more than the fibroblasts, and the proportion of heparan sulfate was greater. These findings are consistant with earlier findings from our laboratory (Shimada et al., 1981; Gill et al., 1981).

Incubation of the *F. heparinum* preparation together with the $^{35}$S-labeled cell layer resulted in a release of almost as much

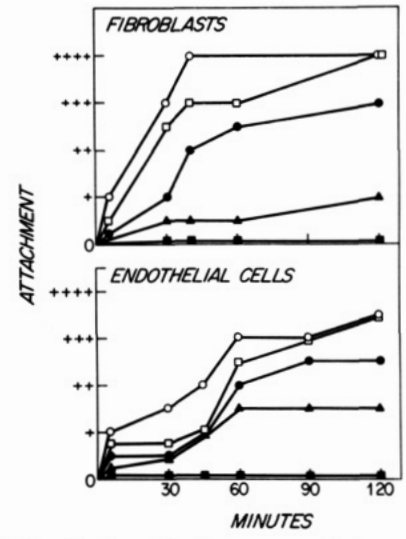


FIGURE 4: Cell reattachment in the presence of glycosaminoglycan-degrading enzymes. Suspended cells ($10^5$) were visually assessed for reattachment in the presence of PBS (○), 15 units of chondroitin ABC lyase (□), or 0.25 (●), 0.45 (▲), and 0.9 units (■) of *F. heparinum* preparation. Units of activity are expressed as micromoles of substrate degraded per hour.

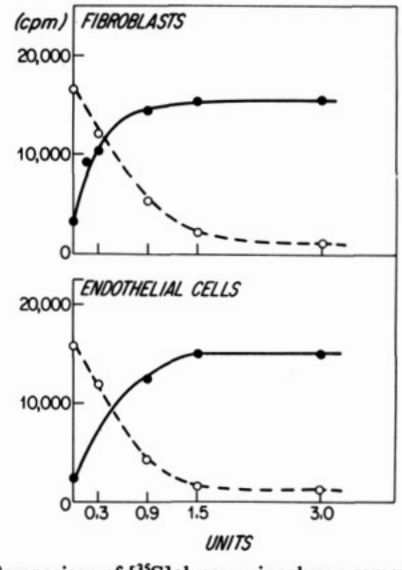


FIGURE 5: Comparison of [$^{35}$S]glycosaminoglycan removal to $^{3}$H-cell reattachment. Varying amounts of *F. heparinum* preparation were used to determine the release of $^{35}$S from monolayers (●) and to measure the reattachment of suspended $^{3}$H-labeled cells (○).

$^{35}$S-labeled material as could be released by trypsin, while use of chondroitin ABC lyase was less effective. As previously described, the use of the *F. heparinum* preparation had no visible effect on either fibroblasts or endothelial cell monolayers. Only a small amount of $^{35}$S-labeled material remained after incubation of cell monolayers with the *F. heparinum* preparation. Furthermore, the size of this material indicated that considerable degradation had occurred on all of the surface proteoglycan and that the remainder represented small oligosaccharide stubs of the original (Figure 1). This absence of any intact glycosaminoglycan demonstrated that all the cell-layer glycosaminoglycan or proteoglycan had been accessible for degradation. Similarly, use of chondroitin ABC lyase left no intact chondroitin sulfate. Thus, cells remained attached even when there were no intact glycosaminoglycan chains.

The possibility remained that the small stubs might be involved in maintaining attachment. Should this be the case,

one might expect that attachment would be weakened following the use of the *F. heparinum* preparation. However, release of cells by trypsin or EGTA was not facilitated by this pretreatment of cell monolayers (Table II), confirming that heparan sulfate probably had no role in maintenance of attachment.

In contrast to the apparent lack of involvement in maintenance of attachment, heparan sulfate did appear to be involved in reattachment of suspended cells (Figures 2–4), while chondroitin sulfate was not involved. Thus, the small stubs of heparan sulfate remaining after complete *F. heparinum* treatment of cells were not effective in promoting reattachment. However, partial removal of heparan sulfate by small amounts of the *F. heparinum* preparation was found to correlate well with partial cell attachment (Figure 5).

We have now provided evidence that heparan sulfate is involved in the process of cell attachment, even though this material is not necessary to maintain attachment once it has taken place. Thus, the observations that heparan sulfate is enriched at attachment sites (Rollins et al., 1983) can be explained by the function of this molecule in promotion of the original attachment. However, it is still not clear whether the function of heparan sulfate is to interact directly with attachment sites or whether it is to interact with some intermediate that in turn is involved with the attachment sites. The latter might be indicated, since substances such as platelet factor 4 and fibronectin have already been shown to be potential mediators of attachment with heparan sulfate (Laterra et al., 1983; Lark et al., 1984). Moreover, we have described preliminary results indicating that fibroblasts will reattach in the presence of the *F. heparinum* preparation if fibronectin is present on attachment surfaces (Silbert et al., 1981). This suggests that the role of heparan sulfate in attachment might be to maintain fibronectin in position on the cell surface.

**Registry No.** Heparan sulfate, 9050-30-0; dermatan sulfate, 24967-94-0; chondroitin sulfate, 9007-28-7.

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