Cytokine gene expression in aortic adventitial inflammation associated with advanced atherosclerosis (chronic periaortitis)

A L Ramshaw, D E Roskell, D V Parums

Abstract

Aims—To determine whether aortic adventitial chronic inflammation associated with advanced atherosclerosis (“chronic periaortitis”) is associated with any detectable cytokine gene expression.

Methods—RNA was extracted from six fresh surgical specimens of atherosclerotic aortic adventitia. Controls included four normal aortas and an HUT 78 T cell line. Reverse transcriptase and the polymerase chain reaction (PCR) were used to amplify mRNA for interleukins-1α (IL-1α), -2 (IL-2), -4 (IL-4), IL-2 receptor-α (IL-2R-α), tumour necrosis factor α (TNF-α) and gamma interferon (IFN-γ) with β-actin as an internal control.

Results—No TNF-α mRNA was detected in any of the inflamed aortic tissue samples, in contrast to the aortic T lymphocytes propagated in culture in IL-2 conditioned medium (aortic cultured T cells) and peripheral blood mononuclear cells from these patients. In contrast, IFN-γ, IL-1α, IL-2, IL-2 receptor and IL-4 PCR products were detected for each inflamed aortic tissue RNA sample with IFN-γ mRNA expression increasing with increasing degrees of adventitial inflammation. Only β-actin mRNA was present in the normal aorta.

Conclusions—These findings indicate the active nature of aortic adventitial chronic inflammation associated with human advanced atherosclerosis (“chronic periaortitis”) and show its possible progressive potential to the clinically important diseases termed “idiopathic retroperitoneal fibrosis” and “inflammatory aneurysm”.


Chronic inflammatory cells are present in the aortic and arterial intima at all stages of atherogenesis. As the lesion becomes more advanced and the media thins, a spectrum of chronic inflammation is seen in the adventitia. The inflammatory cells consist of lymphocytes and plasma cells and lymphoid follicles are common. A varying degree of fibrosis is seen. When present in the aorta, the condition is termed “chronic periaortitis.” Although the degree of inflammation seen usually produces no aortic wall thickening or clinical consequences, in its most severe form the condition may represent “inflammatory aneurysm” or “idiopathic retroperitoneal fibrosis” in dilated and undilated aortas, respectively.

Chronic periaortitis is thought to arise as a local immune response to oxidised lipids elaborated by macrophages in the atheromatous plaque. Immunohistochemistry has shown that, whereas the chronic inflammatory cells in the intimal atheroma consist of macrophages and T lymphocytes, in the inflamed adventitia B lymphocytes predominate, including plasma cells, surrounded by CD4 positive T lymphocytes and macrophages. MHC class II molecule expression is abundant, particularly on endothelial cells (CD31 positive). Interleukin (IL)-2 and IL-4 receptor expression is seen throughout the tissue and B cell proliferation is seen within the lymphoid follicles.

Immunohistochemical analysis of cytokine dependent adhesion molecules in atherosclerosis and chronic periaortitis has shown strong anti-E-selectin (formerly endothelial leucocyte adhesion molecule-1 (ELAM-1)) staining confined to endothelial cells in the aortic media and adventitia, extensive anti-intercellular adhesion molecule-1 (ICAM-1) staining on endothelial cells in the intima and adventitia, and anti-vascular cell adhesion molecule-1 (VCAM-1) staining associated with B cell aggregates with moderate staining of vessels in lymphoid follicles.

Cytokines are small soluble protein mediators with specialised functions that have a central role in the regulation of all immune responses. These molecules are fundamental in the initiation, maintenance, and progression of inflammation, and are required for recruitment of cells, for activation and proliferation. There has been recent interest in the role of cytokines in atherogenesis. The predominant cytokine mediators thought to be involved include platelet derived growth factor (PDGF), IL-1, monococyte chemoattractant protein-1 (MCP-1), tumour necrosis factor (TNF)-α, interferon (IFN)-γ and granulocyte-macrophage colony stimulating factor (GM-CSF). These mediators are thought to control the intimal inflammation as well as the altered functions of smooth muscle cells. While cytokines are clearly important in the inflammatory component of plaque formation, they must also have a role in chronic periaortitis where the inflammation is much more intense than that present in the intima.

To determine the presence and relative
abundance of cytokines, the protein product can be measured by western blot analysis, by immunohistochemistry, and by immune assays such as the enzyme linked immunosorbent assay (ELISA). Cytokine mRNA can be detected by northern blotting, by RNA dot blotting, and by in situ hybridisation, or by the polymerase chain reaction (PCR) following reverse transcription. It is often preferable to study mRNA expression, which in many instances is a reflection of the protein product. Of the techniques for measuring cytokines, PCR is the most reproducible as well as being extremely sensitive, requiring only small amounts of cellular RNA.

PCR was first described for indirect amplification of RNA transcripts by Veres et al. Specific mRNA is amplified following the synthesis of copy DNA (cDNA) using the reverse transcriptase enzyme. cDNA is subsequently amplified by PCR using sequence specific primers. This method has been applied to the analysis of cytokine mRNA expression by a process called message amplification pheno- typing (MAPPing). Most work using PCR assisted RNA amplification has been carried out for cultured cells. Recently, workers have begun to investigate the presence of cytokines in aortic atherosclerosis using immunohistochemistry and ELISA techniques.

To determine which cytokines may be modulating and controlling this B cell dominant, active inflammatory response seen in chronic periaortitis, we chose to screen tissues for mRNA expression using PCR assisted amplification of RNA. Due to the large number of known cytokines, this investigation was limited to cytokines which have been shown to be particularly important in chronic inflammation and which may be relevant to T and B lymphocyte responses. These include the pro-inflammatory cytokines TNF-α, IFN-γ, IL-1α, as well as the cytokines IL-2 and IL-4, and the IL-2 receptor, involved in lymphocyte activation and proliferation. The resultant PCR products were taken to indicate which cytokines were present in vivo in chronic periaortitis and which might therefore play a part in the maintenance and progression of this chronic inflammatory process, which we believe is secondary to aortic advanced atherosclerosis.

### Methods

Samples included six abdominal aortic tissues with chronic periaortitis (I to VI), two post mortem “normal” aortas (A and B), and two “normal” aortas received as surgical pathology specimens (C and D) (table 1). The histology was observed on paraffin wax sections from blocks of tissue taken adjacent to the tissue for RNA extraction.

Peripheral blood was collected 24 hours preoperatively from three patients with aneurysm (cases I, III, and V (table 1)) and from three laboratory volunteers (a 27 year old woman; a 29 year old man; and a 28 year old man). Mononuclear cells were isolated by Ficoll–Paque (Pharmacia) density gradient centrifugation.

### CONTROL CELL LINES

Two cell lines were used (HUT 78 and MCF-7). The HUT 78 human lymphoma T cell line is highly inducible for cytokines when stimulated with phytohaemagglutinin-P (PHA). HUT 78 cells were therefore used as a positive control for the analysis of cytokine mRNA expression. The MCF-7 breast cell line (a largely non-secretory cell) was used as a negative control.

HUT 78 cells were stimulated to increase the synthesis of specific cytokine mRNA. Phytohaemagglutinin (10 µg/ml) (PHA) (Sigma) in sterile water was added to HUT 78 cells in exponential growth. 10° PHA stimulated HUT 78 cells, 10° unstimulated HUT 78 cells, and 10° MCF-7 cells were harvested and prepared for RNA extraction.

### AORTIC CULTURED T LYMPHOCYTES

To compare the in vivo and in vitro expression of cytokines, two cases of abdominal aortic aneurysm tissue (IV and V (table 1)) were cultured in vitro in IL-2 conditioned medium. Biopsy samples from abdominal aortic aneurysms were cut into small pieces (1 mm³) and placed in 2 ml of media (RPMI 1640 (Flow), 2 mM glutamine (Flow), 10% heat inactivated normal human serum, 10 µg/ml gentamycin (Flow), 10 units/ml recombinant human IL-2 (Cetus)) per well in a 24 well plate (Flow). The cultures were placed at 37°C, 5% CO₂ in a humid incubator. Controls were carried out by placing tissue pieces in wells without IL-2. Media was replenished every three to four days.

About 3 x 10⁷ cells were harvested after four to six weeks of growth. Cytospins were prepared and immunocytochemistry was performed using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method. Monoclonal antibodies included 3D4 (anti-CD3, T cell associated), T3–10 (anti-CD4, T helper cell associated), Tu102 (anti-CD8, T cytotoxic/suppressor cell associated), HD37/4KB128 (anti-CD19/22, B cell associated) and EBM11 (anti-CD66, macrophage-associated). Immunocytochemistry showed that the aortic tissue cultured cells were predominantly T cells. They were 80% CD4 positive (T helper cell associated), 15% CD8 positive (T cytotoxic/suppressor cell

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Tissue samples for PCR analysis of mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue no</td>
<td>Degree of inflammation</td>
</tr>
<tr>
<td>Chronic periaortitis</td>
<td>(fresh surgical abdominal aortic aneurysm tissue)</td>
</tr>
<tr>
<td>I</td>
<td>Mild</td>
</tr>
<tr>
<td>II</td>
<td>Severe</td>
</tr>
<tr>
<td>III</td>
<td>Severe</td>
</tr>
<tr>
<td>IV</td>
<td>Moderate</td>
</tr>
<tr>
<td>V</td>
<td>Mild</td>
</tr>
<tr>
<td>VI</td>
<td>Severe</td>
</tr>
<tr>
<td>Normal aorta</td>
<td></td>
</tr>
<tr>
<td>(Post mortem tissue &lt;18 hours)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Absent</td>
</tr>
<tr>
<td>B</td>
<td>Absent</td>
</tr>
<tr>
<td>(Surgical tissue &lt;1 hour)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Absent</td>
</tr>
<tr>
<td>D</td>
<td>Absent</td>
</tr>
</tbody>
</table>
Cytokine gene expression in aortic adventitial inflammation associated with advanced atherosclerosis (chronic periarteritis) 723

Tissue and cell preparation
Biopsy and necropsy samples were minced (1 cm² of tissue was cut into 1–2 mm³ pieces) with a sterile scalpel. After rinsing briefly in sterile phosphate buffered saline (PBS) to remove excess red blood cells, the tissue was transferred to a 50 ml centrifuge tube (Falcon UK) containing 2 ml of 4 M guanidine-thiocyanate solution (Sigma UK). Tissues were homogenised at top speed for 20–40 seconds using a Polytron (Kinematica, Switzerland). Samples were stored at −70°C until required for RNA extraction.

Cultured cells and blood mononuclear cells (about 1–3 × 10⁵ cells) were centrifuged at 200 × g for five minutes in a 15 ml centrifuge tube and then washed in sterile phosphate buffered saline (PBS). Cells were vortexed for one minute in 2 ml of 4 M guanidine-thiocyanate solution and stored at −20°C.

RNA isolation and detection
RNA extraction from cell and tissue samples was based on the acid-guanidinium-thiocyanate-phenol-chloroform method of Chomczynski and Sacchi. The integrity of the RNA was assessed by electrophoresis following glyoxal treatment in a 1% agarose gel stained with ethidium bromide.

Reverse transcription
First-strand cDNA was synthesised from each sample of RNA in DEPC-water using the GeneAmp RNA PCR kit (Perkin-Elmer Cetus). Reverse transcription was carried out in a 750 µl microfuge tube in a total volume of 20 µl reaction mix (1 mM each of deoxynucleotide (d)ATP, dCTP, dGTP, dTTP (Gibco, BRL); 2.5 mM MgCl₂; PCR buffer containing 50 mM KCl, 10 mM TRIS-HCl pH 8.5; 5 µM oligo d(T)₁₆ (Pharmacia LKB); 20 U/ml RNAase inhibitor (Pharmacia LKB); 50 U/ml reverse transcriptase (cloned Moloney Murine Leukemia Virus (MMLV) reverse transcriptase)(Gibco BRL)) and 0.5 µg of RNA. Reactions were carried out at 42°C for 30 minutes, heat denatured at 99°C for five minutes, and placed on ice. Volumes of 10 µl were then removed for amplification of cDNA using specific primers.

Oligonucleotide primers (100 ng) specific for β-actin, IFN-γ, TNF-α, IL-1α, IL-2, IL-2 receptor-α and IL-4 were used in this study and are described in table 2. These primers, which span introns to control for amplification of contaminating DNA, were based on published sequences. IFN-γ and TNF-α primers were obtained from Clontech, USA. IL-2, IL-2 receptor-α, IL-4 and β-actin primers were synthesised in the Department of Biochemistry and Molecular Biology, University of Leeds. IL-1α primers were obtained from the GeneAmp RNA PCR kit (Cetus, USA).

Amplification by PCR
Polymerase chain reaction (PCR) assisted amplification of RNA was carried out as described by Veres et al and Brenner et al. Directly in the reverse transcription reaction tubes by adding 80 µl reaction mix (2 mM MgCl₂, PCR buffer, 2-5 U AmpliTaq DNA polymerase from Thermophilus Aquaticus (Amersham), 0.2 µM sense and anti-sense oligonucleotide primers) to each sample. Tubes were mixed and centrifuged at 10 000 × g for 30 seconds; 100 µl of light mineral oil was overlaid onto each sample to prevent evaporation during incubation.

PCR was carried out in a thermal cycler (Ericonphil, Lazer Ltd UK). Conditions were optimised and standardised for each primer pair using PHA-stimulated HUT 78 T cells samples. Optimal temperatures (for denaturation, for annealing, and for primer extension) were determined for each primer pair, and carried out over 35 cycles. There was about one minute ramp time between temperatures to allow the samples to equilibrate. PCR products were stored at 4°C. PCR assisted mRNA amplification was repeated at least once for each sample type. Controls for false positive results were carried out for each set of primers by substituting water for RNA. RNA specificity was tested by omitting reverse transcriptase for each primer pair. PCR primer sequences and the expected molecular weight of each cDNA product are shown in table 2.

Table 2 Oligonucleotide primer pair sequences

<table>
<thead>
<tr>
<th>cDNA</th>
<th>5' sense primer</th>
<th>3' antisense primer</th>
<th>Amplified fragment size (base pairs)</th>
<th>Reference for cDNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5'-TGACGGGGTGCAACCCACATGCTGCGCCCATCTA-3'</td>
<td>3'-CTATAGAGGAAACGTGGGAAAGG-5'</td>
<td>661</td>
<td>35</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-GTAGGCTCTGCGAACCTGGAAACAGGAT-3'</td>
<td>3'-AGATGCTCTGCGAACCTGGAAACAGGAT-5'</td>
<td>485</td>
<td>36</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-ATGAGCACGTGACATCTGCTA-3'</td>
<td>3'-TCAACAGGCTCCATGATCCATTCGAC-5'</td>
<td>702</td>
<td>37</td>
</tr>
<tr>
<td>IL-1α</td>
<td>5'-GTCTCTGCTGACATGGAATCTTCCAT-3'</td>
<td>3'-CTAATTCATTGCTGGGGAACATTTGTT-5'</td>
<td>420</td>
<td>38</td>
</tr>
<tr>
<td>IL-2</td>
<td>5'-ATCTGACGAATGCACTTCTGATTC-3'</td>
<td>3'-TCAATCCAGCCATGGGACATTTGTT-5'</td>
<td>462</td>
<td>39</td>
</tr>
<tr>
<td>IL-2Rα</td>
<td>5'-TCAACAGGCTCCATGATCCATTCGAC-3'</td>
<td>3'-CTATAGAGGAAACGTGGGAAAGG-5'</td>
<td>391</td>
<td>40</td>
</tr>
<tr>
<td>IL-4</td>
<td>5'-ATGAGGCTCTGACATCTGCTA-3'</td>
<td>3'-TCAACAGGCTCCATGATCCATTCGAC-5'</td>
<td>462</td>
<td>41</td>
</tr>
</tbody>
</table>
Figure 1  Low power view of an atherosclerotic aortic biopsy specimen (case IV, aortic wall thickness 4 mm) showing the moderate infiltrates of mononuclear cells in the aortic adventitia (left), media (centre) and in the base of the intimal atheroma (right) (haematoxylin and eosin).

ANALYSIS OF PCR PRODUCTS

PCR products were size fractionated by electrophoresis through 2% agarose gels to determine the presence, size, and degree of amplification of a particular transcript. Specificities of the resultant bands were validated by their predicted size in comparison with standard molecular weight markers (pHC624-TaqI and pMJ3-NciI). Semi-quantitative analysis was carried out by determining the relative PCR band intensity for a given primer pair. Band intensity was graded as absent (no detectable product (−)), low (+), moderate (+++) or strong (++++) according to the fluorescence intensity of the ethidium bromide staining. Gels were photographed using ultraviolet transillumination.

Results

Normal aortic tissues (specimens A–D) showed no evidence of atheroma or inflammation. The atheromatous aortic aneurysm samples (specimens I to VI) all showed atherosclerosis with a varying degree of adventitial inflammation and aortic wall thickness as described in table 1. The histology and grading of inflammation (fig 1) was as described before.

A strong signal for β-actin cDNA was detected in all samples, confirming the integrity of the RNA. Cytokine mRNA profiles are shown in table 3 and fig 2. To confirm that all fragments were derived from amplification of cDNA products, duplicate tests were analysed without the addition of reverse transcriptase. No PCR products were detected in these tests.

RNA isolated from PHA-stimulated HUT 78 cells resulted in strong PCR product bands for each transcript, while the unstimulated HUT 78 RNA resulted in weaker products for each cytokine. For the MCF-7 RNA, strong β-actin and weak IL-1α PCR products were detected. PCR products from the aortic cultured T cell RNA (from cases IV and V) were detected for all of the primer pairs, including TNF-α. Only PCR products corresponding to β-actin were detected from the "normal" aortic

Table 3  Relative gene expression detected in cell lines, aortic tissues, and cultured cells

<table>
<thead>
<tr>
<th>RNA sample</th>
<th>β-actin</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-1α</th>
<th>IL-2</th>
<th>IL-2R</th>
<th>IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUT 78 (+PHA)</td>
<td>+ + + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>HUT 78 cell line</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>MCF-7 cell line</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Inflamed aorta (inflammation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAA tissue I (mild)</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>AAA tissue II (severe)</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>AAA tissue III (severe)</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>AAA tissue IV (mild)</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>AAA tissue VI (severe)</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Normal aorta</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Tissue A</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Tissue B</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Tissue C</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Tissue D</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Patient blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBM I</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>PBM III</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>PBM V</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Voluteer blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBM I</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>PBM L</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>PBM R</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Cultured T cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cells tissue IV</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>T cells tissue V</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>No reverse transcriptase</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

The relative PCR band intensity for a given primer pair was graded as; absent (no detectable product (−)), low (+), moderate (+++) or strong (++++).
Cytokine gene expression in aortic adventitial inflammation associated with advanced atheroma (chronic periortitis)

Figure 2. Representative patterns of cytokine gene expression in Hu78 stimulated T cell line; AAA tissue (case III); normal aorta (case A), and negative control for β-actin; IFN-γ, TNF-α; IL-1α, IL-2; IL-2 receptor; IL-4 and IL-4 receptor. Molecular weight marker lanes are included.

Discussion

We have detected cytokine gene expression in chronic periortitis and compared the results with non-diseased aortic tissue and cell controls. PCR assisted mRNA analysis permitted a rapid, highly sensitive, and reproducible semi-quantitative initial screening of RNA samples for the presence of cytokines. The in vivo presence of cytokine and cytokine receptor mRNA transcripts was investigated for six cases of inflamed atherosclerotic aortic tissue. TNF-α was not detected in chronic periortitis by this method, but the other pro-inflammatory cytokines IL-1α, IL-2, IL-4 and the IL-2 receptor were detected in all six cases (I to VI) but were not detected in normal aorta (A–D); mRNA expression of IFN-γ increased with increasing severity of the aortic adventitial inflammation. Only β-actin mRNA was present in the normal aorta.

IL-1 is important for acute and chronic inflammation, in activating cells and in cell recruitment. High concentrations of IL-1α have also been found in synovial tissue in rheumatoid arthritis and in thyroid gland in Grave’s disease. The concentrations of IL-1 in the tissue may reflect the state of chronic inflammation—either active and developing, or resolving. Of course, abundance does not necessarily imply importance, and the in vivo role of any of these cytokines remains to be elucidated.

The finding of IL-2 mRNA in the inflamed aortic wall is consistent with the presence of activated and proliferating T cells in chronic periortitis. IL-2 and IL-4 are important in T and B cell differentiation and proliferation and the IL-2 receptor is upregulated during inflammation and activation states. Furthermore, consistent with the mRNA results, immunohistochemistry revealed IL-2 receptor—a protein expression in tissue with chronic periortitis, but not in the non-diseased aortas.7

The presence of the cytokine transcripts in the blood is probably due to the natural killer and T cell populations. These cells are known to synthesise and secrete cytokines such as IFN-γ, IL-1, IL-2 and IL-4. While the detection of cytokines in the tissue is not thought to be artefactual through blood contamination, the absence of TNF-α in the tissue samples, contrasting with its presence in the blood samples, indicates that the level of sensitivity of this assay was too low to detect such contamination.

Further differences in mRNA expression were demonstrated by comparing in vivo and in vitro results. TNF-α was not detected in the tissue with chronic periortitis but it was detected in the aortic cultured T cells after three weeks’ propagation in vitro. Many cytokine investigations have been carried out on mRNA isolated from tissue cells propagated in vitro. While these in vitro studies may indicate the potential cytokine synthesis and secretion, they may be due to non-specific activation in cell culture.

We have previously documented the presence of cytokine inducible molecules,
HLA-DR, HLA-DQ, VCAM-1, E-selectin and ICAM-1 in chronic periaortitis, which may provide circumstantial evidence for the presence of cytokine. TNF-α is of importance in the induction of ICAM-1 and E-selectin and it acts synergistically with IL-4 to increase VCAM-1 mediated T cell adhesion to cultured endothelial cells. TNF-α has been detected in macrophages in carotid artery atheroma using immunohistochemistry but not in tissue sections of whole atherosclerotic aorta using ELISA assays. The absence of TNF-α in chronic periaortitis may be due to a lack of stimulatory signals, or perhaps to the presence of inhibitory signals. Oxidised low density lipoprotein (LDL), which is abundant in the atherotic plaque, inhibits the cellular expression of TNF-α in vitro. It remains to be seen if this is the mechanism responsible for the possible absence of TNF-α in the inflamed aortic wall. Culturing the aortic tissue cells in vitro in the presence of oxidised LDL might provide further insight into its effects on cytokine expression.

TNF-α is believed to be an important cytokine due to its abundance in chronic inflammatory diseases such as rheumatoid arthritis. TNF-α has, in fact, been implicated as one of the most important regulators in chronic inflammation. The lack of TNF-α in chronic periaortitis indicates that a "full-blown" progressive chronic inflammatory response can, however, develop in the absence of this mediator. Other cytokines may compensate for the apparent absence of TNF-α in chronic periaortitis. In addition, the absence of TNF-α, which is chemotactic for polymorphonuclear cells, may be responsible for the lack of these cells in chronic periaortitis. In contrast, polymorphonuclear cells are seen in rheumatoid arthritis where TNF-α is also abundant. These studies confirm the view that chronic periaortitis is an active, progressive, local immune response associated with advanced atherosclerosis. Further research needs to be directed towards understanding the similarities and the differences in the genesis and perpetuation of the inflammatory processes occurring in the intima as part of atherosclerosis and in the adventitia in chronic periaortitis. Immunohistology and in situ hybridisation techniques would permit the identification of cells responsible for the production of cytokines that influence fibrosis, such as acidic and basic fibroblast growth factor (FGF), and for platelet derived growth factor (PDGF), transforming growth factor (TGF) α and β, monocyte chemotactic protein 1 (MCP-1) and macrophage colony stimulating factor (M-CSF), IL-6 and IL-8 as well as IFN-γ, IL-1, IL-2 and IL-4. These further studies on the cytokine network in atherosclerosis may lead to a greater understanding of how the cellular (and non-cellular) elements of the lesion interact at all stages. We may then hope for scientifically based therapeutic strategies for disease prevention and diagnosis. It may be possible to identify sites in the cytokine network that are susceptible to therapeutic intervention using antibodies, recombinant cytokines, cytokine receptor antagonists or by using gene transfection to inhibit cytokine mediated inflammation in atherogenesis. This work was supported by the British Heart Foundation and the Oxford Medical Research Fund.

Cytokine gene expression in aortic adventitial inflammation associated with advanced atheroma (chronic periaortitis)

28 Kelly FMA, Bliss E, Morton JA, Burns J, JOD McGee. Monoclonal antibody EBMA/1: high cellular specificity
29 Chomezynski P, Sacchi N. Single step method of RNA
isolation by acid guanidinium thiocyanate-phenol-
clones for human α-, β- and γ-actin mRNAs: skeletal but
not cytoplasmic actins have an amino-terminal cysteine
that is subsequently removed. Mol Cell Biol 1983;3:
787–95.
factor: precursor structure, expression and homology to
34 Holbrook NJ, Smith KA, Fornace AJ, Comeau CM, Wiskocil RL, Crabbee GR. T-cell growth factor: complete
nucleotide sequence and organization of the gene
in normal and malignant cells. Proc Natl Acad Sci USA
36 Yokota T, Otuka T, Mosmann T, Banchereau J, DeFrance T, Blanchard D, et al. Isolation and characteri-
zation of a human interleukin CDNA clone, homologous
to mouse B-cell stimulatory factor 1, that expresses B-
cell- and T-cell-stimulating activities. Proc Natl Acad Sci
USA 1986;83:3894–8.
38 Rees RC. Cytokines as biological response modifiers.
rheumatoid joint: implications for treatment. Ann Rheum
40 Orsulak JR. Cytokine production by CD3+ large granular
lymphocytes. In: Reynolds CW, Woltrout RH, eds. Functions of the natural immune system. New York:
42 Thorhill MH, Wellcome SM, Mahiout DL, Lanchbury JSS, Kyan-Aung U, Haskard DO. Tumour necrosis fac-
tor combines with IL-4 or IFN-γ to selectively enhance
endothelial cell adhesiveness for T cells. J Immunol
43 Hamilton TA, Gouping MA, Chisholm GM. Oxidized low
density lipoprotein suppresses the expression of tumor
necrosis factor-α mRNA in stimulated murine peritoneal
44 Paul WE. Pleiotropy and redundancy: T cell-derived
lymphotokines in the immune response. Cell 1989;57:
521–4.
45 Brown KA. The polymorphonuclear cell in rheumatoid
46 Ross R. The pathogenesis of atherosclerosis: a perspective
Cytokine gene expression in aortic adventitial inflammation associated with advanced atherosclerosis (chronic periaortitis).

A L Ramshaw, D E Roskell and D V Parums

doi: 10.1136/jcp.47.8.721

Updated information and services can be found at:
http://jcp.bmj.com/content/47/8/721

_These include:_

**Email alerting service**

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/