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Atherosclerosis as a Proliferative Disease of Arterial Intimal Cells: An ultrastructural, immunohistochemical, and in situ hybridization study

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SUMMARY: Coronary arteries obtained from 35 autopsied cases were studied with the aid of electron microscopy, immunohistochemistry, and in situ hybridization. Smooth muscle cells were the main cellular components of the fibrocellular intimal thickening of the coronary artery. Atherosclerotic lesions were characterized by proliferation of smooth muscle cells as well as foam cells. Ultrastructural and immunohistochemical studies indicated that foam cells originated from smooth muscle cells and macrophages. Immunohistochemistry showed low levels of expression of the myc oncogene product in the lesions of fibrocellular intimal thickening and increased expression of that in the early stage of the atherosclerotic lesions. Both smooth muscle cell and macrophage were responsible for the expression of the myc oncogene. In situ hybridization demonstrated the presence of m-RNA of myc oncogene in the intimal cells of the uncomplicated atherosclerotic lesions.

INTRODUCTION

Atherosclerotic process involves degeneration and proliferation of arterial intimal cells. Intimal cells include various types of cells such as smooth muscle cells, monocytes, and neutrophils. According to the present view cell proliferation of the arterial intima is one of the key factors in the pathogenesis of atherosclerosis. Recent molecular biological studies have indicated that cellular oncogenes are activated and then followed by the expression of the gene products which can transform normal cells into malignant neoplasms. In the field of atherosclerosis research, protooncogene-related factors such as platelet-derived growth factor (PDGF) and monocyte-derived growth factor (MDGF) have also been reported to play some important roles in atherogenesis. In this connection, the present study was carried out to examine whether atherosclerotic lesions are associated with abnormal expression of the myc oncogene which has been reported to possess regulating roles in the control of cell growth.

MATERIALS AND METHODS

Samples were collected from 35 autopsied cases with the age of ranging from 4 to 86 years. For morphological studies, samples of the anterior descending branch of the left coronary arteries were collected from the...
formalin-fixed heart, embedded in paraffin, and cut into 4-micrometer thickness. Specimens were stained with hematoxylin-eosin and elastica van GIESON to locate the arteriosclerotic lesions. Immunohistochemical staining was carried out on paraffin sections by the peroxidase-antiperoxidase (PAP) method of STERNBERGER 16). Dewaxed sections were treated with 0.3 % hydrogen peroxide to block endogenous peroxidase. After exposure to non-immune serum, the sections were reacted with either the primary anti-sera or non-immune sera, link antibodies, followed by peroxidase-anti-peroxidase complex for 1 hour at room temperature. The sections were washed three times with 0.1 M PBS (pH 7.5) for 5 minutes after each antibody application and were treated with dianaminobenzidine in hydrogen peroxide. Primary antibodies for lysozyme, beta-lipoprotein, and actin (DAKO) OKMI (ORTHO), Myc-oncogene product (Oncor Inc WAKO) were used in this study. In situ hybridization was carried out according to the method of Cox et al. 4). For prehybridization treatments, slide glasses were soaked in chromic acid mixture and coated with gelatin chrom alum springs. Four micrometer thick sectioned specimens were mounted on the treated slides, dried at 40°C and deparaffinized. Specimens on slides were digested with 1 microgram proteinase K per ml in 0.1 M Tris-HCL, 50 mM EDTA, pH 8.0, for 30 min at 37 °C, treated with 0.25 % (v/v) acetic anhydride and 0.1 M triethanolamine, pH 8.0 for 10 min, and dehydrated in increasing concentrations of alcohol. Cloned c-myc DNA containing exon I, II, III, was generously offered by Japanese Cancer Research Resources Bank. Fragments of sma I (4251)/sac I (5403) containing exon 2 (4506-5278) were inserted into psp65. They were transfected to HB101 (E coli) by rubidium method (11) to obtain large quantities of psp65 containing myc exon 2 by CsCL method 11). Linear templates were truncated by sma I digestion at the sma I site (4251). Biotinated RNA probes were synthesized by in vitro transcription of template with sp 6 RNA polymerase and Biotin 11-UTP. Obtained probes were diluted in 50 % formamide and 10 mM DTTT and then heated at 80°C for 30 seconds. Probe-containing buffer was made by mixing the probe and buffer at the concentration ratio of 1 : 4. Sixty microliter of probe-containing buffer was applied to each slide, which was incubated at 50°C overnight in moist chamber. For posthybridization washing, groups of slides were incubated in 50% formamide solution (50% formamide solution is the same hybridization buffer as that from which Dextran Sulfate and Yeast RNA were removed) at 50°C for one hour. Groups of slides were then treated with RNase A (20 microgram/ml) in 0.5 M Nacl, 10 mM Tris, and 1 mM EDTA at 37 °C for 30 minutes, washed in 2 X SSC and 0.1 X SSC. Biotinated probes on slides were visualized with BluGENETM (BRL).

For electron microscopic examinations, small pieces of the coronary arteries were fixed in 3% glutalaldehyde solution, pH 7.4, postfixed in cacodylate-buffered osmium tetroxide, dehydrated in increasing concentrations of alcohol, and embedded in spur resin. Thick sections were stained with alkaline toluidine blue and examined under a light microscope to locate the lesions. Ultrathin sections were cut with a Porter-Blum MT-ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a JEM 2000 EX electron microscope.

**RESULTS**

Light microscopy: 16 lesions of fibrocellular intimal thickening, 5 uncomplicated atherosclerotic lesions and 7 advanced atherosclerotic lesions were selected from 35 autopsied cases by histological examination. In the coronary arteries with fibrocellular intimal thickening, a large number of spindle-shaped cells and collagen and elastic fibers were seen. The tunica media was tightly packed with smooth muscle cells (Fig. 1). In early and uncomplicated atherosclerotic lesions, frequent foam cells were seen near the lumen. Interstitial space of the thickened intima in this area was more widened than that of the fibrocellular intimal thickening lesions. Atherosclerotic lesions had fibrous cap which was composed of numerous foam cells, avascular connective
tissues and elongated or spindle-shaped intimal cells (Fig. 2). In the thickened intima of advanced atherosclerotic lesions, acellular area surpassed cellular portion in quantity and deposition of cholesterol crystal and calcium was frequently noted.

Electron microscopy: The lesions of fibrocellular intimal thickening consisted of large numbers of mature and activated smooth muscle cells (Fig. 3). Luminal surface of the thickened intima was covered with flat or cuboidal endothelial cells with abundant organelles. Monocytes or macrophages having varying amount of dense bodies and cytoplasmic foot process were occasionally seen in the superficial portion of the thickened intima. Some macrophages containing a small amount of lipid droplets were occasionally seen (Fig. 4). The tunica media was exclusively composed of mature smooth muscle cells with basement membrane and abundant myofilament. The interstitium of the tunica media contained a small amount of amorphous material probably of mucopolysaccharide, collagen fibrils and cell debris. In the atherosclerotic lesion, endothelial cells had abundant microvillous structure, Golgi apparatus and endoplasmic reticulum. The central portion of the atheroma contained large amounts of cell debris, extracellular lipid granules, cholesterol crystal, and calcium. Most of the foam cells in the deeper portion...
of the atherosclerotic lesions showed characteristic features of smooth muscle cells such as fusiform densities, pinocytotic vesicles and basement membrane (Fig. 5). Degenerate cells without stainable lipid were sometimes seen to possess nuclear pyknosis and cytolysis. The tunica media in the atherosclerotic lesions contained more cell debris and collagen fibrils than that of the coronary arteries with fibrocellular intimal thickening.

Immunohistochemistry and in situ hybridization: The lesion with fibrocellular intimal thickening contained beta-lipoprotein positive cells in the superficial layer of the thickened intima. These foam cells were positively reacted with OKM1, but not with lysozyme (Fig. 6). Positive staining of beta-lipoprotein was seen in the extracellular granules and spindle-shaped cells in the deeper portion of the atherosclerotic lesions. These spidle-shaped cells and smooth muscle cells of the tunica media showed the same reaction to antibody for actin (Fig. 7). Staining of Myc oncogene product was weakly positive for the intimal smooth muscle cells and negative for the smooth muscle cells of the tunica media in the coronary arteries with fibrocellular intimal thickening (Fig. 8). Strong positive staining results of Myc oncogene product was demonstrated in foam cells in the superficial

Fig. 5. Smooth muscle cell type of foam cell (SM) in the deeper portion of the thickened intima of atherosclerotic lesion. Open arrow: Basement membrane (× 4700).

Fig. 6. Note collection of macrophages (M) reactive for OKM1 (PAP stain ×280).

Fig. 7. Positive reaction for actin (arrow) is seen in the spindle-shaped cells in both deep intima and medial smooth muscle cells (PAP stain ×300).

Fig. 8. Fibrocellular intimal thickening (IT) showing slight staining for Myc oncogene product (PAP stain ×280).
portion of the thickened intima (Fig. 9). In atherosclerotic lesions, strongly positive staining of Myc oncogene product was seen in lipid-containig macrophages or the foam cells of macrophages and that of smooth muscle cells (Fig. 10), while weak staining of myc oncogene product was seen in intimal cells without lipid droplets and smooth muscle cells in the inner media. However, frequency and degree of staining of Myc oncogene product was decreased in the advanced atherosclerotic lesions. Biotinated c-myc RNA was more positively demonstrated in intimal cells with or without lipid droplets in the atherosclerotic lesions than in those of fibrocellular intimal lesions (Fig. 11).

**DISCUSSION**

Present electron microscopic and immunohistochemical studies confirmed that atherosclerotic lesions are characterized by proliferation of smooth muscle cells without stainable lipid droplets, and foam cells derived from macrophages and smooth muscle cells. In the present study, foam cells in the superficial layer showed intense reactivity with OKM 1 which is the basis for its use as a marker of the macrophages. However, these OKM 1-positive cells are weakly positive or none at all with lysozyme which is positively found in histiocytes, monocytes, and mature myeloid cells. YAMASHITA et al. indicated that
lysozyme is immunohistochemically demonstrated in macrophages of immune-related granulomatous lesions of the lymphnodes but not in those of foreign body-type granulomatous lesions of the lymphnodes. Therefore, it is suggested that macrophages in the atherosclerotic lesions possess the same function as those of foreign body type granulomatous lesions. The foam cells in the deeper portion of the thickened intima had electron microscopic and immunohistochemical character of smooth muscle cells.

The myc oncogene, which was originally identified in retroviruses, are reported to be responsible for mouse erythroleukemia and adult T-cell leukemia. Expression of the C-myc oncogene product has been reported to increase in colonic and testicular tumors. In contrast, Ciclitira et al. reported that c-myc is expressed in both gastric and colonic mucosa of normal, inflammatory, metaplastic and dysplastic histological appearances. Present result by PAP method also demonstrated that myc oncogene is expressed in both smooth muscle cells and macrophages in the human atherosclerotic lesions. Martinet et al. indicated that the human activated monocytes express the c-sis protooncogene and release a mediator showing PDGF-like activity. Although question remains that polyclonal antibody of myc oncogene product might react with an unrelated antigen, and in situ hybridization demonstrated the expression of messenger RNA of myc oncogene especially in the uncomplicated atherosclerotic lesions. We also reported that dietary manipulation and anti-atherosclerotic drug promoted the regression of atherosclerotic lesions in which myc oncogene was highly expressed.

In view of the present results, it is difficult to conclude which hypothesis is correct in atherogenesis, response to endothelial injury or monoclonal theory. Thomas et al. reported that individual atherosclerotic foci are monoclonal phenomena arising as secondary features in lesions whose origins are unrelated to monoclonism. Therefore, it is plausible that c-myc oncogene may be a maker of cellular proliferation.

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