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Sulfated Glycoproteins and Extracellular Matrix of Cultured Human Pulmonary Endothelial Cells

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Endothelial cells derived from human pulmonary arteries incorporate (^{3}H)-glucosamine and $^{35}SO_4$ into glycosaminoglycans and into the carbohydrate side chains of glycoproteins. These ^{3}H/^{35}S-carbohydrate chains were isolated from cells and culture medium after Pronase digestion. The ^{3}H/^{35}S-glycosaminoglycans were separated from the ^{3}H/^{35}S glycopeptides by chromatography on Sephadex G-50. The distribution of cellular glycosaminoglycans and glycopeptides indicated that 30–60% of the cellular ^{35}S-glycopeptides may be associated with the matrix components that are synthesized by the cell and attached to a plastic substratum. Human pulmonary arterial endothelial cells were grown on collagen or on a matrix derived from vascular smooth muscle cells in order to investigate how smooth muscle cell extracellular matrix components may regulate the synthesis of endothelial cell glycoconjugates. Endothelial cells grown on plastic release various proportions of the glycoconjugates they synthesize into the culture medium. However, these same cells, when grown on substratum composed of extracellular matrix materials, synthesized altered proportions of cell-associated glycosaminoglycans and reduced the levels of total glycosaminoglycans they released into the culture medium. Thus the growth of endothelial cells on a matrix of smooth muscle cell components indicates that the glycosaminoglycan materials released into the culture medium by cells grown on a plastic substratum may not be an accurate reflection of the levels or composition of extracellular matrix materials made by endothelial cells in vivo.

Key words: sulfated glycoconjugates, endothelial cell

Glycoproteins and proteoglycans have been suggested to play a major role in endothelial cell function and in the maintenance of the subendothelial layer. Endothelial cells normally synthesize a subendothelial basement membrane that defines areas of smooth muscle growth and which may play a role in platelet adhesion and plaque formation in cases of endothelial cell injury. The oligosaccharide side chains of glycoproteins and the glycosaminoglycan chains of proteoglycans have been implicated as recognition signals involved in cell-surface interactions, as regulators of surface membrane component turnover, and as important structural components of extracellular

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matrices [1]. The sulfation of these carbohydrate side chains may regulate the synthesis and degradation of these glycoconjugate materials, which to a large extent determine the ionic composition, permeability, degree of hydration, and mechanical properties of extracellular matrices. Recently a class of glycoproteins were found to have sulfated oligosaccharide side chains that were presumably N-linked to protein [2–5]. This class of glycoprotein is synthesized by cultured vascular endothelial cells [6]. Changes in the synthesis and sulfation of endothelial cell glycoconjugates may be regulated in part by components of the cells or their matrices, with which they interact. Components of the subendothelial matrix have been shown to modulate the growth and morphology of epithelial cells [7]. The effect of epithelial cells on the glycosaminoglycan composition of connective tissue has also been studied [8]. This report describes the synthesis of sulfated glycoproteins and glycosaminoglycans by human pulmonary arterial endothelial cells in culture, and the effect of various extracellular materials on the synthesis of these sulfated glycoconjugates.

MATERIALS AND METHODS

Cell Culture and Radioisotope Labeling

Endothelial cells were obtained from arteries dissected from whole human lungs, maintained in culture, and identified as previously described [9]. Smooth muscle cells from porcine aorta and human pulmonary arteries were obtained from explant cultures as described by Coltoff-Schiller et al [10] and maintained in Medium 199 (GIBCO) containing 10% neonatal calf serum (Biocell Labs) and 50 μg/ml gentamicin (Sigma). Cell cultures were labeled with 5 μCi/ml D-[6-^{3}H]glucosamine and 50 μCi/ml $H_2{}^{35}SO_4$ (New England Nuclear) in 5 ml Ham's F-12 nutrient medium for 18–24 hours. After labeling, the culture medium was removed, and the released glycoconjugates were precipitated with 10% cold trichloroacetic acid. Cell layers plus some plastic were removed from the culture vessel by vigorous scraping with a metal spatula in 2 ml of methanol. Cell layers and trichloroacetic acid-insoluble materials from the medium were extracted with chloroform:methanol:water (10:10:3, v/v) and centrifuged at 10,000g for 20 min. The supernatants were removed, and the denatured glycoconjugate pellets were dried under N_2 and were then digested with Pronase CB (1 mg/ml, Calbiochem). The resulting digests were chromatographed on Sephadex G-50 in 0.1 M ammonium acetate as previously described [5]. The ^{3}H/^{35}S-glycosaminoglycans eluted from Sephadex G-50 with the excluded volume (Vo) and were separated from the oligosaccharide side chains of glycoproteins, which eluted as ^{3}H/^{35}S-glycopeptides in the retarded fractions. Fractions containing the ^{3}H/^{35}S-glycosaminoglycans or ^{3}H/^{35}S-glycopeptides were combined and lyophilized prior to further analysis.

Glycosaminoglycan Analysis

^{3}H/^{35}S-Glycosaminoglycans were analyzed by treatments with Streptomyces hyaluronidase and chondroitinase ABC (Miles Labs) as previously described [11], and the materials resistant to these enzymatic probes were separated from the smaller degradation products by precipitation with cetylpyridinium chloride. The material resistant to both Streptomyces hyaluronidase and chondroitinase ABC was identified as heparan sulfate. This method of analysis yielded similar results when compared to an analytical scheme that used a sequential series of degradative treatments [11, 12].

RESULTS

Endothelial cells in culture incorporate (^{3}H)glucosamine and $^{35}SO_4$ into glycosaminoglycans and oligosaccharide chains that can be isolated after pronase digestion and separated by chromatography on Sephadex G-50, as shown in Figure 1. The larger glycosaminoglycan components elute with columns excluded volume (Vo) and are composed of (^{3}H/^{35}S)heparan sulfate, (^{3}H)hyaluronic acid, and (^{3}H/^{35}S)chondroitin and dermatan sulfates. The smaller size ^{3}H/^{35}S-glycopeptides elute in the retarded fractions of Sephadex G-50 columns, and are composed of the oligosaccharside side chains of glycoproteins with smaller peptide cores similar to that previously described as synthesized by embryonic lung [5, 13]. These ^{3}H/^{35}S-glycopeptides are not precipitated in the presence of 1% cetylpyridinium chloride, whereas the various ^{3}H/^{35}S-glycosaminoglycans synthesized by pulmonary endothelial cells are quantitatively precipitated with cetylpyridinium chloride. The ^{3}H/^{35}S-glycopeptides are not degraded by treatment with Streptomyces hyaluronidase, chondroitase ABC, endo-β-galactosidase, and nitrous acid; however, these sulfated glycopeptides are degraded by neuraminidase, β-galactosidase, and β-N-acetylglucosaminidase, as judged by gel filtration analysis on Biogel P-6. Thus human pulmonary endothelial cells incorporate $^{35}SO_4$ into presumably complex-type carbohydrate chains of glycoproteins [14] as well into heparan sulfate, chondroitin sulfate, and dermatan sulfate.

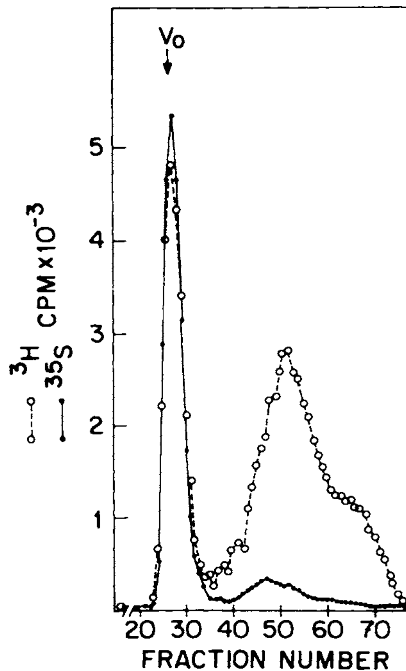

Fig. 1. Sephadex G-50 column chromatography of glycoconjugates after digestion with Pronase. Human pulmonary endothelial cells (1.1 × 10^6 cells) were incubated with (^{3}H)glycosamine and $^{35}SO_4$ and digested with Pronase, and the digests were chromatographed on a column of Sephadex G-50 as described under Materials and Methods. Aliquots from each fraction were taken to monitor radioactivity. The position of elution marker blue dextran (Vo) is indicated.

In order to study the distribution of glycopeptides and glycosaminoglycans, cell-associated materials were extracted with salt, urea, and detergent, and the extracts were then analyzed as described under Materials and Methods. As shown in Table I, 20–30% of the sulfated glycosaminoglycans and 12% of the sulfated glycopeptides may be extracted with 1 M NaCl from the cell particulate fraction. After salt extraction less than 7% of the total glycoconjugates are extracted with 8M urea, except for heparan sulfate, of which 16% is released. The particulate residue after salt and urea extraction contains 20–50% of the total glycoconjugates measured, and this entire fraction was solubilized in 1% sodium docecyl sulfate. Thus this total cell fraction contained about 95%, 70%, and 80% of the cell layer associated hyaluronic acid, chondroitin and dermatan sulfates, and heparan sulfate, respectively. This total cell fraction contained 90% of the total cell layer ^{3}H-glycoproteins, but only about 40% of the total cell layer ^{35}S-glycoproteins. The remainder of the labeled materials were found to be still associated with the plastic surface of the tissue culture flask, and could be removed by treatment with 2% sodium dodecyl sulfate to yield a fraction rich in ^{35}S-glycoproteins and poor in (^{3}H)-hyaluronic acid.

In order to investigate the regulation of endothelial cell sulfated glycoconjugates by extracellular matrix components, endothelial cells were first cultivated on a matrix derived from rat tail collagen. This material formed a hydrated matrix upon which the

TABLE I. Distribution of Endothelial Cell Sulfated Glycoconjugates

	Glycopeptides cpm ^{3}H:^{35}S	Heparan sulfate cpm ^{3}H:^{35}S	Hyaluronic acid cpm ^{3}H	Chondroitin and dermatan sulfates cpm ^{3}H:^{35}S
Cells[a]		(%)		
10,000g supernatant	7,200:40	800:120	1,400	2,700:1,500
	(5,1)	(4,2)	(11)	(16,12)
1 M NaCl extract	55,000:540	6,800:1,500	2,800	4,800:3,600
	(42,12)	(35,22)	(23)	(28,29)
8 M urea extract	4,200:40	3,200:940	900	500:850
	(3,1)	(16,14)	(7)	(3,7)
1% sodium dodecyl sulfate extract	52,000:1,220	5,000:2,620	6,600	3,500:2,450
	(39,26)	(26,39)	(54)	(20,20)
Material remaining on flask[b]	14,000:2,800	3,600:1,540	600	5,600:4,020
	(11,60)	(19,23)	(5)	(33,32)
Total cpm	132,400:4,640	19,400:6,720	12,300	17,100:12,420
(Total %)	(100,100)	(100,100)	(100)	(100,100)

[a]Cells were labeled with [^{3}H]glucosamine and $^{35}SO_4$, washed with medium M199, and then removed from the 75 cm^2 Corning plastic flask by gentle scraping with a Teflon spatula in 0.1 M Tris-HCl (pH 8.0) at 4°C. Cells were homogenized, centrifuged at 10,000g for 20 min, and the cell pellet was sequentially extracted for 20 min on ice with 1 M NaCl in 0.1 M Tris-HCl (pH 8.0), 8 M urea M 0.1 M Tris-HCl (pH 8.0), and 1% sodium dodecyl sulfate in 0.05 M Tris-HCl (pH 8.3). After each extraction the insoluble residue was recovered by centrifugation at 10,000g for 30 min. Extracts were dialyzed vs 0.2 M Tris-HCl (pH 8.0), boiled for 10 min, and digested with Pronase.

[b]After cells were removed from the plastic flask, the materials remaining attached to the plastic surface were extracted with 2% sodium dodecyl sulfate in 0.05 M Tris-HCl (pH 6.8). These soluble materials were extracted with 10 volumes chloroform:methanol (1:1, v/v) and the insoluble residue was digested with Pronase as described under Materials and Methods.

cells attached and into which the cells later extended processes. As shown in Table II, cells on a collagen matrix synthesized 50% lower levels of ($^3H/^{35}S$)heparan sulfate and ($^3H/^{35}S$)-chondroitin and dermatan sulfates. The most dramatic effect was seen in the markedly lower level of $^3H/^{35}S$-glycosaminoglycans that were found in the culture medium. In contrast, the cells on collagen incorporated approximately the same level of (^3H)glucosamine into glycoproteins. The level of $^{35}SO_4$ incorporated into glycoproteins was reduced 22% and 57% for proteins on the cell layer and in the medium, respectively, whereas the decreases in $^{35}SO_4$ incorporated into ^{35}S-glycosaminoglycans were 50–60% on the cell layer and 79–84% in the medium.

In order to assess the influence of smooth muscle cell extracellular components upon the synthesis of endothelial cell products, smooth muscle cell cultures were extracted with 0.5% Triton X-100, and the insoluble residue was used as a matrix for endothelial cell growth. As shown in Figure 2, pulmonary endothelial cells synthesized nearly equivalent amounts of (^3H)mannose- or (^{35}S)metionine-labeled materials found associated with the cell layers or with the medium. However, there were differences in individual components. Angiotension-converting enzyme activities were lower on cells grown on the matrix derived from porcine smooth muscle cells, whereas angiotensinase II activities were more variable for cells growing on plastic. Endothelial cells, after 5 days of growth on plastic or on this Triton-insoluble matrix, were incubated in the presence of (^3H)glucosamine and $^{35}SO_4$ for 18 hrs, and the various glycosaminoglycan and glycopeptide materials were analyzed as described above. As shown in Figure 3B, endothelial cells cultured on detergent-insoluble matrices prepared from porcine aorta smooth muscle cell cultures showed a 4-fold increase in the incorporation of $^{35}SO_4$ into heparan sulfate, and a smaller but consistant increase in (^3H)heparan sulfate synthesis compared to endothelial cells cultured on plastic. Much smaller differences in the synthesis of the other $^3H/^{35}S$-glycoconjugates were observed. Endothelial cells and smooth muscle cells generally have decreased incorporation of $^{35}SO_4$ into glycosaminoglycans at increased cell densities. Thus small differences in $^3H/^{35}S$-glycoconjugate biosynthesis between cells grown on plastic and smooth muscle cell matrix may be due to similar phenomena. When similar experiments were carried out using matrices prepared from human arterial smooth muscle cells (Fig. 3C and D), there was a 3-fold increase in the synthesis of (^3H)hyaluronic acid by matrix grown cells with only

TABLE II. Effect of Collagen Substratum on Glycoconjugate Biosynthesis

	cpm/μg DNA						
	Glycopeptides		Heparan sulfate		Hyaluronic acid	Chondroitin and dermatan sulfate	
	^3H	^{35}S	^3H	^{35}S	^3H	^3H	^{35}S
Plastic substratum							
Cell layer	175	73	62	205	280	47	819
Medium	96	101	11	83	208	51	552
Total	271	174	73	288	488	98	1,371
Collagen matrix							
Cell layer	191	57	34	102	269	41	500
Medium	90	43	4	13	48	8	115
Total	281	100	38	115	317	49	615

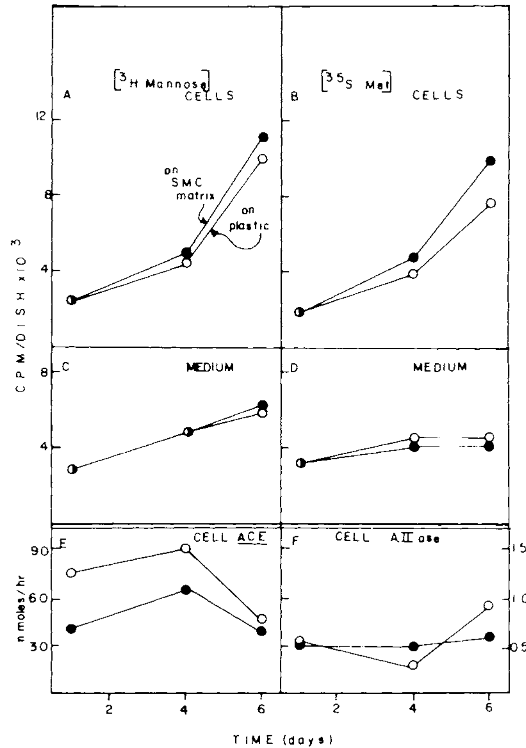

Fig. 2. Glycoprotein synthesis by endothelial cells. Cells were grown on a tissue culture plastic substatum (○——○) or on a Triton X-100-insoluble matrix derived from vascular smooth muscle cells (●——●). Panels A and C represent the incorporation of (^{3}H)mannose into trichloroacetic acid-insoluble glycoproteins. Panels B and D represent the incorporation of (^{35}S)methionine into trichloroacetic acid-insoluble proteins. Panel E is the cell layer activity of angiotensin converting enzyme (A.C.E.), and Panel F is the cell layer activity of angiotensinase II (AIIase). The time cells were grown in culture is indicated.

small changes in ^{3}H/^{35}S heparan sulfate. The values shown in Figure 3 varied less than 10% in duplicate experiments. However, as shown in Figure 3A and C, the incorporation of (^{3}H)glucosamine and $^{35}SO_4$ into human endothelial cell glycoconjugates can vary with different preparations of endothelial cells, and may represent individual differences. As with cells grown on collagen, there were consistent overall decreases in the level of labeled products released from the cell layer, with decreases in chondroitin and dermatan sulfates of 50% and decreases in heparan sulfate of 30%.

DISCUSSION

The biosynthesis of several classes of sulfated glycoconjugates was measured in vascular endothelial cells derived from human pulmonary arteries. In addition to the sulfated glycosaminoglycans heparan sulfate, chondroitin sulfate, and dermatan sulfate,

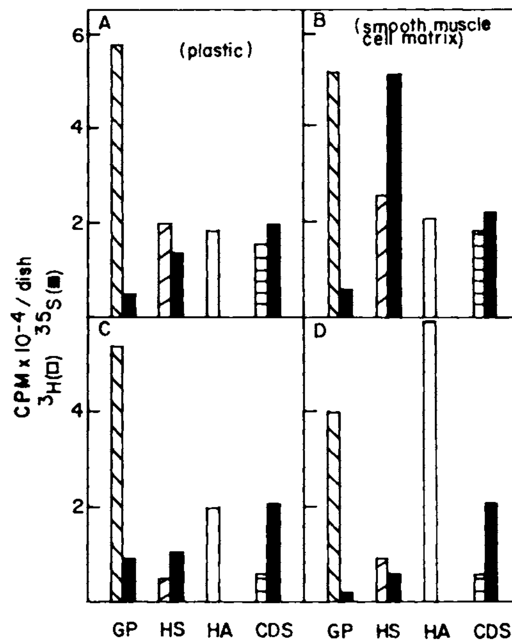

Fig. 3. Incorporation of (^{3}H)glucosamine and $^{35}SO_4$ into glycosaminoglycans and glycopeptides by human pulmonary arterial endothelial cells in culture. Cells were grown for 6 days on a plastic substratum (panels A and C) or on a Triton-insoluble smooth muscle cell matrix (panels B and D) as described in Figure 2. The smooth muscle cells were cultured from porcine aorta (Panel B) or human pulmonary artery (Panel D). Endothelial cells were labeled, and the incorporation into various glycopeptides and glycosaminoglycans on the cell layer was measured as described under Materials and Methods. The glycoconjugates are GP, glycopeptides; HS, heparan sulfate; HA, hyaluronic acid; CDS, chondroitin and dermatan sulfates.

endothelial cells synthesize a class of sulfated oligosaccharide chains that appear to be different from known glycosaminoglycans. These sulfated oligosaccharides, isolated as glycopeptides, are not susceptible to chemical and enzymatic treatments, which degrade endothelial cell glycosaminoglycans. In addition, these sulfated glycopeptides are less charged than endothelial glycosaminoglycans and do not precipitate in the presence of cetylpyridinium chloride. Endothelial cells in culture on a plastic substratum retain varying levels of the glycoconjugates they synthesize on the cell layer, the remainder being released into the culture medium. A population of glycosaminoglycans and glycopeptides were found to be very tightly associated with the plastic substratum used for cell culture. This endothelial cell basal material was enriched in the ^{35}S-glycopeptides synthesized by the cells, and also contained 20% of the cellular (^{3}H/^{35}S)-heparan sulfate and 30% of the cellular (^{3}H/^{35}S)chondroitin and dermatan sulfates. This same material was relatively poor in (^{3}H)hyaluronic acid, containing only 5% of the cellular (^{3}H)hyaluronic acid synthesized. Gordon and Bernfield have shown that basal lamina differ between tissue types with adult epithelia and embryonic epithelial sheets being rich in heparan sulfate, whereas epithelia that are at branch points or folds are rich in hyaluronate [15].

The effect of extracellular matrix materials, as a cell culture substrate, on the synthesis of sulfated glycoconjugates were also investigated. Cells grown on matrices of collagen or smooth muscle cell materials generally synthesized less total ($^3H/^{35}S$)-glycosaminoglycans, with markedly lower levels of labeled glycosaminoglycans being released from the cell into the culture medium. The synthesis of total (^3H)-glycopeptides did not vary greatly when cells were grown on these types of matrices, although there were fewer ^{35}S-glycopeptides synthesized and released from cells grown on extracellular matrix components. It has been reported [16] that mouse mammary epithelial cells do not seem to alter their rate of proteoglycan synthesis when cultured on plastic or a type I collagen gel, but rather reduce the degradation and thus increase the accumulation of heparan sulfate. The results of several studies using a Triton X-100-insoluble matrix to support the growth of endothelial cells suggest that the most frequent changes in cell-associated glycosaminoglycan synthesis are changes in hyaluronic acid and heparan sulfate. However, since matrices prepared in this manner may have lost important detergent-soluble smooth muscle cell components, further studies using more intact matrices may provide a better model of in vivo endothelial glycoconjugate synthesis.

The role of sulfation in the regulation of glycoconjugate function is not well established. Sulfate groups may contribute to the anionic charge of the saccharide chains and serve as possible recognition sites in cell surface interactions, as do phosphorylated mannosyl residues [17]. Sulfation has been suggested to play a role in the regulation of the turnover of cellular glycoproteins, since sulfated carbohydrate residues are more resistant to glycosidase attack [18]. The changes in the incorporation of (^3H)glucosamine and $^{35}SO_4$ into specific glycoconjugates may be differentially regulated by extracellular signals in the form of matrix macromolecules. The $^{35}SO_4$ incorporated into both heparan sulfate and chondroitin and dermatan sulfates is presumably derived from the same precursor pool. Thus changes in (^{35}S)heparan sulfate synthesis, under conditions where (^{35}S) chondroitin and dermatan sulfate synthesis remain nearly constant, do not appear to be due simply to changes in precursor pool sizes. Lower molecular weight extracellular matrix components produced by the cell's degradative mechanisms may also have a role in changing glycoconjugate synthesis. Soluble factors released by the degradation of matrix material on which the cells were grown could alter the synthesis and sulfation of endothelial cell glycoconjugates. Thus the interaction of smooth muscle cell extracellular matrix components with endothelial cells may have a role in regulating the synthesis of specific endothelial glycosaminoglycans and glycoproteins in a manner that reflects the dynamic state of the vascular tissue.

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