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Pro-α3(V) collagen chain is expressed in bone and its basic N-terminal peptide adheres to osteosarcoma cells

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Abstract

The third α-chain of type V collagen, α3(V) chain, was initially identified in the placenta more than 20 years ago, but was poorly characterized with regard to its expression and function. We generated a specific monoclonal antibody against the N-terminal domain of the pro-α3(V) chain and examined gene expression using immunohistochemical methods combined with in situ hybridization. The pro-α3(V) chain was seen in funis and amnion, but not chorionic villi and deciduas of mouse placenta. In mouse embryo, the transcripts of the pro-α3(V) gene were seen in tissues that were related to bone formation as well as developing muscle and nascent ligament previously reported (J. Biol. Chem. 275, 8749–8759, 2000). However, immunohistochemistry showed that pro-α3(V) protein accumulated rather in the developing bone of mouse embryo. On the other hand, the N-terminal globular domain of the pro-α3(V) chain has a unique structure that contains a highly basic segment of 23 amino acids. The peptide derived from the basic segment showed a specific adhesive feature to osteosarcoma cells but not to chondrosarcoma cells. The four heparin binding sites in the basic segment equally contribute toward adhesion to the osteosarcoma cells. Our data suggested that N-terminal globular domain of the pro-α3(V) chain influence bone formation of osteoblasts through proteoglycan on the cell surface during development or regeneration.

Keywords: Pro-α3(V) collagen; Bone formation; Heparin binding; Gene expression

1. Introduction

The collagens are major constituents of the extracellular matrix. Among them, fibrillar collagen, which include five different molecular types, I, II, III, V and XI, participate in the formation of fibrils (Vuorio and de Crombrugghe, 1990; van der Rest and Garrone, 1991; Brown and Timpl, 1995). Collagen V is a minor component of connective tissues but it plays an important role in matrix organization. Mutations in the collagen V gene produce abnormal fibril aggregates in the tissue (Andrikopoulos et al., 1995; Toriello et al., 1996; De Paepe et al., 1997; Michalickova et al., 1998). Type V collagen co-polymerizes with the major collagen, type I, and regulates the diameter of collagen fibers in non-cartilage tissues. Collagen V is widely distributed in vertebrate tissues as an $[\alpha1(V)]_2\alpha2(V)$ heterotrimer (Fessler and Fessler, 1987; Fichard et al., 1994). Other forms of collagen V include an $[\alpha1(V)]_3$ homotrimer secreted by a line of Chinese hamster cells (Haralson et al., 1980) that may also exist in normal tissues (Moradi-Améli et al., 1994; Kumamoto and Fessler, 1980), and a poorly characterized α1(V)α2(V)α3(V) heterotrimer that

Abbreviations: PBS, Phosphate-buffered saline; RT-PCR, Reverse transcription-polymerase chain reaction; IPTG, Isopropyl-β-D-thiogalacto-pyranoside; GST, Glutathione S-transferase; ELISA, Enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's medium.

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is primarily isolated from placenta (Rhodes and Miller, 1981; Niyibizi et al., 1984) but also reported in uterus, skin, and synovial membranes (Fessler and Fessler, 1987; Abedin et al., 1982).

Collagen specifically interacts with other macromolecules in the extracellular matrix such as fibronectin, laminin and proteoglycan (Hynes and Yamada, 1982; Timpl and Brown, 1994; Scott, 1988) and with cell-surface receptor, integrins (Kramer and Marks, 1989; Plow et al., 2000; Ruggiero et al., 1996). The interactions are important in regulating cell behavior, including proliferation, migration, and differentiation during development and physiological and pathological conditions. Heparin is abundant in the tissues as a form of heparin sulfate proteoglycans. Heparin binding sites are found in the extracellular matrix proteins such as collagens, fibronectin, tenascin, and laminin. Common structural motifs have been proposed from the analysis of different heparin binding sites (Cardin and Weintraub, 1989).

Type V collagen, $[\alpha1(V)]_2\alpha2(V)$ form, was shown to possess a site that binds heparin/heparan sulfate under physiological conditions (Cardin and Weintraub, 1989; LeBaron et al., 1989). This site is located within the NH_2-terminal half of the α1(V) chain (Yaoi et al., 1990; Delacoux et al., 1998). Delacoux et al. (2000) narrowed the region down to a 12 kDa fragment that contains a cluster of seven basic amino acids. Unlike α1(V) chains, α2(V) and α3(V) chains do not bind heparin under physiological or denaturing conditions (Mizuno and Hayashi, 1996). Triple helical type V collagen trimers bind to heparin with decreasing affinity in the order $[\alpha1(V)]_3 > [\alpha1(V)]_2\alpha2(V) > \alpha1(V)\alpha2(V)\alpha3(V)$, indicating that α1(V) chains mediate heparin binding, but α2(V) and α3(V) chains do not (Mizuno and Hayashi, 1996).

Mann (1992) presented the first partial human amino acid sequence of the N-terminus of α3(V) collagen chain. Recently, Imamura et al. (2000) provided the primary structure of the human and mouse pro-α3(V) collagen chains. The structure of the pro-α3(V) chain was shown to be closely related to that of the pro-α1(V) chain. It has a unique acidic domain in the N-terminal globular domain, which is also contained in the pro-α1(V) chain as well as pro-α1(XI) and pro-α2(XI) chains. They showed expression of the pro-α3(V) gene in the epimysial sheaths of developing muscles, within nascent ligaments adjacent to forming bones and in joints using in situ hybridization. Alternatively, Chernousov et al. (2000) reported that rat pro-α4(V) chain must be the counterpart of mouse and human pro-α3(V) chains based on its high similarity of structure as shown in the genomic databases (Gopalakrishnan et al., 2004). Their group also found a new heparan sulfate binding site that mediates Schwann cell adhesion in the unique N-terminal domain of rat pro-α4(V) (Erdman et al., 2002). They showed that the major binding proteins are glypican-1 and perlecan using pull down assays and immunofluorescent staining (Rothblum et al., 2004).

In this study, we generated a specific monoclonal antibody directed against the unique N-terminal domain of the pro-α3(V) chain. We examined the expression of the pro-α3(V) chain using immunohistochemical methods and in situ hybridization. The pro-α3(V) chain was observed in tissues related to bone formation. Furthermore, the peptide derived from the basic segment flanking the acidic domain of the pro-α3(V) chain binds to osteosarcoma cells.

2. Results and discussion

2.1. Transcripts of pro-α3(V) collagen gene

The pro-α3(V) chain was originally isolated from human placenta but is distributed in many more tissues than previously expected (Rhodes and Miller, 1981; Abedin et al., 1982; Imamura et al., 2000). Initially, to examine the pro-α3(V) gene expression temporally and spatially in mouse, we performed RT-PCR. As shown in Fig. 1A, pro-α3(V) transcripts were detectable from E16.5, whereas pro-α1(V) transcripts were readily detectable in embryos at E12.5. In different tissues of E18.5, relatively high levels of pro-α3(V) expression were seen in placenta, kidney, vertebrae, calvaria and tongue (Fig. 1B). Weak bands were also detectable in tail and heart, but almost undetectable in liver, lung, intestine, limb, brain and skin (Table 1). By contrast, pro-α1(V) gene was expressed in many tissues tested (data not shown) (Wu et al., 1998).

To characterize the expression pattern of the pro-α3(V) gene in detail, we applied in situ hybridization. In situ hybridization was performed on sagittal sections of E16.5 mouse embryos using specific probes for pro-α3(V) basic/acidic domain, pro-α1(V) acidic domain and type II collagen (Yoshioka et al., 1995). Although pro-α1(V)

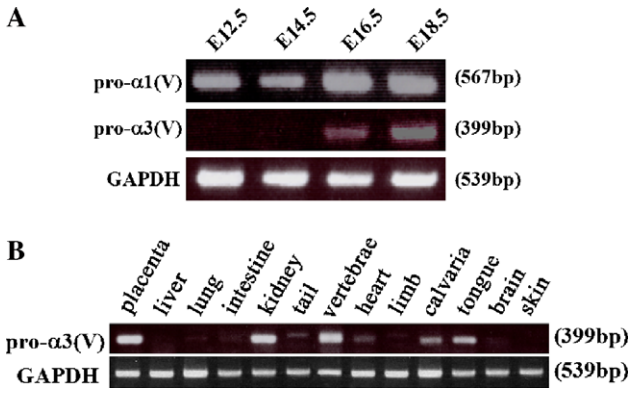

Fig. 1. RT-PCR analysis of pro-α3(V) collagen gene expression in mouse embryo. A: RNAs from whole mouse embryos at E12.5–E18.5 were used. RT-PCRs using specific primers for the mouse pro-α3(V), pro-α1(V) collagen and GAPDH genes were performed. B: RNAs from different tissues of the E18.5 mouse embryo were used. GAPDH was used as an internal control. Expected size of the PCR product is shown on the right of each panel.

Table 1
Expression of α3(V) collagen gene in the placenta and the mouse embryo

	RT-PCR	In situ hybridization	Immunohistochemistry
Placenta	+	/	/
Chorionic villi	nd	–	–
Funis	nd	+	+
Amnion	nd	+	+
Deciduas	nd	–	–
Liver	–	–	–
Lung	–	–	–
Intestine	–	–	–
Kidney	+	–	–
Renal facia	nd	+	+
Tail	±	–	–
Vertebrae	+	+	+
Heart	±	–	–
Vessel	nd	–	–
Limb	–	–	–
Calvaria	+	+	+
Tongue	+	+	–
Brain	–	–	–
Skin	–	–	–
Superficial facia of developing muscle	nd	+	+

+, Positive; ±, weakly positive; –, negative; nd, not determined.
The E18.5 mouse embryos were used for RT-PCR, and E16.5 for in situ hybridization and immunohistochemistry.

expression was widely distributed throughout developing connective tissues (Fig. 2C), pro-α3(V) expression was restricted (Fig. 2B). Type II collagen was strongly expressed in cartilage, especially prehyper/hypertrophic chondrocytes (Figs. 2D and 3H). Pro-α3(V) mRNA was expressed in osteoblasts of calvaria (Fig. 3C) and in the associated periosteal cells surrounding first ossification center of vertebrae (Fig. 3D). The former represent bones formed by intramembranous ossification, and the latter by endochondrial ossification. By contrast, pro-α1(V) expression was seen in calvaria and its surrounding connective tissue (Fig. 3E), and type II collagen was never seen in calvaria (Fig. 3G). In vertebrae, pro-α1(V) mRNA was expressed in perichondrial cell layers surrounding cartilage primordium (Fig. 3F).

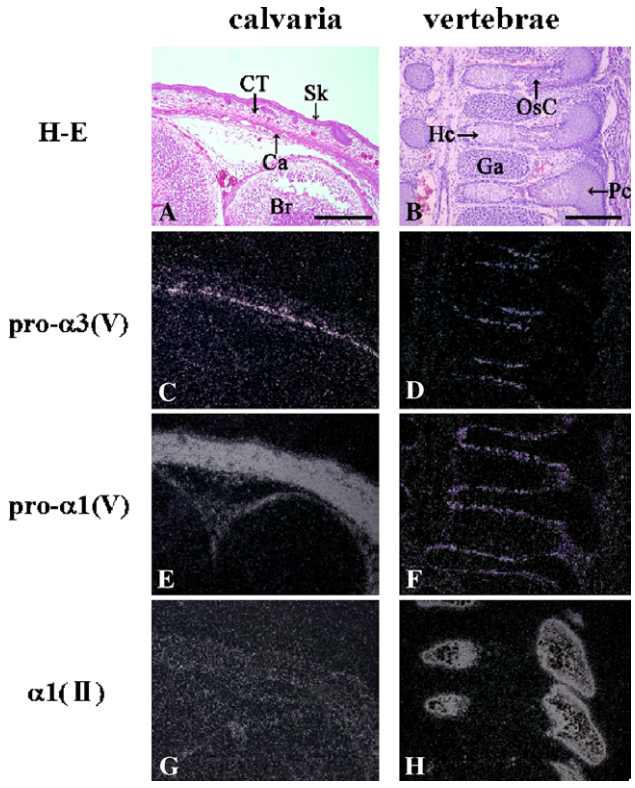

Fig. 3. In situ hybridization of the tissues related to the bone formation of E16.5 mouse embryos. Photomicrographs are H–E stained with brightfield (A and B) or hybridization with darkfield (C–H). Panels are shown at high magnification of Fig. 2 to clearly show the portion of calvaria (A, C, E and G) and vertebrae (B, D, F and H). The sections were hybridized with a radioactively labeled pro-α3(V) (C and D), pro-α1(V) (E and F) and α1(II) (G and H) collagen antisense riboprobe. Specific regions were labeled as follows: skin (Sk), subcutaneous connective tissue (CT), calvaria (frontal bone) (Ca), midbrain (Br), first ossification center (OsC), hypertrophic chondrocyte (Hc), prehypertrophic chondrocyte (Pc), ganglion (Ga). Scale bar: 100 μm.

2.2. Distribution of pro-α3(V) collagen chain in bone-related tissues

To detect the protein in tissues by immunohistochemistry, we prepared a specific monoclonal antibody directed against the basic/acidic domain of pro-α3(V). The antibody

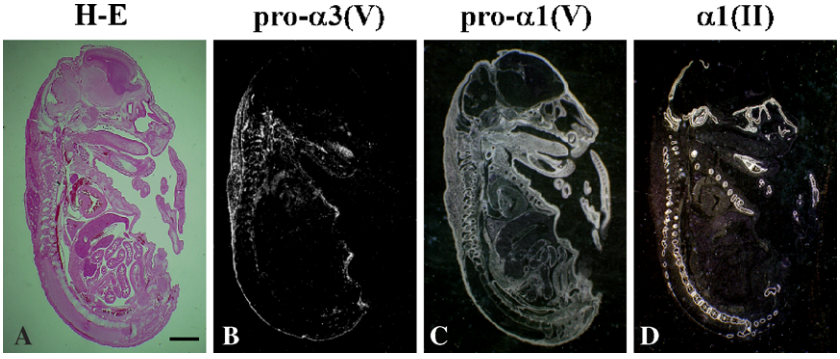

Fig. 2. In situ hybridization of E16.5 mouse embryos. Photomicrographs are hematoxylin and eosin (H–E) staining with brightfield (A) or in situ hybridization with darkfield (B–D). Sagittal sections from E16.5 embryos were hybridized with a radioactively labeled pro-α3(V) (B), pro-α1(V) (C) and α1(II) (D) collagen antisense riboprobe. Scale bar: 1 mm.

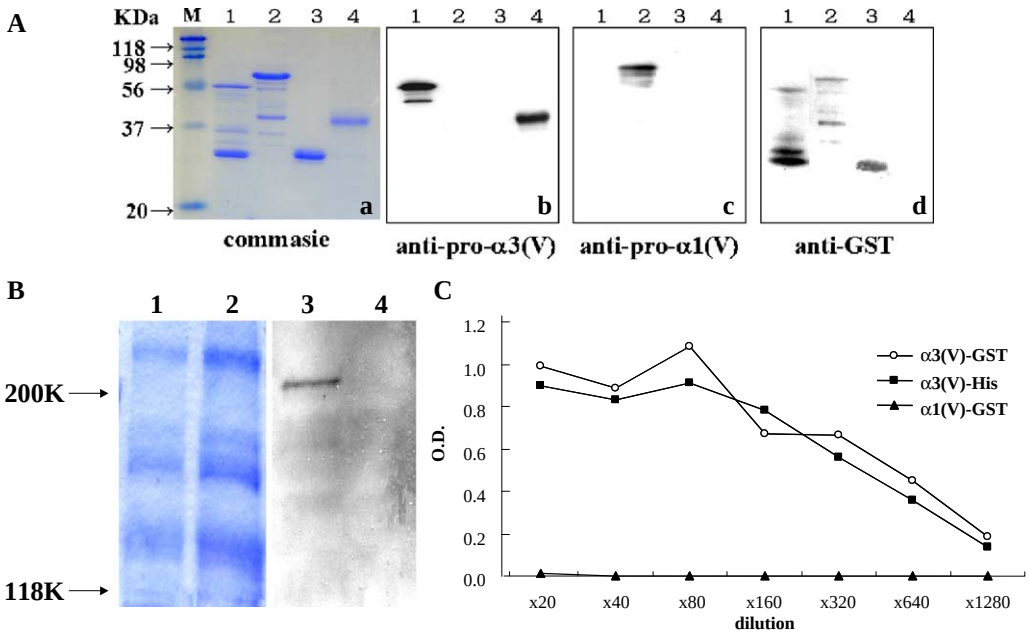

Fig. 4. Characterization of monoclonal antibody against the basic/acidic domain of pro-α3(V) chain. A: Western blot analysis using recombinant proteins. Purified recombinant proteins were run on a 15% SDS-PAGE gel and stained with Coomassie brilliant blue (panel a). The proteins blotted on the filters were hybridized with anti-pro-α3(V) antibody (panel b), anti-pro-α1(V) antibody (panel c) and anti-GST antibody (panel d). The samples in each lane were as follows—lane 1: pro-α3(V)–GST fusion protein, lane 2: pro-α1(V)–GST fusion protein, lane 3: empty vector (GST), lane 4: pro-α3(V)–His-tag fusion protein, lane M: marker. The positions of the molecular mass standards are shown on the left. B: Western blot analysis using native collagenous protein. Proteins were run on a 10% SDS-PAGE gel and stained with Coomassie brilliant blue (lanes 1 and 2). The proteins blotted on the filters were hybridized with anti-pro-α3(V) antibody (lanes 3 and 4). The samples were digested with collagenase (lanes 2 and 4). The positions of the molecular mass standards are shown on the left. C: ELISA using anti-pro-α3(V) antibody. The pro-α3(V) monoclonal antibody reacted with the pro-α3(V)–GST (open circles) and pro-α3(V)–His (closed squares) recombinant protein applied to a plate as a serial dilution, but not with similar dilutions of the pro-α1(V)–GST (closed triangles) fusion proteins.

was purified by protein G affinity column chromatography and Western blot and ELISA confirmed its specificity. As shown in Fig. 4A and C, the anti-pro-α3(V) antibody recognized the GST fusion and His-tagged pro-α3(V) recombinant polypeptide but not the tagged pro-α1(V) recombinant polypeptide. The antibody also recognized a collagenous peptide of approximately 220 kDa from a fraction of neutral salt-extracted osteosarcoma cells (Fig. 4B). An anti-pro-α1(V) polyclonal antibody directed against the acidic domain was also generated.

Sagittal sections from E16.5 mouse embryos, as used in the in situ hybridization experiments, were analyzed by immunohistochemistry. At low magnification, the pro-α3(V) chain was observed in tissues that are related to bone formation, such as the calvaria, vertebra, maxilla, mandibula, and clavicula (Fig. 5C). At this developmental stage, alkaline phosphatase activity was detected strongly in the frontal region of the calvaria, perichondrium, and bone matrix (Fig. 5B). Both staining patterns were essentially consistent. Pro-α1(V) collagen was detected in most connective tissues, including bone-related tissues (Fig. 5D). In the calvaria region, strong expression of pro-α3(V) was observed at high magnification, in the matrix of the calvaria and aponeurosis (Fig. 6E), whereas pro-α1(V) was observed in the matrix of the calvaria and subcutaneous tissue, but not in the aponeurosis (Fig. 6G). In the vertebral

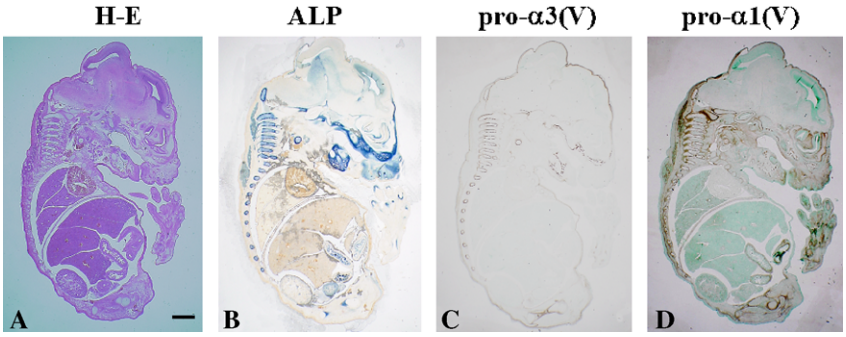

Fig. 5. Immunohistochemical localization of pro-α3(V) chain in E16.5 mouse embryos. Photomicrographs were shown for sagittal serial sections of an E16.5 mouse embryo. Paraffin sections were stained with H–E (A), alkaline phosphatase activity (B), anti-pro-α3(V) antibody (C) and anti-pro-α1(V) antibody (D). Scale bar: 1 mm.

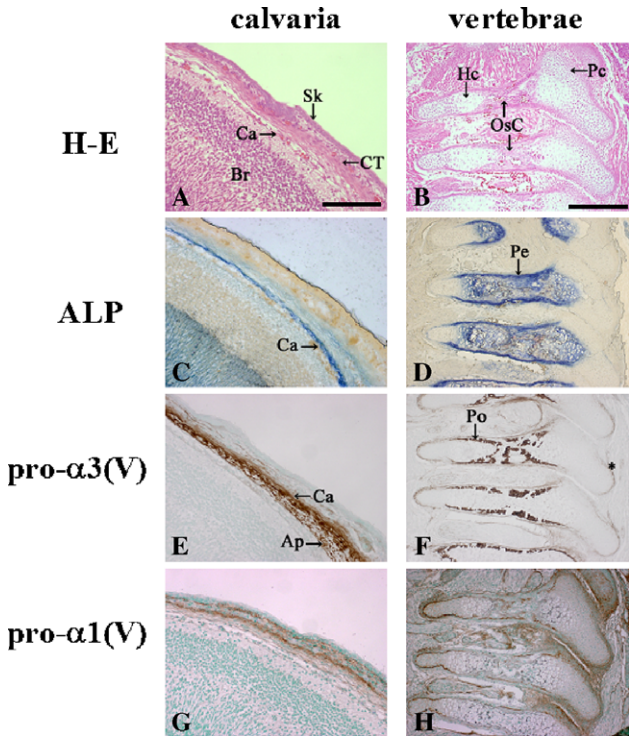

Fig. 6. Immunohistochemical localization of pro-α3(V) chain in the tissues that are related to bone formation in E16.5 mouse embryos. Photomicrographs are H–E stained (A and B) or immunohistochemical stained (C–H). Panels are shown at a higher magnification than Fig. 5 to clearly show the portion of calvaria (A, C, E and G) and vertebrae (B, D, F and H). The sections were stained for alkaline phosphatase activity (C and D), anti-pro-α3(V) (E and F) and anti-pro-α1(V) (G and H) antibodies. Specific regions were labeled as follows: skin (Sk), subcutaneous connective tissue (CT), calvaria (frontal bone)(Ca), midbrain (Br), aponeurosis (Ap), first ossification center (OsC), hypertrophic chondrocyte (Hc), prehypertrophic chondrocyte (Pc), perichondrial (Pe), periosteal (Po). Scale bar: 100 μm.

region, pro-α3(V) was detected in the periosteum and bone matrix, where ossification initially occurs (Fig. 6F). This staining pattern is consistent with that of alkaline phosphatase (Fig. 6D). Weak staining of the pro-α3(V) chain was also seen in ligamentous attachments. On the other hand, staining for pro-α1(V) was more intense in the periosteum and ligamentous attachments than in the bone matrix (Fig. 6H). This pattern differed from that of alkaline phosphatase.

2.3. Expression in non-bone-related tissues

The α3(V) chain was originally isolated from the placenta. As shown in Fig. 1B, the transcripts of pro-α3(V) were detected in the placenta using RT-PCR. However, Chernousov et al. (2000) did not detect pro-α3(V) in the placenta of rat. This was one of the reasons for them to call the collagenous peptide, the α4(V) chain, not the α3(V) chain. We examined the distribution of the mouse pro-α3(V) chain in the placenta using in situ hybridization and immunohistochemical methods. Signal from the transcripts were seen in the soft connective tissue, Wharton's jelly, in funis and amnion (Fig. 7B and E). However, they could never be seen in chorionic villi and decidua. The immunohistochemical data were consistent with those of in situ hybridization (Fig. 7C and F).

Imamura et al. (2000) reported that the pro-α3(V) gene was expressed in the epimysial sheaths of developing muscles and within nascent ligaments adjacent to forming bones and joints in the E15.5 mouse embryo. Similarly, we could detect the signals in those regions in the E16.5 mouse embryo. The staining of pro-α3(V) could be detected in the superficial fascia of developing muscle and renal fascia around the kidney and adrenal gland (Fig. 7I and L). However, we could not clearly detect a signal in other regions where expression was positive by RT-PCR or in situ hybridization (Table 1). This discrepancy may be due to the fast turnover or disseminated distribution of the protein in these tissues.

2.4. Cell adhesion of pro-α3(V) chain

To elucidate the biological function of type V collagen in osteoblast, we used an osteosarcoma cell line to examine whether the N-terminal domain of pro-α3(V) chain is involved in cell adhesion. The N-terminal globular domain of the pro-α3(V) chain can be divided into subdomains (Fig. 8). Among them, the acidic domain of pro-α3(V) chain, which is flanked by a highly basic segment of 23 amino acids at the N-terminus, is unique because it is only found in mouse and human pro-α3(V) and rat pro-α4(V) chains (Imamura et al., 2000; Chernousov et al., 2000).

We tested the adhesive ability of the basic/acidic domain of pro-α3(V) chain using recombinant proteins to ROS and RCS cells. As shown in Fig. 9, ROS cells adhere to the basic/acidic domain of pro-α3(V) recombinant protein but not to the acidic domain of pro-α1(V). The adhesion of RCS cells was hardly recognizable in both acidic domains of pro-α1(V) and basic/acidic domain of pro-α3(V). This adhesive function was restricted to the region of 239–269 amino acids where the basic segment is contained. This segment has four repeats of the heparin binding consensus sequence BBXB (B and X are a basic and any amino acids, respectively) (Fig. 12A) (Cardin and Weintraub, 1989). To assess the heparin binding activity of the recombinant protein, heparin affinity chromatography was performed. As shown in Fig. 10A and B, pro-α3(V) (239–369) and pro-α3(V) (239–269) fragments, containing the basic segment, bound to the heparin column and were eluted at a high NaCl concentration, reflecting high affinity binding to heparin. In contrast, the pro-α1(V) acidic domain and pro-α3(V)(269–369) that deletes the basic segment, dramatically abolished the heparin binding activity. Thus, the basic segment in the N-globular domain of pro-α3(V) chain is important to bind heparin.

These results suggest that ROS cells adhesion to pro-α3(V) peptide might be mediated by cell-surface heparan sulfate proteoglycans. The contribution of glycosaminogly-

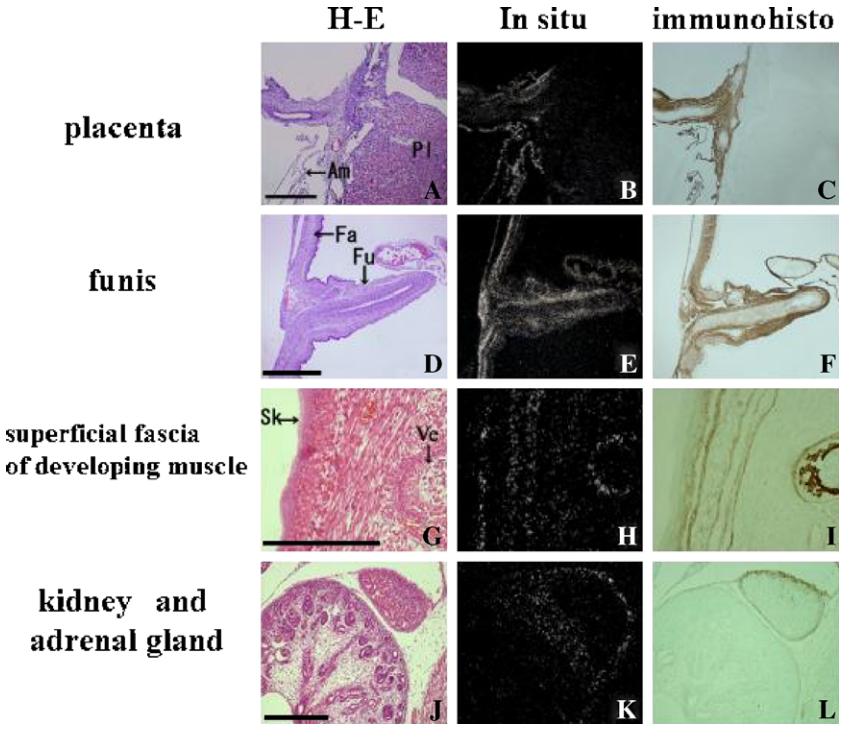

Fig. 7. Expression in non-bone-related tissues. Photomicrographs are H–E stained (A, D, G and J), in situ hybridization with darkfield (B, E, H and K) and immunohistochemical staining (C, F, I and L). The sections were hybridized with a radioactively labeled pro-α3(V) collagen antisense riboprobe (B, E, H and K) and stained for anti-pro-α3(V) collagen antibody (C, F, I and L). The tissues are placenta (A–C), funis attached to fetal abdomen (D–F), superficial fascia of developing muscle (G–H) and kidney and adrenal gland (J–L). Specific regions were labeled as follows: chorionic villi in placenta (Pl), amnion (Am), funis (Fu), superficial fascia in fetus (Fa), skin (Sk), vertebrae (Ve). Scale bar: 100 μm.

can to ROS adhesion was examined by plating the cells onto the recombinant proteins of pro-α3(V) basic/acidic domain in the presence of heparin, chondrotin sulfate and dextran sulfate. As shown in Fig. 11, heparin and dextran sulfate, added at a concentration of 10 μg/mL, inhibited cell adhesion by 76%. Chondrotin sulfate inhibited adhesion

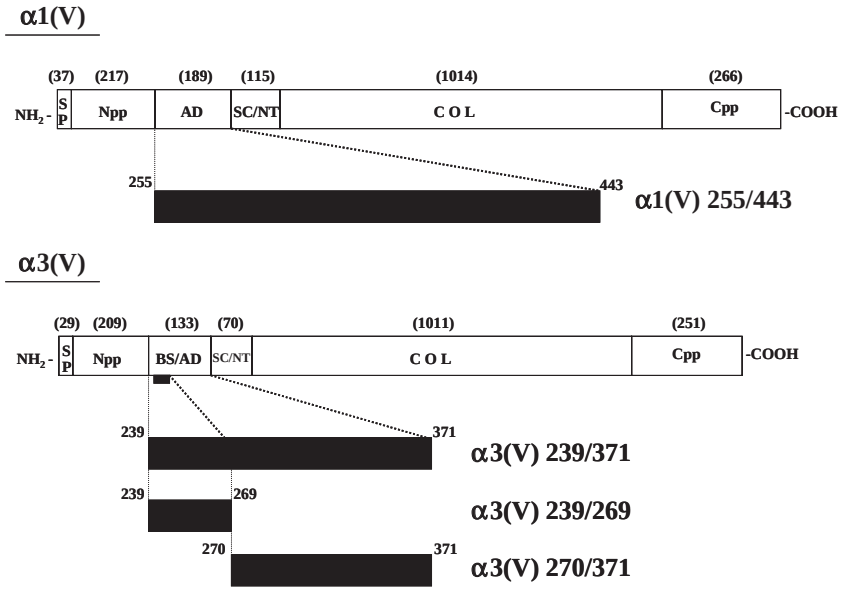

Fig. 8. The constructs of recombinant protein of the mouse pro-α3(V) and pro-α1(V) collagen polypeptides. The recombinant protein covered the basic/acidic domain of pro-α3(V) and the acidic domain pro-α1(V) chains were generated. The domains of prepro-α3(V) and prepro-α1(V) chain are consist of a signal peptide (SP), N-terminal propeptide (Npp), basic/acidic (BS/AD) or acidic domain (AD), short collagenous segment (SC), *N*-telopeptide (NT), central continuous collagenous domain (COL) and C-terminal propeptide (Cpp). A short bar in the pro-α3(V) chain indicates a basic segment flunked acidic domain. Vertical arrows and numbers in parenthesis indicate the cleaved sites of pro-α1(V) chains and number of amino acid residues in individual domains, respectively. The recombinant protein numbers indicate the number of amino acid residues from the N-terminal end.

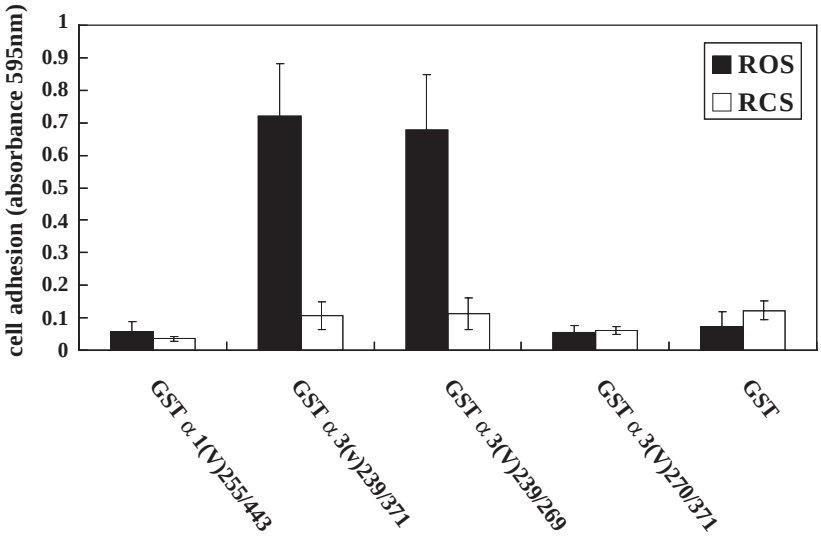

Fig. 9. Cell adhesion to the recombinant proteins of pro-α3(V) and pro-α1(V) collagen polypeptides. ROS and RCS cells were plated on dishes coated with the recombinant proteins. Cell adhesion was quantitated by staining with crystal violet.

by about 30%. The interaction between ROS cells and basic segment might be mediated by a cell-surface heparan sulfate proteoglycan receptor. Erdman et al. (2002) showed the N-terminal binding activity to Schwann cells. Rothblum et al. (2004) suggested that this binding is mediated via glypican-1, that is a lipid-anchored proteoglycan of the plasma membrane. In osteoblasts, the same or similar proteoglycan might also contribute to the interaction with the basic segment.

2.5. Adhesion activity of the four heparin binding sites

To know which heparin binding site is the most important in cell adhesion among the four heparin binding sites, we replaced each heparin binding site by site-directed mutagenesis. These amino acids were replaced by alanine (Fig. 12A). The mutated fusion proteins were expressed in bacteria and tested by the heparin binding assay and cell adhesion assay. As shown in Fig. 12B, M1, M2, M3 and M4 mutated proteins were eluted from the heparin column at NaCl concentration of 0.8–1.5 M. However, the binding affinities were decreased compared with that of wild type pro-α3(V)(247–269), which eluted at the concentration of 1.0–2.0 M. Similarly, as shown in Fig. 12C, each mutated protein, M1, M2, M3 and M4, showed approximately 60–70% of the adhesive activity to ROS compared with that of wild type pro-α3(V)(247–269). These results indicate that each heparin binding site

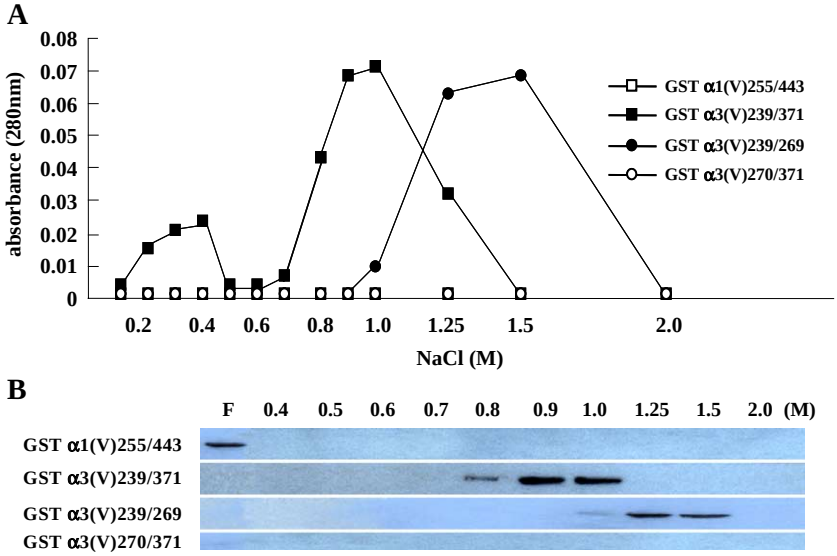

Fig. 10. Heparin binding of the recombinant pro-α3(V) and pro-α1(V) collagen polypeptides. GST fusion proteins of pro-α3(V) and pro-α1(V) collagen polypeptides were applied to a heparin–sepharose column and eluted by step gradient at the indicated concentrations from 0 to 2.0 M NaCl. A: Elution data are shown in the graph. B: Aliquots of column fractions were subjected to Western blot analysis with anti-GST antibody. F indicates the fraction of flowthrough.

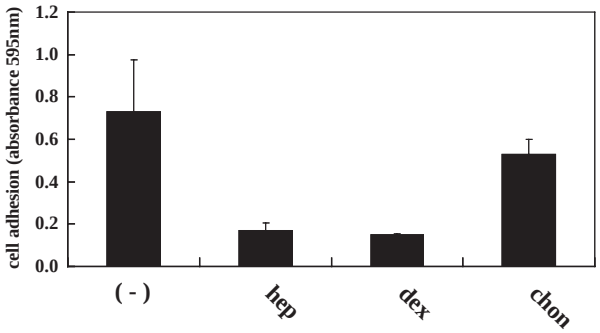

Fig. 11. Inhibition of cell adhesion by glycosaminoglycan. ROS cells were suspended in serum-free medium containing 10 μg/mL of heparin (hep), dextran sulfate (dex), and chondroitin sulfate (chon), and plated onto wells coated with pro-α3(V)(239–369) recombinant protein. The values shown are the mean±SD of five independent wells.

in the pro-α3(V) chain equally contributes with regard to adhesive interactions with ROS cells.

Delacoux et al. (2000) showed that a heparin binding site is present in the collagenous domain of the α1(V) chains. The sequence contains a cluster of seven basic amino acids that are different from the consensus sequence of the heparin binding site previously reported (Cardin and Weintraub, 1989). However, its binding affinity is significantly lower than that of the pro-α3(V) chain. The former is eluted at 0.35 M NaCl concentration whereas the latter is at 1.0–2.0 M. This basic segment would play a specific role through a strong electrostatic interaction with molecules such as heparan sulfate proteoglycan in vivo. This portion of the protein is retained in the fibrils that contain the rat α4(V) chain synthesized by Schwann cells (Chernousov et al., 1996; Chernousov et al., 2000) and is exposed on the fibril surface (Linsenmayer et al., 1993). However, Gopalakrishnan et al. (2004) have recently reported that this portion of the recombinant human pro-α3(V) chain was removed by BMP-1 in a fibroblast culture system. The processing of the N-terminal domain of the pro-α3(V) chain (or rat α4(V) chain) may depend on the cell type or the circumstances of the tissue. The processing of the pro-α3(V) chain in bone is unknown. Whether the basic segment is retained in the fibrils or removed, it might still affect the osteoblast in the developing bone.

2.6. Conclusion

Using immunohistochemistry and in situ hybridization, this study has demonstrated that the pro-α3(V) chain is expressed in tissues that are related to bone formation as well as in the funis and amnion of the placenta. The peptide derived from the basic segment of the N-terminal domain of the pro-α3(V) chain showed specific adherence to osteosarcoma cells but not to chondrosarcoma cells. The unique segment should play an important role in bone formation. Further study is required to determine the precise role of this chain in osteoblasts, and to characterize its function in bone formation during development and regeneration.

3. Materials and methods

3.1. Animals

Mice and rabbits were purchased from commercial sources (Yoshitomi, Fukuoka, Japan; Kyudo, Saga, Japan).

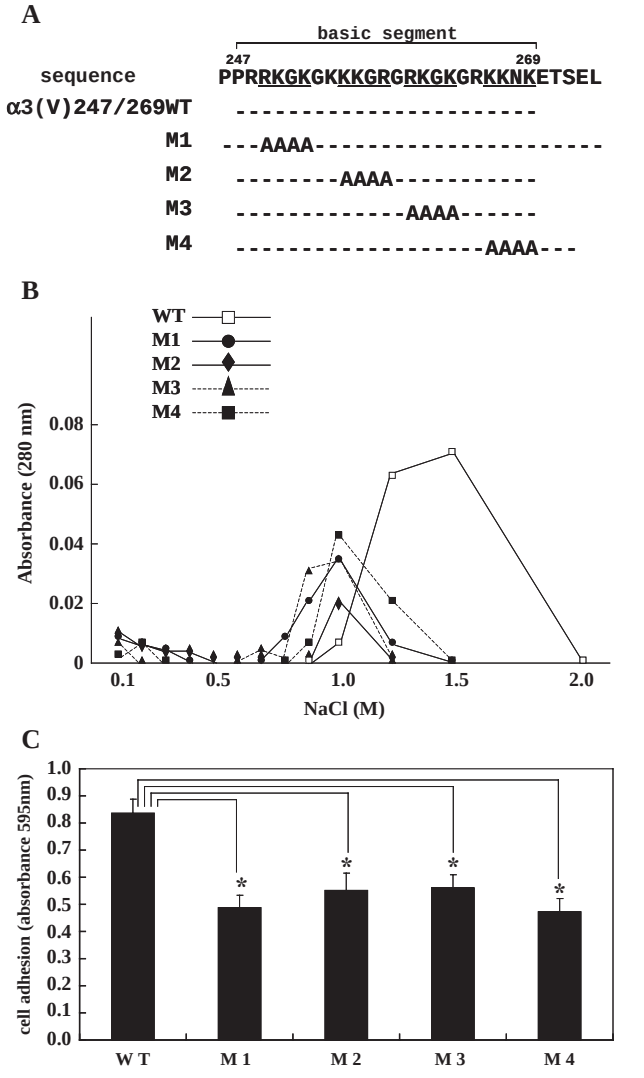

Fig. 12. Assessment of four heparin binding sites in the basic segment of pro-α3(V) chain for heparin binding affinity and ability of cell adhesion. A: Amino acid sequence of the constructs with an alanine mutation. Note that it was difficult to generate a 23 amino acids-size pro-α3(V)M1 and pro-α3(V)M4 using the PCR procedure because of annealing problems with the primers. Therefore, pro-α3(V)M1 and pro-α3(V)M4 are six and three amino acids, respectively, longer than the others. B: Heparin binding assay using substituted mutant proteins. Bound proteins were eluted by step gradient of indicated concentrations of 0–2.0 M NaCl. Elution data are shown in the graph. C: Cell adhesion assay using substituted mutant proteins. ROS cells were plated on dishes coated with mutated protein. The results are displayed graphically. The values shown are the mean±SD for four independent wells. *$p<0.01$.

The animals were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory Animals based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3.2. Staging of mouse embryos and preparation of sections

Gestational age was initially determined by the date of formation of the copulation plug and confirmed by crown-rump length. For in situ hybridization and immunohistochemistry, mouse embryos were fixed overnight in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated, and embedded in paraffin, and 7 μm consecutive sections were prepared.

3.3. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA samples were prepared from E12.5, 14.5, 16.5, and 18.5 mouse whole embryos, and from different tissues of the E18.5 mouse embryo, using Isogen (Wako, Osaka, Japan) according to the manufacturer's instructions. Total RNAs were used as templates to synthesize cDNAs using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers. The PCR program was as follows: 1 min at 94 °C for denaturation, 1 min at 55 °C for annealing, and 1 min at 72 °C for extension; repeated for 30 cycles with a final extension of 7 min 30 s at 72 °C. The gene-specific primers used in the PCR reactions were:

pro-α1(V)255/443: (forward) 5′-CAGGACCCTAACCCGGATGA-3′
(reverse) 5′-CTCAAAGATGGTGTCCTGGT-3′
pro-α3(V)239/371: (forward) 5′-GATGAACCAGAAACCCCTGC-3′
(reverse) 5′-AGCACCAGGAAAGATCTGGA-3′
GAPDH: (forward) 5′-AGAGGTGCTGCCCAGAACATCATC-3′
(reverse) 5′-GTGGGGAGACAGAAGGGAACAGA-3′

3.4. In situ hybridization

In situ hybridizations were carried out using [^{35}S]-labeled riboprobes on tissue sections of mouse embryos as described previously (Iyama et al., 2001; Sumiyoshi et al., 2001). The fragments of the acidic domain of the pro-α1(V) and the basic/acidic domain of pro-α3(V) mouse collagen cDNA were generated by the RT-PCR procedure mentioned above. The amplified fragments were cloned into TA-Easy vector (Promega, Madison, WI). Type II collagen cDNA was described elsewhere (Yoshioka et al., 1995). After linearization at appropriate restriction sites, sense and antisense probes were generated by in vitro transcription with T3 and T7 polymerases. In situ hybridization was performed on deparaffinized and proteinase K-treated sections. The sections were incubated with [^{35}S]-labeled antisense and sense cRNA riboprobe at 52 °C for 16 h and washed several times with increasingly stringent conditions. The slides were dipped in Kodak NTB-2 (Tokyo, Japan), dried for 1 h and exposed for 7 days at 4 °C. Sections were counterstained with hematoxylin and eosin. Photographs were taken by a camera attached to a microscope (Olympus BX-50 and Keyence VB-6000/6010).

3.5. Expression of recombinant proteins

cDNAs encoding the different region of the basic/acidic domain of the pro-α3(V) collagen (Fig. 7) were generated by RT-PCR procedure using the pro-α3(V)239/371 fragment as a template.

Pro-α3(V)239/269:(forward) It is the same as the forward primer of pro-α3(V)239/371.
(reverse) 5′-CTTGTTTTTCTTTCTTCCCT-3′
pro-α3(V)270/371: (forward) 5′-GAGACCTCAGAGCTGAGTCC-3′
(reverse) It is the same as the reverse primer of pro-α3(V)239/371.

Various mutant cDNAs covering the basic segment of the pro-α3(V) collagen were also generated using the pro-α3(V)239/269 fragment as a template.

Pro-α3(V)247/269WT: (forward) 5′-CGTCGTCGAAAGGGCAAAGG-3′
(reverse) 5′-CTTGTTTTTCTTTCTTCCCT-3′
pro-α3(V)247/269M1: (forward) 5′-CCTCGTCGTGCAGCGGCCGCAGGGAAGAAA-3′
(reverse) 5′-CAGCTCTGAGGTCTCCTTGT-3′
pro-α3(V)247/269M2: (forward) 5′-CGTCGTCGAAAGGGCAAAGGGAAGGCAGC, AGCGGCGGGT-3′
(reverse) It is the same as the reverse primer of pro-α3(V)247/269WT.
pro-α3(V)247/269M3: (forward) It is the same as the forward primer of pro-α3(V)247/269WT.
(reverse) 5′-CTTGTTTTTCTTTCTTCCCGCGGCGGCTGCACC-3′
pro-α3(V)247/269M4: (forward) It is the same as the forward primer of pro-α3(V)247/269WT.
(reverse)5′-TGAGGTCTCCGCGGCTGCCGCTCTTCCCTT-3′

The amplified fragment was subcloned into the TA-Easy vector (Promega). Following digestion at appropriate restriction sites, the fragments were subcloned into pGEX-4T vector (Amersham-Bioscience) to produce glutathione S-transferase (GST) fusion proteins. And His-tag pro-α3(V) recombinant protein was also prepared (Novergen, Darmstadt, Germany).

Nucleotide sequences of the inserts and junctions linked to the vector were determined by automated DNA sequencing (ABI PRISM 310 Genetic Analyzer, Applied Biosys-

tems, Foster City, CA) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Large-scale preparation of bacterial sonicates for the purification of GST fusion proteins were performed according to the manufacturer's protocol (Amersham-Bioscience). In brief, a single colony of *E. coli* BL21 cells containing the recombinant pGEX plasmid was grown overnight and used to inoculate 2 × YT medium containing 100 μg/mL ampicillin and 2% glucose. The cells were grown at 37 °C until OD_{600} of 0.5 was obtained, and protein expression was induced by incubation for an additional 3 h in 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were collected by centrifugation, resuspended in PBS, sonicated, solubilized in 1 × PBS and 1% Triton-X-100, and bound to glutathione sepharose 4B. The glutathione fusion protein matrix was washed three times in five bed volumes of 1 × PBS and eluted with glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris–HCl pH 8.0). The purified proteins were collected and concentrated by using Centricon (Millipore, Bedford, MA).

3.6. Production of anti-pro-α3(V) and anti-pro-α1(V) antiboies

Antibody against the pro-α3(V) basic/acidic domain was established by the rat lymph node method (Kishiro et al., 1995). In brief, the purified GST recombinant protein was mixed with an equal volume of Freund's complete or incomplete Adjuvant (Wako). Initial subcutaneous injections contained 100 μg of recombinant protein with complete Freund's adjuvant. Two booster injections, which contained the same amount of protein with incomplete Freund's adjuvant, were given 1 and 2 weeks after the initial injection. The rats were bled 3 weeks after the second booster injection. The inguinal lymph nodes were dissected out for production of monoclonal antibody. Antigen against the pro-α1(V) acidic domain was prepared by immunizing rabbits using purified GST recombinant protein as described previously (Iyama et al., 2001). Specificity of the antibodies was confirmed by ELISA (enzyme-linked immunosorbent assay) as previously described (Iyama et al., 2001) and Western blot analysis (see below).

3.7. Cell culture

Rat ROS 17/2.8 osteosarcoma cells and rat RCS chondrosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (10 IU/mL), streptomycin (10 μg/mL) and glutamine (200 μg/mL). The cell lines were maintained at 37 °C in a humidified environment of 5% CO_2.

3.8. Western blot analysis

A fraction containing collagenous protein was prepared from the cell layer of ROS cells. Cells were cultured in medium described above plus 50 μg/mL L-ascorbic acid and 50 μg/mL β-aminopropionitrile to promote collagen synthesis and prevent cross-linking. Cells were lysed in extraction buffer containing 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.5% NP-40, 2.5 mM EDTA and 0.2 mM PMSF. An aliquot of the sample was digested with bacterial collagenase (Form III Wakojunyaku, Tokyo, Japan) before loading onto the gel. The sample for digestion was first neutralized with 0.5 M NaOH and then incubated with bacterial collagenase solution (250 U/mL collagenase, 50 mM Tris–HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$) containing 5.5 mM $CaCl_2$ for 5 h at 37 °C.

Native collagenous and recombinant proteins were electrophoresed on 10% and 15% polyacrylamide gels, respectively. Proteins were transferred electrophoretically at 60 V for 5 h onto fluoro transmembrane (Japan Genetics, Tokyo, Japan). The blotted membrane was treated with a blocking solution (PBS containing 4% nonfat milk) at 4 °C overnight. The membrane was subsequently incubated at room temperature for 1 h with the primary antibody. After being washed, the membrane was incubated with anti-rat or rabbit-IgG-HRP at 1:5000 dilution (Wako) at room temperature for 1 h and finally immunoreactive signals were detected with ECL Plus Western Blotting Detection Reagents (Amersham Biosciences).

3.9. Immunohistochemistry

For immunostaining of pro-α3(V) and pro-α1(V) collagen, deparaffinized sections were pretreated for antigen retrieval by autoclave heating (121 °C, 110 kPa) in 10 mmol/L citrate buffer (pH 4.0) for 5 min (Iyama et al., 2001). These sections were blocked for endogenous peroxidase activity with 1% H_2O_2 methanol for 30 min and then washed in PBS. Subsequently, sections were pretreated for removal of glycosaminoglycan by 500 U/mL hyaluronidase (Sigma) for 20 min at 37 °C. Thereafter, sections were immersed in 5% normal rabbit serum in PBS for 30 min, covered with anti-pro-α3(V) monoclonal or anti-pro-α1(V) polyclonal antibody, and incubated for 18 h at 4 °C. Immunoreactions were performed using a Vectastain peroxidase ABC kit (Vector Laboratories, Burlingame, CA). The antigenic sites were demonstrated by reacting the sections with a mixture of 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Dojin Chemicals, Tokyo, Japan) in 0.05 mol/L Tris–HCl pH 7.6, containing 0.01% H_2O_2 for 7 min. After washing in distilled water, the nuclei were stained with methylgreen, and then the sections were dehydrated in ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific).

3.10. Heparin affinity chromatography

Purified recombinant protein was subjected to heparin affinity chromatography on prepacked HiTrap-Heparin HP columns (Amersham Biosciences). The columns were

equilibrated with 50 mM Tris–HCl pH 7.5 at a flow rate of 0.5 mL/min. The proteins were diluted in equilibration buffer, and 500 μg of each protein was applied to the column. The column was eluted at a flow rate of 0.5 mL/min with 50 mM Tris–HCl pH 7.5 followed by a linear gradient of 0–2.0 M NaCl in 50 mM Tris–HCl pH 7.5.

3.11. Cell adhesion assay

The recombinant proteins were used to coat 24-well plates (2 μg/cm^2). The proteins were diluted in 20 mM Tris–HCl pH 7.5, 100 mM NaCl to a final concentration of 10 μg/mL, and incubated in the wells at 37 °C for 18 h. The solution was removed, and the plates were blocked with a solution of 1% bovine serum albumin in 20 mM Tris–HCl pH 7.5, 100 mM NaCl at 37 °C for 1 h. The blocking solution was removed, and the wells were washed with PBS. ROS and RCS cells were removed from the dishes by trypsinization. ROS and RCS cells were harvested by centrifugation, resuspended in serum-free medium, and added to the protein-coated wells. The plates were incubated for 3 h at 37 °C. At the end of the incubation period, the medium was aspirated, and the wells were washed with DMEM to remove nonadherent cells. Attached cells were fixed with 3% paraformaldehyde in PBS and stained with 0.5% crystal violet in 10% ethanol for 20 min. After extensive washing with water, bound dye was solubilized with 1% SDS, and the absorbance was read at a wavelength of 595 nm.

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