Recently, leptin, the product of the Ob gene, was identified as an adipocyte-secreted protein.[8-10] The human leptin receptor (Ob-R) was recognized, and the intracellular domain of the long form of human leptin receptor contained sequence motifs of the carboxyl terminal, suggestive of intracellular signal-transducing capability. In vitro study, treatment of human umbilical venous endothelial cells (HUVECs) with leptin enhanced the formation of capillary-like tubes in an in vitro angiogenesis assay and neovascularization in an in vivo model of angiogenesis. These results indicate that leptin, via activation of the endothelial Ob-R, generates a growth signal involving a tyrosine kinase-dependent intracellular pathway and promotes angiogenic processes.[11,12] However, there is a lack of information regarding the role of leptin in atherosclerotic vascular diseases.

It is hypothesized that such a local leptin signaling pathway and high concentration of leptin within vasa vasorum and plaque itself, exerting paracrine effects, may influence inflammatory and vascular neovascularization coupling with functional upregulation of VEGF. To test the hypothesis that leptin may influence neovascularization coupling with VEGF and MMP-9, we investigated whether there is colocalization between Ob-R, VEGF and MMP-9 in neovascularization of atherosclerotic lesions of the aorta. The present study is the first report on vascular tissue and opens a promising perspective concerning future investigations of leptin-dependent modulation of atherogenesis and vascular neovascularization under pathophysiological conditions.

MATERIALS AND METHODS

Tissue preparation and histologic examination

Immediately following 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 coronary artery morphologic integrity. The atherosclerotic change was confirmed microscopically. Two normal aortic segments obtained by surgical repair for traumatic aortic dissection were for control study (median age: 28 years old). To preserve the integrity of the adventitia and perivascular tissues, aortic specimens were carefully removed in a segment along with adjacent tissues and rinsed with phosphate buffered saline (PBS). Each segment was embedded in paraffin and cut in 5 μm sections, which were stained with hematoxylin-eosin.

Immunohistochemistry for Ob-R,VEGF, MMP-9 and TIMP-1

Paraffin sections (5 μm) were made and transferred to glass slides. Slides were deparaffinized and rehydrated through the following solutions: xylene twice for 5 minutes, 100% ethanol twice for 10 dips and 95% ethanol twice for 10 dips. Endogenous peroxidase activity was blocked for 10 minutes 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 minutes at room temperature. The serum was blotted off and the primary antibody (goat polyclonal antibodies for Ob-R; rabbit polyclonal antibodies for VEGF; goat polyclonal antibodies for MMP-9; goat polyclonal antibodies for TIMP-1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was diluted in 1% normal goat serum and PBS/0.05% Tween 20, applied and incubated overnight at 4°C in a humidity chamber. Antibodies directed against the carboxyl (C-20) or the amino terminus (N-20) of Ob-R recognized a 170-kDa protein, the size of which is consistent with the human leptin receptor.[12]

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 minutes at room temperature. Slides were rinsed in running tap water, blotted and streptavidin-horseradish peroxidase diluted 1/500 in PBS/ 0.05% Tween 20 and 1% normal goat serum was applied and incubated for 30 minutes at room temperature. The slides were rinsed in tap water and color developed in 3-amino-9-ethylcarbazole substrate solution for 15 minutes at room temperature, then counterstained in methylene blue for 30 seconds and covered with coverslip.

Stock solutions:

Tween 20 (Pierce Chemical, Rockford, Ill, USA)

Normal goat serum (Dako, Carpinteria, CA, USA)
Biotinylated mouse IgG (Dako, Carpinteria, CA, USA)
Biotinylated rabbit IgG (Dako, Carpinteria, CA, USA)
Streptavidin-horseradish peroxidase (Dako, Carpinteria, CA, USA)
3-amino-9-ethycarbasole (Sigma, St. Louis, MO, USA)

Microscopic three-dimensional computerized tomography (3-D CT) reconstruction

To visualize the three-dimensional spatial pattern of intimal neovascularization using a microscopic 3-D CT,[2] coronary arteries with whole heart (n=2) obtained from human were utilized since there was technical difficulty in injecting radiopaque liquid polymer into the microvasculature of the aortic vessel wall. The non-infarct related right coronary artery of a sudden cardiac death patient (68-year-old male) due to acute anterior myocardial infarction (Fig. 2) and the right coronary artery of a chronic stable angina patient (59-year-old male) who had died due to lung cancer were used. Following removal of the heart, glass cannulae were tied at the coronary orifices and injected with 500 ml of heparinized saline (0.9% sodium chloride with 5,000 units of heparin) at a pressure of 70 mmHg to clear the coronary network of remaining blood. The anterior cardiac vein was ligated to exclude adventitial venular supply from the left anterior descending coronary arteries. A specially-prepared, low viscosity, radiopaque liquid polymer compound (MV-122, Canton Biomedical Products, Boulder, Col, USA) was then injected through the cannulae. The heart was immersed in 10% buffered formalin and placed in a refrigerator at 4°C overnight to allow polymerization of the plastic compound. On the following day, the coronary arterial segments (about 2 cm in the length) were removed from the heart by careful dissection, placed in 70% ethanol and dehydrated through ascending alcohol concentrations. At successive 24-hour intervals, glycerin concentrations were raised from 30, 50, 75%, and finally 100% pure glycerin in order to completely dehydrate the coronary segments. The specimens were then rinsed in acetone and left in open air for 24 hours. Finally, the coronary segments were embedded in wax for scanning. The main luminal polymer was removed for the identification of radiopaque filled microvasculature and plaque calcification.

Specimens were scanned by a micro-CT system consisting of a spectroscopy X-ray tube, a fluorescent crystal plate, a microscopic objective and a charge coupled device camera (CCD).[13-15] The charge in each pixel was digitized and stored as an array in a computer as 500−1000 projections of the specimen in 360 degree rotations.

Three-dimensional images were reconstructed using a modified Feldhaup cone beam filtered back projection algorithm and the resulting 3-D images were displayed using Mayo Analyze software (Version 7.5, Biomedical Imaging Resource, Mayo Foundation, Rochester, MN, USA). Volume rendering provided a variety of display representations of 3-D image data sets. Volume rendered transmission displays, voxel gradient shading and maximum intensity projections were displayed at various angles and threshold values of voxels. Average voxel size was 21 m, and images of up to 800 slices were rendered for each arterial specimen (each with a matrix of 10−20 mm cubic voxels × 16 bits of gray scale).

RESULTS

Control specimens

At histologic examination, control aortic specimens revealed early atherosclerotic changes in intimal areas as a fatty streak. Intimal lesions were composed of macrophages/foam cells and VSMCs. However, there was no intimal neovascularization within early atherosclerotic lesion.

Control aortic tissues revealed little Ob-R (N-20, C-20) immunoreactivity in intimal SMCs and infiltrated inflammatory cells (Fig. 1). VEGF immunoreactivity in the endothelial lining was distributed weakly with little non-endothelial immunoreactivity. Immunoreactivity for MMP-9 and TIMP-1 was small, predominantly in the foam cells and VSMCs.

Atherosclerotic specimens

Radiopaque liquid polymer was injected through the coronary ostium using a glass cannulae and was filled in the adventitial vasa vasorum originated from a branch of the coronary artery. Atherosclerotic coronary arteries scanned by a micro-CT showed a dense plexus of microvessels in the adventitia and the noncalcified plaque itself. Neovascularization of the

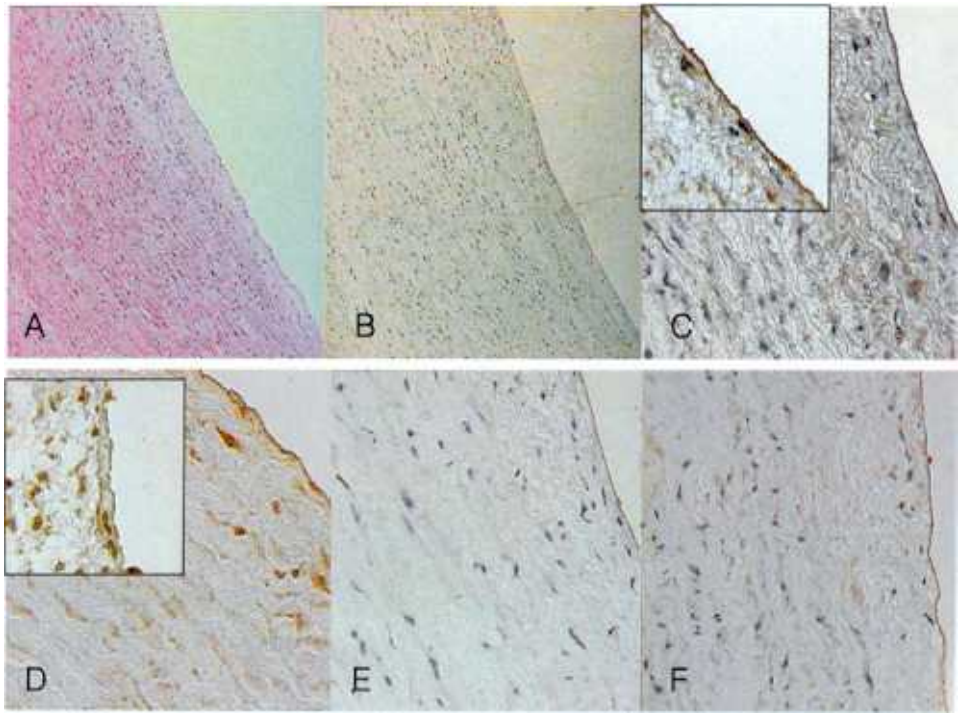

Fig. 1. Hematoxylin & eosin stain of normal aorta (panel A; final magnification, 100 ×). Expression of the leptin receptor using a goat polyclonal antibody directed against the amino (N-20, panel B; final magnification, 100 ×) or carboxy (C-20, panel C; final magnification 400 × & 1000 ×) terminus of human Ob-R and immunoreactivity for VEGF (panel D; final magnification 400 × & 1000 ×), MMP-9 and TIMP-1 (panel E and F; final magnification, 400 ×) in normal aorta. Immunoreactivity for Ob-R and VEGF was seen weakly in normal endothelial lining with little non-endothelial immunoreactivity.

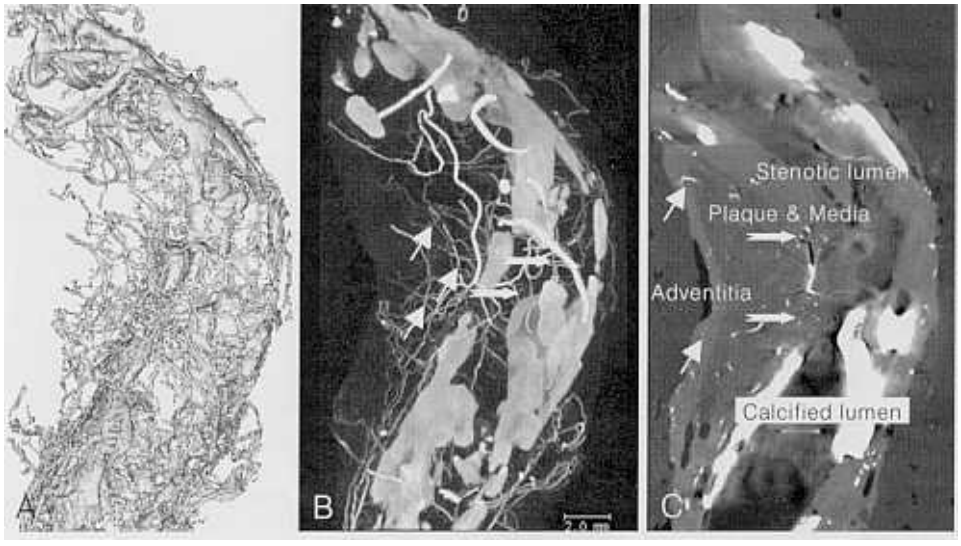

Fig. 2. Micrographs showing the spatial distribution of vasa vasorum and intimal neovascularization (white arrows) in voxel gradient shading (panel A), maximum intensity projection (panel B) and transverse sectional image (panel C) from atherosclerotic coronary artery using a microscopic CT. Coronary artery obtained from autopsy specimen shows a dense plexus of microvessels in the adventitia (short white arrows) and plaque itself (long white arrows) (voxel size, 21 m. bar size, 2 mm).

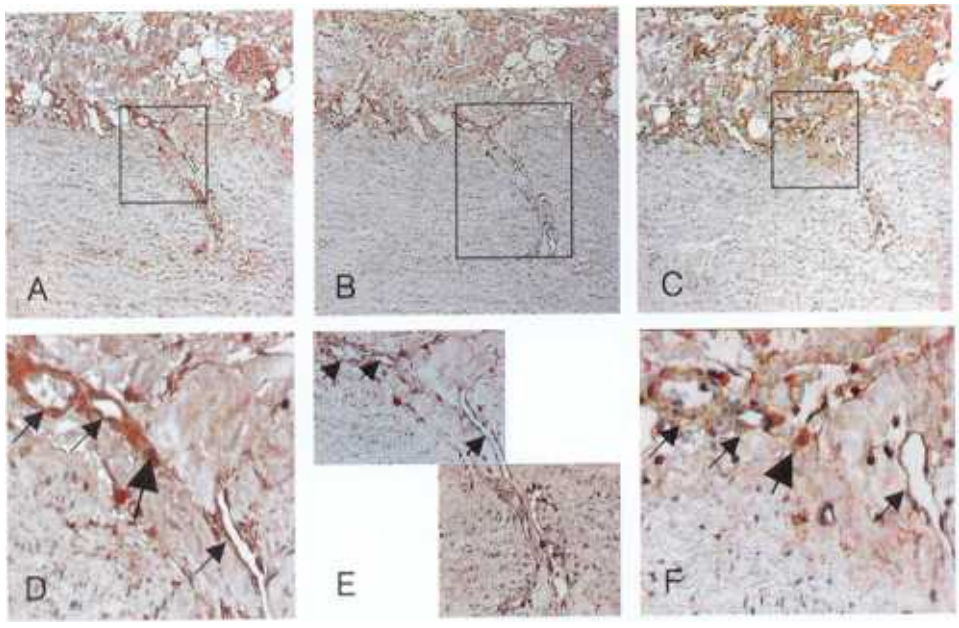

Fig. 3. Intimal thickening and neovascularization with extension from inner medial layer to atherosclerotic plaque base. Furthermore, immunoreactivity for VEGF (panel A & D), C-20 of Ob-R (panel B & E), MMP-9 (panel C & F) were evident in endothelial lining (thin black arrows) and perivascular cells of neovessels (thick black arrows) (final magnification, 100× & 400×).

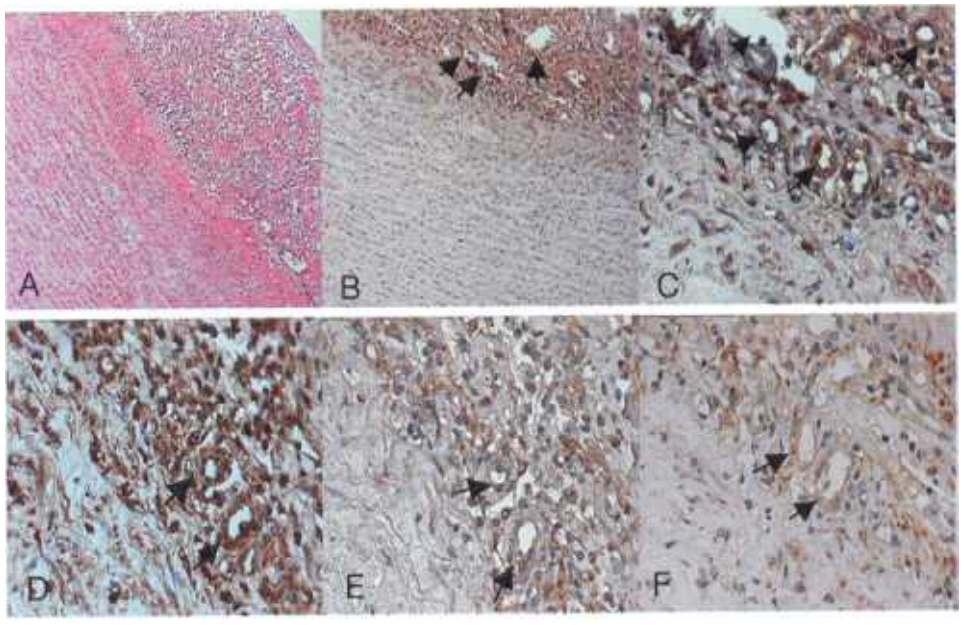

Fig. 4. Hematoxylin & eosin stain of atherosclerotic lesion (panel A; final magnification, 100×). Immunoreactivity for N-20 (panel B; final magnification, 100×), C-20 (panel C; final magnification, 400×) of Ob-R, VEGF (panel D; final magnification, 400×), MMP-9 and TIMP-1 (panel E and F; final magnification, 400×) in atherosclerotic lesion. A strong immunostaining was seen in atherosclerotic plaque, mainly in foam cells, vascular smooth cells and the vascular endothelial lining of intimal neovessels (black arrows). Compared with fig. 1, a strong immunostaining was colocalized, mainly in foam cells, vascular smooth cells and the vascular endothelial lining of intimal neovessels.

adventitial vasa vasorum with extension into theintima of atherosclerotic lesions was observed. Intraplaque neovessels were communicating with adventitial vasa vasorum and mainly located within noncalcified atherosclerotic plaque. Three-dimensional images, voxel gradient shading, maximum intensity projection and transverse section, suggested the intimal neovascularization within plaque (Fig. 2). In atherosclerotic aortas, intimal thickening with neovascularization at the plaque base, even though extension of microvasculature from the inner medial layer suggested communication between intimal neovascularization and vasa vasorum (Fig. 3).

Compared with control aorta specimens, the increase in the cellularity of the intima was accompanied by the infiltration of inflammatory cells, such as foam cells and neovascularization at the plaque base. The atherosclerotic aortic plaque base demonstrated marked increases in Ob-R (C-20, N-20) immunoreactivity, predominantly in foam cells, VSMCs and the vascular endothelial lining of intimal neovessels (Fig. 4). VEGF immunoreactivity in normal endothelial lining was distributed within control aortic specimens. Contrary to control aorta, atherosclerotic plaque base revealed marked increases in VEGF immunoreactivity, mainly in foam cells, VSMCs and the vascular endothelial lining of intimal neovessels. Compared with Ob-R immunoreactivity in corresponding sections, tissue expression of Ob-R (N-20, C-20) and VEGF were colocalized in atherosclerotic plaques. These expressions were evident in non-endothelial cells, foam cells, and VSMCs. Furthermore, immunoreactivity for Ob-R and VEGF was coexpressed within the neovascular endothelial cells. Atherosclerotic lesions demonstrated marked increases in immunoreactivity for MMP-9 and TIMP-1, predominantly in plaque areas. Moreover, MMP-9 and TIMP-1 immunoreactivity was evident in the foam cells and VSMCs.

DISCUSSION

The present study of atherosclerotic arteries showed that a dense plexus of microvessels in the adventitia, noncalcified plaque and intraplaque neovessels were communicating with adventitial vasa vasorum and were mainly located within noncalcified atherosclerotic plaque.

Angiogenesis, the formation of new blood vessels by capillary sprouting from preexisting vessels, is a major physiological event that occurs for example in the female reproductive system throughout the menstrual cycle and in pregnancy, as well as during wound healing. The angiogenic process is under the control of proangiogenic factors, including VEGF, fibroblast growth factor (FGF), and antiangiogenic factors such as endostatin and angiostatin.[16] In atherosclerotic plaques, VEGF immunoreactivity was observed in foam cell-rich regions adjacent to the lipid core or the neovascularized basal regions of plaque consisting predominantly of VSMCs. Ananyeva et al,[17] recently demonstrated that Ox-LDL induced the release of acidic FGF from acidic FGF-transfected VSMCs. VEGF receptor (flt-1) expression in monocytes and VSMCs has recently been confirmed; this receptor mediates chemotaxis and tissue factor production associated with the upregulation of MMPs to contribute to the lateral expansion of new blood vessels.[18-21] Furthermore, vascular localization of VSMCs derived MMPs may permit their spatially-controlled role in degradation of the subendocardial basement membrane, and that is a crucial step in the initial stage of angiogenesis.[19]

Recently, leptin, the product of the Ob gene, was identified as an adipocyte-secreted protein.[8-10] Loffreda et al,[22] recently reported that leptin stimulates murine macrophage phagocytic activity in vitro and in vivo. These effects were dependent on expression of Ob-R, the long form of the leptin receptor, as macrophages isolated from the diabetes mouse were insensitive to these effects of leptin. Moreover, leptin selectively enhanced the secretion of tumor necrosis factor α, interleukin (IL)-6, and IL-12 from isolated macrophages in response to lipopolysaccharide. Given the known regulatory effects of these cytokines, it thus appears that leptin may help to amplify selected proinflammatory responses via expression of Ob-R.[23] Experiments using human umbilical venous endothelial cells demonstrated that treatment of HUVECs with leptin enhanced formation of capillary-like tubes in an in vitro angiogenesis assay and neovascularization in an in vitro model of angiogenesis comparable with VGEF. These results indicated that leptin, via activation of the endothelial Ob-R, generated a growth signal involving a tyrosine kinase-dependent intracellular pathway and promoted angiogenic processes.[11] Otherwise, leptin-induced signaling

in endothelial cells induced reactive oxygen species as a second messenger and might activate atherogenic processes, as well as a potent stimulatory hormone on human peripheral blood monocytes as a proinflammtory cytokine.[24,25] Since leptin is secreted into the plasma, thin-walled endothelial cell tubes could be a target for leptin with a vasoactive substance.[25] Futhermore, its receptor, Ob-R, was expressed in this human vasculature and in primary cultures of human endothelial cells, while leptin and its receptor interaction suggested a physiological mechanism whereby leptin induced angiogenesis.[11,26]

It is hypothesized that a high concentration of leptin within vasa vasorum and plaque itself, exerting paracrine effects, may influence inflammatory and vascular neovascularization coupling with functional upregualtion of the VEGF and FGF. In our study, the expression of immunoreactivity for Ob-R and VEGF in atherosclerotic lesions were often seen, mainly in the endothelial lining of neovessels within plaque. These findings suggested that intimal neovascularization might be evident in atherosclerotic arteries according to the atherosclerotic inflammatory process and thickening of the vascular wall. Our histochemical data might indicate that increased activity of Ob-R plays an important role in the pathogenesis of intimal neovascularization as with other angiogenic factors. Our observation of a prominent colocalization between VEGF, Ob-R and MMP-9 supports this hypothesis, and these factors participate in neovascularization of atherosclerotic lesions. We investigated whether the leptin receptor is expressed in human atherosclerotic aorta using polyclonal antibodies to synthetic peptides based on the sequence of a human leptin receptor. The limitation of this study is that we did not investigate the expression of aortic tissue using a Western blot for leptin and leptin receptor. However, several recent studies suggested the expression of a leptin receptor in cell culture study and nonvascular tissue.[11,26] The present study is the first report on vascular tissue and opens a promising perspective concerning future investigations of leptin-dependent modulation of atherogenesis and vascular neovascularization under pathophysiological conditions.

REFERENCES

1. Barger AC, Beeuwkes R, Lainey LL, Silverman KJ. Hypothesis: Vasa vasorum and neovascularization of human coronary arteries. N Engl J Med 1984;310:175-7.
2. Kwon HM, Sangiorgi G, Ritman EL, Mckenna C, Holmes DR, Schwartz RS, et al. Enhanced coronary vasa vasorum neovascularization in experimental hypercholesterolemia. J Clin Invest 1998;101:1551-6.
3. Pels K, Labinaz M, Hoffert C, O'Brien E. Adventitial angiogenesis early after coronary angioplasty; correlation with arterial remodeling. Arterioscler Thromb Vasc Biol 1999;19:229-38.
4. Williams JK, Heistad DD. The vasa vasorum of the arteries. J Mal Vasc 1996;21(Suppl C):266-9.
5. Depre C, Havaux X, Wijins W. Neovascularization in human coronary atherosclerotic lesions. Cathet Cardiovasc Diagn 1996;39:215-20.
6. Moulton KS, Heller E, Konerding MA, Flynn E, Palinski W, Folkman J. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. Circulation 1999; 99:1726-32.
7. Ramos MA, Kuzuya M, Esaki T, Miura S, Satake S, Asai T, et al. Induction of macrophage VEGF in reponse to oxidized LDL and VEGF accumulation in human atherosclerotic lesions. Arterioscler Thromb Vasc Biol 1998; 18:1188-96.
8. Pelleymounter M, Cullen M, Baker M, Hecht R, Winters D, Boone T, et al. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 1995;269: 540-3.
9. Halaas J, Gajiwala K, Maffei M, Cohen S, Chait B, Rabinowitz D, et al. Weight-reducing effects of the plasma protein encoded by the obese gene. Science 1995;269: 543-6.
10. Campfield L, Smith F, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central networks. Science 1995; 269:546-9.
11. Bouloumie A, Drexler HCA, Lafontan M, Busse R. Leptin, the product of Ob gene, promotes angiogenesis. Circ Res 1998;83:1059-66.
12. Tartagia L. The leptin receptor. J Biol Chem 1997;272: 6093-6.
13. Flannery BP, Dickman HW, Roberge WG, D'Amico KL. Three-dimensional x-ray micro tomography. Science 1987; 237:1439-44.
14. Avula RTV, Dunsmuir JH, Beighley PE, Thomas PJ, Faridani A, Ritman EL. A micro tomographic technique enhanced by novel image reconstruction algorithm: application to rat coronary vessels [abstract]. FASEB J 1994; 8:A854.
15. Ritman EL, Dunsmuir JH, Faridani A, Finch DV, Smith KT, Thomas PJ. Local reconstruction applied to x-ray tomography. In: Edited by Chavent G, Papanicolaou G, Sack P, Symes W. IMA volumes in mathematics and its applications: inverse problems in wave propagation. vol 90. New York (NY): Springer-Verlag; 1997. p.443-52.
16. Ware JA, Simons M. Angiogenesis in ischemic heart disease. Nature Med 1997;3:158-64.

17. Ananyeva NM, Tjurmin AV, Berliner JA, Chisolm GM, Liau G, Winkles JA, et al. Oxidized LDL mediates the release of fibroblast groawth factor-1. Arterioscler Thromb Vasc Biol 1997;17:445-53.
18. Clauss M, Weich H, Breier G, Knies U, Rock W, Waltenberger J, et al. The vascular endothelial growth factor receptor flt-1 mediates biological activities. J Biol Chem 1996;271:17629-34.
19. Wang H, Keiser JA. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. Circ Res 1998; 83:832-40.
20. Nielson B, Timshel S, Kjeldsen L, Sehested M, Pyke C, Borregaard N, et al. 92 kDa type IV collagenase (MMP-9) is expressed in neutrophils and macrophages but not in malignant epithelial cells in human colon cancer. Int J Cancer 1996;65:57-62.
21. Arbiser JL, Moses MA, Fernandez CA, Ghiso N, Cao Y, Klauber N, et al. Oncogene H-ras stimulates tumor angiogenesis by two distinct pathways. Proc Natl Acad Sci USA 1997;94:861-6.
22. Loffreda S, Yang SQ, Lin HZ, Karp CL, Brengman ML, Wang DJ, et al. Leptin regulates proinflammatory response. FASEB J 1998;12:57-65.
23. Bemelmans MH, van Tits LJ, Buurman WA. Tumor necrosis factor:function, release and clearance. Crit Rev Immunol 1996;16:1-11.
24. Bouloumie A, Marumo T, Lafontan M, Busse R. Leptin induces oxidative stress in human endothelial cells. FASEB J 1999;13:1231-8.
25. Nakada M, Yada T, Soejima N, Maruyama I. Leptin promotes aggregation of human platelets via the long form of its receptor. Diabetes 1999;48:426-9.
26. Sierra-Honigmann MR, Nath AK, Murakami C, Garcia-Cardena G, Papapetropoulos A, Sessa WC, et al. Biological action of leptin as an angiogenic factor. Science 1998; 281:1683-6.