

# Potential role of leptin in angiogenesis: leptin induces endothelial cell proliferation and expression of matrix metalloproteinases *in vivo* and *in vitro*

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Abbreviations: ox-LDL, oxidized low density lipoprotein; SD rat, Sprague-Dawley rat; SEM, scanning electron microscopy; PBS, phosphate buffered saline

## Abstract

Leptin, the product of *ob* gene, is an endocrine hormone that regulates adipose tissue mass. Recently, leptin has been found to generate a growth signal involving a tyrosine kinase-dependent intracellular pathway and promote angiogenic processes via activation of leptin receptor (Ob-R) in endothelial cells. However, it is not clear how leptin functions to promote multi-step processes involved in the neovascularization at the atherosclerotic plaque. We have examined the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) and Ob-R in human atherosclerotic lesions, leptin-mediated angiogenesis *in vivo* and *in vitro*. Immunohistochemical analysis of human atherosclerotic aorta revealed an increased expression of Ob-R in the intima of neorevascularized regions and of both MMPs and TIMPs predominantly in the endothelial lining of intimal neovessels and macrophages/foam cells. In the rat corneal angiogenesis assay, leptin elicited a comparable sensitivity of angiogenic activity to those of vascular endothelial growth factor (VEGF). The immunohistological anal-

ysis of the leptin-treated rat cornea showed definitive rises in Ob-R, MMPs and TIMPs expression as well as those of VEGF receptor (VEGFR-1). Leptin (10-40 ng/ml) induced proliferation of the human umbilical vein endothelial cells (HUVECs) and elevation of MMP-2, MMP-9, TIMP-1, and TIMP-2 expression in a dose-dependent manner. Leptin also induced increases of MMP-2, MMP-9, TIMP-1, and Up-regulated the human coronary artery smooth muscle cells (HCASMCs). These findings suggest that leptin, a hormone with pluralistic properties including a mitogenic activity on vascular endothelial cells, plays a role in matrix remodeling by regulating the expression of MMPs and TIMPs. Taken together, our findings further provide evidences for leptin's role as an angiogenesis inducer in the normal organ (rat cornea) and in aberrant vasculature under duress like atherosclerosis.

**Keywords:** leptin, angiogenesis, MMPs, atherosclerosis

## Introduction

Leptin, the product of *ob* gene secreted by adipocytes (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pellemounter *et al.*, 1995), has been known to generate a growth signal involving a tyrosine kinase-dependent intracellular pathway and act as a functional link between adipocytes and the vasculature. Recently, treatment of HUVECs with leptin enhanced formation of capillary-like tubes in an *in vitro* angiogenesis assay and neovascularization in an *in vivo* model of angiogenesis (Bouloumié *et al.*, 1998). Atherosclerotic diseases are known to be closely associated with high levels of LDL and obese state of patient and animals. However, any involvement of leptin in the progression of atherosclerotic diseases has not been well understood although neovascularization within the plaque has been known to be essential in the atheromatous plaque formation (Kahlon *et al.*, 1992; O'Brien *et al.*, 1994).

It is not clear whether a circulating leptin could exert paracrine effects on the plaque forming cells and participate in the progression of atherosclerosis. We have investigated the effects of leptin on vascularization *in vitro* and *in vivo* and on the microenvironment around the plaque by examining expression of tissue modeling enzymes (MMPs) and inhibitors (TIMPs). We have compared the effects of leptin in the aberrant

tissue by examining the expression of Ob-R, VEGF and MMPs/TIMPs in atheromatous plaques and in normal tissue using angiogenesis-sensitive rat corneal model.

## Materials and Methods

### Cell culture and proliferation assay

Normal HUVECs and HCASMCs were purchased from Clonetics and maintained in SmGM2 and EGMMV bullet kits (Clonetics). HUVECs and HCASMCs from passage 5 to 13 were used in this study. For the proliferation assay, the cells in 24 wells were arrested for 24 h in serum-free medium and then incubated in serum-deprived medium supplemented with 2% FBS for 72 h in the presence of increasing concentration of leptin (10 to 40 ng/ml). Total cell number was counted after trypsinization using a cell counter.

### *In vitro* angiogenesis assay

Before the HUVECs were plated, the 24 well plates coated with growth factor-reduced basement membrane proteins (Matrigel, 0.3 ml/well) were solidified at 37°C for 1 h. Leptin was added to Matrigel solution prior to polymerization. For angiogenesis assay with HUVECs, the trypsinized cells were plated on the surface of Matrigel and cultured in serum-deprived medium ( $8 \times 10^4$  cells/well).

### Measurement of VEGF<sub>165</sub> and bFGF by ELISA

Expression of VEGF<sub>165</sub> and bFGF by HUVECS and HCASMCs after stimulation of leptin (10 to 40 ng/ml) were measured by ELISA kit (R&D). The 100  $\mu$ l of standard or cultured medium was added into antibody-coated wells of the microplate and incubated at 25°C for 2 h. The wells were washed 4 times with washing buffer and incubated at 25°C for 2 h. The substrates were added to the washed well and reacted for 20 min at 25°C. After adding the 50  $\mu$ l of stop buffer, the concentrations of VEGF<sub>165</sub> and bFGF were measured with spectrophotometric reader at 450 nm.

### Zymography

Enzymatic activities of MMP-2 and MMP-9 were investigated using zymography analysis. The protein content of the culture medium was measured by the method of Bradford using bovine serum albumin as standard. Aliquots of culture media containing 50  $\mu$ g of protein from HUVECs and HCASMCs, with or without leptin treatment, were loaded on an 11% SDS-PAGE gel containing 0.1% gelatin for electrophoresis. Gels were reacted with collagenase buffer for 16 h at 37°C, stained with 0.25% Coomassie brilliant blue, and destained with 30% isopropanol in 10% acetic acid to visualize the MMP bands.

### Western blot analysis

Fifty  $\mu$ g of protein was subjected to 8-12% gradient SDS-PAGE gel and transferred to immobilon-P membrane (Millipore, Bedford, MA) at 12 V for 1 h. The membrane was blocked in 5% non-fat dry milk in TBS-T at 25°C for 1 h. TIMP-2 antibody (5  $\mu$ g/ml, 1 : 2000 dilution) and secondary antibody (goat anti-rabbit/mouse IgG, horseradish peroxidase-conjugated, Amersham) was used for the detection of TIMP-2. Signals were detected with an ECL kit (Amersham), and exposed to X-ray film (Kodak).

### Analysis of the expression of MMPs by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cells and 5  $\mu$ g of purified total RNA was incubated with reverse transcriptase, dNTP, random hexamer and reaction buffer in a final volume of 20  $\mu$ l at 37°C for 1 h. After a final denaturation at 94°C for 10 min, 10  $\mu$ l of cDNA was subjected to PCR consisting of a denaturation at 95°C for 1 min, followed by 1 min of annealing at 55°C and 1 min of elongation at 72°C for 35 cycles. The primers for amplification of MMP-1, -2, -9 and TIMP-1 were as follows; MMP-1: sense 5'-ggaaacacatggtgtgagtc-3', antisense: 5'-gttagaagagttatcccttgcc-3'; MMP-2: sense 5'-caggctcttctccttcacac-3', antisense 5'-aagccacggcttggtttctc-3'; MMP-9: sense 5'-tgggtacgtgacctatgacat-3', antisense 5'-gccagcccacctccactcctc-3'; TIMP-1: sense 5'-accacagacggccttctgcaattc-3', antisense 5'-ggctatctgggaccgcagggactgc-3'. The amplified cDNA was size fractionated by agarose gel electrophoresis.

### *In vivo* angiogenesis assay

Adult SD rats were anesthetized with ketamine and the eye was topically anesthetized with oxybuprocaine. With the use of an operating microscope, a central intra-stromal linear keratotomy was performed with a surgical blade. A lamellar micropocket was then dissected and extended to within 2 mm of the superior limbus. Dehydrated pellet (2 mm diameter) made of 70% hydratable hydrogel containing PBS (n=4) or 125 ng of leptin (n=4) were placed into the preformed corneal pocket. The eyes were routinely examined by slit lamp biomicroscopy on day 7 after implantation. To analyze whether the effect of cytokines on angiogenesis was direct or due to infiltration by inflammatory cells, histological studies were performed on sections after hematoxylin-eosin staining.

### Tissue preparation and histologic examination

Immediately following the removal of human aortic segments (6 aortic dissections with atherosclerotic change and 6 aortic aneurysms with atherosclerotic change, median age; 54 years old) excluding dissection and aneurysmal segment, each segment was perfused with

10% formalin to maintain vascular integrity. The atherosclerotic change was confirmed microscopically. Two normal aortic segments obtained by surgical repair for traumatic aortic dissection were used as control (mean age: 28 years old).

Paraffin sections (5  $\mu$ m) from human aortas and rat cornea were made and transferred to slide glasses. Slides were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked for 10 min at room temperature in 50% volume  $H_2O_2$ /50% volume methanol and rinsed in running tap water. Non-specific protein binding sites were blocked by applying 5% normal goat serum diluted in PBS/0.05% Tween 20 (pH 7.2-7.4) to slides for 10 min at room temperature. The primary antibody (goat polyclonal antibodies for Ob-R; rabbit polyclonal antibodies for VEGF; goat polyclonal antibodies for MMP-2; goat polyclonal antibodies for TIMP-2; rabbit polyclonal antibodies for flk-1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was diluted in 1% normal goat serum in PBS/0.05% Tween 20, applied and incubated overnight at 4°C in a humidity chamber. Antibodies were directed against the carboxyl (C-20) or the amino terminus (N-20) of Ob-R recognized a 170 kD protein, the size of which is consistent with the human leptin receptor. On day 2, the primary antibody was rinsed off in tap water, blotted and the biotinylated secondary antisera cocktail including goat anti-mouse IgG and goat anti-rabbit diluted 1/400 was incubated on the slides for 30 min at room temperature. Slides were rinsed in running tap water, blotted and streptavidin-horseradish peroxidase diluted 1/500 in PBS/0.05% Tween 20 containing 1% normal goat serum was applied and incubated for 30 min at room temperature. The slides were rinsed in tap water and color developed in 3-amino-9-ethylcarbazole substrate solution for 15 min at room temperature, then counterstained in methylene blue for 30 s and covered with coverslip.

### Statistical tests

Significance of values were determined with the 2-tailed student's *t* test. All results are expressed as the mean  $\pm$  SEM.

## Results

### Cell proliferation study

The effect of leptin on HUVEC and HCASMC proliferation was shown in Figure 1. Treatment with leptin (10 to 40 ng/ml) stimulated HUVECs proliferation, but had no significant effect on HCASMCs proliferation. These differential effects of leptin on HUVECs and HCASMCs are similar to the effect of VEGF.

### In vitro angiogenesis assay

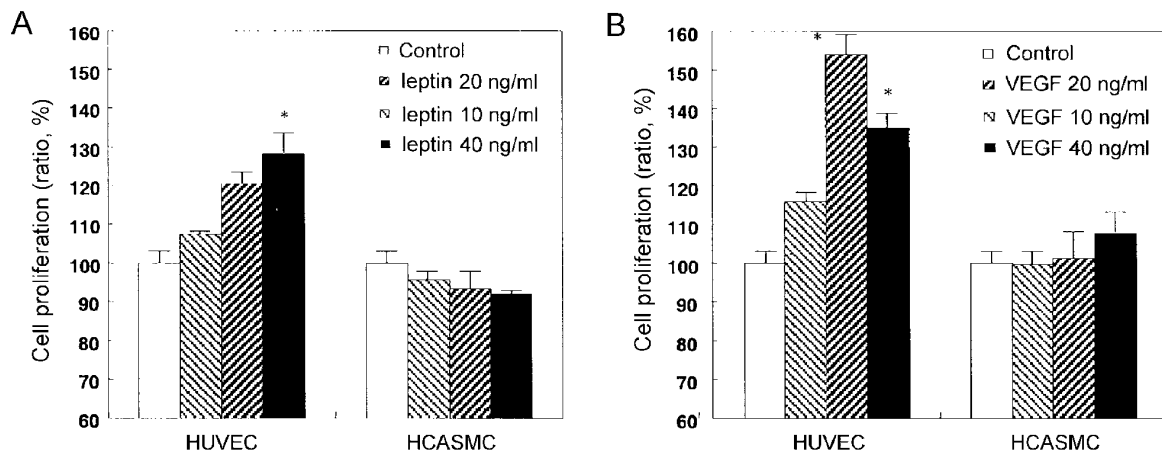
As shown in Figure 2, HUVECs cultured on Matrigel containing leptin (100 ng/ml) were stretched and elongated forming a capillary-like structure within 4 h, but HUVECs on untreated gel failed to form the capillary structure.

### Effect of leptin on VEGF and bFGF expression

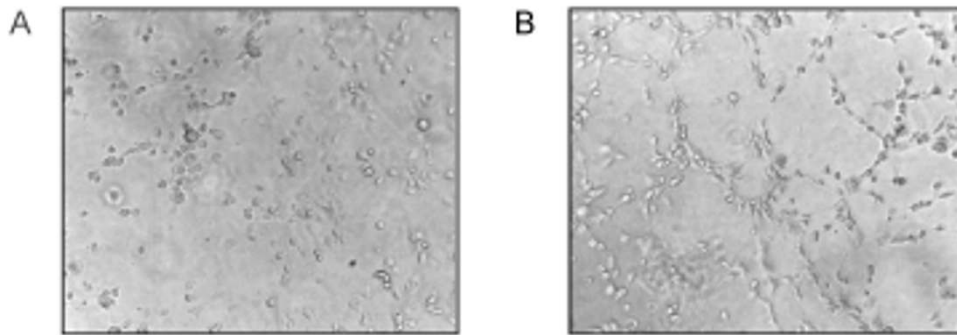
The concentrations of VEGF and bFGF after stimulation with leptin (10 to 40 ng/ml) for 48 h were measured to determine whether the angiogenic effect of leptin is dependent on the other growth factors. As shown in Figure 3, leptin enhanced the VEGF expression in a dose-dependent manner from HUVECs, but not HCASMCs. There was no significant change of bFGF expression in both HUVEC and HCASMC (data not shown).

### Effect of leptin on MMPs protein expression

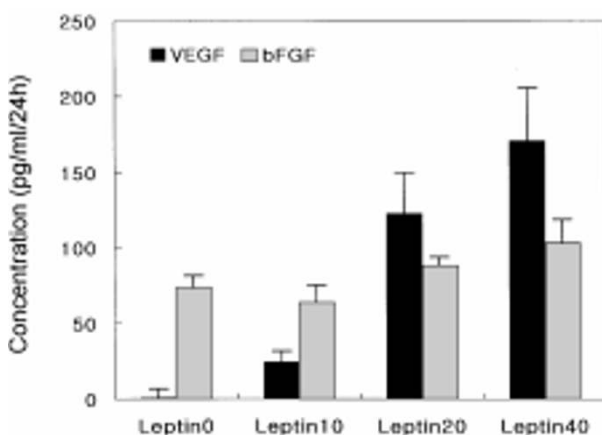
MMP-2 and MMP-9 productions by HUVEC and HCASMC were analyzed using zymography. The activities of MMP-



**Figure 1.** Effect of leptin on HUVECs and HCASMCs proliferation. HUVECs and HCASMCs were cultured in the presence of increasing concentration of human recombinant leptin (A) or VEGF<sub>165</sub> (B) for 72 h in serum-deprived medium. Data are presented as mean  $\pm$  SEM. Three independent experiments were performed.



**Figure 2.** Effect of leptin on the formation of capillary-like structures in cultured HUVECs in growth factor-reduced Matrigel. HUVECs plated on Matrigel and cultured in serum free medium in the absence (A) or presence of 100 ng/ml human recombinant leptin (B).



**Figure 3.** Effects of leptin on VEGF and bFGF secretion. Leptin up-regulates the secretion of VEGF, but not bFGF by HUVECs.

2 and MMP-9 increased after treatment with leptin. The activities of pro- and active MMP-2 were increased 1.5-fold at the leptin concentration of 10 ng/ml, and 4-fold at a higher concentration (20 or 40 ng/ml) in HUVECs (Figure 4A). Total amount of MMP-9 secretion was much lower than MMP-2 secretion and the effect of

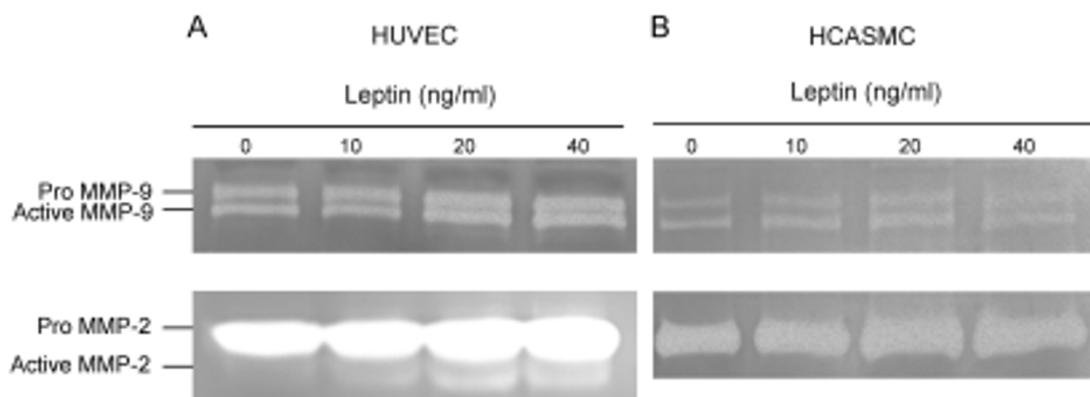
leptin on MMP-9 secretion was less prominent. The change of MMP-2 in HCASMCs was not as prominent as in HUVECs. However, at a high concentration of leptin (40 ng/ml), the activity of pro MMP-2 was increased as shown in Figure 4B.

#### Effect of leptin on HCASMCs MMPs and TIMPs mRNA expression

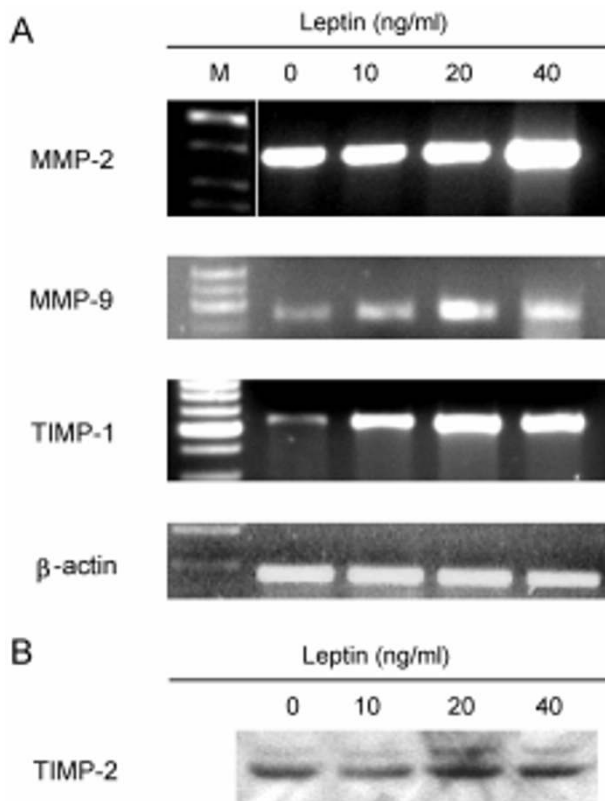
The mRNA levels of MMP-2, MMP-9, and TIMP-1 by HCASMCs were upregulated after leptin treatment (Figure 5A). The TIMP-2 protein expression by Western blot analysis was also increased in the HCASMCs treated with leptin (Figure 5B).

#### Expression of Ob-R, VEGFR-1, and MMPs/TIMPs in neovascularized atheromatous plaque

Control aortic tissues revealed little Ob-R (N-20, C-20) immunoreactivity in intimal SMCs and infiltrated inflammatory cells. The VEGFR-1 immunoreactivity was also weakly distributed in the endothelial lining with little non-endothelial immunoreactivity. Compared to the control aorta, the atherosclerotic aorta showed the increased cellularity accompanied by the infiltration of inflamma-



**Figure 4.** Effects of leptin on human MMP-2 and MMP-9 secretion. Leptin enhances MMP-2 secretion from both HUVECs and HCASMCs. Enzymatic activity of human MMP-2 and MMP-9 were analyzed using SDS-PAGE gelatin zymography.



**Figure 5.** Effects of leptin on MMP-2, MMP-9, TIMP-1, and TIMP-2. The PCR products from cDNA derived by RT-PCR of HCASMCs with primers specific for MMP-2, MMP-9, and TIMP-1 were shown (A). Equal loading of RNA was confirmed by the PCR products of  $\beta$ -actin. The expression of TIMP-2 was analyzed by the Western blot analysis (B).

tory cells, such as foam cells and neovascularization at plaque base (Figure 6A). Atherosclerotic aortic plaques demonstrated marked increase in Ob-R (C-20, N-20) immunoreactivity, predominantly in foam cells, VSMCs and the vascular endothelial lining of intimal neovessels (Figure 6B, C). Tissue expression of Ob-R (N-20, C-20) and VEGFR-1 were co-localized in atherosclerotic plaques especially in non-endothelial cells such as foam cells and vascular smooth muscle cells (VSMCs) (Figure 6D). Furthermore, immunoreactivity of Ob-R and VEGFR-1 were co-expressed within neovascular endothelial cells. The immunoreactivities for MMP-9 and TIMP-1 were evident in the neovascular endothelial cells suggesting that the expression of MMP has a role in the neovascularization in atheromatous plaque (Figure 6E, F).

#### **In vivo angiogenesis assays and immunohistochemistry**

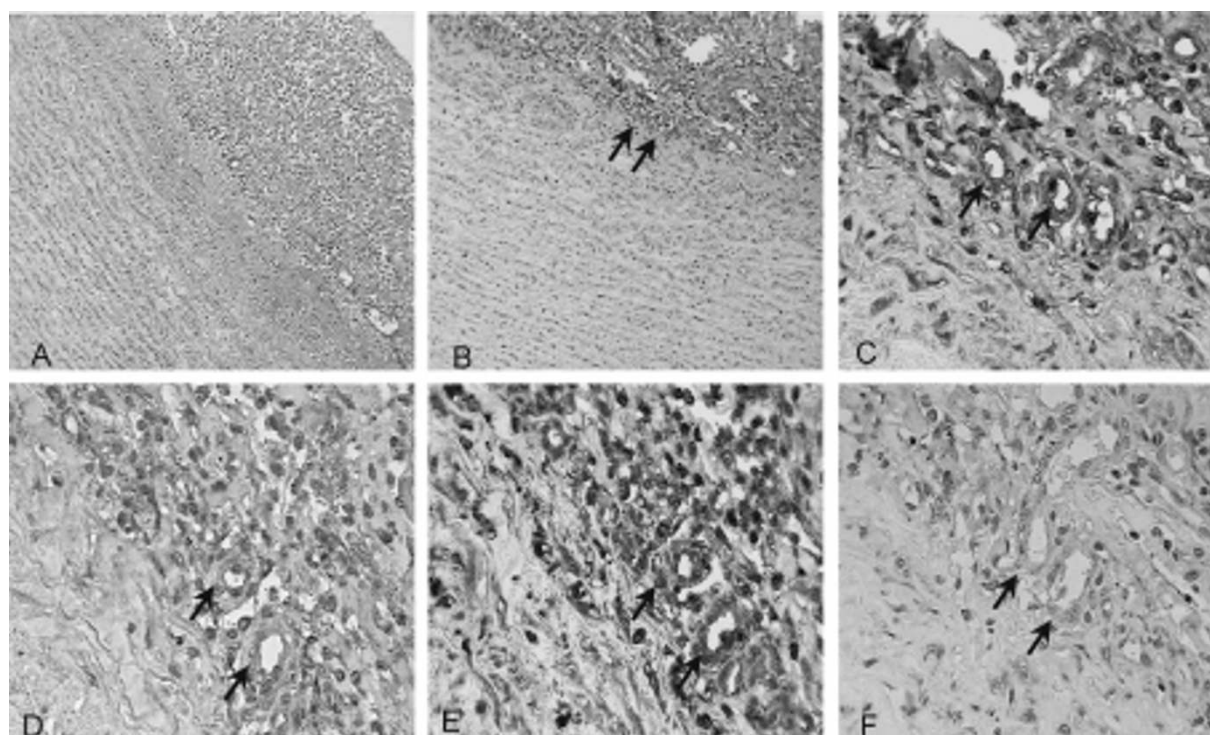
The former results suggest that leptin induces angiogenesis through the endothelial proliferation and expression of MMPs. Thus, we used a rat corneal model

to evaluate the angiogenic property of leptin *in vivo* and to investigate whether the expression of MMP is related with angiogenic activity of leptin. Leptin at a dose of 125 ng/pellet promoted a new vessel growth, progressing over half way to the lens by day 7 (Figure 7). Histologic studies stained with hematoxylin & eosin demonstrated that rat cornea treated with Hydron pellet containing leptin revealed blood filled neovessels compared with rat corneae treated with PBS pellets. Comparing pellet with vehicle alone (Figure 8A), immunoreactivity of MMP-2, MMP-9, TIMP-2 and VEGFR-1 were markedly increased around the leptin-treated pellets (Figure 8B).

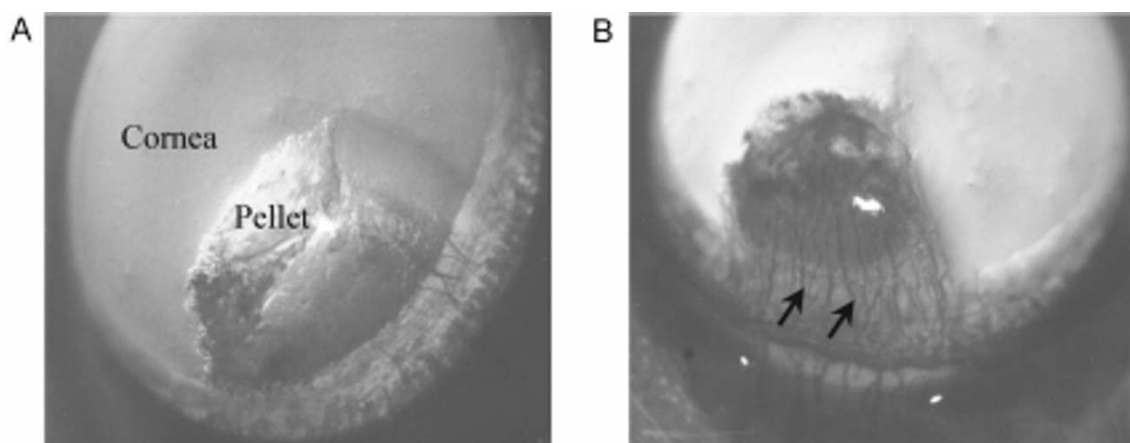
#### **Discussion**

In the present study, we have demonstrated the angiogenic activity of leptin and its possible mechanisms including indirect effect through the VEGF and effect on the MMP. Leptin, the product of the *ob* gene secreted by adipocytes, has been known to play a key role in the regulation of body weight, reproduction, hematopoiesis, and proinflammatory response (Bennett *et al.*, 1996; Gainsford *et al.*, 1996; Conway and Jacobs, 1997; Loffreda *et al.*, 1998; Santos-Alvarez *et al.*, 1999). Leptin acting as a functional link between adipocytes and the vasculature, might also play an important extrahypothalamic role in the modulation of adipose tissue mass. Recently, Sierra-Honigsmann *et al.* (1998) demonstrated that treatment of HUVECs with leptin enhanced formation of capillary-like tubes in the *in vitro* angiogenesis assay and neovascularization in the *in vivo* model of angiogenesis. We hypothesized that the overexpression of leptin has a role in the growth of atheromatous plaques through its effect on neovascularization.

In atherosclerotic plaques, the immunoreactivity of Ob-R increased especially in foam cells and VSMCs. Interestingly, the immunoreactivity of VEGFR-1 was co-localized in atheromatous plaques and observed in foam cell-rich regions adjacent to the lipid core or the basal regions of plaque consisting predominantly of VSMCs. Ananyeva *et al.* (1997) recently demonstrated that ox-LDL induced the release of aFGF from aFGF-transfected VSMCs. VEGF receptor (flt-1) expression in monocyte and VSMCs has recently been confirmed; this receptor mediates chemotaxis and tissue factor production associated with up-regulation of MMPs to contribute to the lateral expansion of new blood vessels. However, the immunoreactivities of Ob-R, MMPs and their inhibitors (TIMPs) increased in atherosclerotic plaques simultaneously with VEGFR-1, suggesting that the expression of VEGF is related with the increased activity of leptin. The experimental study with rat corneas also showed a prominent co-localization of Ob-R and VEGFR-1 suggesting that the coupling of these factors



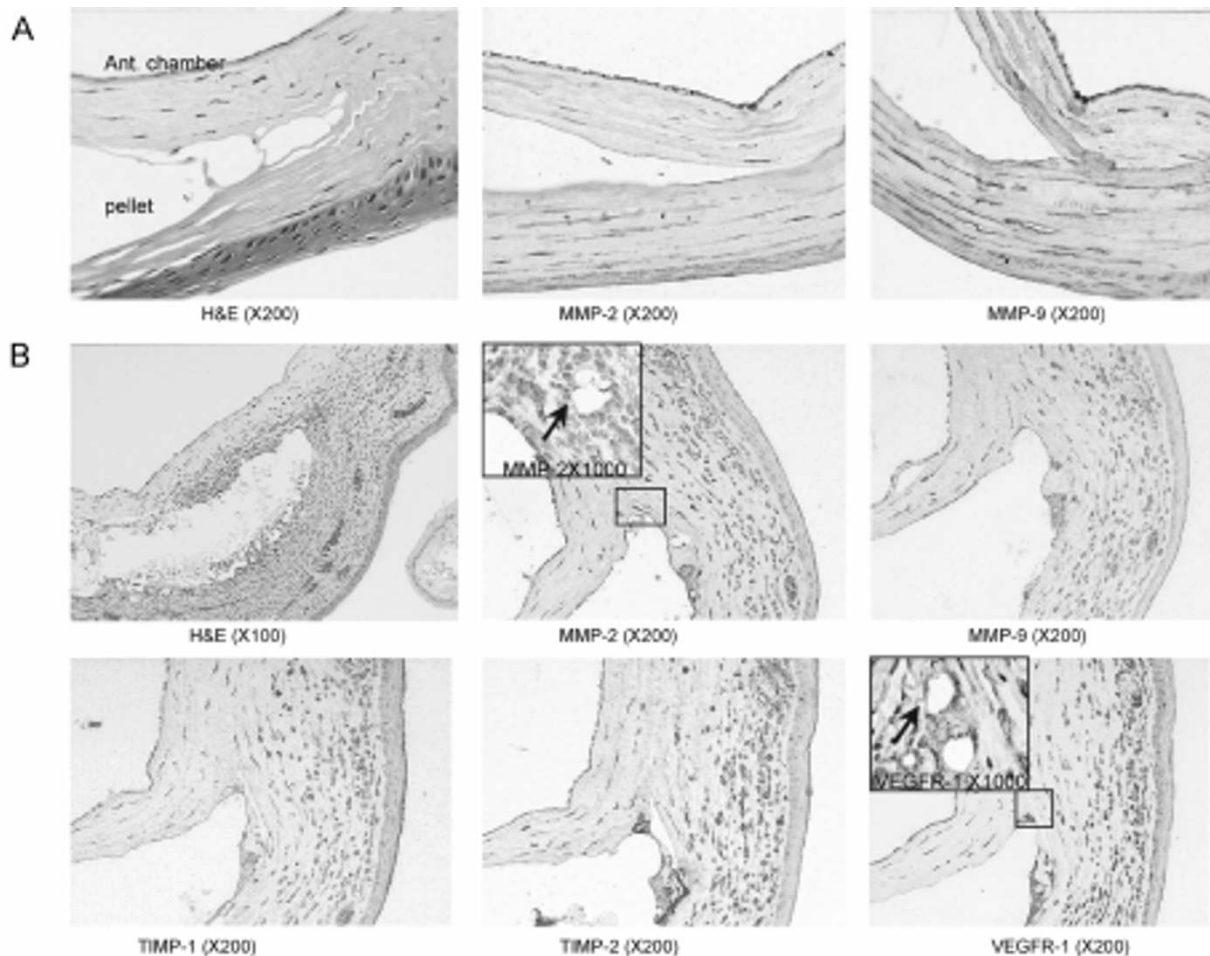
**Figure 6.** Hematoxylin & eosin stain of atherosclerotic lesion (panel A; final magnification, 100 x). Expression of the leptin receptor using a goat polyclonal antibody directed against the amino (N-20)(panel B; 100 x), carboxyl (C-20)(panel C; 400 x) terminus of human Ob-R, immunoreactivity for VEGFR-1 (panel D; 400 x), MMP-9 and TIMP-1 (panel E and F; 400 x) in atherosclerotic lesion. A strong immunostaining was seen in atherosclerotic plaque, mainly in foam cells, vascular smooth muscle cells and the vascular endothelial lining of intimal neovessels (black arrows).



**Figure 7.** *In vivo* angiogenic activity of leptin. Corneal response 7 days after implantation of a Hydron pellet containing PBS (A) or 125 ng of leptin (B). Arrow indicates neovessels progressing from the limbus to the pellet.

participates in neovascularization. The *in vitro* study used to elucidate the effect of leptin on VEGF and bFGF expression showed that leptin increased the secretion of VEGF in HUVECs in a dose-dependent manner, but it had little effect on the secretion of bFGF. However, leptin has little effect on the secretion of VEGF<sub>165</sub> or bFGF in HCASMC. The differential effects on VEGF according to the cell type need a further study.

One of the initial steps in the angiogenesis process involves the degradation of the subendothelial basement membrane and surrounding extracellular matrix (ECM). Following matrix breakdown, endothelial cells migrate and proliferate to form new vessels, and proteolytic enzymes that degrade ECM mediate this process. The MMPs, a family of zinc-dependent proteinase, can digest various ECM components, the requirement for



**Figure 8.** Immunohistochemical staining was performed with antibody for MMP-2, MMP-9, TIMP-1, TIMP-2, and VEGFR-1 (flt-1) in rat cornea on day 7 after implantation of a Hydrion pellet containing PBS (A) or 125 ng of leptin (B). Comparing with Hydrion pellet containing PBS, rat cornea with leptin-treated pellet reveals blood filled neovessels. Immunoreactivity for MMP-2, MMP-9, TIMP-2 and VEGFR-1 are markedly increased around the pellets containing leptin. Arrows indicate neovessels. Lacunae in corneal tissue represent the sections of vessels. In controls, only a few vessels are noted, whereas in leptin-treated animals, marked angiogenesis is obvious.

cellular migration. Wang and Keiser (1998) demonstrated the enhancement of MMP expression in arterial SMCs after VEGF stimulation. The vascular localization of MMP may permit their spatial role in the degradation of subendothelial basement membrane, which is a crucial step in angiogenesis and atherosclerosis (Stetler-Stevenson, 1999). MMPs are involved in more than the breakdown of connective tissue barriers necessary for new vessel formation. They also function to promote angiogenesis by regulating endothelial cell attachment, proliferation and migration of endothelial cells, either directly or by release of growth factors sequestered in the ECM. Moreover, new evidence suggests that MMPs may also generate or release angiogenesis inhibitors such as angiostatin from the ECM (Cornelius *et al.*, 1998). Among MMPs, MT-1-MMP and MMP-2 are active on the endothelial cell surface and function immediately in extracellular matrix turnover and regulation of cell-

ECM interactions. These changes in turnover of the ECM may result in phenotypic changes associated with the angiogenic response (Stetler-Stevenson, 1999). TIMP-2 binding to the active site of MT-1-MMP and the COOH-terminal hemopexin-like domain of pro-MMP-2 results in the formation of a ternary complex. If this occurs in proximity to a second MT-1-MMP molecule, proteolytic modification of the pro-fragment of MMP-2 initiates an activation of this protease. The activated protease may then dissociate from the cell surface and contribute to degradation of the extracellular matrix prerequisite for endothelial sprout invasion. Although recent studies suggest the expression of leptin receptor in cell culture study and nonvascular tissue, there was no information about the interaction between leptin and MMPs/TIMPs (Bouloumié *et al.*, 1998; Sierra-Honigsmann *et al.*, 1998).

In the present study, we demonstrated that the leptin receptor was expressed in the human atheromatous



plaques with neovascularization using polyclonal antibodies to synthetic peptides based on the sequence of human leptin receptor and leptin induced the proliferation of endothelial cell and expression of MMPs/TIMPs. *In vitro* study, the leptin increased the secretion of VEGF in HUVECs in a dose dependent manner and treatment with leptin on HUVECs and HCASMCs enhanced the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 by Western blotting and RT-PCR. These findings suggested that the leptin might be used as an angiogenic factor or mitogenic factor of vascular endothelial cells and play a role in matrix remodeling of angiogenic process and on neovascularization micro-environment. The present study might open a promising perspective in regard to the future investigations of leptin-MMPs dependent modulation of atherogenesis and vascular neovascularization under pathophysiological conditions.

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