Local Expression of Inflammatory Cytokines in Human Atherosclerotic Plaques

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Human atherosclerotic plaques are heterogeneous tissues containing a number of different cell types, including macrophages, smooth muscle, endothelial and other undefined mesenchymal-appearing cells. Significant numbers of macrophages are found in human atherosclerotic plaques and have been postulated to be a major source of growth factor production during atherogenesis. In vitro evidence suggested that macrophages synthesize PDGF and might therefore contribute to the growth of the vessel wall in atherosclerosis. However, examination of PDGF synthesis in human atheroma by in situ hybridization revealed that while smooth muscle, mesenchymal, and endothelial cells synthesize this growth factor macrophages did not.

Our inability to detect PDGF mRNA in macrophages was not due to any problems with hybridization to this cell type. In situ hybridization studies on human atherosclerotic plaques have demonstrated that plaque macrophages contain many different mRNAs other than PDGF including tissue factor, factor XIII, apoprotein E, transforming growth factor beta, and tumor necrosis factor. Recent studies have indicated that macrophages may be a major source as well of another group of inflammatory cytokines which are members of the RANTES/SIS cytokine family. In situ hybridization studies on human carotid endarterectomy specimens using probes specific for the inflammatory cytokines RANTES, LD78, HIMAP, and MCP-1 revealed numerous cells containing the mRNAs encoding for these proteins (5%, 13%, 8%, and 16% of plaque cells respectively). This is in contrast to generally low level expression found in normal human arteries (<1% of normal medial cells contain these mRNAs). Cells expressing these cytokines were often found associated with inflammatory zones in human atherosclerotic plaques. Serial section immunohistochemistry suggests that macrophages and/or T cells may synthesize these proteins. In addition to localization to macrophages MCP-1 expression was also detected in smooth muscle cells and mesenchymal-appearing cells with many of the morphological characteristics of cells previously seen to express PDGF. In vitro evidence suggests that these proteins may be chemotactic to monocytes and lymphocytes. The finding of increased expression of these mRNAs in human atheroma suggests they may play a role in monocyte trafficking into the atherosclerotic plaque.

Atherosclerotic lesions are heterogeneous tissues containing a number of different cell types including monocytes, macrophages, smooth muscle cells, T cells, endothelial cells, and a variety of undefined mesenchymal-appearing cells(1,2). Within the plaque there can be necrosis, calcification, and focal areas of thrombus organization which may originate either from plaque rupture or intraplaque hemorrhage resulting from disruption of the vasa vasorum within the intima. In an attempt to understand the pathophysiology of human atherosclerosis we have undertaken studies examining human...
atherosclerotic tissue using in situ hybridization (ISH) (3, 4). Since ISH is a histological technique cellular relationships are maintained and it is possible to precisely identify cell types expressing the gene of interest. Thus, potentially important interactions between cells that express different proteins may be uncovered.

PDGF is an important growth factor that might be involved in intimal smooth muscle proliferation in atherosclerotic plaques (I). In vitro studies indicated that PDGF is a mitogen (5) and chemoattractant (6) for smooth muscle cells. The discovery of PDGF mRNA in extracts of human atherosclerotic plaques by northern blots (7) suggested that local production of PDGF may contribute to intimal smooth muscle proliferation. However, it was unclear which cell type(s) might be producing PDGF in vivo in the plaque since many of the cell types found in these tissues including macrophages (8), endothelial cells (9) and arterial smooth muscle cells (10, 11) synthesize PDGF in vitro.

We were able to identify specific cells in the human atherosclerotic plaque containing PDGF-A and -B chain mRNA using in situ hybridization (4). Endothelial cells lining the vasa vasorum of the plaque hybridized to PDGF-B chain specific riboprobes. While a few endothelial cells were found to contain PDGF-A chain mRNA, this was not a common finding. Not all endothelial cells in these tissue sections hybridized to the PDGF-B chain probe suggesting that a subset of these cells might be involved in PDGF synthesis. The major cell types expressing both the PDGF-A and -B chain mRNAs were smooth muscle cells and a population of mesenchymal-appearing intimal cells (MIC) which may be smooth muscle derived. Many MICs had a stellate shape, displayed variable amounts of cytoplasm, and had large pale hematoxylin staining nuclei but did not stain well with any of the cell type-specific antibodies directed against smooth muscle cells (alpha actin antibody, HHF-35), endothelial cells (Ulex lechtin), human macrophages (HAM-56), or T cell (Leu3 or UCHL1) markers. PDGF-beta receptor mRNA was found almost exclusively in MICs. Furthermore, MICs containing PDGF-beta receptor mRNA were found in regions of the plaque rich in cells expressing PDGF-A and -B chain mRNAs. Preliminary studies suggest that these cells might also contain PDGF-alpha receptor mRNA. The apparent co-localization of PDGF and PDGF receptors in the same region of the plaque and in similar cell types suggests that PDGF may be acting through autocrine and/or paracrine mechanisms to stimulate the proliferation and/or differentiation of these cells.

MIC containing PDGF-A chain mRNA were often found in areas of organizing thrombi. Fibrin deposition and thrombus organization have been suggested to play a role in plaque development (12). The "thrombogenic" or "encrustation" hypothesis proposed by von Rokitansky (1852) suggested that plaques might develop by the abnormal deposition of fibrin or blood products on the surface of blood vessels. Additional support for this hypothesis came with the observation by Deguid (13) of a continuum between organizing thrombi and fibrotic intimal thickening in many atherosclerotic plaques. Studies of arterial thrombi in contact with the vessel wall have indicated that arterial thrombi eventually developed into fibrous intimal thickenings as part of the normal healing process.

PDGF mRNAs were not detected in macrophages by in situ hybridization in these studies (4). Macrophages identified as either foamy cells or hemosiderin-containing cells based on their morphology after hematoxylin and eosin staining did not hybridize to the PDGF probes. Serial sections from a number of atherosclerotic plaques were analyzed using in situ hybridization, for PDGF-A or -B chain mRNA, and immunohistochemistry using the monoclonal antibody HAM56, to identify macrophages. A comparison of these sections suggested that few if any macrophages made PDGF. Instead the greater proportion of cells containing PDGF mRNA were identified as MIC or endothelial cells. This was confirmed in additional studies in which in situ hybridization and immunohistochemistry was performed on the same slides in a joint procedure. PDGF mRNAs were not detected in cells staining with macrophage markers.

A recent report suggested that macrophages in human lesions may contain PDGF proteins (14). Using a specific monoclonal antibody directed against the PDGF-B chain (PGF-007) these authors performed double-label immunohistochemistry to localize PDGF staining with markers for either macrophages (HAM56) or smooth muscle cells (HHF35) in human atherosclerotic plaque or developing lesions from a hypercholesterolemic primate. It was concluded that many macrophages contained PDGF proteins in the human atherosclerotic plaque and suggested there was no evidence for smooth muscle or endothelial PDGF production.

It is not clear why PDGF containing endothelial cells were not observed by immunohistochemistry since these cells clearly contained the mRNA for PDGF-B (4). This apparent discrepancy might be explained by the observation that the HAM56 antibody stains both capillary endothelium as well as monocyte/macrophage appearing cells (4, 15). Thus some of the HAM56/PDGF co-stained cells observed by Ross and co-workers might have been endothelial cells and not macrophages. In addition questions have to be raised concerning the specificity of the PGF-007 antibody. Western blots on extracts of human atheroma would help establish whether this antibody reacts with a single molecular species or possibly cross reacts with other proteins in these tissues. This is an especially important question in light of recent observations indicating that activated human macrophages synthesize other proteins that share PDGF-like epitopes and cross react with PDGF antibodies, but are different gene products with very little homology to PDGF on the nucleic acid level (16). Additional work will have to be done to
determine under what conditions macrophages may make PDGF during the development of human atherosclerotic plaques, in part to explain why these cells contain protein but apparently no mRNA for PDGF-B chain.

Our inability to detect PDGF mRNA in macrophages was not due to any problem inherent in hybridizing to these cells. We were able to detect many high and low copy number mRNAs in macrophages in these tissues including: apoprotein E, factor XIII, tissue factor (3), transforming growth factor beta, tumor necrosis factor, cholesterol ester transfer protein and other mRNAs. Recent studies have indicated that macrophages may be a major source as well of another group of inflammatory cytokines which are members of the RANTES/SIS cytokine family. The RANTES/SIS cytokine family includes monocyte chemotactic protein-1 (MCP-1), HIMAP, LD78, and RANTES. These are secreted molecules with potent chemotactic activity for monocytes and T cells (17, 18). Atherosclerotic plaques exhibited marked hybridization for these cytokine mRNAs, especially in macrophage-rich inflammatory regions. By comparison, minimal expression was noted in control arteries without atherosclerotic disease (Table 1).

MCP-1 mRNA was found in normal human atherosclerotic plaques associated with macrophages, smooth muscle cells, MICs, and possibly T cells. By light microscopy lipid containing macrophages were often seen to hybridize with the MCP-1 probes. Positive MCP-1 hybridization was seen in inflammatory zones (24% of 1, 999 cells counted) and in areas of thrombus deposition and organization (33% of 1,035 cells counted). These areas contained many macrophages with a few scattered UCHL1 positive T cells as determined by serial section immunohistochemistry. While we can not eliminate the possibility of synthesis by T cells these data suggest that most of the MCP-1 positive cells in these areas were macrophages. LD78 and HIMAP were found in similar regions of the plaque but appeared to be localized to populations of T cells instead of macrophages. Cells identified morphologically as foamy macrophages did not contain mRNAs for LD78 or HIMAP. The numbers of cells expressing these cytokines appeared to be more closely related to the number of UCHL1 positive T cells in these tissues than the numbers of macrophages (Table 1).

MCP-1 mRNA was also found in mesenchymal-appearing cells which had a similar morphology and staining characteristics as the PDGF-producing MIC and were found in the same areas. HIMAP, LD78, or RANTES mRNAs were not detected in MIC. MCP-1 is the human homologue of the mouse PDGF-inducible gene JE (19). The apparent co-localization of PDGF and MCP-1 expression in the same regions of the plaque suggests that autocrine or paracrine stimulation of MIC by PDGF may in turn upregulate MCP-1 synthesis by these cells. Areas of organizing thrombi contain not only MICs expressing PDGF and PDGF receptors but also cells making MCP-1, LD78, HIMAP, and RANTES mRNA. Thrombus deposition may not only contribute to smooth muscle proliferation through an increase in PDGF expression but may also stimulate the synthesis and release of inflammatory cytokines which contribute to the ongoing inflammatory process by attracting mononuclear cells to these sites.

MCP-1, HIMAP, and LD78 mRNA containing cells were detected in cells populating early human fatty streaks. MCP-1 was localized to macrophages and endothelial cells overlying these lesions (20), HIMAP and LD78 appeared to be synthesized by T cells, while cells synthesizing RANTES were not detected. MCP-1 mRNA and protein are upregulated in vitro in human umbilical vein endothelial cells and smooth muscle cells by minimally oxidized LDL and beta VLDL (21, 22). Elevated lipids may play a role initiating atherosclerotic lesion development by stimulating MCP-1 production which might start the inflammatory process. As monocytes and T cells begin to arrive in the lesion they too begin to synthesize additional inflammatory cytokines (ie. MCP-1, LD78 and HIMAP) leading to increased monocyte chemotaxis into the lesion. With infiltration of monocytes, a positive feedback mechanism might exist whereby infiltrating monocyte/macrophages secrete MCP-1 in large amounts, thereby attracting more peripheral blood monocytes to the developing atherosclerotic plaque beginning the cycle of inflammation and intimal development.

Table 1. A comparison of the proportion of cells expressing MCP-1, HIMAP, LD78, and RANTES mRNAs in atherosclerotic plaques compared to normal internal mammary artery (IMA) as determined in situ hybridization. Serial sections were hybridized with 35S-labeled riboprobes encoding for MCP-1, HIMAP, LD78, or RANTES. To aid in the identification of positive cells, serial sections were immunostained with antibodies specific for macrophages (HAM56), T cells (UCHL1), endothelial cells (Ulex lechitin), or smooth muscle cells (SMC) (HHF35). The proportion of positive cells was calculated relative to total cells counted in 9 human carotid endarterectomy specimens.

<table>
<thead>
<tr>
<th>% of Cells Counted</th>
<th>Atheroma</th>
<th>Normal IMA</th>
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<tbody>
<tr>
<td></td>
<td>Intima</td>
<td>Media</td>
</tr>
<tr>
<td>LD78</td>
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<tr>
<td>HIMAP</td>
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</tr>
<tr>
<td>SMC</td>
<td>15</td>
<td>92</td>
</tr>
</tbody>
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References

(1) Ross R: The pathogenesis of atherosclerosis: an up-


(7) Barrett TB and Benditt EP: sis (platelet-derived growth factor B chain) gene transcript levels are elevated in human atherosclerotic lesions compared to normal artery. Proc Natl Acad Sci USA, 84: 1099-1103, 1987


