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Letter

Cell density-dependent modulation of perlecan synthesis by dichloro(2,9-dimethyl-1,10-phenanthroline)zinc(II) in vascular endothelial cells

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ABSTRACT — Proteoglycans that are synthesized by vascular endothelial cells contribute to the proliferation, migration, and blood coagulation-fibrinolytic system in vascular endothelial cells. Clarification of the molecular mechanisms for proteoglycan synthesis allows understanding of the regulation of endothelial functions. The research strategy of bioorganometallics analyzes biological systems using organic-inorganic hybrid molecules as tools. The present study found dichloro(2,9-dimethyl-1,10-phenanthroline) zinc(II) and its ligand-modulated perlecan expression in vascular endothelial cells, which depends on the cell density.

Key words: Bioorganometallics, Proteoglycan, Perlecan, Vascular endothelial cell, Zinc complex, 2,9-Dimethyl-1,10-phenathroline

INTRODUCTION

Proteoglycans are extracellular matrix components that consist of a core protein and one or more glycosaminoglycan (GAG) side chains, which are covalently bound to the core protein (Ruoslahti, 1988). There are two proteoglycan types that are synthesized by the vascular endothelial cells: one is the heparan sulfate proteoglycan, while the other is the dermatan sulfate proteoglycan (Yamamoto *et al.*, 2005). The former includes perlecan as its major component and a transmembrane syndecan family, such as syndecan-1 and -4, while the latter type is mostly all biglycan, which is composed of a small leucine-rich proteoglycan.

These proteoglycans contribute to the antithrombogenic properties of the vascular endothelial cells through regulation of the blood coagulation-fibrinolytic system. Specifically, heparan sulfate and dermatan sulfate chains activate antithrombin III (Mertens *et al.*, 1992) and heparin cofactor II (Tollefsen *et al.*, 1983), respectively, leading to anticoagulant activity. Although we have reported that some cytokines and growth factors regulate the expression of vascular endothelial proteoglycans in a cell density-dependent manner (Kaji *et al.*, 2000), the regulation was not proteoglycan-type specific.

Bioorganometallics as a research strategy analyzes biological systems using organic-inorganic hybrid molecules as tools (Fujie *et al*., 2016). Organic-inorganic hybrid molecules consist of metal(s) and organic ligand(s). The molecules exhibit unique biological activities and can be used to analyze biological systems. We have studied the cytotoxicity of organic-inorganic hybrid molecules (Hara *et al*., 2019, 2018; Fujie *et al*., 2019), and have demonstrated the hypoxia-inducible factor-1α/β pathway (Hara *et al*., 2017c) and the p38 MAPK pathway (Hara *et al*., 2018) as the intracellular signaling pathways that mediate the synthesis of syndecan-4, a small heparan sulfate proteoglycan, in vascular endothelial cells.

We have previously reported that the cytotoxicity of dichloro(2,9-dimethyl-1,10-phenanthroline)zinc(II) (Zn-DMP) is low among cells tested with zinc complexes (Hara *et al.*, 2016). In the present study, we investigated that the effects of Zn-DMP on proteoglycan synthesis in vascular endothelial cells of different cell densities to examine whether this compound can be used for analysis of endothelial proteoglycan synthesis.

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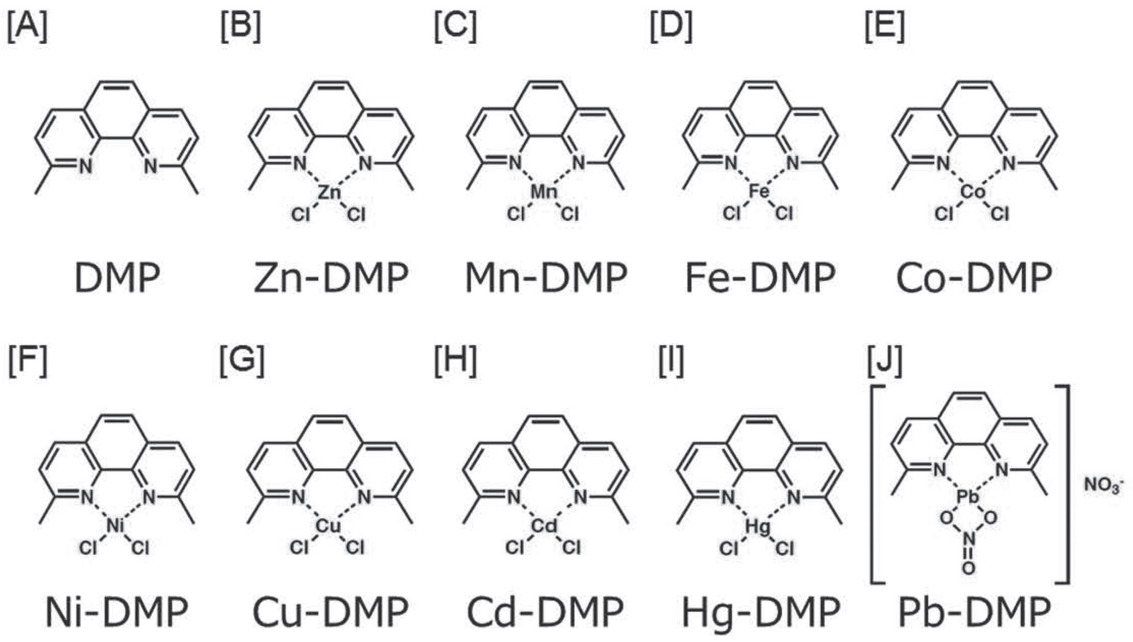

Fig. 1. Structures of DMP and metal complexes used in this study. [A] 2,9-Dimethyl-1,10-phenanthroline (DMP), [B] dichloro(2,9-dimethyl-1,10-phenanthroline)zinc(II) (Zn-DMP), [C] dichloro(2,9-dimethyl-1,10-phenanthroline)manganase(II) (Mn-DMP), [D] dichloro(2,9-dimethyl-1,10-phenanthroline)iron(II) (Fe-DMP), [E] dichloro(2,9-dimethyl-1,10-phenanthroline)cobalt(II) (Co-DMP), [F] dichloro(2,9-dimethyl-1,10-phenanthroline)nickel(II) (Ni-DMP), [G] dichloro(2,9-dimethyl-1,10-phenanthroline)copper(II) (Cu-DMP), [H] dichloro(2,9-dimethyl-1,10-phenanthroline)cadmium(II) (Cd-DMP), [I] dichloro(2,9-dimethyl-1,10-phenanthroline)mercury(II) (Hg-DMP), and [J] 2,9-dimethyl-1,10-phenanthrolinebis(nitrato) lead(II) (Pb-DMP).

MATERIALS AND METHODS

Materials

The bovine aortic endothelial cells and bovine vascular smooth muscle cells were purchased from Cell Applications (San Diego, CA, USA). Tissue culture dishes and plates were from AGC Techno Glass (Shizuoka, Japan). Dulbecco's modified Eagle's medium (DMEM) and Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (CMF-PBS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) and chondroitin ABC lyase (EC 4.2.2.4, derived from *Proteus vulgaris*) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). 2,9-dimethyl-1,10-phenanthroline (DMP) was obtained from Tokyo Chemical Industry (Tokyo, Japan) and DMP complexes (shown in Fig. 1) were synthesized, as previously described (Nakamura *et al.*, 2017). The QIAzol lysis reagent was purchased from QIAGEN (Valencia, CA, USA), and GeneAce SYBR qPCR mix α was obtained from Nippon Gene (Tokyo, Japan). The high-capacity cDNA reverse transcription kit was purchased from Thermo Fisher Scientific. The anti-perlecan antibody (sc377219) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while the anti-biglycan antibody (ab94460) was purchased from Abcam (Bristol, UK). The horseradish peroxidase-conjugated anti-rabbit IgG (#7074) and anti-mouse IgG (#7076) were obtained from Cell Signaling Technology (Beverly, MA, USA). Heparinase II (derived from *Flavobacterium heparinum*) and heparinase III (EC 4.2.2.8, derived from *F. heparinum*) were purchased from IBEX Technologies (Montreal, QC, Canada). DEAE Sephacel and Amersham™ Hybond™ P PVDF 0.2 were obtained from GE Healthcare UK Ltd (Amersham Place, UK). [^{35}S]Na_2SO_4([^{35}S]sulfate) was purchased from Perkin Elmer (Waltham, MA USA). Furthermore, other reagents of the highest available grade were obtained from Nacalai Tesque (Kyoto, Japan).

Cell culture and treatments

Vascular endothelial cells and vascular smooth muscle cells were cultured until confluence in a humidified atmosphere of 5% CO_2 at 37°C in DMEM that was supplemented with 10% FBS. They were then transferred into dishes or plates and cultured until confluence ("dense culture") or into dishes or plates at 1×10^4 cells/cm^2 and

cultured for 24 hr ("sparse culture"). After incubation, the medium of the dense and sparse culture of the vascular endothelial cells and vascular smooth muscle cells was discarded, and the cells were washed twice with serum-free DMEM, after which the following experiments were performed.

Incorporation of [^{35}S]sulfate into GAGs

The dense and sparse cultures of the vascular endothelial cells and vascular smooth muscle cells were treated with metal complexes with DMP, DMP, or $ZnCl_2$ (10, 20, and 30 μM) for 12, 24, and 48 hr in the presence of [^{35}S] sulfate (1 MBq/mL). After incubation, [^{35}S]sulfate incorporation into GAGs was determined by the cetylpyridinium chloride precipitation method, as had been reported in our previous study (Kaji *et al.*, 2000).

Real time reverse transcription polymerase chain reaction (RT-PCR)

The dense and sparse culture of the vascular endothelial cells were treated with Zn-DMP (10, 20, and 30 μM) for 12 and 24 hr, respectively. Extraction of the total RNA from the vascular endothelial cells and the synthesis of the complement DNA were previously described (Hara *et al.*, 2017a). RT-PCR was performed using the GeneAce SYBR qPCR mix α with 1 ng/μL of cDNA and 0.1 μM of primers; bovine perlecan, 5′-ATGGCAGCGATGAAGCGGAC-3′ (forward), and 5′-TTGTGGACACGCAGCGGAAC-3′ (reverse); bovine biglycan, 5′-GCTGCCACTGCCATCTGAG-3′ (forward), and 5′-CGAGGACCAAGGCGTAG-3′ (reverse); bovine beta 2 microglobulin (B2M), 5′-CCATCCAGCGTCCTCCAAAGA-3′ (forward), and 5′-TTCAATCTGGGGTGGATGGAA-3′ (reverse) in the CFX Connect™ real-time PCR system (BioRad). Levels of perlecan, biglycan, and B2M mRNAs were quantified using the relative standard curve method. The fold change in the intensity value of the target gene was normalized to that of the B2M.

Proteoglycan core protein extraction and western blot analysis

The dense and sparse culture of the vascular endothelial cells were treated with Zn-DMP (10, 20, and 30 μM) for 48 hr. Proteoglycans were extracted and concentrated from both the cell layer and conditioned medium of vascular endothelial cells under dissociative conditions, as previously described (Hara *et al.*, 2017a). Concentrated proteoglycans were dissolved with either heparinase II/III (0.083 IU/mL of heparinase II and 0.083 IU/mL of heparinase III in 100 mM of Tris-HCl buffer (pH 7.0) that contains 10 mM of calcium acetate and 18 mM of sodium acetate) for 3 hr at 37°C to determine the core proteins of perlecan, or chondroitinase ABC (1.67 U/mL of chondroitinase ABC in 50 mM of Tris-HCl buffer (pH 8.0) that contains 0.1 mg/mL of bovine serum albumin and 3 mM of sodium acetate) for 3 hr at 37°C to determine the core proteins of biglycan. The proteoglycans were lysed in sodium dodecyl sulfate (SDS) sample buffer [(50 mM Tris-HCl buffer solution that contains 2% SDS and 10% glycerol (pH 6.8)] and then incubated at 95°C for 3 min. Proteoglycans were separated by SDS-polyacrylamide gel electrophoresis on a 4-12% polyacrylamide gel and transferred onto a PVDF membrane at 2 mA/cm^2 followed by incubation for 1 hr. Membranes were blocked for 1 hr with 5% skim milk in 20 mM Tris-HCl buffer solution (pH 7.5) that contained 150 mM of NaCl and 0.1% Tween 20 (TTBS) or 2% BSA-TTBS solution, and incubated overnight with a primary antibody at 4°C. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence western blot detection reagent, Chemi-Lumi One Super, and scanned using the Amersham Imager 600 (GE Healthcare). Representative blots from the two independent experiments are shown.

Statistical analysis

Data were analyzed for statistical significance by Dunnett's or Tukey's tests, when possible. *P* values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Figure 1 shows the structures of the DMP compounds used in this study. We first investigated whether Zn-DMP influences the total amount of proteoglycans synthesized by vascular endothelial cells and vascular smooth muscle cells. As shown in Fig. 2, Zn-DMP significantly enhanced the accumulation of [^{35}S]sulfate-labeled proteoglycans in both the cell layer and conditioned medium of a dense culture of vascular endothelial cells (Figs. 2A and 2C). However, the accumulation of [^{35}S]sulfate-labeled proteoglycans was significantly suppressed by Zn-DMP in both the cell layer and conditioned medium of the corresponding sparse culture (Figs. 2B and 2D). On the other hand, the accumulation of [^{35}S]sulfate-labeled proteoglycans was unchanged by Zn-DMP in the cell layers of both dense and sparse cultures of vascular smooth muscle cells (Figs. 2E, 2F, 2G, and 2H). In the conditioned medium of dense and sparse cultures of vascular smooth

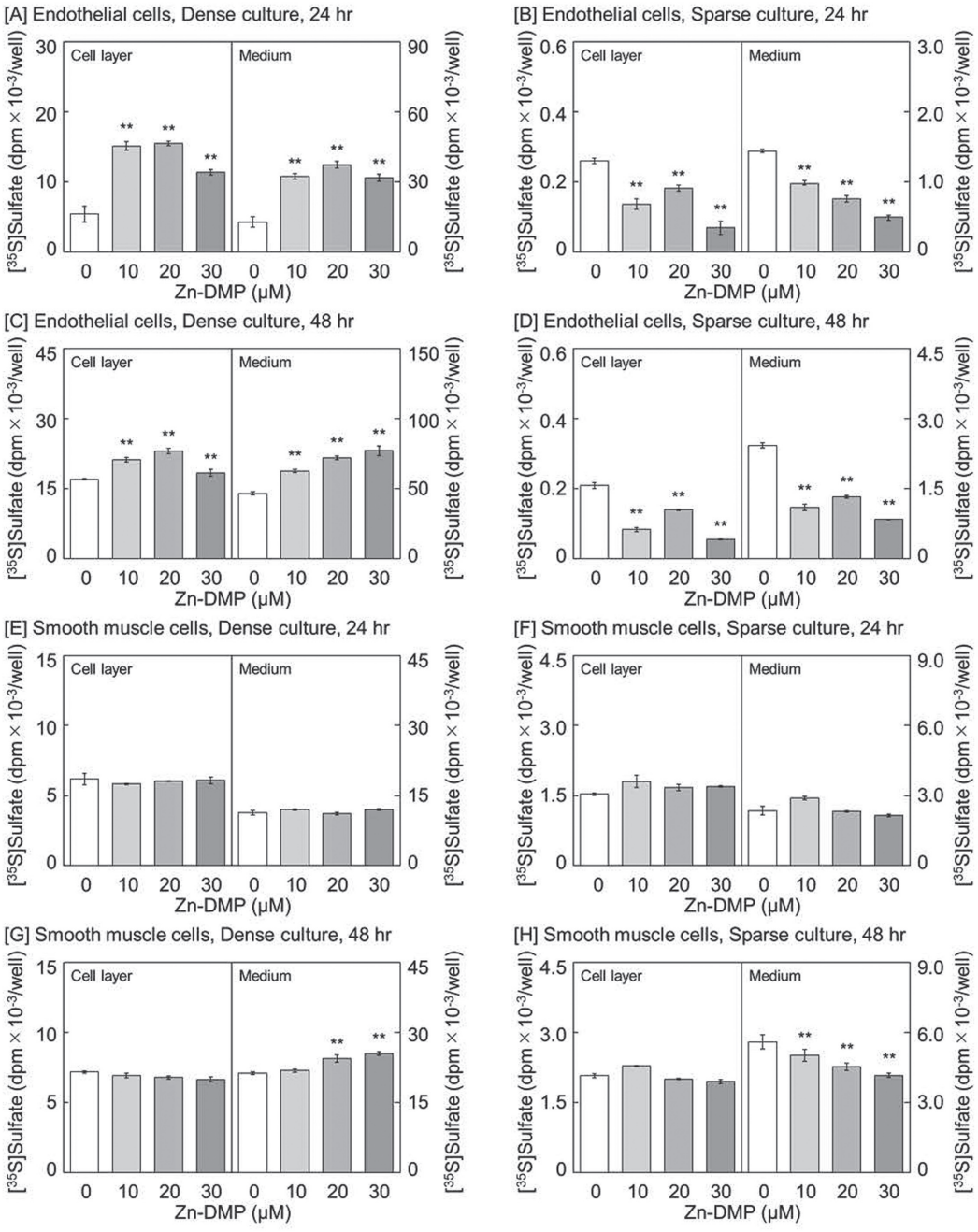

Fig. 2. The accumulation of [^{35}S]sulfate-labeled proteoglycans in the cell layer and conditioned medium of vascular endothelial and smooth muscle cells after treatment with Zn-DMP. Dense and sparse cultures of bovine aortic endothelial and smooth muscle cells were incubated with Zn-DMP (10, 20, and 30 μM) at 37°C for 24 and 48 hr in the presence of [^{35}S]sulfate. Values of the 4 samples are represented as means ± S.E. Significant difference from the corresponding control is represented by ** $P < 0.01$. [A] Dense cultures of vascular endothelial cells after 24-hr treatment with Zn-DMP. [B] Sparse cultures of vascular endothelial cells after 24-hr treatment with Zn-DMP. [C] Dense cultures of vascular endothelial cells after 48-hr treatment with Zn-DMP. [D] Sparse cultures of vascular endothelial cells after 48-hr treatment with Zn-DMP. [E] Dense cultures of vascular smooth muscle cells after 24-hr treatment with Zn-DMP. [F] Sparse cultures of vascular smooth muscle cells after 24-hr treatment with Zn-DMP. [G] Dense cultures of vascular smooth muscle cells after 48-hr treatment with Zn-DMP. [H] Sparse cultures of vascular smooth muscle cells after 48-hr treatment with Zn-DMP.

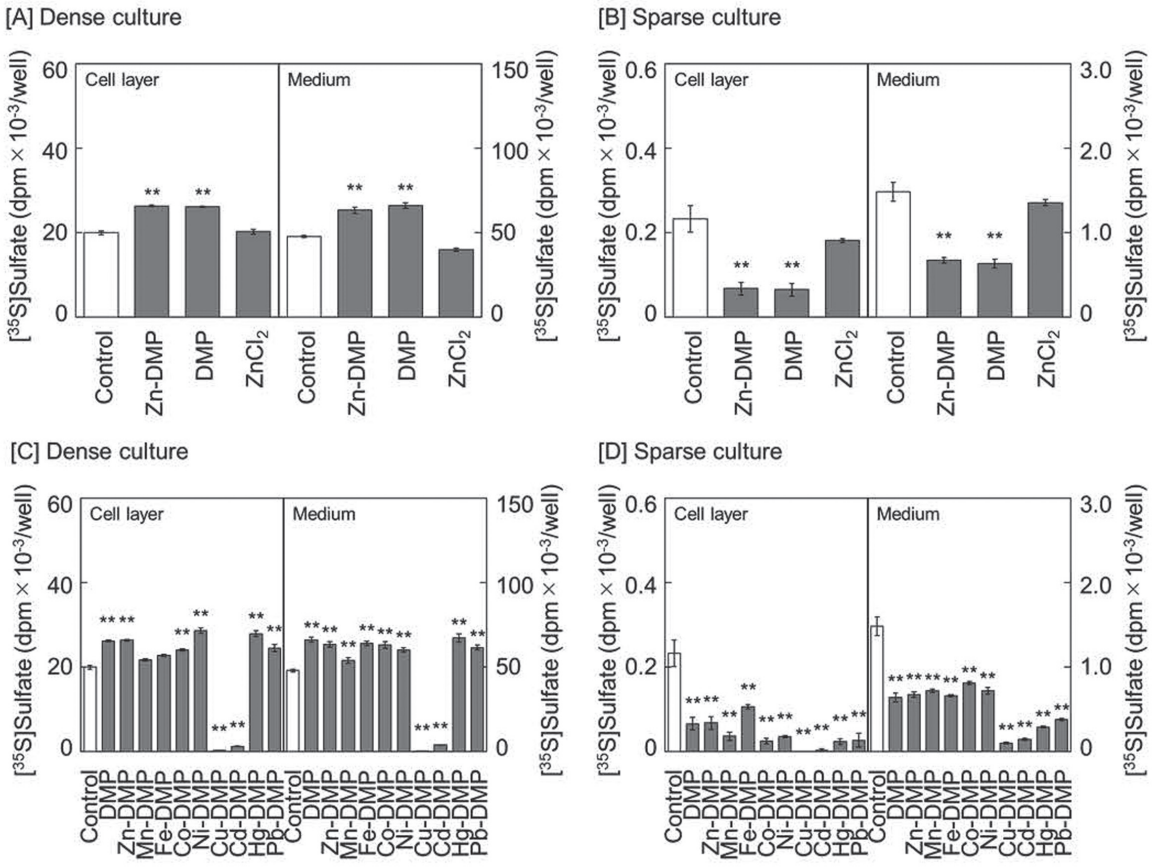

Fig. 3. The accumulation of [^{35}S]sulfate-labeled proteoglycans in the cell layer and conditioned medium of vascular endothelial cells after treatment with Zn-DMP and its related compounds. Dense and sparse cultures of bovine aortic endothelial cells were incubated with Zn-DMP or its related compounds (10 μM each) at 37°C for 48 hr in the presence of [^{35}S]sulfate. Values of the 4 samples are represented as means ± S.E. Significant difference from the corresponding control is represented by ** $P < 0.01$. [A] Dense and [B] sparse cultures of vascular endothelial cells after treatment with Zn-DMP, DMP, and $ZnCl_2$. [C] Dense and [D] sparse cultures of vascular endothelial cells after treatment with DMP, Zn-DMP, Mn-DMP, Fe-DMP, Co-DMP, Ni-DMP, Cu-DMP, Cd-DMP, Hg-DMP, and Pb-DMP.

muscle cells, the accumulation of [^{35}S]sulfate-labeled proteoglycans was unchanged after 24-hr treatment with Zn-DMP (Figs. 2E and 2F); however, after 48-hr treatment, the zinc complex significantly increased the accumulation of [^{35}S]sulfate-labeled proteoglycans in a dense culture of vascular smooth muscle cells, but significantly decreased it in the corresponding sparse culture (Figs. 2G and 2H). These results suggest that Zn-DMP is a zinc complex that modulates proteoglycan synthesis in vascular endothelial cells depending on cell density; additionally, vascular endothelial cells, as a cell type, are susceptible to this zinc complex.

To determine the functional structure of Zn-DMP on endothelial proteoglycan synthesis, dense and sparse cultures of vascular endothelial cells were treated with Zn-DMP components—the ligand DMP and inorganic zinc—along with DMP complexes consisting of manganese, iron, cobalt, nickel, copper, cadmium, mercury, or lead (Mn-DMP, Fe-DMP, Co-DMP, Ni-DMP, Cu-DMP, Cd-DMP, Hg-DMP, and Pb-DMP, respectively). Both Zn-DMP and DMP significantly increased the accumulation of [^{35}S]sulfate-labeled proteoglycans to the same degree in both the cell layer and conditioned medium of a dense culture of vascular endothelial cells (Fig. 3A). Similarly, in a sparse culture of the cells, both Zn-DMP and DMP significantly decreased to the same degree the accumulation of [^{35}S]sulfate-labeled proteoglycans in both the cell layer and conditioned medium (Fig. 3B). Thus, it is suggested that DMP, which is the ligand structure of Zn-DMP, is responsible for the modulation of proteoglycan

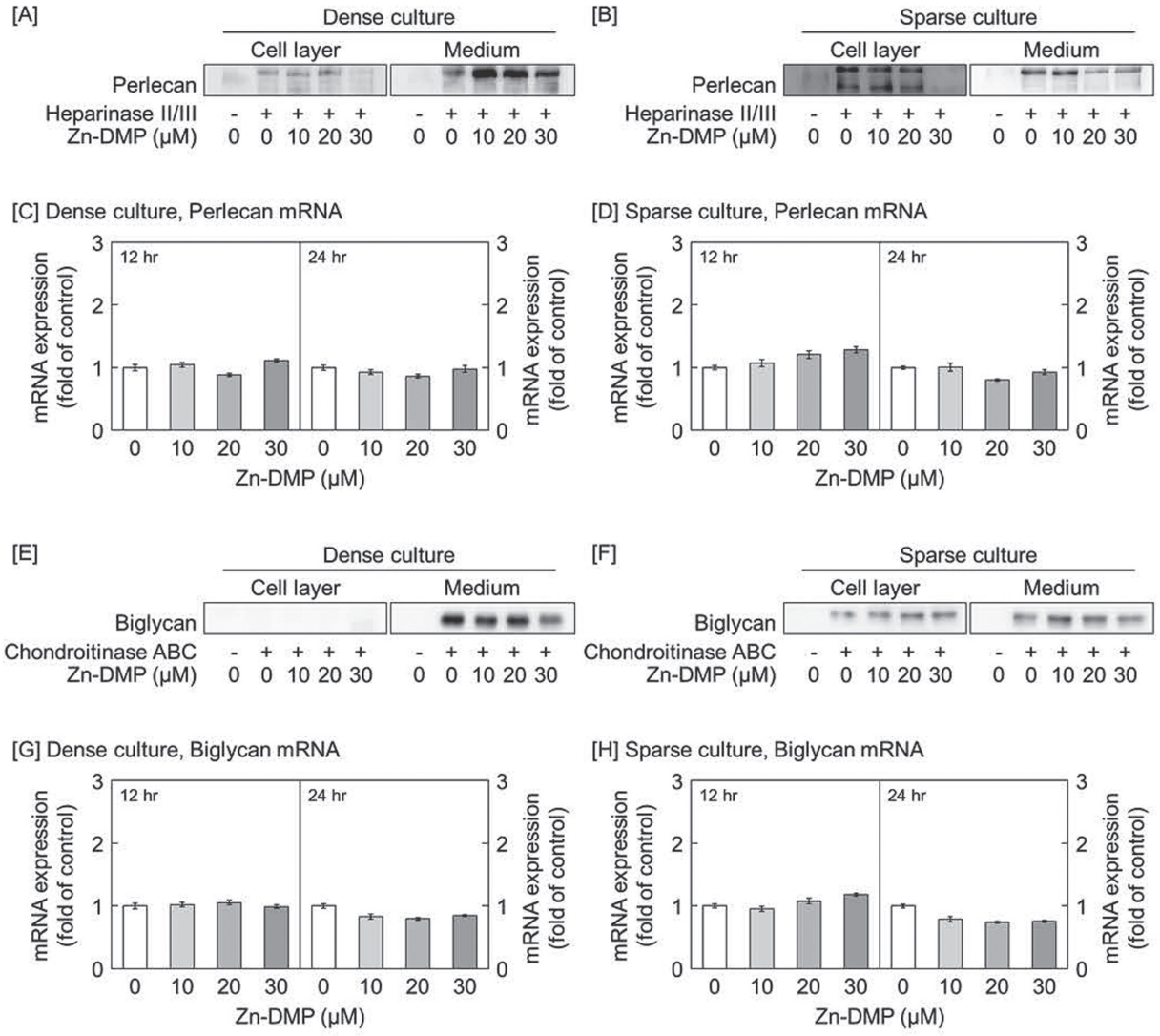

Fig. 4. Expression of perlecan and biglycan core proteins and mRNAs in vascular endothelial cells after treatment with Zn-DMP. Dense and sparse cultures of bovine aortic endothelial cells were incubated with Zn-DMP (10, 20, and 30 μM) at 37°C for 12, 24, and 48 hr. Values of the 4 samples are represented as means ± S.E. [A] Perlecan core protein expression in dense cells and [B] in sparse cells. [C] Perlecan mRNA expression in dense cells and [D] in sparse cells. [E] Biglycan core protein expression in dense cells and [F] in sparse cells. [G] Biglycan mRNA expression in dense cells and [H] in sparse cells.

synthesis; further, intramolecular zinc does not disturb this modulation. On the other hand, Co-DMP, Ni-DMP, Hg-DMP, and Pb-DMP as well as DMP and Zn-DMP modulate the accumulation of [^{35}S]sulfate-labeled proteoglycans in both the cell layer and conditioned medium of dense and sparse cultures (Figs. 3C and 3D); Mn-DMP and Fe-DMP failed to increase the accumulation of [^{35}S] sulfate-labeled proteoglycans in the cell layer of dense cells. Cu-DMP and Cd-DMP exhibited cytotoxicity, as had been reported previously (Nakamura *et al.*, 2017). These results suggest that there are metals that partly disturb the modulation of endothelial proteoglycan synthesis by DMP, regardless of cell density.

Since a large heparan sulfate proteoglycan perlecan and a small dermatan sulfate proteoglycan biglycan are the major proteoglycan types synthesized by cultured vascular endothelial cells (Yamamoto *et al.*, 2005; Kaji *et al.*, 2006; Hara *et al*., 2017b), we determined which of the

two is modulated by Zn-DMP (Fig. 4). With Zn-DMP, the accumulation of perlecan core protein in both the cell layer and conditioned medium was increased and decreased in dense cells (Fig. 4A) and sparse cells (Fig. 4B), respectively, although the expression of perlecan mRNA was not changed (Figs. 4C and 4D). However, the expression of both the biglycan core protein (Figs. 4E and 4F) and mRNA (Figs. 4G and 4H) was unchanged by exposure to Zn-DMP (Figs. 4E-4H). This suggests that modulation of endothelial cell proteoglycan synthesis occurs in perlecan core protein synthesis, without an increase in perlecan mRNA expression.

Although regulation of proteoglycan synthesis in vascular endothelial cells can operate in a cell-density-dependent manner (Kaji *et al.*, 2000), little is known about the intracellular signal pathways that mediate cell-density-dependent regulation. Bioorganometallics may be an effective strategy to analyze such pathways as we have previously demonstrated (Hara *et al*., 2017c, 2018). Further studies will be required for clarification of the intracellular signal pathways that mediate the regulation of synthesis of perlecan, an essential molecule for the proliferation of vascular endothelial cells (Lord *et al*., 2014), using organic inorganic hybrid molecules.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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