Autocrine/Paracrine Involvement of Parathyroid Hormone-Related Peptide in Vascular Leiomyoma

SHINJI NAITO*,###, KAZUHIRO SHIMIZU**, KOZO AKINO***, AKIRA OHTSURU[#],
MASAHISA WATANABE^{##}, SHUNICHI YAMASHITA[#] AND ICHIRO SEKINE^{###}

*Division of Pathology, Research Laboratory, National Ureshino Hospital, Saga 843–0393, Japan

**Department of Dermatology, Nagasaki University School of Medicine, Nagasaki 852–8523, Japan

***First Department of Anatomy, Nagasaki University School of Medicine, Nagasaki 852–8523, Japan

[#]Department of Molecular Medicine, Nagasaki University School of Medicine, Nagasaki 852–8523, Japan

^{##}Department of Dermatology, National Ureshino Hospital, Saga 843–0393, Japan

^{###}Department of Molecular Pathology, Nagasaki University School of Medicine, Nagasaki 852–8523, Japan

Abstract. Vascular leiomyomas are believed to arise from the smooth muscle of blood vessels and are characterized by the proliferation of vascular smooth muscle cells (VSMC) and numerous slit-like vascular lumens. Parathyroid hormone (PTH)-related peptide (PTHrP) plays an important role in local autocrine and/or paracrine regulation of cellular growth and function in VSMC. To investigate the interaction between VSMC and endothelial cells, we evaluated the distribution of PTHrP and PTH/PTHrP-receptor in 10 vascular leiomyomas of the skin by immunohistochemistry and *in situ* hybridization (ISH) of paraffin-embedded specimens. Both immunohistochemistry and ISH revealed that PTH/PTHrP-receptors are expressed in endothelial cells lining areas with slit-like vascular lumens and very weakly expressed in proliferating VSMC in all vascular leiomyomas. On the other hand, PTHrP itself was localized mainly in proliferating VSMC. These results support the hypothesis that PTHrP acts through the PTH/PTHrP-receptor via an autocrine and/or paracrine mechanism from VSMC to endothelial cells in the formation of characteristic microenvironments of vascular leiomyoma cell composition.

Key words: PTHrP, PTH/PTHrP-receptor, Vascular leiomyoma

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VASCULAR leiomyomas are believed to arise from the vascular smooth muscle cells (VSMC), occurring more frequently in females than males. Clinically, it is a painful lesion similar to a glomus tumor, angiolipoma or traumatic neuroma [1, 2]. Microscopically, they are made up of intersecting fascicles of smooth muscle cells and numerous slit-like vascular lumens of various sizes. The smooth muscle cell bundles seem to be derived from the wall of medium-size blood vessels that lack elastic fibers. Thus, vascular leiomyoma is an angiogenic tumor character-

ized by proliferating VSMC and slit-like vascular lumens. On the other hand, it has been demonstrated that endothelial cells and VSMC play important roles in the formation of new capillaries [3]. Based on these histological findings, the vascular leiomyoma may be a useful model to examine the involvement of cell-cell interaction between VSMC and endothelial cells in angiogenesis.

It has been demonstrated that some growth factors such as fibroblast growth factor (FGF), vascular endothelial growth factors (VEGF) and tumor necrosis factor- α (TNF- α) play important roles in angiogenesis in neoplastic and regenerative tissue [3]. PTHrP is also considered to be a potential regulator of VSMC proliferation and to play an important role in the neointimal formation observed in vascular stenosis after balloon angioplasty [4]. PTHrP and the

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Correspondence to: Shinji NAITO, M.D., Ph.D., Division of Pathology, National Ureshino Hospital, 2436 Ureshino-machi, Fujitsu-gun, Saga 843–0393, Japan

PTH/PTHrP-receptor are co-expressed in VSMC [5, 6] and PTHrP is thought to be involved in the control of vascular tone [7]. On the other hand, PTHrP expression is induced by some growth factors such as endothelin-1, angiotensin II, epidermal growth factor (EGF) and transforming growth factor- β (TGF- β) [8–12]. These findings suggest that locally produced PTHrP may play a crucial role not only in the contractile effects but also in the mitogenic effects of these growth factors in VSMC. Furthermore, the promoter regions of the human PTHrP gene are GC rich and include many GC “boxes”, indicating that the expression of PTHrP gene is regulated by a transcription factor such as specificity protein 1 (Sp-1). In addition, its transcription has a rapid turnover, which may be due to the AUUUA motifs within the 3' untranslated region [13–15]. These characteristics are commonly observed in the mRNA of cytokines participating in cell proliferation and differentiation, suggesting that PTHrP might play a role as a cytokine in regulating VSMC proliferation.

The present study aims to evaluate the distribution of PTHrP and the PTH/PTHrP-receptor in vascular leiomyoma of the skin and to ascertain their possible role in the initiation and progression of this tumor considered from the PTH/PTHrP-receptor axis.

Materials and Methods

Materials

A total of 10 cases of typical vascular leiomyoma of the skin were registered at the Department of Molecular Pathology, Atomic Bomb Disease Institute, Nagasaki University School of Medicine during the period from 1990 to 2001. The archival materials used in this study have been approved to use for research only by the steering committee of the Pathology Department in Nagasaki University School of Medicine.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue was used for immunohistochemistry of PTHrP, PTH/PTHrP-receptor, Factor VIII and smooth muscle actin. Paraffin-embedded tissue was cut into 3 μ m sections, deparaffinized in xylene, and rehydrated in

phosphate-buffered saline. For detection of PTHrP and the PTH/PTHrP-receptor, deparaffinized sections were treated with microwave oven heating for antigen retrieval (boiled in 10 mM citrate buffer, pH 6.0, for 5 min, 3 times). Deparaffinized sections were then preincubated with normal goat serum to prevent non-specific binding and incubated overnight at 4°C with an optimal dilution (5 μ g/ml) of anti-PTHrP antibody (monoclonal; Oncogene Science, Uniondale, NY, U.S.A.) and anti-PTH/PTHrP-receptor antibody (polyclonal; Berkeley Antibody Co., Richmond, CA, U.S.A.). PTHrP monoclonal antibody, which recognizes the middle portion of PTHrP, is a unique epitope which does not cross-react with PTH. PTHrP antibody was detected by the alkaline phosphatase method with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT). HHF35 (Enzo Diagnostics, Inc., NY, U.S.A) is a monoclonal mouse antibody which recognizes actin isotypes alpha and gamma (42 kd) of skeletal, cardiac and smooth muscles. The antibody for Von Willebrand factor (Factor VIII-related antigen) is a rabbit polyclonal anti-human antibody (Dako, Kyoto, Japan). The antibody for proliferating cell nuclear antigen (PCNA) is a monoclonal mouse anti-proliferating cell nuclear antigen (Dako, Kyoto, Japan) and reacts with PCNA from all vertebrate species. The antibody for vascular endothelial growth factor (VEGF) is a goat polyclonal antibody, with an epitope corresponding to an amino acid sequence mapping at the amino terminus of VEGF of human origin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). HHF35, Factor VIII, PCNA and VEGF are detected by the avidin-biotin-peroxidase complex method with deaminobenzidine (DAB) colorization. Negative controls were prepared by replacing the primary antibody with non-immunized mouse serum. An immunoabsorption test was also performed to confirm the specific immunoreactivity of the antibody.

In situ hybridization

Deparaffinized sections were treated with 20 μ g/ml proteinase K (Boehringer Mannheim) in PBS for 30 min at 37°C, and immersed in 0.2N HCL for 15 min. Using a digoxigenin RNA labelling kit (Boehringer Mannheim), antisense RNA probe for human PTH/PTHrP receptors were made from PTH/PTHrP-

receptor cDNA BstXI-BstXI fragments into pcDNA I vector. Hybridization was performed as described previously [4, 16]. PTH/PTHrP-receptor mRNA was detected by anti-digoxigenin-AP, Fab fragments (Boehringer Mannheim) and stained with 5-bromo-4-chloro-3 indolylphosphate p-toluidine salt nitroblue tetrazolium chloride (BCIP/NBT).

Results

The main clinical characteristics of 10 patients are summarized in Table 1. All ten cases of vascular leiomyomas showed typical clinical and histological features, characterized by the proliferation of smooth muscle cells of vessels (Figs. 1, 2A). The results of immunohistochemistry were as follows: Factor VIII was positive in the endothelial cells lining on the slit-like lumens of blood vessels (Fig. 2B). HHF35, which was used to prove that proliferating spindle cells were smooth muscle cells, was positive in proliferating VSMC observed in all vascular leiomyomas (Fig. 2C). PTHrP was overexpressed in the cytoplasm of proliferating VSMC in all vascular leiomyomas (Fig. 2D). PTH/PTHrP-receptor protein was strongly positive in the cytoplasm of endothelial cells within the tumor blood vessels and was very weakly expressed in proliferating VSMC with myofibroblasts (Fig. 2E). However, it was not expressed in the endothelial cells of normal tissue adjacent to tumors (Fig. 2I). In addition, PTH/PTHrP-receptor mRNA was expressed in endothelial cells by ISH. However, it was very weakly expressed or not expressed at all in proliferating VSMC (Fig. 2F).

The expression of PCNA, which is related to cell proliferation, was seen in the nuclei of endothelial cells and VSMC in vascular leiomyomas (Fig. 2G). VEGF was immunopositive in VSMC composed of vascular leiomyomas (Fig. 2H). Similar immunohistochemistry results were observed in all cases. The expression of PTHrP and PTH/PTHrP-receptor in normal blood vessels adjacent to vascular leiomyomas was very weak or undetectable.

Discussion

Although PTHrP and the PTH/PTHrP-receptor are localized to VSMC in various normal tissues and are believed to play an important role in the local modulation of VSMC function, their role in angio-

Fig. 1. Histologic features of vascular leiomyoma A number of vessels are present with a slit-like lumen. Proliferating smooth muscle bundles are observed ($\times 100$, hematoxylin and eosin staining).

Table 1. Clinical characteristics of 10 patients with vascular leiomyoma

Case No.	Age (yrs)	Sex	Location (skin) of Tumor	size (mm)
1	39	F	left 3rd finger	10 × 10 × 1.5
2	53	M	left 1st finger	5 × 5 × 5
3	71	M	right plantaris	7 × 7 × 7
4	18	M	right auricle	3 × 3 × 3
5	65	F	right wrist	5 × 6 × 6
6	41	M	left elbow	3 × 5 × 7
7	60	M	left elbow	10 × 11 × 10
8	57	F	left dorsal hand	5 × 10 × 7
9	26	M	right knee	6 × 9 × 6
10	36	F	right knee	4 × 5 × 4

Fig. 2. Immunohistochemical localization of PTHrP and PTH/PTHrP receptor in vascular leiomyoma. **A.** A large vein with a thick muscular wall is observed ($\times 200$, hematoxylin and eosin staining). **B.** Immunohistochemical expression of Factor VIII protein in the endothelial cells lining the slit-like lumen of vascular vessels in vascular leiomyoma ($\times 200$, DAB colorization). **C.** Immunohistochemical expression of HHF35 was observed in VSMC proliferating around the slit-like vascular lumen ($\times 200$, DAB colorization). **D.** Overexpression of PTHrP protein was observed in the cytoplasm of proliferating VSMC ($\times 200$, BCIP/NBT colorization). **E.** PTH/PTHrP-receptor protein was strongly expressed in the cytoplasm of endothelial cells lining the slit-like lumen of vascular vessels and very weakly expressed in proliferating VSMC with myofibroblastic cells ($\times 200$, AEC colorization). **F.** PTH/PTHrP receptor mRNA was detected in the endothelial cells lining the slit-like lumen (arrowheads). It was not detected in VSMC ($\times 200$, BCIP/NBT colorization). **G.** Immunohistochemical expression of PCNA was observed in the nuclei (arrowheads) of endothelial cells and proliferating VSMC ($\times 200$, DAB colorization). **H.** VEGF immunoreactivity was observed in the cytoplasm of proliferating VSMC in vascular leiomyomas ($\times 200$, DAB colorization). **I.** The PTH/PTHrP-receptor was hardly detected in the endothelial cells of the normal tissue adjacent to tumors ($\times 200$, AEC colorization).

genic tumors is still unknown. In this study, we demonstrated that PTHrP expression was observed in proliferating VSMC comprising vascular leiomyoma of the skin. On the other hand, PTH/PTHrP-receptor was strongly expressed in endothelial cells lining slit-like vascular lumens and weakly expressed in proliferating VSMC of vascular leiomyoma. The PTH/PTHrP-receptor is a receptor containing seven transmembrane domains and PTHrP binding to its receptor stimulates changes in several intracellular second messengers, such as cyclic adenosine monophosphate (cAMP) and intracellular free Ca^{2+} in signal transduction pathways for cell proliferation [17]. Therefore, our current results suggest that the PTH/PTHrP-receptor axis might be involved in the formation of characteristic histopathological features such as proliferating slit-like vascular lumens and VSMC in vascular leiomyoma through paracrine and/or autocrine mechanism. Regarding to human endometrium, it has been already reported that a local PTHrP autocrine and/or paracrine mechanism between epithelial cells and stromal cells may play an important role in endometrial proliferation, which is similar to our results [18].

It is still not clear whether the PTH/PTHrP-receptor is expressed in vascular endothelial cells, although Akino *et al.* have demonstrated it in the endothelial cells of tumor vasculature [16]. A likely explanation is that a small number of PTH/PTHrP-receptors exist in normal endothelial cells which rapidly disappear after binding to PTHrP. Indeed, the expression of PTH/PTHrP-receptor was not observed in the endothelial cells of the normal tissue adjacent to tumors in this study. In tumor cells, such as osteosarcoma cell lines and gastric cancer, PTHrP gene expression is up-regulated by certain growth factors such as EGF and TGF- β produced by tumor cells [9, 12]. Thus, it seems likely that PTH/PTHrP-receptor in endothelial cells of vascular leiomyoma may also be up-regulated by the same mechanism, and that its overexpression may enable us to detect it by immunohistochemical means. On the other hand, regarding its weak expression in VSMC, it is possible that increased PTHrP results in down-regulation of PTH/PTHrP-receptor in VSMC, or that PTH/PTHrP-receptor is only weakly expressed. Although the effect of intracellular second messengers induced by PTHrP on cell proliferation is complicated, Loesberg *et al.* demonstrated that increased levels

of intracellular cAMP inhibited VSMC proliferation [19]. Therefore, in view of the overexpression of PTHrP in vascular leiomyoma that increases cAMP levels, VSMC proliferation must be inhibited in these tissues even before down-regulation of PTH/PTHrP-receptor. This suggests that the weak expression of PTH/PTHrP-receptor in the proliferating VSMC may not be due to down-regulation. On the other hand, the low levels of PTH/PTHrP-receptor expression may abrogate the effects of PTHrP and result in the loss of inhibition of VSMC proliferation. However, this idea is insufficient to explain VSMC proliferation in vascular leiomyoma. As for other vascular diseases such as Kaposi's sarcoma and angiosarcoma, it has been reported that some growth factors such as platelet derived growth factor-BB (PDGF-BB), bFGF, nerve growth factor (NGF) and VEGF play an important role in their initiation and progression by an autocrine and/or paracrine mechanism [20–25]. Expression of VEGF in proliferating VSMC of vascular leiomyomas was also observed in the present study (Fig. 2H). Thus, some of these growth factors may play an important role in the proliferation of VSMC in vascular leiomyoma as well. The expression of PCNA observed in the nuclei of endothelial cells and VSMC in vascular leiomyoma might result from the activation of proliferation that is stimulated by these growth factors, although its expression was slightly weak in this study, suggesting the slow growth of a benign tumor. As discussed above, these growth factors are also known to up-regulate PTHrP gene transcription in VSMC [8–12]. PTHrP might be acting in the initiation and progression of vascular leiomyoma in an analogous way to such growth factors possibly by cooperating with them.

The vessel sprout is the first step for the neovascular formation which is completed by surrounding of VSMC and/or pericytes [26]. The proliferation and migration of endothelial cells are observed in this process. In addition, adhesion molecules and matrix metalloproteinase, which affect cell migration and proliferation, have been demonstrated to play an important role in various steps of neovascular formation [26, 27]. Interestingly, PTH is known as a regulator of cell adhesion and extracellular matrix proteins [28] and PTHrP exerts its effects via PTH/PTHrP common receptors [29]. We recently reported that PTHrP was a potential paracrine factor acting via the PKA pathway to enhance angiogenesis

through capillary tube formation by endothelial cells in malignant pituitary tumors [30]. These findings suggest that PTHrP might play an important role in neovascular formation, particularly in the formation of the vascular lumen, by regulating the adhesion molecules and matrix metalloproteinase.

In conclusion, we have demonstrated the characteristic distribution of PTHrP and PTH/PTHrP-

receptor in vascular leiomyoma. PTHrP was expressed in proliferating VSMC and PTH/PTHrP-receptor was localized in endothelial cells and VSMC in vascular leiomyoma. These findings suggest that the PTH/PTHrP-receptor axis is involved in the development of vascular leiomyoma via either paracrine or autocrine actions, or both.

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