Proteoglycan Synthesis is Not Influenced by Zinc in Proliferating Bovine Aortic Endothelial Cells in Culture

Yasuyuki Fujiwara,[a] Anna H. Plaas,[b] and Toshiyuki Kaji*[, a, c]

[a]Department of Environmental Health, Faculty of Pharmaceutical Sciences, Ho-3 Kanagawa-machi, Kanazawa 920–1181, Japan, [b]Department of Internal Medicine, College of Medicine, University of South Florida, 12901 Bruce B. Downs Blvd., Tampa, FL 33612–4799, U.S.A., and [c]Organization for Frontier Research in Preventive Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920–1181, Japan

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Zinc promotes the proliferation of bovine aortic endothelial cells depending on endogenous fibroblast growth factor-2 whose activity is modulated by proteoglycans. In this study, we investigated the effects of zinc on the synthesis of proteoglycans in proliferating cells. It was demonstrated that zinc neither influenced the core protein synthesis nor the glycosaminoglycan chain formation during the synthesis of proteoglycan molecules, suggesting that zinc stimulation of endothelial cell proliferation is independent of proteoglycan synthesis.

Key words —— endothelial cell, heparan sulfate, proliferation, vascular, proteoglycan

INTRODUCTION

Vascular endothelial cells cover the luminal surface of blood vessel walls in a monolayer and function as a barrier between blood and subendothelial tissue. In case this monolayer is damaged, the endothelial cells are stimulated by fibroblast growth factor (FGF)-2 that leaks from the damaged endothelial cells, and the endothelial cells proliferate to reendothelialize the damaged site.[1] Thus, the proliferation of these cells is important for the prevention of vascular disorders that are mediated by the contact of the blood with the underlying tissue.

Proteoglycans are macromolecules that consist of a core protein and one or more glycosaminoglycan (GAG) side chains.[2] Proteoglycans are classified into two types based on the GAG structure: heparan sulfate proteoglycans (HSPGs) and chondroitin/dermatan sulfate proteoglycans (CS/DSPGs). FGF-2 is bound to a specific heparan sulfate sequence of a hexasaccharide containing 2-*O*-sulfated iduronic acid [IdoA(2S)] and *N*-sulfated glucosamine (GlcNS).[3] A dodecasaccharide containing 6-*O*-sulfated GlcNS [GlcNS(6S)] residues as well as the binding region is required for receptor signaling.[4]

It was shown that lead, a toxic heavy metal, inhibits the repair of the damaged monolayers of endothelial cells by lowering the cell response to endogenous FGF-2 due to the inhibition of perlecan synthesis, the core protein of a large heparan sulfate proteoglycan.[5] However, it was suggested that lead did not change the length[6] and the microstructure[7] of heparan sulfate chains. In contrast to lead, zinc stimulates the proliferation of vascular endothelial cells; this proliferation which is dependent on endogenous FGF-2,[8] results in repair of damaged monolayers of the cells.[9]

However, the effects of zinc on endothelial proteoglycan synthesis have yet not been elucidated. To determine whether zinc stimulation of endothelial cell proliferation is mediated by alteration in the synthesis of proteoglycan molecules and/or the formation of GAG chains, the synthesis of proteoglycan core proteins and disaccharide composition of GAGs were investigated using a culture system of proliferating bovine aortic endothelial cells after exposure to zinc.

MATERIALS AND METHODS

Materials —— Vascular endothelial cells derived from bovine aorta were purchased from Dainihon Pharmaceutical (Osaka, Japan); Dulbecco's modified Eagle's medium (DMEM), from Nissui Pharmaceutical (Tokyo, Japan); ASF 301 medium, from Ajinomoto (Tokyo, Japan); and tissue culture dishes and plates, from Iwaki (Chiba, Japan). [^{35}S]Na_2SO_4 (carrier free) and Tran^{35}S-label, a metabolic labeling reagent that consists of ~70% L-[^{35}S]methionine, ~15% L-[^{35}S]cysteine, and other ^{35}S-labeled com-

*To whom correspondence should be addressed: Department of Environmental Health, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920–1181, Japan. Tel. & Fax: +81-76-229-6208; E-mail: t-kaji@hokuriku-u.ac.jp

pounds, were purchased from MP Biomedicals (Irvine, CA, U.S.A.). DEAE-Sephacel, benzamidine, Tris base, phenylmethanesulfonyl fluoride, sodium cyanoborohydride (95% pure), ammonium hydroxide, acetic acid, boric acid, glycerol, dimethylsulfoxide (highest purity grade available), unsaturated glucuronic acid-6-*O*-sulfated *N*-acetylgalactosamine [GlcA-GalNAc(6S)], D-galactose 6-sulfate (gal6S), and unsaturated disaccharide standards of heparan sulfate were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). Chondroitinase ABC (EC 4.2.2.4, derived from *Proteus vulgaris*), chondroitinase ACII (EC 4.2.2.5; derived from *Arthrobacter aurescens*), heparinase II (derived from *Flavobacterium heparinum*), heparinase III (EC 4.2.2.8 derived from *Flavobacterium heparinum*), chondroitin sulfate A, and a standard kit of unsaturated chondroitin/dermatan sulfate disaccharide units were purchased from Seikagaku (Tokyo, Japan). Pronase and proteinase K (EC 3.4.21.64; derived from fungi, *Tritirachium album Limber*) were from Hoffmann-La Roche (Basel, Switzerland) and Invitrogen (Carlsbad, CA, U.S.A.), respectively. PD 10 columns (disposable Sephadex G-25M) and Microcon 3 (3000 MW cutoff) ultrafiltration devices were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.) and Millipore (Billerica, MA, U.S.A.), respectively. 2-aminoacridone hydrochloride was purchased from Molecular Probes (Eugene, OR, U.S.A.). Urea and sodium dodecyl sulfate (SDS) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cetylpyridinium chloride (CPC), zinc sulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Determination of Cell Number —— Vascular endothelial cells were cultured in DMEM supplemented with 10% fetal bovine serum in 100-mm dishes in a humid atmosphere of 5% CO_2 until confluent growth was obtained. The cells were then transferred into 24-well culture plates at a density of 1×10^4 cells/cm^2 and cultured for 24 hr in DMEM supplemented with 10% fetal bovine serum ("sparse culture"). After washing the cells with the medium, it was replaced by fresh serum-free ASF 301 medium, and the cells were incubated with zinc sulfate (10 μM) at 37°C for 24 hr. After incubation, the medium was discarded and the cell layer was washed twice with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (CMF-PBS). Subsequently, the cell layer was dispersed using 0.25% trypsin-0.02% EDTA in CMF-PBS, and the trypsinized cell suspension was harvested. The cultured well was washed with 10% fetal bovine serum in CMF-PBS and the washing was pooled with the corresponding cell suspension. The trypsinized cell suspension was pipetted carefully, and the cell number was counted using a hematocytometer.

Incorporation of [^{35}S]Sulfate into GAGs —— Sparse cultures of vascular endothelial cells were prepared in 24-well plates and incubated at 37°C for 3, 6, 24, or 48 hr in fresh ASF 301 medium with zinc sulfate (1, 2, 5, or 10 μM) in the presence of both [^{3}H]glucosamine (100 kBq/ml) and [^{35}S]sulfate (370 kBq/ml). After incubation, the conditioned medium was harvested and the cell layer was washed with CMF-PBS; the washing was pooled with the corresponding conditioned medium. The cell layer was incubated at 37°C for 5 min with 0.25% trypsin-0.02% EDTA in CMF-PBS. The trypsinized cell suspension was harvested and the culture well was washed with CMF-PBS; the washing was pooled with the corresponding cell suspension. The cell suspension and the conditioned medium were used for the determination of radioactive GAGs by the CPC precipitation method.[10]

DEAE-Sephacel Ion Exchange Chromatography —— Sparse cultures of vascular endothelial cells were prepared in 100-mm dishes and incubated at 37°C for 24 hr in serum-free ASF 301 medium with zinc sulfate (10 μM) in the presence of [^{35}S]sulfate (740 kBq/ml). After incubation, the conditioned medium was harvested and solid urea was added to obtain a concentration of 8 M. The cell layer was washed twice with ice-cold CMF-PBS and harvested after extraction with 8 M urea solution containing 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 10 mM *N*-ethylmaleimide, 2 mM EDTA, 0.1 M phenylmethanesulfonyl fluoride, 0.1 M NaCl, 50 mM Tris base, and 2% Triton X-100 (pH 7.5) at 4°C for 15 min; the cell layer was harvested by scraping with a rubber policeman. The medium and the cell extracts were chromatographed on PD-10 columns equilibrated in 8 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.1 M NaCl, 0.5% Triton X-100, and 50 mM Tris base to obtain macromolecules with a high molecular mass (> 3 kDa). To separate proteoglycans into HSPGs and CS/DSPGs on the basis of differences in charge density, the macromolecules were applied to a DEAE-Sephacel column (5 ml of resin) in the urea buffer, and unbound radioactive molecules were removed from the column by washing with 30 ml of the buffer. Bound radioactive molecules were eluted from the column

with a liner gradient of 0.1–0.7 M NaCl in the urea buffer (total volume of 50 ml).

Analysis of Proteoglycan Core Proteins —— Sparse cultures of vascular endothelial cells were incubated at 37°C for 24 hr in fresh ASF 301 medium with zinc sulfate (10 μM) in the presence of Tran^{35}S-label reagent (1 MBq/ml). After incubation, proteoglycans were extracted from the cell layer and the conditioned medium under a dissociative condition in the presence of 8 M urea. The extract was concentrated on 0.3-ml DEAE-Sephacel minicolumns and precipitated with 3.5 volumes of 1.3% potassium acetate in 95% ethanol; the precipitation was repeated three times. The precipitated proteoglycans were digested with heparinase II (30 mU/ml) in 0.2 M Tris–HCl buffer (pH 7.0) containing 0.1 M calcium acetate at 37°C for 4 hr to degrade heparan sulfate chains or chondroitinase ABC (1.7 U/ml) in 50 mM Tris–HCl buffer (pH 8.0) with 0.1% bovine serum albumin and 3 mM sodium acetate at 37°C for 4 hr to degrade chondroitin/dermatan sulfate chains. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on a 4–12% acrylamide gradient slab gel with a 3% stacking gel according to Laemmli's procedure.[11] The radiolabeled proteoglycan cores were visualized by autoradiography of dried gels exposed to Kodak XAR-2 film at –70°C.

Analysis of Disaccharide Composition of GAGs —— Proteoglycans, accumulated in the cell layer and in the conditioned medium of sparse cultures of vascular endothelial cells treated with zinc sulfate (10 μM) for 24 hr, were extracted under a dissociative condition in the presence of 8 M urea. The extract was concentrated on 0.3-ml DEAE-Sephacel minicolumns and precipitated with 3.5 volumes of 1.3% potassium acetate in 95% ethanol. The dried precipitate was digested overnight with proteinase K (800 μg/ml) in 0.1 M sodium acetate buffer (pH 7.2) at 60°C. After the inactivation of proteinase K at 100°C for 10 min, buffer salts and protein digests were removed by centrifugation of the digests in Microcon 3. The GAGs that were separated on the filter were recovered in 0.1 M ammonium acetate (pH 7.3) and digested at 37°C for 18 hr with both chondroitinase ABC (0.2 U/ml) and chondroitinase ACII (1 U/ml). The chondroitin/dermatan sulfate hydrolase products were recovered using Microcon 3, and dried for further processing. The undigested materials on the filter (heparan sulfate chains) were recovered in 0.1 M ammonium acetate (pH 7.0) containing 0.01% bovine serum albumin and digested at 37°C for 8 hr with both heparinase II and III (30 mU/ml, each). The heparan sulfate hydrolase products were recovered using Microcon 3 ultrafiltration devices and were then dried. The dried heparan sulfate and chondroitin/dermatan sulfate samples were fluorotagged separately at 90°C for 30 min with 2-aminoacridone hydrochloride (0.1 M) as described previously.[12,13] The fluorotagged disaccharide units of heparan sulfate were immediately separated on the separating gels (19.5% acrylamide, 0.52% *N*,*N*′-methylenebisacrylamide, 2.5% glycerol, 0.1 M Tris base, 0.09 M boric acid, 0.05% ammonium persulfate, and 0.1% TEMED) with a stacking gel (7.5% acrylamide, 0.2% *N*,*N*′-methylenebisacrylamide, 2.5% glycerol, 0.1 M Tris base, 0.09 M boric acid, 0.05% ammonium persulfate, 0.6% agarose, and 0.1% TEMED). Similarly, the fluorotagged disaccharide units of chondroitin/dermatan sulfate were separated on the separating gels (19.5% acrylamide, 0.52% *N*,*N*′-methylenebisacrylamide, 2.5% glycerol, 0.05% ammonium persulfate, 0.1% TEMED, and 0.045 M Tris-acetate buffer of pH 7.0) with a stacking gel (7.5% acrylamide, 0.2% *N*,*N*′-methylenebisacrylamide, 2.5% glycerol, 0.05% ammonium persulfate, 0.6% agarose, 0.1% TEMED, and 0.045 M Tris-acetate buffer of pH 7.0). Electrophoresis was carried out in a running buffer (0.1 M Tris-borate, pH 8.3) at 4°C. The fluorescent images were obtained using a gel documentation system (Atto, AE-6914). The bands of unsaturated heparan sulfate and chondroitin/dermatan sulfate disaccharides were quantitatively analyzed by using NIH Image analysis software that uses the bands of unsaturated GlcA-GalNAc(6S) and gal6S, respectively, as internal standards. The disaccharides were identified by comigration when compared with unsaturated disaccharide standards.

Statistical Analysis —— Data were analyzed for statistical significance by analysis of variance and Bonferroni's multiple *t*-test, where possible. *p*-Values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

First, we determined the number of vascular endothelial cells after exposure to zinc. Treatment with zinc at a concentration of 10 μM for 24 hr significantly increased the cell number by 21% (control, 13.16 ± 0.43 cells $\times 10^3/cm^2$; zinc treatment, 15.89 ± 0.49 cells $\times 10^3/cm^2$; $n = 4$; $p < 0.05$).

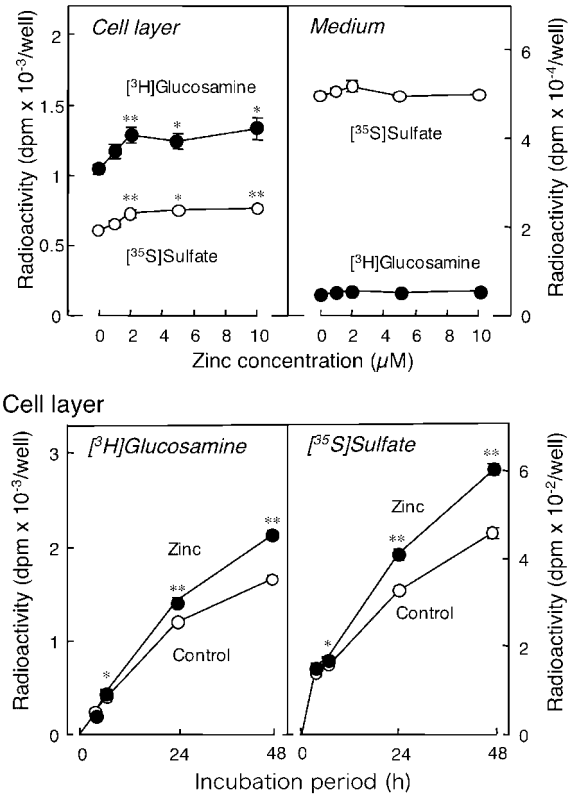

Fig. 1. Accumulation of [^{3}H]Glucosamine- and [^{35}S]Sulfate-Labeled Glycosaminoglycans in the Cell Layer and the Conditioned Medium of Cultured Vascular Endothelial Cells after Treatment with Zinc

Sparse cultures of bovine aortic endothelial cells were incubated at 37°C with at 1, 2, 5, or 10 μM zinc sulfate for 24 hr (upper panels) or with 10 μM zinc sulfate for 3, 6, 24, or 48 hr (lower panels) in the presence of [^{3}H]glucosamine and [^{35}S]sulfate. Values are means ± S.E. of four samples.

Figure 1 shows the effect of zinc on the accumulation of GAGs in the cell layer and in the conditioned medium of proliferating vascular endothelial cells. Zinc at a concentration of 2 μM or more significantly increased the accumulation of [^{3}H]glucosamine- and [^{35}S]sulfate-labeled GAGs in the cell layer after a 24-hr treatment. When the cells were treated with 10 μM zinc, the increase occurred at or later than 6 hr. However, the radioactivity was 118–128% of the control after treatment with zinc at a concentration of 2 μM or more for 24 hr; this corresponded to the increase in the cell number. These results suggest that the zinc-enhanced increase in the amount of cell layer-associated GAGs reflects an increase in the cell number. On the other hand, zinc did not change the accumulation rate of radiolabeled GAGs in the conditioned medium.

It is possible that zinc may influence the synthesis of a specific type of proteoglycans. To examine this possibility, [^{35}S]sulfate-labeled proteoglycans were separated into HSPGs and CS/DSPGs by DEAE Sephacel ion exchange chromatography (Fig. 2). The major type of proteoglycan in the cell layer was HSPGs, eluted by 0.4 M NaCl, and in the conditioned medium was CS/DSPGs, eluted by 0.55 M NaCl.[14] The elution profile after zinc treatment was almost similar to that of the control, suggesting that zinc did not influence the type of cell layer-associated proteoglycans. In the conditioned medium, CS/DSPGs predominantly accumulated in the control; however, zinc did not change the elution profile.

Proteoglycan core proteins were analyzed by SDS-PAGE to examine the possibility of zinc influencing the accumulation of the specific type of proteoglycans (HSPGs and CS/DSPGs) in the cell layer (Fig. 3). Prior to zinc treatment, the cell layer had two types of core proteins: HSPG core proteins that have a molecular mass of approximately 400 kDa and CS/DSPG core proteins that have a molecular mass of approximately 50 kDa; these are identified as the perlecan core and the biglycan core, respectively.[14] When quantified using densitometry, zinc showed an increase of approximately 20% for both types of the core proteins; this appeared to correspond with the increase in the cell number.

It is possible that zinc did not influence the synthesis of all the proteoglycan molecules, but it only changed the microstructure of the GAG chains. Figures 4 and 5 show the disaccharide compositions of heparan sulfate and chondroitin/dermatan sulfate chains, respectively, accumulated in the cell layer and the conditioned medium of proliferating vascular endothelial cells prior to and after treatment with zinc. The disaccharide units of GlcA/IdoA-*N*-acetylglucosamine (GlcA/IdoA-GlcNAc), GlcA/IdoA-GlcNS, and 2-*O*-sulfated GlcA/IdoA-GlcNS [GlcA/IdoA(2S)-GlcNS] were detected as the components of heparan sulfate in the cell layer. In addition to these, GlcA/IdoA-GlcNS(6S) and GlcA/IdoA(2S)-GlcNS(6S) were also detected in the conditioned medium. Zinc did not change the percentage of each disaccharide unit. Similarly, zinc did not change the microstructure of chondroitin/dermatan sulfate, which was detected to comprise the disaccharide units of GlcA/IdoA-GalNAc, GlcA/IdoA-GalNAc(6S), GlcA/IdoA-4-*O*-sulfated GalNAc [GlcA/IdoA-GalNAc(4S)], or GlcA/IdoA-4-*O*-, 6-*O*-sulfated GalNAc [GlcA/IdoA-GalNAc(4S,6S)].

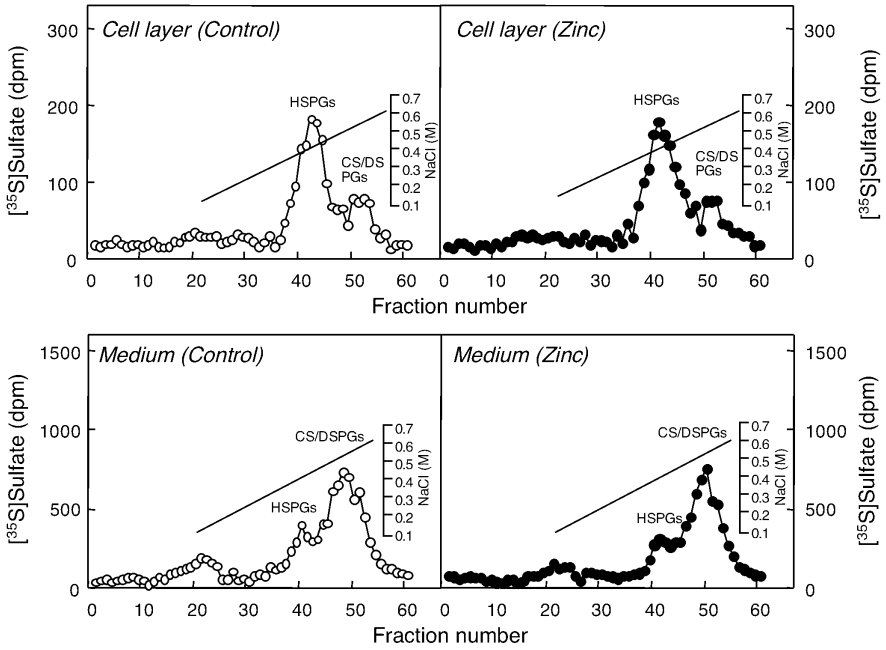

Fig. 2. DEAE-Sephacel Ion Exchange Chromatography of [^{35}S]Sulfate-Labeled Proteoglycans Extracted from the Cell Layer and the Conditioned Medium of Vascular Endothelial Cells after Exposure to Zinc

Sparse cultures of bovine aortic endothelial cells were incubated at 37°C with 10 μM zinc sulfate for 24 hr in the presence of [^{35}S]sulfate.

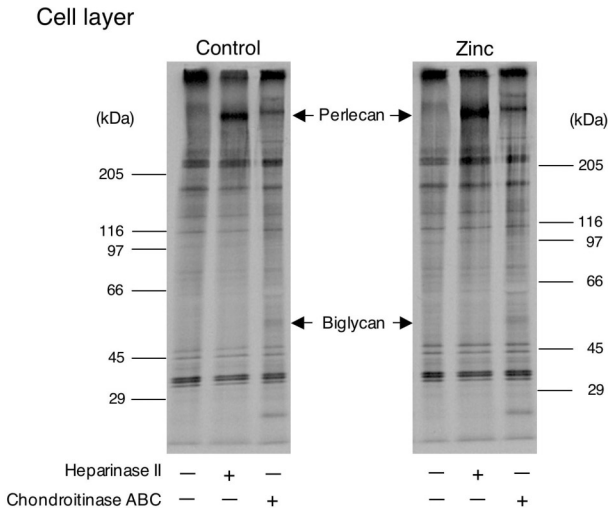

Fig. 3. SDS-Polyacrylamide Gel Electrophoresis of Heparan Sulfate and Chondroitin/Dermatan Sulfate Proteoglycan Core Proteins Metabolically Labeled with ^{35}S-Labeled Amino Acids Extracted from the Cell Layer of Vascular Endothelial Cells after Exposure to Zinc

Proteoglycans extracted from the cell layer of sparse bovine aortic endothelial cells after digestion with heparinase II or chondroitinase ABC were run on a 4–12% gradient slab gel. Arrows indicate the bands of perlecan and biglycan core proteins.

DISCUSSION

Proliferation of vascular endothelial cells strongly depends on endogenous FGF-2.[15,16] Although the biological activities of FGF-2 are mediated by the high affinity cell surface receptor for FGF-2 (FGFR), heparan sulfate chains of perlecan play a crucial role in exhibiting the FGF-2 activity.[17] The interaction between FGF-2 and FGFR is modulated by heparan sulfate chains through the formation of a FGF-2•heparan sulfate chain•FGFR ternary complex.[18] The heparan sulfate chains may induce FGF-2 oligomerization that promotes FGFR dimerization and signal transduction.[19] The number of HSPG molecules, particularly perlecan molecules, and the structure of their heparan sulfate chains can influence the activity of FGF-2 in vascular endothelial cells. In this study, we investigated the synthesis and the structure of proteoglycans in vascular endothelial cells because zinc stimulates the proliferation of these cells depending on endogenous FGF-2.[8] The results suggest that zinc influences neither the synthesis of perlecan core proteins nor the microstructure of heparan sulfate chains. This clearly indicates that zinc stimulation of vascular endothelial cell proliferation is not mediated by changes of proteoglycan synthesis but is caused by some other mechanism(s).

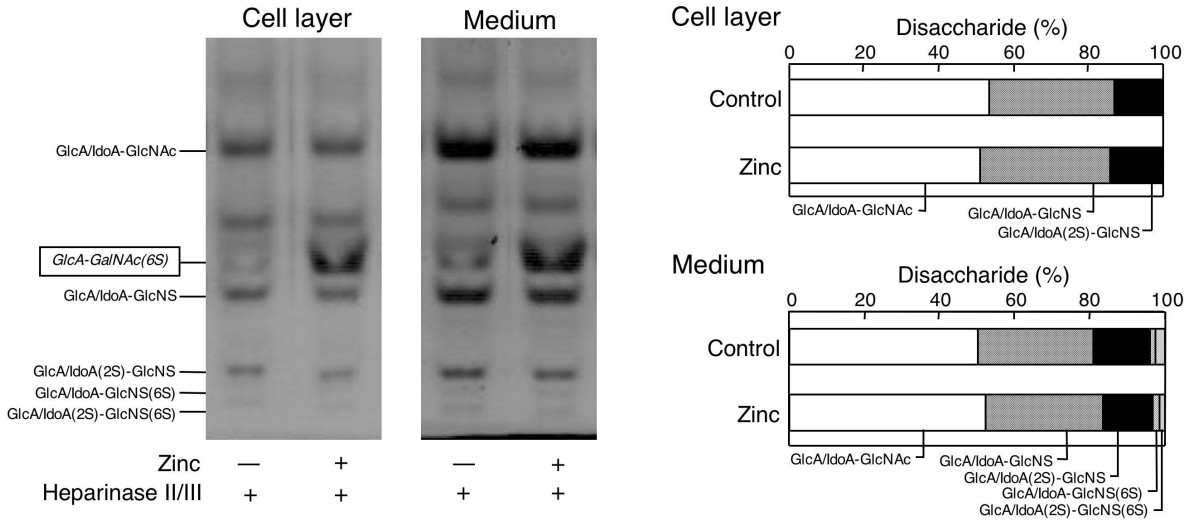

Fig. 4. Fluorophore-Assisted Carbohydrate Electrophoresis Analysis of Heparan Sulfate Extracted from the Cell Layer and the Conditioned Medium of Vascular Endothelial Cells after Treatment with Zinc

Sparse cultures of bovine aortic endothelial cells were incubated at 37°C with 10 μM zinc sulfate for 24 hr.

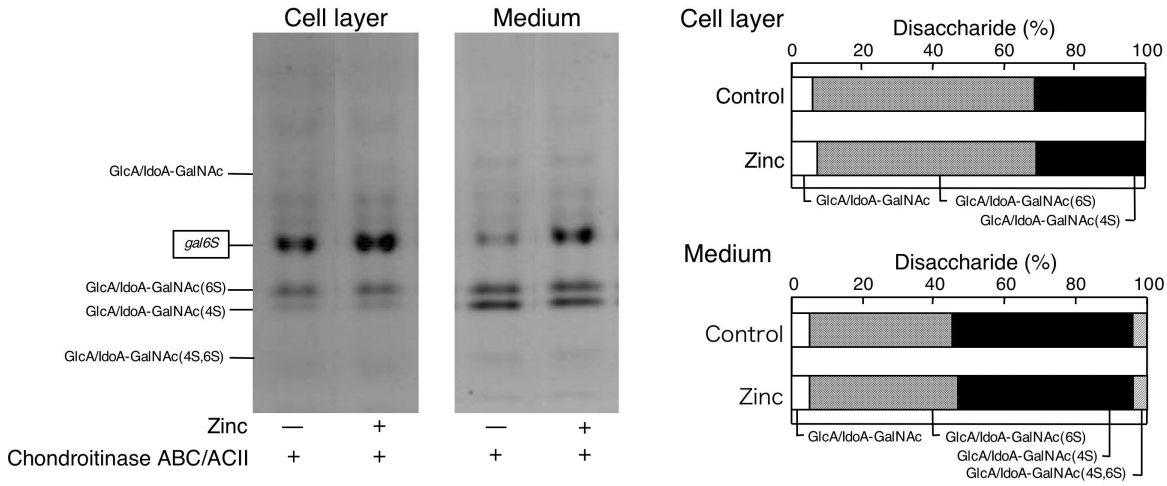

Fig. 5. Fluorophore-Assisted Carbohydrate Electrophoresis Analysis of Chondroitin/Dermatan Sulfate Extracted from the Cell Layer and the Conditioned Medium of Vascular Endothelial Cells after Treatment with Zinc

Sparse cultures of bovine aortic endothelial cells were incubated at 37°C with 10 μM zinc sulfate for 24 hr.

We have shown that lead, a heavy metal, inhibits vascular endothelial cell proliferation by lowering the response to endogenous FGF-2 through the suppression of perlecan synthesis[6] although the length[6] and disaccharide composition of the heparan sulfate chains are not changed.[7] This suggests that zinc and lead have opposite effects on the proliferation of vascular endothelial cells because zinc intensifies the activity of endogenous FGF-2, and lead suppresses this activity; however, the mechanisms by which these metals change the FGF-2 activity is different. In particular, inhibition by lead is mediated by suppression of the perlecan synthesis whereas stimulation by zinc is induced by mechanisms other than proteoglycan synthesis. It has been suggested previously that lead-induced inhibition of endothelial HSPG synthesis may be due to excess accumulation of intracellular Ca^{2+}.[20] On the other hand, zinc stimulates the proliferation of vascular endothelial cells through the lipoxygenase pathway that mediates the stimulation of endogenous FGF-2,[8] suggesting that zinc may directly act on intracellular mechanisms for enhancing the FGF-2 activity. However, the exact mechanism remains to be

elucidated.

Further, the effects of zinc and lead on the proliferation of vascular smooth muscle cells are different. Zinc alone does not influence vascular smooth muscle cell proliferation but potentiates the stimulation by FGF-1,[21] FGF-2,[21] and thrombospondin.[22] In contrast, lead alone stimulates the proliferation probably by the activation of the intracellular calcium-dependent pathway but does not interact with FGF-1, FGF-2, and platelet-derived growth factor.[23] In summary, zinc and lead have specific effects on vascular smooth muscle cells as well as on endothelial cells. In the system, other heavy metals including copper, manganese, and nickel have no effect on the vascular endothelial[8] and smooth muscle cells.[23] Further studies should be carried out to clarify the mechanisms by which zinc and lead exhibit specific activities on the proliferation of vascular endothelial and smooth muscle cells.

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