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EXPRESSION OF INSULIN-LIKE GROWTH FACTOR RECEPTOR MRNA IN RABBIT ATHEROSCLEROTIC LESIONS

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We have found by 'in situ' hybridization a high level of expression of insulin- like growth factor I receptor (IGF-I R) gene in foam cells of atherosclerotic rabbit aortas. By reaction with either anti- rabbit macrophage (RAM11) or anti smooth muscle cell antibodies we also found that most cells expressing increased amounts of IGF-I R mRNA were of smooth muscle cell origin. Thus, increased IGF-I R mRNA levels might be related to the genesis of the atheroma plaque. © 1995 Academic Press, Inc.

Smooth- muscle cell (SMC) proliferation is a key event in the development of atherosclerotic lesions (1). The primary stimulus for SMC growth is believed to be Platelet- derived growth factor (PDGF), but now it is well established that many other growth factors and other chemicals acting on SMC are present in atherosclerotic lesions (2). However, the ability of cells to respond to growth factors is determined not only by the presence of growth factors but may also depend on the availability of the appropriate receptors. In this respect, the overexpression of PDGF receptors in atherosclerotic lesions of

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Abbreviations: LDL, low density lipoproteins; HDL, high density lipoproteins; PDGF, Platelet- derived growth factor; IGF-I, Insulin- like growth factor I; IGF-I R, Insulin-like growth factor I receptor; IGF- II, Insulin- like growth factor II; SMC, smooth muscle cells.

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diverse origin has been demonstrated (3,4) . The coexpression of PDGF protein and receptor genes suggests the possibility of a local autocrine or paracrine mechanism of SMC control growth. In vitro, SMC require insulin-like growth factor for progression to the S phase of the cell cycle (1,5,6). Recently, the induction of Insulin- like growth factor I has been reported in rat aorta after balloon denudation (7). The biological effects of IGF-I are mediated by its specific cell surface receptor (IGF-I R). The purpose of this work was to study the variations of IGF-I R mRNA in atherosclerotic aorta of hypercholesterolemic rabbits. Our results indicated that there is an increase in IGF-I R levels in the atherosclerotic neointima. This result suggests the existence of an auto or paracrine mechanism of regulation of SMC growth similar to that described for PDGF and involving IGF-I and its receptor.

MATERIAL AND METHODS

Female New Zealand White rabbits (2-3 kg) were fed chow containing 2% (w/w) cholesterol (Sigma) as previously described (8). Diets were maintained for 8 weeks. Animals were sacrificed by ether anaesthesia and aortas were dissected out. For the study of the influence of plasma cholesterol levels, rabbits that had been on a cholesterol- rich diet for 8 weeks were changed to a diet consistent in standard chow for the periods fo time indicated. Serum lipoproteins and cholesterol levels were determined as described (9).

mRNA 'in situ' hybridization. 10 μm- thick cryostat sections prepared from paraformaldehyde- fixed aortas, were set on slides coated with poly- L- lysine (MW= 150,000, Sigma) and stored at -70° C until use. Before hybridization, slides were quickly warmed to room temperature, rinsed with phosphate- buffered saline, treated with proteinase K (5 μg/ml) in 100 mM Tris pH 7.5, 50 mM EDTA for 10 minutes, rinsed with water and acetylated in 0.25% acetic anhydride. Prehybridization mixture (50% formamide, 20% dextran sulfate, 5 x Denhardt's, 10 mM sodium phosphate, 5 x SSC and 200 μg/ml herring sperm DNA) was added and preparations were incubated for 2 hours at 37 °C in a wet chamber. Probes were labelled with digoxigenin by random priming (Boehringer Manheim kit) following manufacturer's instructions. Hybridizations were done at 37°C for 12 hours. Slides were sequentially washed at 48 °C for 30 minutes in 2 x SSC/ 50% formamide, 2x SSC, 2 x SSC/ 0.5 x PBT and PBT (PBT is phosphate buffered saline- 1% Tween 20). After a 2-hour room- temperature incubation with PBT containing a 1:500 dilution of an antidigoxigenin antibody (Boehringer Manheim) slides were washed with PBT for 10 min, rinsed in 100 mM Tris pH 9.5, 100 mM NaCl, 50 mM Mg Cl_2. Developing was carried out in the presence of 1 mM levamisole and 0.1 % Tween 20. Preparations were either mounted in an aqueous medium (Crystalmount, Biomeda) or dehydrated with increasing alcohol concentrations, cleared in xylene and mounted with Eukitt.

In all the experiments, a labeled pBR322 was used as a negative control of hybridization.

Immunocytochemistry and cholesterol distribution in tissue sections. 10 μm cryostat sections from paraformaldehyde- fixed aortas were stained immunohistochemically with the following antibodies: Anti- PCNA monoclonal antibody (clone PC-10, Oncogene Science), RAM 11 (monoclonal antibody to rabbit macrophages, Dako, Denmark) and hm 19/2 (monoclonal antibody to smooth muscle cells, Boehringer Manheim). After incubation with the primary antibody, the slides were rinsed in PBS and incubated with FITC- conjugate goat anti-mouse immunoglobulin (Sigma). Glycerol- PBS (1:1) mounted specimens were examined under a Nikon epi- fluorescence microscope. Negative controls were carried out following the same protocol, but without adding primary antibody.

Histological distribution of cholesterol- rich elements was studied on cryostat sections from paraformaldehyde- fixed aortas. Sections were placed on uncoated slides, mounted in an aqueous medium and examined by simple polarizing microscopy (10).

Ultrastructural analysis. Rabbits under anaesthesia were perfused with 1% glutaraldehyde and 1% paraformaldehyde in 0.12 M phosphate buffer. Small aorta fragments were dissected out, postfixed in 2% osmium tetroxide, dehydrated and embedded in Araldite . Semithin sections (1 μm) were stained with toulidine blue and used for light cytological examination. Ultrathin sections stained with uranyl acetate and lead citrate were used for ultrastructural analysis.

RESULTS

Plaques resembling atherosclerotic lesions were present in rabbit aortas after eight weeks of cholesterol-rich diet. Plaque analysis by either light or polarizing microscopy indicated that cholesterol- laden foam cells constitute the majority of the plaque, which is in accordance with our previously reported results (8). Immunocytochemistry with an anti- PCNA antibody revealed a high degree of proliferation within the plaque (not shown).

In order to study the expression of IGF-I R mRNA within the lesions, 10 μm sections were hybridized with a digoxigenin-labelled probe specific for the IGF-I R gene. Control arteries, prepared from rabbits feed on a low- cholesterol diet, did not show detectable levels of IGF-I R mRNA (Fig. 1A). However, a high level of expression was found in the intima of atherosclerotic lesions, even at very early stages of plaque development (Fig. 1B). In advanced lesions, the signal was localized in the superficial and deep zones, the intermediate region exhibiting a very low level of mRNA, which is in accordance with the low cellular content of the central region of the atherosclerotic plaque. This pattern of expression was different at the edges of the lesion, where an intense hybridization signal was detected all the way through the intima (Fig. 1C). In the early stages of

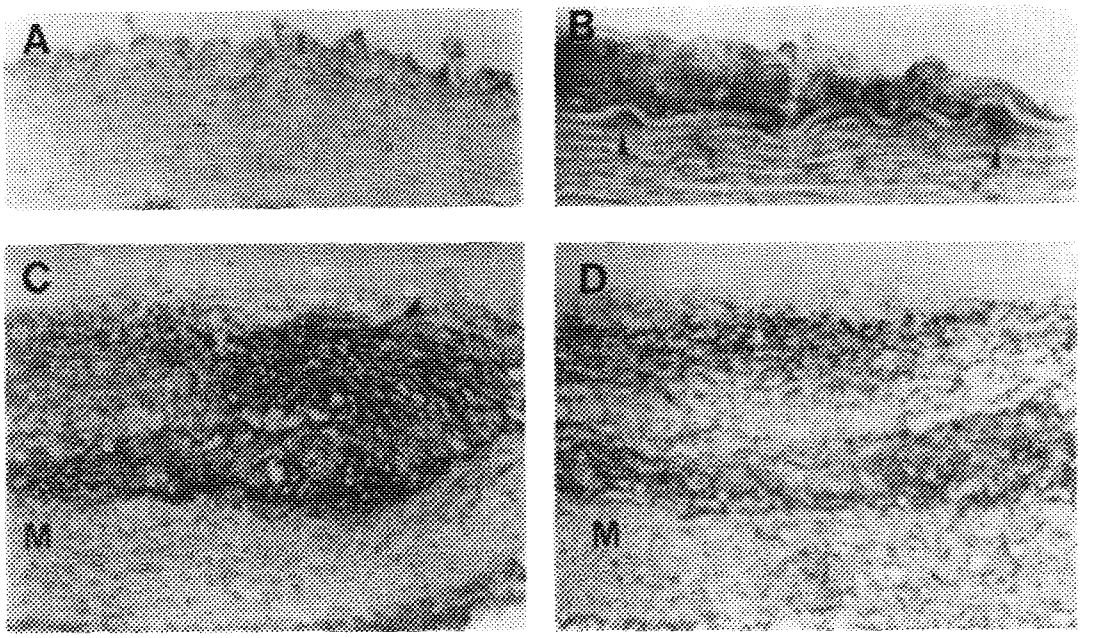

Figure 1. Detection of IGF-I mRNA by in situ hybridization in A. Control plaque from a normolipidemic rabbit. B. Small atherosclerotic plaque from a hyperlipidemic rabbit. Note some patches of hybridization signal in the tunica media (arrowheads). C. Large atherosclerotic plaque. D. Typical hybridization of large atherosclerotic plaque from a rabbit that after being eight weeks on a hypercholesterolemic diet was on a normal diet for twelve weeks. In panels C and D the location of tunica media is labeled with an M.

lesion development some cells in the media, next to the elastic internal lamina, also showed high IGF-I mRNA levels (Fig. 1B).

To determine the influence of plasma cholesterol levels in the expression of IGF-I R mRNA, we studied a group of three rabbits which, after eight weeks on a cholesterol- rich diet, had been for twelve weeks on a normal diet. In these rabbits, serum cholesterol dropped to normal levels within 12 weeks. The changes in serum lipids were not followed by a decrease in either number or size of the atherosclerotic lesions, which was in accordance with our previous results (9). Samples derived from aortic lesions of these rabbits were hybridized to an IGF-I R specific probe as indicated. A typical hybridization is shown in the figure 1 D and is indistinguishable from those obtained with the aortic lesions of rabbits under hypercholesterolemic treatment. Thus, the maintenance of elevated levels of IGF-I R mRNA in foam cells do not require high plasma cholesterol levels.

To ascertain the type of cells responsible for the high levels of IGF-I R mRNA, we performed immunocytochemical analysis with two different antibodies, RAM 11 and HM 19/2, specific for macrophages and SMC respectively. The analysis with the antibody RAM 11, specific for rabbit macrophages revealed the presence of a layer of immunoreactive cells just beneath the endothelium that covers the atherosclerotic plaque (Fig. 2A). Light and electron microscopic examination confirmed the identification of these cells as macrophage- derived foam cells (not shown). Apart from this subendothelial layer of macrophages, most cells within the plaque displayed the typical fine structure of vascular smooth muscle cells with the presence of lipid droplets in their cytoplasms (Fig. 3A). The lipid droplets were also visible in some cells of the intima located beneath the elastica interna (Fig. 3B). Immunofluorescence analysis with the antibody HM19/2,

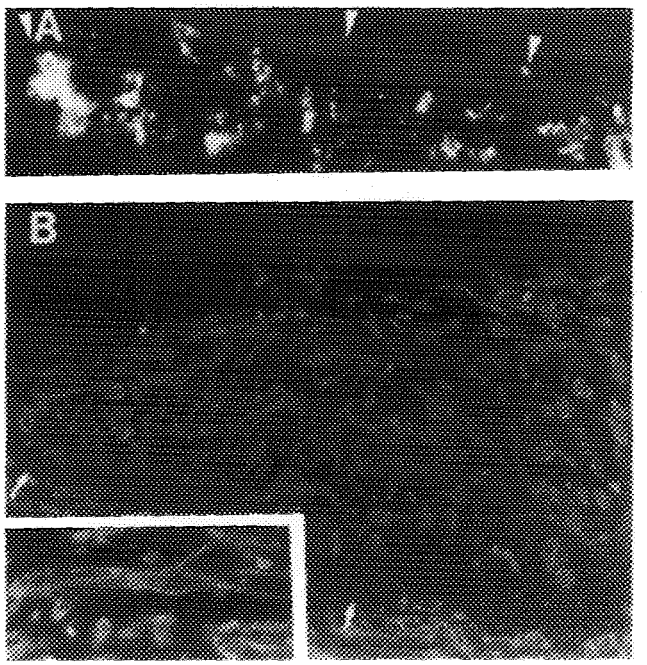

Figure 2. A. Immunofluorescent macrophages with the antibody RAM-11 in the subendothelial layer of an atherosclerotic plaque. Arrows indicate the position of the endothelium.
B. Immunofluorescence localization of smooth muscle cells in the atherosclerotic plaque with HM 19/2 antibody. White arrows indicate the position of the elastica interna. A detail of a smooth muscle cell of the plaque is shown in the inset.

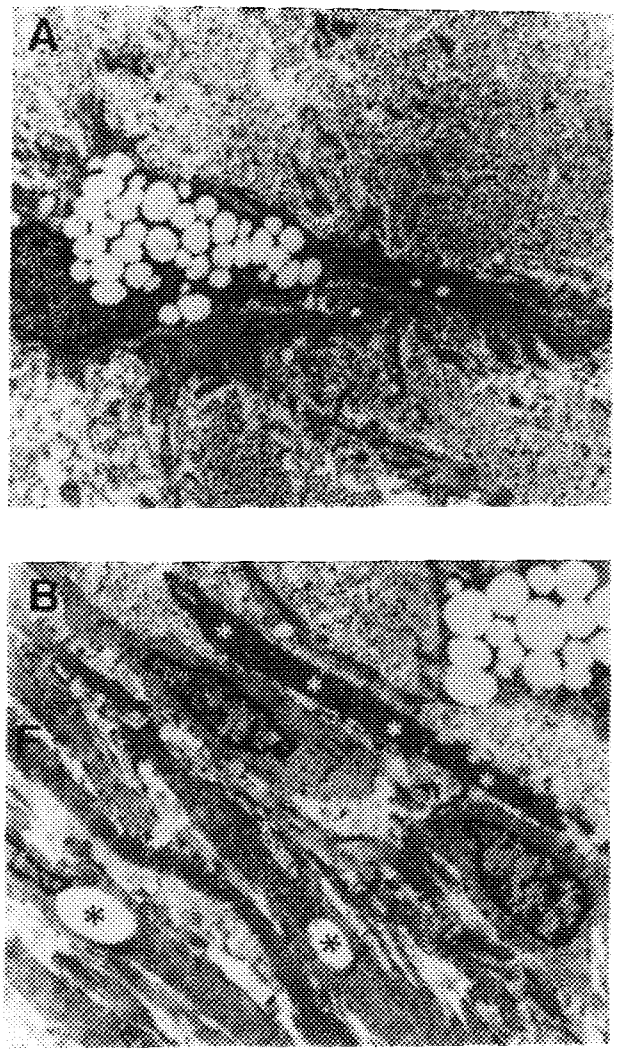

Figure 3. A. Electron micrograph of a typical smooth muscle cell of the atherosclerotic plaque. Note the presence of lipid droplets and of bundles of myofilaments.
B. Low magnification electron micrograph showing the transition between the atherosclerotic plaque of the intima and the tunica media. White asterisks indicate the elastica interna. Some of the SMC of the tunica media also contain isolated lipid droplets (black asterisks).

a specific marker for SMC showed numerous branched SMC (Fig. 2B). The comparative analysis of in situ hybridization and immunofluorescence analysis of the same plaque (Figures 1C and 2B) clearly indicated that there is a correspondence between SMC localization and the presence of IGF-I R mRNA and thus SMC were the major source of IGF-I R mRNA.

DISCUSSION

We have found by "in situ" hybridization that cells of atherosclerotic lesions from the aorta of hypercholesterolemic rabbits express very high levels of IGF-I R mRNA from very early stages of plaque formation. The high level of expression is specific for the regions affected by lesions. In fact, IGF-I R mRNA is below our level of detection either in control aortas of normolipidemic rabbits or in those regions of the aorta of hypercholesterolemic rabbits which did not present morphological evidence of lesions. Our immunocytochemistry with antibodies specific for either macrophages or SMC indicated that most of the cells expressing IGF-I R mRNA are of SMC origin. This distribution of cells within the lesion is very similar to that reported previously for cardiac allograft atherosclerosis in rabbits (11).

SMC belong to a type of cells which require IGF-I for growth (see ref 12 for a review). In experiments carried out in 3T3 cells the overexpression of IGF-I R allows cell growth in the absence of competence growth factors such as PDGF or EGF. In consequence, it has been suggested that a major role for competence growth factors might be to participate in an autocrine loop based on the IGF-I IGF-I R interaction, which is obligatory for the proliferation of the cells (13,14). IGF-I is present in plasma and is also made by some cells in the aorta including SMC (15,16). Increased levels of IGF-I mRNA have also been found that in catheter- induced rat aortic lesions (7). Taken together, those results suggest that the expression of high levels of IGF-I R mRNA could be part of an autocrine loop leading to SMC proliferation and hence to the formation of atherosclerotic plaque. To this respect, it is interesting to note the degree of proliferation of the intimal cells, as indicated by the positive reaction with the antiPCNA antibody.

On the other hand, IGF-I R mRNA is overexpressed in some of the SMC from the media. If these cells are a subpopulation of medial SMC known to undergo DNA synthesis (17) or if IGF-I R expression is also related to SMC migration to the intima is a question that deserves further studies.

In our studies hypercholesterolemia is the condition which in the end produces the induction of IGF-I R mRNA levels. However, when plasma cholesterol levels were back to normal we did not see any change in the expression of IGF-I R mRNA. In the

periods of time studied there is no evident regression of the lesions either, which is in accordance with our previous results (9). The persistence of the increased expression of IGF-I R might be related to the degree of differentiation of the cells. Perhaps the presence of intracellular lipids would suffice for the induction of IGF-I R expression. To this respect it has to be considered that lipoproteins are mitogens for SMC. In absence of lipoproteins, SMC do not proliferate even in presence of a mixture of PDGF and IGF-I(18). Also, in SMC cultures, lipoproteins are able to induce genes related to proliferation such as PDGF-R (19). Moreover, our preliminary experiments have shown that LDL induce IGF-I R mRNA in cell cultures of the rat aortic cell line A10 (Polanco et al., unpublished). The confirmation of this hypothesis would help to establish a new link between lipid metabolism and the development of atherosclerotic plaques.

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REFERENCES

1. Ross, R. 1986. New Eng. J. Med. 314, 488- 500.
2. Ross, R. 1993. Nature **362**, 801- 809.
3. Rubin, K., Hansson, G.K., Rönnstrand, L., Claesson-Welsh, L., Felström, B., Tingström, A., Larsson, E., Klareskog, L., Heldin, C.H., and Terracio, L. 1988. Lancet. **8599**, 1353-1357.
4. Wilcox, J.N., Smith, K.N., Williams, L.T., Schwartz, S.M., and Gordon, D. 1988. J. Clin. Invest. **82**, 1134-1143.
5. Stiles, C.D., Capone, G.T., Scher, C.D., Antoniades, H.N., Van Wyk, J.J., and Pledger, W.J. 1979. Proc. Natl. Acad. Sci. USA. **76**, 1279-1283.
6. Pardee (1990), A.B. 1990. Science **246**, 603-608.
7. Cercek, B., Fishbein, M.C., Forrester, J.S., Helfant, R.H., and Fagin, J.A. 1990. Circ. Res. **66**, 1755-1760.
8. Crespo, P., González, C., Ordovás, J.M., Ortiz,J.M., Rodríguez, J.C. and León, J. 1990. Biochem. Biophys. Res. Commun. **168**, 733-740.
9. Crespo, P., Ros, M.A., Ordovás, J.M., Rodríguez, J.C., Ortiz, J.M. and León, J. 1992. Biochem. Biophys. Res. Commun. **183**, 514-523.
10. Miklossy, J., Van der Loos,H. 1987. Brain Res. **426**, 377-380.
11. Sasaguri,S., Eishi,Y, Tsukada,T., Sunamori,M., Suzuki,A., Numano,F., Hatakeyama,S and Hosoda, Y. 1990. J. Heart Transplant. **9**, 18-24.
12. Goldring, M.B., and Goldring, S.R. 1991. Eukar. Gene Express. **1**, 301-326.

13. Pietrzkowski, Z., Sell, C., Lammers, R., Ullrich, A., and Baserga, R. 1992. Mol. Cell. Biol. **12**, 3883-3889.
14. Pietrzkowski, Z., Lammers, R., Carpenter, G., Soderquist, A.M., Limardo, M., Phillips, P.D., Ullrich, A., and Baserga, R. 1992. **3**, 199-205.
15. Thyberg, J., Hedin, U., Sjölund, M., Palmberg, L., and Bottger, B.A. 1990. Arteriosclerosis. **10**, 966-990.
16. Cascells, W. 1992. Circulation. **86**, 723-729.
17. Scwartz, S.M., Heimark R.L., and Majesky, M.W. 1990. Physiol. Rev. **70**, 1177- 1209.
18. Björkerud, S. and Björkerud, B. 1994. Arterioscler. Thromb. **14**, 288-298.
19. Ververis, J.J., Ku, L., and Delafontaine, P. 1993. Circ. Res. **72**, 1285-1292.