Modulation of Sulfated Proteoglycan Synthesis by Bovine Aortic Endothelial Cells during Migration

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Abstract. The rates of $^{35}$S-sulfate incorporation into proteoglycan were compared in multi-scratch wounded and confluent cultures of bovine aortic endothelial cells to determine whether proteoglycan synthesis is altered as cells are stimulated to migrate and proliferate. Incorporation was found to be stimulated in a time-dependent manner, reaching maximal levels 44–50 h after wounding, as cells migrated into wounded areas of the culture dish. Quantitative autoradiography of $^{35}$S-sulfate-labeled single-scratch wounded cultures demonstrated a 2–4-fold increase in the number of silver grains over migrating cells near the wound edge when compared to cells remote from the wound. Furthermore, when cell proliferation was blocked by inhibition of DNA synthesis, the increase in $^{35}$S-sulfate incorporation into proteoglycan after wounding was unaffected. These data indicate that cell division is not required for the modulation of proteoglycan synthesis to occur after wounding. Characterization of the newly synthesized proteoglycan by ion-exchange and molecular sieve chromatography demonstrated that heparan sulfate proteoglycan constitutes ~80% of the labeled proteoglycan in postconfluent cultures, while after wounding, chondroitin sulfate proteoglycan and/or dermatan sulfate proteoglycan (CS/DSPG) increases to as much as 60% of the total labeled proteoglycan. These results suggest that CS/DSPG synthesis is stimulated concomitant with the stimulation of endothelial cell migration after wounding.

Recent studies have indicated that the metabolism of proteoglycan and collagen, two common components of the extracellular matrix, may be modulated during the growth and migration of endothelial cells (ECs). For example, Madri and Stenn (24) have shown that qualitative changes in the distribution of collagens and laminin occur during EC migration in vitro, and have suggested that continual secretion of collagen is required for migration. ECs in culture have also been shown to undergo a secondary pattern of growth in which typical, highly contact-inhibited monolayers of polygonal cells give rise to fibroblastoid cells that undergo the monolayer, in a process that is thought to require renewed growth and/or migration (9, 41). Such atypical, sprouting cultures have been shown to synthesize type I collagen (9), whereas nonsprouting monolayers are known to synthesize only types III (38), IV, and/or type V (17, 23) collagen. Oohira and his co-workers (32) have described the apparent loss of high molecular weight heparan sulfate proteoglycan (HSPG) in cultures of sprouting EC. Similarly, histochemical evidence suggests that HSPG may be depleted at the tips of growing capillaries (3).

Our interest in the control of EC migration and proliferation stems in part from recent observations concerning the role of endothelial regeneration in effecting arterial intimal repair, a process that may be of key importance in the development of vascular disease (37). To study the process of intimal repair and its relationship to atherogenesis in vivo, several investigators have used, as an injury model, balloon catheterization of the aorta (5, 13, 14, 26, 29). Biochemical and morphological studies (35, 47) have reported that proteoglycan-rich connective tissue accumulates in re-endothelialized regions of balloon-catheterized rabbit aorta in which EC migration has occurred, but not in uninjured areas or regions of the vessel that remain de-endothelialized. Furthermore, lipids and lipoprotein, which are known to bind proteoglycans (18, 42), accumulate within the re-endothelialized aortic intima when these animals are placed on a lipid-rich diet, while such accumulation is minimal within neointima that remains de-endothelialized (13, 26, 28). These findings emphasize the importance of proteoglycan in the atherosclerotic process and raise the interesting possibility that ECs may either contribute to or modify the proteoglycan composition of the arterial wall as they regenerate (i.e., proliferate and migrate) after injury.

In this study, we compare the synthesis of proteoglycan in confluent bovine aortic EC cultures with synthesis in similar cultures that have been stimulated to migrate and proliferate in response to multi-scratch wounding. We present evidence that proteoglycan synthesis increases as cells in wounded cultures are induced to migrate. Furthermore, the reported overall increase in proteoglycan synthesis after wounding appears to be associated with an increased accumulation of chondroitin sulfate and/or dermatan sulfate proteoglycans (CS/DSPG).
Materials and Methods

Materials

Guandaine HCl (GuHCl Grade 1), Tris base, cetyl pyridinium chloride (CPC), EDTA, heparin (Grade I), chondroitin sulfate (Grade II), sodium butyrate, and hydroxyurea were all purchased from Sigma Chemical Co., St. Louis, MO; 6-aminohepxonic acid, benzamide HCl, N-ethylmaleimide, phenylmethylsulfoxonyl fluoride, and all photographic chemicals were from Eastman Kodak Co., Rochester, NY; chondroitin ABC/lyase was from Seikagaku Kogyo Co., Ltd., Tokyo, Japan through Miles Laboratories, Elkhart, IN; collagenase (CLS II) was from Worthington Biochemical Corp., Freehold, NJ; Sephadex, Sepharose and DEAE-Sephacel were from Pharmacia Fine Chemicals, Inc., Piscataway, NJ; Na3[35]SO4 (106 mCi/mmol), [3H]-glucosamine (31 Ci/mmol), (methyl-3H)hydroxymine (20 Ci/mmol), and Triton X-100 were from New England Nuclear, Boston, MA; [3H-5,5'-3H]leucine (55 Ci/mmol) was from the Amersham Corp., Arlington Heights, IL; Grade 3MM filter paper was from Whatman, Ltd., Great Britain, and aphidicolin was the gift of Dr. Philip Liu, University of Washington. All other chemicals were reagent grade.

Cell Culture

Bovine aortic ECs were prepared by collagenase digestion (12, 41) from cow thoracic aorta segments obtained from a local slaughterhouse. Cultures of EC's grown at 37°C in a 5% CO2 humidified atmosphere in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, 1 mM pyruvate, and 1× stock non-essential amino acids (Gibco) and 10% pretested, heat-inactivated fetal bovine serum (Gibco) formed monolayers of small, flattened polygonal cells. Cultures were passaged using 0.25% trypsin-EDTA (Gibco) in calcium- and magnesium-free phosphate-buffered saline (PBS) at a 1:5 split ratio and fed fresh medium weekly.

Cultures of ECs to be used in experiments were prepared by plating early (third to tenth) passage cells at a 1:2 split ratio into 35-mm tissue culture dishes (Falcon Plastics, Oxnard, CA), 2-3 d after such cultures became confluent (6-8 d in culture), the cell layers were multi-scratch wounded (40) with a rake made from nylon bristles, 0.5 mm in diameter.

In some experiments, inhibitors of DNA synthesis were used to block cell proliferation while allowing continued cell migration during the time course of the experiment. In preliminary experiments, 7 mM sodium butyrate, 250 μg/ml hydroxyurea, or 2 μm aphidicolin were found to block EC proliferation (see Results), and were added to cultures in those concentrations from 100× stock solutions in serum-free medium (sodium butyrate and hydroxyurea) or 400× stock solutions in dimethyl sulfoxide (aphidicolin) 16-24 h before labeling. Cultures were maintained in the presence of the inhibitors until they were labeled to assess proteoglycan synthesis, at which time fresh medium was added that contained no inhibitors for the 4-h duration of the labeling period.

To establish the proportion of cells in wounded and confluent cultures that were actively proliferating, cultures were labeled for 24 h with 0.2 μCi/ml [3H]TdR for autoradiography (see below) or for 4 h with 1 μCi/ml [3H]TdR to assay label entry into intracellular precursor pool 35S-sulfate specific activities between 0.5% and 10%.

 Autoradiography

Autoradiography was used to: (a) establish [3H]TdR labeling indices at various times after multi-scratch wounding, and (b) localize regional differences in [3S]-sulfate incorporation into proteoglycan among cells in single-scratch wounded cultures. To prepare [3H]TdR labeled cultures for autoradiography, cultures fixed in 10% formalin were washed 5-6 times with 15 min with 70% ethanol to remove the fixation of the incorporated label. Cultures were coated with NT2B emulsion (Kodak Specialized Research Products, Rochester, NY), dried at room temp, fixed with 70% ethanol, dried, and exposed for 2 wk at 4°C. Autoradiograms were developed for 5 min in D-91 developer and fixed with Rapid-Fix. Tritiated thymidine labeling indices were determined by counting labeled nuclei. For each culture, at least five microscopic fields were counted, each selected at random and containing at least 200 cells. For the localization of incorporated [3S]-radioactivity by autoradiography, single-scratch wounded EC cultures were labeled with 50 μCi/ml [3S]-sulfate in depleted media supplemented with 10% heat-inactivated fetal bovine serum for 3.5 h 2, d after wounding. After labeling, cultures were rinsed with saline and preserved with Holley's fixative (16) containing 0.5% CPC, washed with 8 change of 70% ethanol, and autoradiography performed as described above. Developed autoradiograms of [3S]-labeled single-scratch wounded cultures were lightly stained with Giemsa.

To establish whether cells near the wound edge differed from cells remote from the wound in the amount of cell-associated [3S]-labeled material, silver grains were counted in enlarged micrographs taken at random in three regions of prepared autoradiographs: (a) the region between the front of migrating cells and the razor mark at the original wound edge, (b) areas of the dish remote (at least 8 mm) from any wound edge, and (c) the cell-free central area of the wound, which served to establish a background level of silver grain density.

Proteoglycan Analysis

Biogenesis. Cultures were labeled in fresh medium containing 50 μCi/ml [35S]-sulfate with or without 10 μCi/ml [3H]-glucosamine. After the appropriate labeling period, cultures to be assayed only for macromolecular incorporation of [35S]-sulfate into proteoglycan were harvested by removing and freezing the medium at −20°C, rinsing the cell layer once with PBS (discarded), and incubating the cell layer with 0.75 ml of trypsin-EDTA for 5 min at 37°C. Cells were collected with a pipette and the culture dish was rinsed with 0.75 ml of saline, scraped with a rubber policeman, and the rinse combined with the trypsinate. Aliquots of the cells were counted either with a hemocytometer or with a Coulter counter, and the remaining trypsinate was frozen for further analysis.

Incorporation of radioactivity into sulfated proteoglycan was assayed by precipitation with CPC as described by Wasteson et al. (44). Briefly, 100-μl aliquots of [3S]-labeled media and cell layer isolates were spotted in duplicate on Whatman 3 MM filter paper and allowed to dry. Filter paper strips with dried samples were washed five times for 1 h with 2-4 liters of 1% CPC in 0.05 M NaCl. After washing to remove unincorporated label, the filter paper strips were dried and the amount of radioactivity remaining was determined by liquid scintillation counting. The results of duplicate determinations of radioactivity were averaged, and the total radioactivity per sample was calculated and normalized to cell number. For comparative purposes, incorporation of [3H]-lucine into cell protein was assayed by TCA precipitation essentially as described by Selden et al. (40).

To determine the relationship between media [3S]-sulfate specific activity and the amount of [3S]-sulfate incorporated into proteoglycan, confluent cultures, and cultures wounded 48 h before labeling were pulsed with 8 h with 5, 25, 50, 100, and 250 μCi/ml of [35S]-sulfate (234 Ci/mM) in medium containing 10% fetal bovine serum. Incorporation was directly proportional to media [35S]-sulfate specific activity for both wounded and confluent cultures. Since the ratio between [3S]-sulfate radioactivity incorporated into proteoglycan in wounded and confluent cultures remained constant, a comparison of media [3S]-sulfate specific activity, [3S]-sulfate incorporation rates were considered proportional to proteoglycan biosynthetic rates in these cultures. However, differences in the intracellular precursor pool [3S]-sulfate specific activities between wounded and confluent cultures may exist which, in turn, could influence the apparent proteoglycan biosynthetic rates as determined by [3S]-sulfate incorporation studies.

Proteoglycan Characterization. Cultures harvested for proteoglycan characterization were dissociatively extracted by a method modified from Wight and Hascall (46). Briefly, solid GuHCl was added to pooled culture media to a concentration of 4 M. Cells, layered once with PBS, were extracted for 30 min at 4°C with 4 M GuHCl in 0.05 M sodium acetate buffer, pH 5.8, containing 0.1 M 6-aminoquinol. 5 mM benzidine, 100 μg/ml EDTA, 10 mM N-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, and 2% Triton X-100 (50), collected by scraping with a rubber policeman and stored at −20°C.

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DEAE-Sepharose was used to separate different types of proteoglycans on the basis of differences in charge density (1, 50). To prepare 4 M GuHCl containing samples for ion-exchange chromatography, portions of the samples were first equilibrated with 8 M urea in 0.05 M Tris-HCl buffer, pH 7.5, with 2 mM EDTA, 0.3% Triton X-100 and 0.1 M NaCl by chromatography on Sephadex G-50F (25-ml column) in the same buffer. Sephadex G-50 void volume fractions containing radioactivity were applied to a 3-ml DEAE-Sepharose column that was equilibrated in the urea buffer. Unbound radioactivity eluted as the column was washed with 6 ml of column buffer, and bound labeled macromolecules were eluted with a 0.1-0.7 M (60 ml) linear NaCl gradient in the urea buffer. Fractions of 0.9 ml were collected at a flow rate of ~3.3 ml/h.

The relative hydrodynamic sizes of labeled proteoglycans extracted from EC cultures with 4 M GuHCl and isolated by ion-exchange chromatography were determined by Sepharose CL-4B chromatography (0.7 x 115 cm) in 4 M GuHCl with 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 7.0. Samples of 150-250 μl were run under 40-cm hydrostatic pressure, and fractions of 0.6 ml were collected. Recovery of radioactivity in column samples was greater than 80%. Void (V0) and total (VT) volumes of Sepharose columns were determined by the elution positions of [3H]-DNA and [35S]-sulfate, respectively. Fractions containing major peaks of radioactivity from DEAE-Sepharose or Sepharose chromatography were pooled, dialyzed against distilled water, and freeze-dried. To prepare glycosaminoglycan (GAG) chains from isolated proteoglycan monomers, the samples were treated with papain (30 μg/ml) in 0.1 M sodium acetate buffer with 5 mM EDTA and 5 mM cysteine, pH 7, for 4 h at 65°C. 50 μg/ml each of chondroitin-6-sulfate and heparin was added to the digested samples as carrier, and labeled material was recovered by precipitation with the addition of 4 vol. of 1.3% potassium acetate in 95% ethanol. The molecular weight of purified isolated GAG chains was estimated by chromatography on Sepharose CL-6B as well as comparison of that data with data presented by Wasteson (45). To identify the type of GAG chain present, portions of the resuspended precipitates were digested with chondroitinase ABC (0.05 U/ml in enriched Tris buffer, pH 8.0), for 4 h at 37°C (39), or treated with nitrous acid at low pH (22). The extent of digestion was assessed by separating labeled digestion products from undersized molecules by Sephadex G-50 chromatography (0.7 x 20-cm column) using 0.1 M ammonium acetate in 20% ethanol as the eluting buffer.

**Results**

**The Effect of Wounding on 35S-Sulfate Incorporation into Proteoglycan by ECs**

To determine whether proteoglycan synthesis is affected when ECs are stimulated to proliferate and migrate, wounded monolayers of ECs were labeled with 35S-sulfate at times ranging from 4 to 44 h after multi-scratch wounding. Confluent monolayers of ECs served as controls. Samples of media and cell layers collected from wounded and confluent cultures were harvested at 0, 30, 90, 240, and 480 min after the addition of label, and the amount of radioactivity incorporated into proteoglycan was determined by precipitation with CPC (see Materials and Methods). Proteoglycan-associated radioactivity was detectable in the cell layer by 30 min after labeling and in media by 90 min (not shown). When normalized to cell number, the total incorporation of 35S-sulfate into proteoglycan was linear over the 480-min labeling period, and the rate of incorporation depended upon the amount of time that had elapsed after wounding (Fig. 1). Thus, cultures labeled 44 h after wounding possessed a rate of 35S-sulfate incorporation into proteoglycans that was 2–3-fold greater than that of control cultures or cultures that had been labeled immediately at the time of wounding. However, the rate of 35S-sulfate incorporation into proteoglycan is clearly elevated over that in control cultures between 8 and 16 h after wounding.

**Correlation of Cell Proliferation and Migration with the Time Course of 35S-Sulfate Incorporation into Proteoglycans during Wound Healing**

During the time after multi-scratch wounding of confluent EC monolayers, cells first migrate and then divide to repair the monolayer (40). To determine with which process(es) the alteration in proteoglycan metabolism might correlate, we have compared the time course of cell migration and proliferation with that of 35S-sulfate incorporation into proteoglycan after wounding. In these experiments, cultures were labeled for 6 h with 35S-sulfate to determine when proteoglycan synthesis was maximal in wounded cultures and when it began to decline. Cultures that were multi-scratch wounded 3 d after confluence were labeled at the time of wounding and 20, 44, and 68 h after wounding. Control cultures were labeled 3 and 6 d after reaching confluence, corresponding to the initial and final labeling periods of wounded cultures (Fig. 2a). The results confirmed our previous observation of a progressive increase in the amount of 35S-sulfate incorporated into proteoglycan, with maximal incorporation occurring ~44–50 h after wounding. Incorporation of 35S-sulfate was also found to be significantly decreased by 68–74 h after wounding. Values for the total incorporation of 35S-sulfate into proteoglycan in control confluent cultures continued to decline with increased time after reaching confluence.

In another experiment, the relative incorporation of [3H]-leucine and 35S-sulfate in cultures of wounded and confluent ECs was determined (Table I). While TCA-precipitable [3H]-leucine incorporation in the cell layer of wounded cultures was found to be elevated slightly less than twofold over that in confluent cultures, 35S-sulfate incorporation into proteoglycan, measured in the cell layers of a parallel series of cultures, was increased more than fourfold.

The time course of 35S-sulfate incorporation into proteoglycan was correlated with the time courses of cell migration and proliferation after wounding. The stimulation of cell proliferation was monitored with 4-h pulses of [3H]-TdR 1, 17, 24, 44, and 72 h after wounding (Fig. 2b). In multi-scratch wounded cultures, [3H]-TdR incorporation increases rapidly over 17 h after wounding, reaches a maximal level by 48 h, and remains elevated through 72 h. The 24-fold increase above control values in TCA-precipitable [3H]-TdR incorporation 48 h after wounding represents an increase in the growth fraction from 5–10% in confluent cultures to >90% above control values in TCA-precipitable [3H]-TdR incorporation 48 h after wounding.
in wounded cultures as determined by indices of \(^3\)H-TdR labeled nuclei. Thus, while \(^{35}\)S-sulfate incorporation into proteoglycans is elevated as thymidine incorporation is stimulated and both reach maximal levels by 48 h, thymidine incorporation remains elevated as proteoglycan synthesis begins to decline between 50 and 74 h after wounding. Cell migration (Fig. 2, c–f) begins as early as 2–3 h after wounding (Fig. 2 c), but, in contrast to cell proliferation, has been largely completed by 48 h after wounding (Fig. 2 f).

To determine whether cell division is required for the increase in \(^{35}\)S-sulfate incorporation into proteoglycan to occur, cultures, pretreated for 16–24 h with inhibitors of DNA synthesis, were wounded and labeled for 4 h with \(^{35}\)S-sulfate, 36 h after wounding. The incorporation of \(^{35}\)S-sulfate into proteoglycan in these cultures was compared with confluent cultures treated with the same inhibitors, and with wounded and confluent control cultures that were grown in the absence of inhibitors. Fig. 3 demonstrates that the increase in \(^{35}\)S-
Proteoglycan and Protein

Table I. Relative Incorporation of Radiolabel into Wounded and Confluent Cultures

| Condition          | 35S-Sulfate incorporation* | 3Hleucine incorporation† |
|--------------------|---------------------------|--------------------------|
|                    | cpm/10⁶ cells ± SD        | cpm/10⁶ cells ± SD       |
| Wounded cultures   | 11,438 ± 1,771            | 12,243 ± 2,443           |
| Confluent cultures | 2,578 ± 412               | 6,592 ± 1,815            |
| Fold increase after wounding | 4.44                  | 1.86                     |

* Mean of duplicate determinations of total CPC-precipitable 35S-sulfate radioactivity in cell layers from triplicate cultures labeled with 50 μCi/ml 35S-sulfate for 7 h (wounded cultures labeled 44 h after wounding).
† Mean of TCA-precipitable [3H]leucine radioactivity in the cell layer of triplicate cultures labeled for time periods in * with 5 μCi/ml [3H]leucine. Values obtained from 0 time incorporation controls were subtracted from the calculated means.

![Graph](Image)

Figure 3. 35S-Sulfate incorporation into proteoglycan in multi-scratch wounded and confluent cultures with or without inhibition of cell proliferation. 24 h before the initiation of the experimental cultures in which cell division was to be inhibited, cultures were treated with 7 mM sodium butyrate, 250 μg/ml hydroxyurea or 2 μM aphidicolin. Cultures were labeled for 4 h and 36 h after wounding. All values are the mean of duplicate determinations of 35S-sulfate incorporation in duplicate cultures, normalized to cell number. ![Graph](Image)

Proteoglycan Characterization

To determine whether EC cultures modulated the types of proteoglycans synthesized after wounding, the relative proportion of radioactivity present in different proteoglycan types extracted from wounded and confluent cultures was analyzed by ion-exchange chromatography. Postconfluent cultures (4 wk) were labeled with [3H]glucosamine and 35S-sulfate for 48 h and wounded cultures were similarly labeled from 24–72 h after wounding. Macromolecular radioactivity extracted from media and cell layers with 4 M GuHCl in the presence of protease inhibitors, and detergent was equilibrated in 8 M urea buffer with 0.1 M sodium chloride and applied to a DEAE-Sephadex column. Two 35S-sulfate-labeled peaks (B and C) were eluted from the column with a linear NaCl gradient (0.1–0.7 M) (Fig. 4). Peaks B and C from the media and cell layers of wounded and confluent cultures were pooled and analyzed for GAG content as previously described. Peak B was identified as heparan sulfate–containing material by its susceptibility to degradation by nitrous acid, and peak C was almost totally digested with chondroitinase ABC, indicating

Table II. Effect of Inhibition of DNA Synthesis on EC Migration and Proliferation

| Culture treatment | Distance migrated* (μm ± SD) | Percent control at day 5 (%) | Cell number at day 5 (x10⁴ ± SD) | Percent of density at day 1 (%) |
|-------------------|-----------------------------|------------------------------|----------------------------------|-------------------------------|
| 250 μg/ml hydroxyurea | 1,775 ± 264                | 82                            | 32.50 ± 1.64                     | 91                            |
| 7 mM butyrate     | 2,355 ± 292                 | 109                           | 29.89 ± 1.11                     | 88                            |
| None (control 1)  | 2,174 ± 300                 | 100                           | 198.23 ± 7.43                    | 553                           |
| 2 μM aphidicolin  | 2,339 ± 291                 | 108                           | 68.69 ± 0.26                     | 123                           |
| None (control 2)  | —                           | —                             | 298 ± 2.00                       | 524                           |

* Distance was measured with a calibrated eyepiece reticle 3 d after wounding (single scratch) with a razor blade.
† Cell number was determined in triplicate cultures with duplicate Coulter counting 5 d after plating.
‡ Cells were plated at day 0 and treated with the appropriate inhibitor from that time. Cell number was determined for comparative purposes on day one, 24 h after the addition of inhibitors. Cell number determined as in §.
§ Control values are derived from two separate groups of cultures. For cell density determinations control cultures were plated with hydroxyurea- and butyrate-treated cultures at an initial density of 4 x 10⁴ cells per 2.0-cm² well and cell number determined as in ‡. For determination of cell migration cultures were wounded and the distance migrated determined as in *.
* Cell density controls for aphidicolin-treated cultures were plated at an initial density of 5.9 x 10⁴ cells per 2.0-cm² well and cell number determined as in §, except for duplicate cultures.
Table III. Regional Cell Density and Silver Grain Density in Autoradiographs of 35S-Sulfate-labeled, Single Scratch Wounded EC Cultures

| Culture | Front | Remote | Front/Remote | Front | Remote | Front/Remote |
|---------|-------|--------|--------------|-------|--------|--------------|
|         | nuclei/mm² |        |              | Silver grains/20 µm² |        |              |
| n=3     | 1,108 ± 210  | 1,508 ± 151 | 0.73         | 111.4 ± 19.6 | 58.1 ± 9.3 | 2.07         |
| n=3     | 1,183 ± 215  | 1,558 ± 104 | 0.76         | 111.4 ± 19.9 | 37.6 ± 10.4 | 3.52         |
| n=1     | 1,250        | 1,650 ± 141 | 0.76         | 87.0 ± 12.5  | 33.1 ± 7.0  | 3.17         |
| n=2     | 1,163 ± 124  | 1,638 ± 17.8 | 0.71         | 82.0 ± 19.6  | 33.1 ± 7.0  | 2.97         |

Figure 4. DEAE-Sephacel chromatography of cell layer (a and c) and media (b and d) extracts from wounded (a and b) and confluent (c and d) cultures labeled for 48 h with 35S-sulfate (dotted line) and [3H]glucosamine (solid line). Peaks B and C represent pooled fractions containing, respectively, heparan sulfate and chondroitin sulfate. Horizontal bars on b, c, and d represent pooled fractions of peaks B and C comparable to those in Fig. 4a.

Table IV. Susceptibility of Radioactive Macromolecules Pooled from DEAE-Sephacel Peaks to Enzymic or Nitrous Acid Degradation

| Culture | Compartment | DEAE peak | Percentage chondroitinase-sensitive* | Percentage nitrous acid-sensitive* |
|---------|-------------|-----------|-------------------------------------|----------------------------------|
| Wounded | Cell B      | 8         | 91                                  |                                  |
|         | Media B     | 12        | 75                                  |                                  |
|         | Cell C      | 91        | 8                                   |                                  |
|         | Media C     | 92        | 6                                   |                                  |
| Confluent | Cell B     | 4         | 95                                  |                                  |
|         | Media B     | 10        | 82                                  |                                  |
|         | Cell C      | 87        | 11                                  |                                  |
|         | Media C     | 92        | 7                                   |                                  |

* Labeled GAG, isolated from pooled DEAE-Sephacel peak C by papain digestion was precipitated with ethanol after the addition of carrier. Dried precipitates were digested with 0.5 U/ml chondroitinase ABC in enriched Tris buffer, pH 8.0, for 4 h at 37°C. The percentage of enzyme sensitive radioactivity was determined by separation of digested products from undigested material on Sephadex G-50.

that it contains chondroitin sulfate and/or dermatan sulfate GAG chains (Table IV). Peak B was predominant in cell layer extracts (80% of 35S-label) and media (71%) from postconfluent cultures (Fig. 4, c and d). In contrast, extracts from both cell layers and media of wounded cultures showed an increased proportion of 35S radioactivity in peak C, which contained, respectively, 60% and 57% of the label from these cultures (Fig. 4, a and b).

The relative hydrodynamic size of proteoglycan monomers was determined to find whether the monomers synthesized by wounded cultures differed in size from those isolated from confluent cultures. To this end, portions of the radioactivity present in peaks B and C from cell layer and media extracts were chromatographed on Sepharose CL-4B in 4 M GuHC1 buffer with 0.5% Triton X-100. Chromatographic profiles of the HSPGs in peak B samples, extracted from both cell layers and media of wounded and confluent cultures, revealed three peaks (Fig. 5). A majority (>60%) of the HSPG-associated label from wounded cell layers eluted in two lower molecular weight peaks (BI and BII) of Kav = 0.51 and 0.63, respectively (Fig. 5a), while confluent culture cell layers had less such material (~37%) (Fig. 5c). Instead, confluent culture cell layer HSPG eluted primarily as a high molecular weight peak from Sepharose CL-4B (BI, Kav = 0.19). No differences were apparent between Sepharose CL-4B profiles of HSPG extracted from wounded (Fig. 5b) and confluent (Fig. 5d) culture media.

Individual peaks were isolated after Sepharose CL-4B chromatography, and the elution position on Sepharose CL-6B was determined before and after papain digestion to determine whether the peaks represented intact proteoglycans. The results presented in Table V indicate that peaks BI and BII from both wounded and confluent cell layers and media are proteoglycans with GAG chains of, respectively, 38,000 and 22,000 mol wt (45). In contrast, peak BIII from wounded
Table V. Effect of Papain Digestion on Isolated HSPG Subfractions

| Culture type | Component | Sepharose CL-4B peak | Sepharose CL-6B | Undigested | Papain digested | Percentage nitrous acid-sensitive |
|--------------|-----------|----------------------|----------------|------------|----------------|-------------------------------|
| Wounded      | Cell layers | BI | 0.10 | 0.37 | 96 |
|              |            | BII | 0.27 | 0.50 | 92 |
|              |            | BIII | 0.51 | 0.51 | 72 |
| Media        |            | BI | 0.07 | 0.32 | 98 |
|              |            | BII | 0.25 | 0.51 | 98 |
|              |            | BIII | 0.47 | 0.51 | 68 |
| Confluent    | Cell layers | BI | 0.07 | 0.38 | 93 |
|              |            | BII | 0.23 | 0.48 | 88 |
|              |            | BIII | 0.43 | 0.61 | 76 |
| Media        |            | BI | 0.10 | 0.35 | 98 |
|              |            | BII | 0.21 | 0.48 | 98 |
|              |            | BIII | 0.48 | 0.59 | 48 |

Figure 6. Sepharose CL-4B gel chromatography (in 4 M GuHCl with 0.5% Triton X-100) of 35S-sulfate labeled CS/DSPG (peak C) isolated by DEAE-Sepharose chromatography from cell layer (a and c) and media (b and d) extracts from wounded (a and b) and confluent (c and d) cultures.

cultures may represent a free chain with the same molecular weight as chains isolated from peak BII. However, peak BIII isolated from confluent cultures appears to be a small proteoglycan with GAG chains of an estimated molecular weight of 16,000. Nearly all of the radioactivity in peaks BI and BII was susceptible to nitrous acid degradation. However, a substantial portion of peak BIII samples was not sensitive to nitrous acid. The nature of the nitrous acid insensitive material is currently under investigation.

Sepharose CL-4B profiles of CS/DSPG monomers in peak C samples extracted from both cell layers and media also revealed the presence of three peaks of radiolabeled material (Fig. 6). Cell layer-associated CS/DSPGs, extracted from both wounded (Fig. 6a) and confluent (Fig. 6c) cultures, had similar chromatographic profiles, with the majority of the radioactivity eluting in a single peak (CI) near the void volume of the column (Kav = 0.06). In contrast, the majority of the label in extracts of media-associated CS/DSPG from wounded (Fig. 6b) and confluent (Fig. 6d) cultures eluted in two peaks (CI and CII) within the included volume of the column (Kav = 0.42 and 0.63). Interestingly, a large proportion (~50%) of the labeled CS/DSPG extracted from the media of wounded cultures was found to elute at Kav = 0.63 (CII), while this peak represented ~20% of the radioactivity present in media extracts from confluent cultures. Portions of peak C samples were chromatographed on Sepharose CL-6B before and after papain digestion to determine whether the peaks were proteoglycan in nature (Fig. 7). Media and cell layer peak C samples from both wounded and confluent cultures eluted as a single peak (Kav = 0.50) after papain digestion, indicating the presence of a single GAG chain size class (mol wt ~20,000), which elutes at the same position as peak CII. These results suggest that both peak CI and CII are proteoglycans, while peak CIII may represent a free chain. Finally, most of the radioactivity in each peak C sample was sensitive to chondroitinase ABC (Table IV).

Results from molecular sieve chromatography indicate few qualitative differences in the types of labeled proteoglycan monomers or their derivatives present in wounded and confluent cultures. However, two quantitative differences in the distribution of label among the different size classes of labeled proteoglycans extracted from wounded and confluent cultures exist: (a) there is an increase in the amount of low molecular weight labeled heparan sulfate-containing material present in wounded cell layer when compared to similar material present in confluent monolayers and, (b) there is a greatly enhanced accumulation of low molecular weight chondroitin sulfate/dermatan sulfate, which is apparently free chain, in the media of wounded cultures when compared to media from confluent cultures.

Discussion

We report that there is an increase in the incorporation of radioactive precursors into proteoglycan after wounding of EC monolayers in vitro, that the increase is reflected in an increase in the proportion of labeled CS/DSPG relative to HSPG, and is associated with the movement of cells at the wound edge. It is unlikely that the increase in labeled proteoglycan accumulation in wounded cultures is due to decreased turnover, since differences in synthetic rates are apparent after short labeling periods (Fig. 1). In addition, the increased

Figure 7. Sepharose CL-6B gel chromatography of DEAE-isolated peak C samples from the cell layers (a and c) and media (b and d) extracts from wounded (a and b) and confluent (c and d) cultures, before (solid line) and after (dotted line) papain digestion.
accumulation of lower molecular weight $^{35}$S-sulfate-labeled molecules, particularly apparent free GAG chains (BIII and CIII), in wounded cultures (Figs. 5 and 6) would suggest that turnover of proteoglycans is enhanced rather than decreased as ECs are stimulated to migrate after wounding. If these molecules are the products of metabolic degradation, then the differing distributions of low molecular weight heparan sulfate and chondroitin sulfate containing species (which accumulate, respectively, in the cell layer [Fig. 5a] or the media [Fig. 6b] of wounded cultures) suggest that different catabolic mechanisms or compartments exist for the degradation of HSPGs and CS/DSPGs.

Three lines of evidence suggest that increased CS/DSPG synthesis is associated with the movement of cells at the wound edge. First, autoradiographic evidence (Table III) indicates that incorporation is greater in migrating and proliferating cells near the wound edge than in cells remote from the wound edge. Second, the rate of $^{35}$S-sulfate incorporation into proteoglycan is clearly elevated between 8 and 16 h after wounding, shortly after cells at the wound edge have begun to migrate (~2–3 h), and reaches maximal levels at a time (~48 h) when most cells have moved to fill the wound (Fig. 2, a, c–f). In comparison, cell proliferation, as evidenced by $[^{3}H]$thymidine incorporation, is also maximal by 48 h after wounding, but continues at near maximal levels for at least 72 h after wounding, although levels of $^{35}$S-sulfate incorporation have declined by that time (Fig. 2, a and b). Finally, when cell proliferation is inhibited and cell migration continues, the increase in proteoglycan synthesis after wounding still occurs (Fig. 3).

The wounding model used in these studies was chosen as an advantageous system in which the time course of the response of cells to the release of constraints upon cell migration and proliferation could be followed. Another useful cell culture paradigm uses sparsely plated, logarithmically growing cultures to study the changes in proteoglycan synthesis that occur during increased cell migration and proliferation. While this system may not be directly comparable to wounding, sparsely plated cells might be expected to display similar alterations in metabolic processes related to the stimulation of cell migration and proliferation. While this system may not be directly comparable to wounding, sparsely plated cells might be expected to display similar alterations in metabolic processes related to the stimulation of cell migration and proliferation as cells in wounded cultures. Indeed, recent experiments in our laboratory indicate that sparse cultures of ECs also have elevated levels of proteoglycan synthesis when compared with postconfluent cultures and that the increase of proteoglycan synthesis is associated with a large relative stimulation in CS/DSPG synthesis (unpublished results).

Others have recognized some differences in proteoglycan accumulation by ECs which depended on their growth or migratory state. For example, Ausprunk and her co-workers used histochemical and autoradiographic techniques to describe changes in proteoglycan accumulation as ECs grew and differentiated in rabbit eye microvessels (3) and in chick chorioallantoic membrane (4). They suggest that the proliferating, poorly differentiated cells in growing vessels have little associated heparan sulfate when compared to nongrowing cells in more stable, developed regions of vessels. Similarly, Oohira et al. (32) have described a relative decrease in the proportion of a large, cell-associated HSPG in postconfluent cultures of bovine aortic ECs that have undergone a secondary pattern of growth and movement (spouting). However, while these authors suggest that an alteration in HSPG accumulation may occur as a result of increased degradation of that proteoglycan, they could not exclude the possibility that sprouting cultures may change the amount or type of proteoglycan that is actually synthesized.

Density-dependent changes in the synthesis and deposition of other proteoglycans, such as CSPG and DSPG, have been reported by other investigators. Recently, Montesano and his co-workers (28) have reported that the accumulation of a chondroitin sulfate–rich pericellular matrix is induced in human umbilical vein EC cultures by exposure of the cells to leukocyte interleukins, which are thought to influence EC growth and migration (2, 8). Robinson and Gospodarowicz (36) indicated that an iduronate-enriched DSPG was accumulated in the ammonium hydroxide–insoluble matrix synthesized by postconfluent corneal ECs, but not in the matrix associated with sparsely plated, growing cells. However, since similar material was present in the media of sparse cultures, it seems likely, as the authors suggest, that the selective deposition of DSPG in the matrix of confluent cultures may reflect the increased deposition of collagen which occurs with the increased age of these cultures (43). A similar phenomenon was observed by Winterbourne et al. (48), who reported that a DSPG was recovered in the collagenase digest of bovine aortic ECs grown on hydrated collagen gels.

While proteoglycan distribution and metabolism may be modulated under varying conditions of cell culture (10, 25, 28, 36, 48), the roles of different proteoglycans in the processes of cellular migration and proliferation remain unclear. However, hypothetical functions have been ascribed to HSPG, CSPG, and DSPG largely on the basis of observed differences in their relative culture distributions or ability to bind other matrix molecules. For example, both CSPG and HSPG have been shown to bind to type I fibrillar collagen (10, 31) or procollagen–fibronectin fibers (15). Also, persuasive evidence has been presented to suggest that HSPG may exist as an intercalated membrane proteoglycan (19, 33), which may mediate cell adhesion. In contrast, CSPG has been hypothesized to destabilize focal cellular adhesion (21), and preparations of CSPG from cartilage (20, 34) and from rat yolk sac tumor cells (6) have been shown to inhibit cell attachment to collagen (34). Similarly, Erickson and Turley (11) reported that migrating neural crest cells display marked cell rounding when chondroitin sulfate is added to collagen or fibronectin substrate in vitro, suggesting that decreased cell–substratum adhesion is induced by chondroitin sulfate–containing substrate. While the chondroitin sulfate preparations used in these studies may differ from the CS/DSPG synthesized by ECs, it is nevertheless tempting to speculate that the CS/DSPG synthesized by ECs after wounding is involved in the facilitation of migration by virtue of its inhibition of cell adhesion.

In addition, both HSPG and CS/DSPG are known to function as structural components of the extracellular matrix. Therefore, enhanced synthesis of proteoglycan by wounded EC cultures may also represent the renewed synthesis and deposition of new basement membrane and interstitial matrix, which may be required to complete the process of monolayer wound healing.

In summary, we report that $^{35}$S-sulfate incorporation into proteoglycan is enhanced when confluent monolayers of ECs are stimulated to migrate by wounding. The time-dependent increase in $^{35}$S-sulfate incorporation is associated with cell movement and involves a selective increase in CS/DSPG synthesis. It is, perhaps, important to note that increased
proteoglycan synthesis is one of many changes that occur as cells in monolayer are stimulated to migrate and proliferate after wounding (e.g., see Table I). However, while the functional significance of the modulation of proteoglycan synthesis during EC migration remains unclear, these experiments suggest that ECs, migrating in response to intimal wounding in vivo, may contribute to the physiologically relevant accumulation of connective tissue macromolecules that occurs as an early event in atherogenesis.

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