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DEMONSTRATION OF ACTIVIN-A IN ARTERIOSCLEROTIC LESIONS

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SUMMARY: We previously reported that follistatin, an activin-binding protein, is produced in arteriosclerotic lesions. Here, the expression of activin-A which promotes the growth of vascular smooth muscle cells was examined in arteriosclerotic lesions of WHHL (Watanabe heritable hyperlipidemic) rabbits. Activin-A mRNA was detected in normal aorta by reverse transcriptase-polymerase chain reaction using specific primers for activin-A cDNA and was increased remarkably in arteriosclerotic lesions. In addition, using the cloned rabbit activin-A cDNA, RNA probe was prepared and *in situ* hybridization histochemistry was performed. Activin-A transcripts were detected abundantly in neointima of the diseased artery. Furthermore, immunohistochemistry also detected activin-A at the protein level. These observations suggest that activin-A is a cytokine expressed in arteriosclerotic lesions and might be involved in the pathogenesis of atherosclerosis.

Various cytokines are expressed in blood vessels and implicated in the process of arteriosclerosis (1). These include transforming growth factor (TGF)-ß which plays significant roles in various aspects of cell growth and differentiation (2). Ross et al. (3) reported that transcripts for PDGF-B, c-fms, IL-1 and TGF-ß were increased in the

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Abbreviations: WHHL rabbit, Watanabe heritable hyperlipidemic rabbit; RT-PCR, Reverse transcriptase-polymerase chain reaction.

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advanced lesions of atherosclerosis induced in non-human primates maintained on a hypercholesterolemic diet. They suggested a significant role of cytokines including PDGF-B produced by macrophages on atherosclerosis. On the other hand, vascular smooth muscle cells have TGF-ß receptors (4) and also produce TGF-ß1 (5), which can proliferate themselves by an autocrine mechanism. It is also reported that TGF-ß1 affects migration of vascular smooth muscle cells (6). The expression of TGF-ß1 mRNA in rat aortic tissues was found to be increased in experimental hypertensive rats as compared with normotensive rats (7, 8). These findings suggest some roles of TGF-ß1 in vascular cells and in atherogenesis.

Involvement of other members of TGF-ß family were also reported. BMP (bone morphogenetic protein) -2, a potent stimulator of osteoblastic differentiation, was found to be expressed in calcified human arteriosclerotic plaques (9). Bovine adrenal capillary endothelial cells also express mRNA encoding the BMP-2 and BMP-4 (10).

Activin, a member of TGF-ß family, was originally isolated from ovarian fluids as a stimulator of follicle stimulating hormone (FSH) secretion from pituitary gland (11). This molecule was found to have diverse biological roles besides hormonal effects on the reproductive system. Activin is involved in erythroid differentiation (12), regulation of neural differentiation (13, 14) and in mesoderm-induction (15-17). Follistatin, an activin-binding protein (18), is also presumed to have diverse biological functions. We have reported that neural cells produce follistatin to interact with exogenously derived activin (19), and that the function of an osteoblastic cell-line, MC3T3-E1 cells, was regulated by activin and follistatin (20).

However, there are only few reports about the role of activin and follistatin in vascular tissues (10, 21, 22). Recently, we found that follistatin is produced by vascular smooth muscle cells and in the arteriosclerotic lesions (21). It has been reported that activin-A has a mitogenic effect on vascular muscle cells from rat aorta and this effect of activin-A is different from that of TGF-ß1 (22). Activin-A is produced by vascular endothelial cells and inhibits their growth by an autocrine mechanism (10). Activin-A is also known to be produced by monocyte/macrophage lineage cells (12) which are abundant in arteriosclerotic lesions. These lines of evidence have prompted us to investigate the production of activin-A in the course of atherogenesis. Here, we show the expression of activin-A in arteriosclerotic lesions of WHHL (Watanabe heritable hyperlipidemic) rabbits (23) by reverse transcriptase-polymerase chain reaction (RT-PCR) method, *in situ* hybridization histochemistry and immunohistochemistry.

MATERIALS AND METHODS

Materials

Japanese white rabbits were purchased from Saitama Animal Laboratory (Saitama, Japan). WHHL rabbits were gifts from Dr. M. Shiomi (Institute for Experimental Animals, Kobe University School of Medicine, Kobe, Japan). Rabbit anti-human activin-A polyclonal antibody (24) was a kind gift from Drs. Eto and Shibai (Central Research Laboratories, Ajinomoto Co, Kawasaki, Japan).

Reverse transcriptase-polymerase chain reaction(RT-PCR)

cDNA was synthesized from 1μg of total RNA of aorta of normal and WHHL rabbits essentially according to Gubler and Hoffman (25). To avoid false positive results due to the contamination of genomic DNA, samples with or without treatment of avian myeloblastosis virus (AMV) reverse transcriptase (Seikagaku Kogyo, Tokyo, Japan) were simultaneously prepared. One-tenth of the resulting cDNA was used as template for PCR. Oligonucleotide primers were designed on the basis of DNA sequence of human activin-A cDNA (12) as 5'-GCTAGAATTCATCATCAGCTTTGCCGAGTCAG-3' (the 475 to 496 position of human activin-A and an EcoRI site) and 5'-GCTAAGCTTTCTTACAGCAAATGTTGACCTT-3' (the 1057 to 1078 antisense position and a Hind III site). The temperature program for the amplification was 35 cycles of 1 min at 94°C, 2 min at 52°C and 2 min at 72°C. The products were separated on a 0.7 % agarose gel and visualized with ethidium bromide. They were transferred to a nitrocellulose filter and hybridized to the ^{32}P-labeled activin cDNA (12). RT-PCR products derived from mRNA for the aorta of WHHL rabbits were digested with EcoRI and Hind III, then subcloned into pBluscript vector to construct BS-rbACT. The inserts were completely sequenced by the dideoxy-method (26) with Sequenase (USB, Cleveland, OH.).

In situ hybridization

Antisense and sense RNA probes labeled with digoxigenin-UTP (Boehringer Mannheim Biochemica) were generated from rabbit activin-A cDNA (BS-rbACT) using T7 and T3 RNA polymerases. Probes were used for hybridization at 1μg/ml. *In situ* hybridization histochemistry was carried out essentially as described (27). Briefly, tissues from rabbits were were fixed in 4% phosphate-buffered paraformaldehyde and embedded in paraffin wax. Sections of 4 μm were mounted on coated glass slides. Slides were deparaffined, acetylated with acetic anhydride and hybridized with rabbit activin RNA probes overnight at 42°C. They were subsequently washed in 50% formamide / 2xSSC at 42°C. After rinsing in 2x SSC and 0.1x SSC, slides were reacted in 1% blocking reagent for one hour and then with anti-digoxigenin-AP (Boehringer Mannheim Biochemica) (1:500) diluted in a 1.5 % blocking reagent for one hour. Then color reactions were performed with NBT(p-nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chrolo-3-indolylphosphate p-toluidine salt).

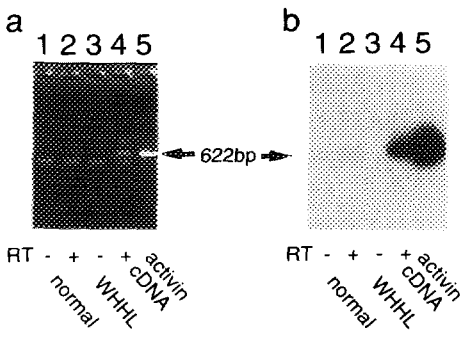
Fluorescence immunohistochemistry

Immunohistochemistry was performed essentially as described (21, 28). Briefly, the tissues obtained from rabbits were fixed, dehydrated, and embedded in paraffin wax. Sections of 2 μm were mounted on coated glass

slides, deparaffinized, and then treated with 10% normal goat serum in phosphate buffered saline to minimize the nonspecific binding of reagents in subsequent steps. They were incubated with 10 μg/ml of the fluorescein-conjugated anti-human activin-A polyclonal antibody (24) overnight at 4°C. Labeling reaction was carried out according to the manufacturer's instruction using fluorescein labeling kit (Boehringer Mannheim Biochemica). The tissues were examined under a fluorescence microscope (Nikon, Inc., Tokyo, Japan).

RESULTS AND DISCUSSION

The expression of activin-A mRNA was detected in arteriosclerotic aorta of WHHL rabbits by RT-PCR method (Fig. 1a). A specific band was detected (Fig. 1a: lane 4) by



c

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                 140        150        160        170        180        190        200
WHHL    N-  TARKTLHFEISKEGSDLSVVERAEVWLFLKVPKANRTRTKVTIRLFQQQKHPQGLLDTGDEAEEVGLKGE
            ************************************************** **** **********
human   N-  TARKTLHFEISKEGSDLSVVERAEVWLFLKVPKANRTRTKVTIRLFQQQKHPQGSLDTGEEAEEVGLKGE

                 210        220        230        240        250        260        270
WHHL        RSELLLSEKVVDARKSTWHIFPVSSSIQRLLDQGKSSLDVRIACEQCQESGASLVLLGKRKKKEEEGEGK
            ******************* ****************************************** **********
human       RSELLLSEKVVDARKSTWHVFPVSSSIQRLLDQGKSSLDVRIACEQCQESGASLVLLGKKKKKEEEGEGK

                 280        290        300        310
WHHL        KKDGGEGGA--EEEKEQSHRPFLMLQARQSEDHPHRRRRRGLECDG -C
            ** ******   ********************************
human       KKGGGEGGAGADEEKEQSHRPFLMLQARQSEDHPHRRRRRGLECDG -C      Homology:  96%
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Fig.1 Detection of activin-A mRNA by RT-PCR.

(a) Poly(A)$^+$RNA which was extracted from aorta of normal white rabbits (lanes 1,2) and WHHL rabbits (lanes 3, 4). RT-PCR of each RNA (1μg) with AMV-reverse transcriptase (lanes 2,4) was performed and electrophorased on 0.7% agarose gel. 622-bp band corresponding to activin-A cDNA products (lane 5) is demonstrated (arrow). RT-PCR patterns without reverse transcription (lanes 1, 3) are shown as negative controls. (b) Southern blot analysis for RT-PCR products. The gel for Fig. 1a was transfered to nylon membrane and hybridized with ^{32}P-labeled activin cDNA probe. (c) Deduced partial amino acid sequence of rabbit activin-A cDNA obtained by RT-PCR. RT-PCR products derived from rabbit aorta of WHHL rabbits was subcloned into pBluscript-SK vector and sequenced by dideoxy method. Deduced amino acid sequence was compared with that of human activin-A.

amplification after reverse transcription but not without the latter (Fig. 1a: lane 3). The size of the band amplified from rabbit aorta cDNA corresponded to that amplified from human activin-A cDNA (Fig. 1a: lane 5). Southern blot analysis of amplified PCR products demonstrates that these bands hybridized with human activin-A cDNA (Fig. 1b: lane 4, 5). Here, we could observe a weak band in aorta of normal rabbits (Fig. 1b: lane 2). Although it is difficult to compare accurately the amount of mRNA by RT-PCR, the signal faintly detected in RNA of normal artery was remarkably increased in the diseased artery. To confirm that the amplified PCR products were *bona fide* activin-A cDNA, we cloned the PCR products into pBluescript SK(-) vector and determined the DNA sequence. The sequence had 90% homology with the human activin-A cDNA. Moreover the deduced amino acid sequence had 96 % homology with human activin-A (Fig. 1c).

Using the rabbit activin-A cDNA as probe, we performed *in situ* hybridization histochemistry of rabbit aorta. Both antisense and sense RNA probes were prepared.

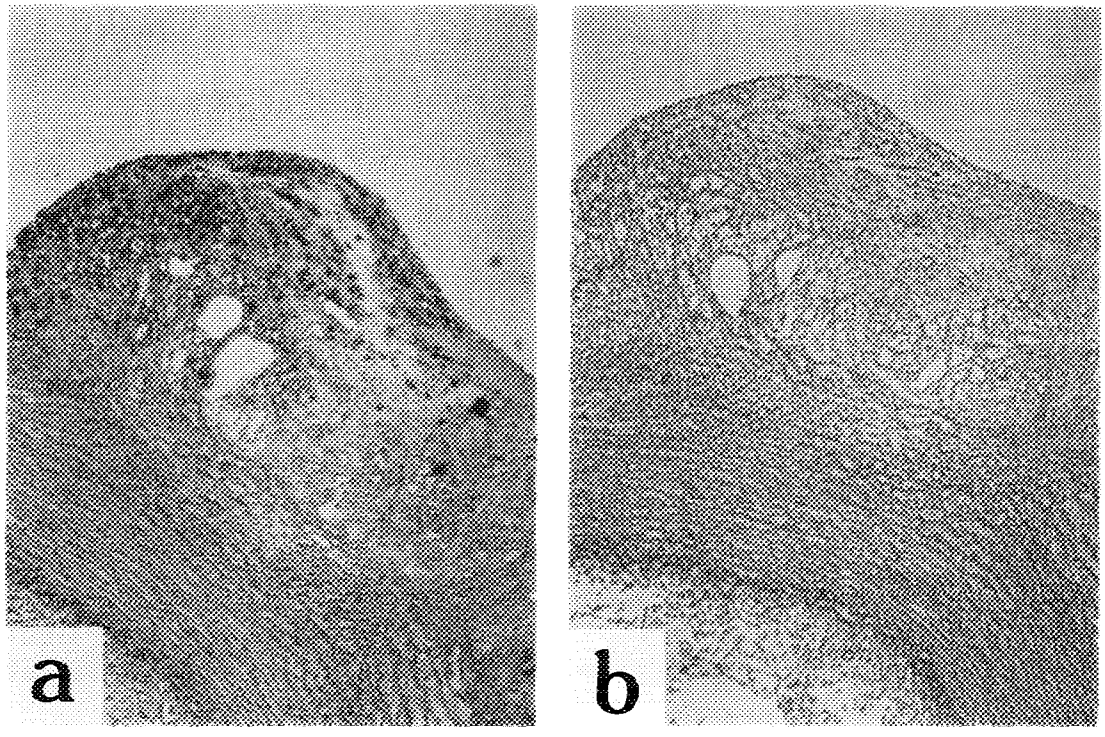

Fig. 2. *In situ* hybridization histochemistry of arteriosclerotic lesions. *In situ* hybridization histochemistry was performed for the arteriosclerotic lesion of WHHL rabbits. Antisense (a) and sense (b) RNA probe derived from rabbit activin-A cDNA was utilized. Original magnification, x 40.

We detected positive signals in the arteriosclerotic lesion of WHHL rabbits by antisense probe (Fig. 2a) but not by sense probe (Fig. 2b). Positive signals are predominantly distributed in neointima of the diseased artery where monocyte-derived macrophages and proliferative smooth muscle cells are abundant. We could not detect positive signals in normal rabbit aorta under the same conditions (data not shown). The activin-A protein was also detected in the arteriosclerotic lesions by fluorescence immunohistochemistry using anti-activin-A antibody (24) (Fig. 3a). This antibody was shown to be suitable for immunostaining and does not cross-react with inhibin A (24) The present work has demonstrated a high level of expression of activin-A in the arteriosclerotic lesions. The expression of activin-A in the healthy aorta is also detectable by RT-PCR method but at a significantly lower degree than diseased artery.

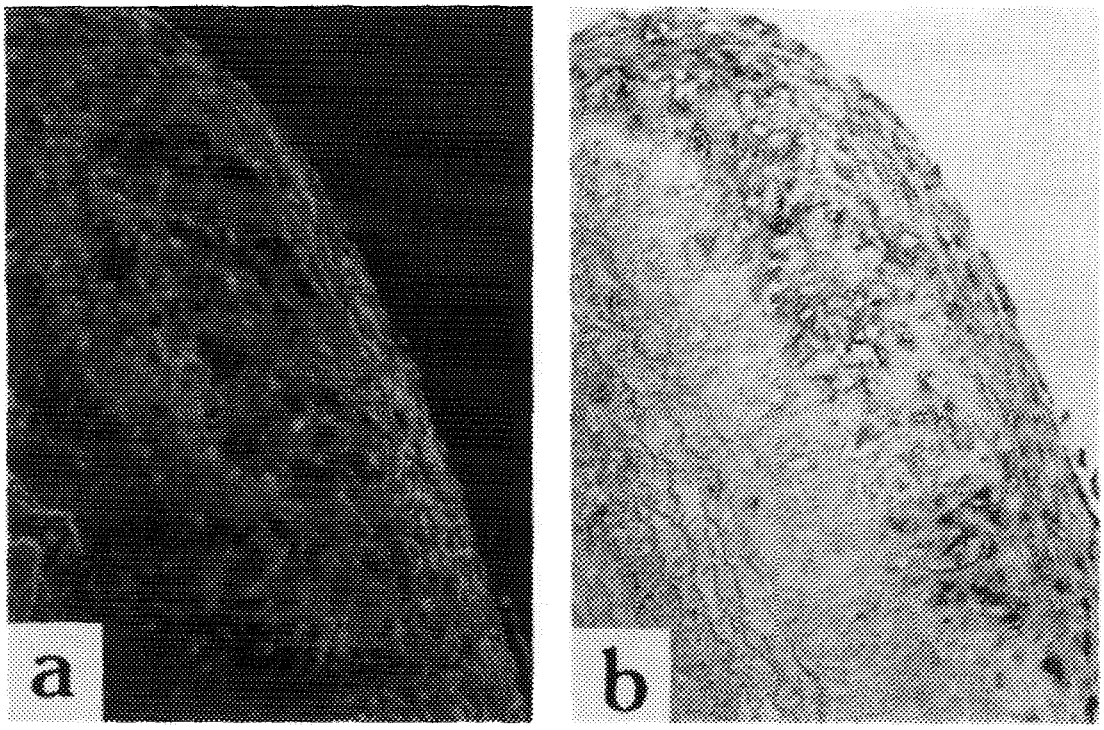

Fig. 3. Immunohistochemistry of arteriosclerotic lesions using anti-activin-A antibody. (a) Immunohistochemistry of activin-A for arteriosclerotic lesions of WHHL rabbits was shown using anti-activin-A anitibody. (b) In situ hybridization histochemistry was performed for the ajacent section of arteriosclerotic lesion shown in Fig. 3a. Original magnification, x 40.

Activin in the arteriosclerotic lesion may exsert its mitogenic effect on vascular smooth muscle cells (22). Alternatively, activin may act on other components in arteriosclerotic lesions, such as endothelial cells and macrophages by paracrine and/or autocrine mechanisms. Activin and its binding protein, follistatin, that is also abundant in these lesions may regulate the proliferation of these cells during the course of atherosclerosis. Further investigations are required to understand the exact roles of activin/follistatin system and the role of TGF-ß family in atherogenesis.

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