Expression and Cellular Localization of the CC Chemokines PARC and ELC in Human Atherosclerotic Plaques

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Local immune responses are thought to play an important role in the development of atherosclerosis. Histological studies have shown that human atherosclerotic lesions contain T lymphocytes throughout all stages of development, many of which are in an activated state. A number of novel CC chemokines have been described recently, which are potent chemoattractants for lymphocytes: PARC (pulmonary and activation-regulated chemokine), ELC (EBI1-ligand chemokine), LARC (liver and activation-regulated chemokine), and SLC (secondary lymphoid-tissue chemokine). Using reverse transcriptase-polymerase chain reaction and *in situ* hybridization, we have found gene expression for PARC and ELC but not for LARC or SLC in human atherosclerotic plaques. Immunohistochemical staining of serial plaque sections with specific cell markers revealed highly different expression patterns of PARC and ELC. PARC mRNA was restricted to CD68⁺ macrophages ($n = 14$ of 18), whereas ELC mRNA was widely expressed by macrophages and intimal smooth muscle cells (SMC) in nearly all of the lesions examined ($n = 12$ of 14). ELC mRNA was also found to be expressed in the medial SMC wall of highly calcified plaques ($n = 4$). Very low levels of ELC mRNA expression could also be detected in normal mammary arteries but no mRNA expression for PARC was detected in these vessels ($n = 4$). *In vitro*, ELC mRNA was found to be up-regulated in aortic SMC stimulated with tumor necrosis factor- α and interferon- γ but not in SMC stimulated with serum. Both PARC and ELC mRNA were expressed by monocyte-derived macrophages but not monocytes. The expression patterns of PARC and ELC mRNA in human atherosclerotic lesions suggest a potential role for these two recently described CC chemo-

kines in attracting T lymphocytes into atherosclerotic lesions. (*Am J Pathol* 1999, 154:365–374)

Atherosclerosis continues to be the major cause of heart disease. Although the histological sequence of events during lesion progression is well documented, the precise regulatory factors and interplay between the major cell types producing those factors remain poorly defined.

The presence of T cells in addition to macrophages in atherosclerotic lesions indicates that there are immunological events occurring in conjunction with inflammatory ones.^{1,2} The earliest visible stage of the disease, the so-called "fatty streak" is characterized by the presence of monocyte/macrophages and T lymphocytes in the intima. After entry into the arterial intima, monocytes differentiate into macrophages, many of which become foam cells following ingestion of modified lipoprotein particles, notably oxidized LDL (low density lipoprotein). Both CD4⁺ and CD8⁺ T cells have been found in human plaques, and many of these cells have been shown to be in an activated state and producing the cytokine interferon- γ (INF- γ), a potent macrophage activating factor.³ The occurrence of activated T cells in plaques supports the idea that there may be a specific immune response in operation during atherosclerosis. Additional evidence for the implication of local immunological mechanisms in atherosclerosis comes from the finding of CD4⁺ T cells in lesions of the apolipoprotein E (ApoE) knockout mouse throughout all stages of development.⁴

T cells and macrophages, in addition to the arterial smooth muscle cells (SMC) and endothelium, are capable of producing a wide range of cytokines and growth factors.⁵ Chemokines are a superfamily of small (8 to 10 kd) cytokines with four conserved cysteine residues, which are potent chemoattractants for leukocytes.^{6,7} To date, four classes of chemokines, containing approximately 40 members, have been identified based on the arrangement of the conserved cysteines. In the CC chemokine family (eg, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1

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(MIP-1 α) these cysteine residues are adjacent, whereas the CXC chemokines (eg, interleukin (IL)-8 and IP (inducible protein)-10) have an intervening amino acid between the first two cysteines.⁷ The C chemokine family contains just one member to date, lymphotactin, which lacks two of the four conserved cysteines.⁸ Fractalkine, the only CX₃C chemokine family member, has three intervening amino acid residues between the first two cysteines and is encoded as a membrane bound molecule with the chemokine domain attached to a mucin-like stalk.⁹ Different chemokine classes tend to display different ranges of leukocyte specificity. CXC chemokines preferentially attract neutrophils, whereas CC chemokines attract monocytes, T and B cells and eosinophils. Chemokine activities are mediated by seven transmembrane domain G protein coupled receptors. To date, at least eight receptors for CC chemokines and five for CXC chemokines have been characterized.⁷

MCP-1, a potent monocyte chemoattractant, is the prototype for the CC chemokine family.¹⁰ MCP-1, 2, 3, and 4, RANTES (regulated on activation normal T expressed and secreted chemokine), MIP-1 α , and MIP-1 β are all chemoattractants for monocytes albeit with differing potencies.^{10–12} MCP-1 was the first CC chemokine found to be expressed in human atherosclerotic lesions^{13,14} and its production either singularly or in combination with other chemokines is postulated to be responsible for the continued influx of monocytes into atherosclerotic plaques. All of the aforementioned CC chemokines are capable of attracting several classes of blood leukocytes^{7,15} and many can bind and signal through a number of different receptors.⁷ Recently, four CC chemokines have been characterized and shown to selectively attract T cells: PARC/DC-CK1 (dendritic cell derived CC chemokine),^{16,17} ELC/MIP-3 β ,^{18,19} SLC,²⁰ and LARC.²¹ Of particular interest in the context of atherosclerosis is the finding that PARC shows a specificity for naive, resting T cells.¹⁷ This property is not shared by other T-cell chemoattractants such as RANTES, MIP1- α , and IL-8.¹⁷ ELC and SLC are known to act through CCR7/EBI1^{22,23} and LARC through CCR6.²² The receptor for PARC has not yet been identified. Given the fact that T cells are present throughout plaque development^{2,4} and their presence is thought to be caused by active recruitment and local polyclonal T-cell activation rather than clonal antigenic expansion,²⁴ we investigated the gene expression of these new lymphocyte specific chemoattractants in human atherosclerotic lesions.

Materials and Methods

Human Tissue Samples

Plaque samples used in this study were carotid endarterectomy specimens ($n = 14$ for ELC and $n = 18$ for PARC) from patients undergoing vascular reconstructive surgery for arterial occlusive disease and internal mammary arteries ($n = 4$). Tissues were either mounted in OCT compound-embedding medium (Agar Scientific Ltd., Essex, UK) and frozen in liquid nitrogen or snap-

frozen in liquid nitrogen and mounted in OCT prior to cryostat sectioning. All specimens were stored at -70°C .

Cell Culture

Human Monocyte-Derived CD1a⁺ Dendritic Cells

Human peripheral blood monocytes were prepared from blood as previously described.²⁵ Monocyte derived dendritic cells were prepared essentially as described by Zhou and Tedder.²⁶ The purified monocyte fraction was resuspended in supplemented RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 20 mmol/L L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin (Gibco BRL, Grand Island, New York). Cells were maintained in T-75 flasks with IL-4 (500 U/ml) and 800 U/ml GM-CSF and 100 U/ml tumor necrosis factor (TNF)- α was added on day 5 (all cytokines from Peprotech, Rocky Hill, NJ). Medium was changed on days 3 and 5. On day 8, cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), 0.1% NaN₃ and stained with phycoerythrin-conjugated anti-human CD1a (DAKO, Glostrup, Denmark). Positively stained cells were sorted on a FACSort (Becton-Dickinson, Bedford, MA) to a purity $>98\%$.

Human CD4⁺ Blood Dendritic Cells

Cells were purified from peripheral blood using the MACS immunomagnetic blood dendritic cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol.

Monocyte Stimulation

The monocyte enriched fraction from a Percoll separation was further enriched by incubation for 1 hour in RPMI 1640 medium, 10% FBS, after which nonadherent cells were washed off. Monocytes were resuspended in RPMI/FBS and treated with 0.1 $\mu\text{g/ml}$ lipopolysaccharide (LPS) (*E. coli* serotype 055:B5) (Sigma, St. Louis, MO) for 18 hours.

THP-1 Cell Culture, Differentiation and Foam Cell Formation

The human monocyte cell-line THP-1 was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 2 mmol/L Glutamax, and 18 $\mu\text{mol/L}$ 2-mercaptoethanol. All media and supplements were obtained from Gibco BRL Life Technologies Ltd. (Paisley, UK).

THP-1 cells were differentiated to anchorage-dependent macrophages by the addition of 0.2 $\mu\text{g/ml}$ phorbol-12-myristate-13-acetate (PMA, Sigma Chemical Co, Dorset, UK) to T225 flasks 7 days after seeding. Cells became anchorage dependent within 24 hours and took on macrophage morphology within 48 hours. At this point either RNA was extracted from the cells or 50 $\mu\text{g/ml}$

Table 1. Primer Pairs Used in PCR Analysis

Gene	Forward primer	Reverse primer	Product size (bp)	Genbank accession numbers
PARC	CTTGCACTGCCCTCCTTGTC	CACTTCTTATTGGGGTCAGC	220	AB 000221
ELC	ATGGCCCTGCTACTGGCC	CAATGCTTGACTCGGACT	339	AB 000887
LARC	AATCAGAAGCAGCAAGCAACT	TTTTACTGAGGAGACGCACAA	206	D 86955
SLC	ATGGCTCAGTCACTGGCT	GGCCCTTTAGGGGTCTGT	400	AB 002409
β -actin	ATCCCTGTACGCCTCTGG	TCCTTCTGCATCCTGTCTG	550	X 00351

oxidized human LDL (ox-LDL) was added in 100 ml of fresh medium for an additional 72 hours. Human LDL was purchased from Calbiochem-Novabiochem Ltd. (Nottingham U.K.) and oxidized with 5 μ mol/L CuSO_4 for 24 hours at 37°C in the absence of EDTA. Uptake of the ox-LDL resulted in accumulation of fat droplets within the cells. This was confirmed by positive staining with oil red-O.

Human Aortic Smooth Muscle Cells

Cryopreserved human aortic SMC were obtained from BioWhittaker (Berkshire, UK). SMC were cultured according to manufacturer's instructions in smooth muscle cell basal medium (SmBM) supplemented with smooth muscle cell growth medium SingleQuot BulletKit-2. The resulting smooth muscle cell growth medium (SmGM-2) contained 0.5 ng/ml human epidermal growth factor, 5 μ g/ml insulin, 2 ng/ml human fibroblast growth factor, 50 μ g/ml gentamicin, 50 pm/ml amphotericin-B, and 5% FBS. Cultures were maintained at 37°C in a humidified 95% air, 5% CO_2 atmosphere in T75 flasks (Costar UK Ltd., Buckinghamshire, UK). SmGM-2 was replaced with fresh medium every 2 to 3 days until confluence was reached, and SMC were subsequently subcultured after treatment with 0.025% trypsin and 0.01% EDTA. SMCs used in these experiments were between passage 4 and 8. Cells stained positive for SMC α -actin.

For cytokine stimulation experiments, SMCs were cultured in T75 flasks for 4 days in SmGM-2 until semiconfluent. SmGM-2 supplements were then reduced to 50% for 24 hours and subsequently made fully quiescent in SmBM containing only 0.5% FBS for an additional 18 hours. SMC were then treated with 10 ng/ml TNF- α and 10 ng/ml INF- γ in SmBM, 0.5% FBS for 8 and 24 hours. For the serum stimulation experiments, the cells were made quiescent as described above and then stimulated with 10% FBS for 1, 2, 4, 8, 24, and 48 hours.

Oligonucleotide Primers

Oligonucleotide primers were designed using DNASTAR software and obtained from Cruachem Ltd. (Glasgow, UK). Primer pairs for the chemokines and β -actin were designed in the coding region of the cDNA (Table 1).

RT-PCR

RNA was isolated from human carotid endarterectomy specimens and cell lines using either Trizol reagent (Gibco BRL) or RNAzol B (Biotech, Houston, TX) as per

manufacturer's instructions. cDNA was reverse transcribed from 1 μ g of DNase (Gibco BRL) treated total RNA using Superscript II (Gibco BRL) as per manufacturer's instructions. For each sample, a parallel RNA was run with no Superscript II. Polymerase chain reaction (PCR) was carried out in 50 μ l of reaction volumes containing 5 μ l of 5 $\times$ diluted cDNA, 1.5 mmol/L MgCl_2 , 0.5 μ mol/L of each primer, 0.2 mmol/L of each NTP, and 5 units of *Taq* polymerase (Gibco BRL). The amplification profile consisted of 30 cycles (35 cycles for LARC) of 94°C for 1 minute, annealing temperatures of 50 to 55°C for 1 minute, and extension for 30 seconds at 72°C with one final cycle of extension at 72°C for 5 minutes. Each PCR reaction was run with the following negative controls: human genomic DNA, water instead of RNA, and a parallel no reverse transcriptase sample for each cDNA used. The positive control for size of products was pBlue-script SK containing the cDNA of interest.

Probes

The chemokine cDNAs used in this study were initially identified at Human Genome Sciences (HGS) (Rockville, MD) by random sequencing of expressed tags in cDNA libraries from human pulmonary artery (PARC), human fetal spleen (ELC), human fetal lung (SLC), and monocytes (LARC). Full length clones were identified and sequenced at HGS and found to be identical to PARC, ELC, SLC, and LARC (clone numbers 35312, 49487, 50795, and 220914, respectively). The cDNA probes used for preparing riboprobes were the full length clones inserted between the *EcoRI* (5') and *XhoI* (3') sites of pBlue-script SK- (Stratagene, Cambridge, UK). Sense and antisense RNA probes were labeled with ^{35}S UTP (Amersham Pharmacia Biotech Buckinghamshire, UK) using T3 or T7 polymerase (Promega, Southampton, UK), respectively. Prior to ethanol precipitation of the probe, 1 μ l of the reaction was run on a 4% polyacrylamide/urea gel (Gibco BRL) to check for full length transcripts. Transcript sizes were approximately 750, 650, 850, and 800 bp for PARC, ELC, SLC, and LARC, respectively.

In Situ Hybridization

Transverse tissue sections (10 μ m) were thaw mounted onto Superfrost+ microscope slides (BDH, Leicester-shire, UK). Sections were fixed in fresh 4% paraformaldehyde in PBS, pH 7.4, acetylated in 0.25% acetic anhydride/0.1 mmol/L triethanolamine/0.1 mol/L NaCl and

dehydrated and delipidated through a graded series of alcohols and chloroform. Sections were air dried and stored at -70°C until use.

RNA sense and antisense probes were resuspended at 25,000 cpm/ μl in hybridization buffer (50% formamide, 0.02% w/v BSA, 0.02% polyvinylpyrrolidone, 0.02% ficoll, 100 $\mu\text{g}/\text{ml}$ polyadenylate, 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, 100 $\mu\text{g}/\text{ml}$ yeast tRNA, $4\times$ SSC, 10% dextran sulphate, 10 mmol/L dithiothreitol), and slides were incubated overnight in a sealed humid chamber at 55°C . After hybridization, the sections were washed with $1\times$ SSC at room temperature for 30 minutes, treated with 20 $\mu\text{g}/\text{ml}$ RNase A in buffer and then with buffer alone (500 mmol/L NaCl, 10 mmol/L Tris, pH 8.0, 1 mmol/L EDTA, 30 minutes each at 37°C), washed with $1\times$ SSC (room temperature, 30 minutes), followed by a high stringency wash ($0.5\times$ SSC at 65°C for 30 minutes) and $0.5\times$ SSC at room temperature for 2×10 minutes. Slides were dehydrated through a series of alcohols, air dried, and dipped in photographic emulsion (Amersham Pharmacia Biotech). Slides were exposed for 3 to 9 weeks at 4°C and developed using Kodak D19 (1:1 water), counterstained in toluidine blue, dehydrated through a graded series of alcohols, and coverslipped for microscope analysis.

Immunohistochemistry

Serial transverse tissue sections (10 μm) were thaw mounted onto Superfrost+ microscope slides, refrozen, and stored at -70°C until use. For immunohistochemistry (IHC), sections were air-dried for 1 hour and fixed in acetone for 10 minutes. IHC was performed using the avidin-biotin-peroxidase complex method. All incubations were done at room temperature unless otherwise stated. Briefly, endogenous peroxidase activity was blocked by incubating slides in 0.6% H_2O_2 in methanol. Nonspecific binding was blocked by incubating sections in normal rabbit serum before applying the primary antibody. Optimal primary antibody concentrations were predetermined by titration and slides were incubated for 60 minutes in the appropriate primary antibody dilutions. Slides were then incubated in biotinylated rabbit anti-mouse secondary antibody (DAKO Ltd., Buckinghamshire, UK) for 30 minutes, washed, and incubated with avidin/biotinylated horseradish peroxidase complex for 30 minutes. Slides were then stained with diaminobenzidine tetrahydrochloride substrate. The sections were counterstained with haematoxylin, dehydrated through a graded series of alcohols, and mounted in DPX. Negative controls were omission of primary antibody and substitution of primary antibody with appropriate isotype. The cellular composition of the plaques was determined using a panel of characterized antibodies: HHF35 (specific for smooth muscle α -actin), CD68, a specific macrophage marker, Von Willebrand factor for endothelial cells, CD1a, a dendritic cell marker (all from DAKO) and CD3 leu, a marker for T cells (Becton Dickinson, Oxford, UK).

Figure 1. Detection of PARC and ELC mRNA in human carotid endarterectomy specimens by RT-PCR. This figure shows the results of PCR analysis after 30 cycles (35 cycles for LARC). Each panel contains molecular weight markers (M), water negative control, six carotid endarterectomy cDNAs (1 to 6) next to their corresponding no RT control, human genomic DNA (G), and pBluescript containing the relevant chemokine cDNA. Molecular weight markers (Sigma) are 2000, 1500, 1000, 750, 500, 300, 150, and 50 bp. The right lane of each panel displays the size of the specific PCR product.

Results

Detection of PARC and ELC in Carotid Endarterectomy Specimens

In order to study PARC, ELC, SLC, and LARC gene expression in human atherosclerotic plaques, we initially screened a number of cDNAs derived from carotid endarterectomy specimens using gene specific primers (Table 1). A clear product for PARC was obtained from all six samples and for ELC in four of six samples. However, products for LARC and SLC were less clear (Figure 1). β -actin was used to assess the quality of the carotid endarterectomy RNA. The plasmid containing the cDNA insert for the gene of interest was used in all four cases as a positive control for PCR product size.

Cellular Localization of PARC and ELC mRNA in Atherosclerotic Plaques

In order to further characterize the gene expression of PARC, ELC, SLC, and LARC in normal and diseased human arteries, we carried out *in situ* hybridization using ^{35}S -labeled riboprobes. After 3 weeks development time in photographic emulsion, strong signals were observed for PARC and ELC in a large number of the carotid endarterectomy sections studied ($n = 14$ of 18 and 12 of 14, respectively). Replicate slides were developed at intervals and, in agreement with reverse transcriptase (RT)-PCR data, no signal was observed for SLC or LARC in the plaque sections even after 9 weeks in emulsion. To determine the cellular composition of lesions and determine the cell types expressing PARC and ELC mRNA, we carried out immunohistochemical staining of serial sections with markers for SMC (α -actin), macrophages (CD68), endothelial cells (Von Willebrand Factor), T cells

Figure 2. Serial sections of a carotid endarterectomy specimen showing PARC mRNA expression in a macrophage-rich area of the specimen. **A:** Dark-field photomicrograph showing *in situ* hybridization for PARC mRNA using the specific antisense probe. **B:** Nonhybridizing PARC sense strand. **C:** Immunohistochemical staining for macrophages using CD68; **D:** bright-field view of **A** showing tissue morphology. **E:** High power photomicrograph showing CD68⁺ macrophage staining in an area of **C** indicated by the square. **F:** A similar region of the specimen indicated by the square in **D** showing a bright field high power view of cells expressing PARC mRNA. Bar, 200 μ m (**A**, **B**, **C**, and **D**); 50 μ m (**E** and **F**).

(CD3), and dendritic cells (CD1a) (all results are not shown). Figure 2 shows PARC mRNA expression in a representative carotid endarterectomy sample. In all positive cases (14 of 18), PARC mRNA expression was detected in macrophage-rich areas of the lesion as determined by positive CD68 staining. Expression was detected around necrotic regions and, where present, in areas of calcification. However, PARC was not expressed in all CD68 positive areas. All specimens were probed using the PARC sense strand and in all cases the sections were devoid of silver grain clusters. mRNA expression for ELC in lesions was found to be more widespread than PARC mRNA and was associated with both intimal SMC and macrophages. Figure 3 shows ELC mRNA expression by macrophages and some diffuse mRNA expression can also be observed in the underlying SMC. In lesions displaying severe calcification ($n = 4$), ELC mRNA was also found to be expressed strongly by medial SMC in addition to lesion associated cells (Figure 4). No expression was observed in the panel of sections

probed with the ELC sense strand. In control mammary arteries, no positive signal was detected for PARC nor SLC or LARC (results not shown). However, low levels of expression of ELC were detected in the medial SMC of mammary arteries. Some signal over background is also visible in the adventitia of these vessels (Figure 5).

mRNA Expression for PARC and ELC in Vitro

In order to gain some information on the regulation of expression of PARC and ELC in SMC and monocyte/macrophages, we carried out RT-PCR on cDNAs derived from a range of stimulated and nonstimulated cells. No PCR products were found for PARC in quiescent SMC or SMC stimulated with serum for 1, 2, 4, 8, 16, 24, or 48 hours. It was also not expressed by naive THP-1 cells or THP-1 cells treated with PMA or incubated with oxidized LDL (results not shown). SMC stimulated with TNF- α and INF- γ also failed to express PARC. We did, however, find

Figure 3. Serial sections of a carotid endarterectomy specimen showing ELC mRNA expression by macrophages. This figure also shows some diffuse mRNA expression in the underlying SMC. **A:** Dark-field photomicrograph showing *in situ* hybridization for ELC using the specific antisense strand. **B:** Nonhybridizing ELC sense strand. **C:** Immunohistochemical staining for macrophages using CD68. **D:** Immunohistochemical staining for SMC using α -actin. **E:** High power photomicrograph of CD68⁺ macrophages in area of the specimen indicated by the square in **C**. **F:** High power bright field view of the area indicated in **A** showing cells expressing ELC mRNA in the lesion. Bar, 200 μ m (**A**, **B**, **C**, and **D**); 50 μ m (**E** and **F**).

PARC to be clearly expressed by human monocyte-derived CD1a⁺ dendritic cells but not by human peripheral blood derived dendritic cells. Human blood-derived monocytes also failed to show a specific PCR product, but clear expression was detected in monocytes that had been activated with LPS (Figure 6).

Only weak/absent PCR products for ELC were found in quiescent SMC or SMC stimulated with serum for 1, 2, 4, 8, 16, 24, and 48 hours. In addition, no mRNA was detected in unstimulated THP-1s, THP-1s that had been activated with PMA, or THP-1s incubated with oxidized LDL (results not shown). However, ELC expression was clearly detected in SMC that had been treated with a combination of TNF- α and IFN- γ for 24 hours. Clear PCR products could also be detected in human blood derived monocytes that had been activated with LPS but not in unstimulated monocytes (Figure 6). No PCR products for ELC were detected in CD4⁺ dendritic cells. All cell preparations and PCR reactions were done in duplicate. Clear

β -actin product can be seen for all the cells used in this experiment.

Discussion

In this study, we have examined the expression of four recently described T cell chemoattractants, PARC, ELC, SLC, and LARC,^{16–22} in human atherosclerosis. We found high expression of both PARC and ELC in lesions but no SLC or LARC gene expression. PARC mRNA was strongly associated with CD68⁺ macrophages, whereas ELC was more widely expressed, co-localizing with both SMC and macrophages in the lesion areas as well as with medial SMC in highly calcified plaques. PARC mRNA was not found to be associated with SMC in normal or diseased arteries, however, very low levels of ELC mRNA expression were detected in mammary artery sections

Figure 4. Serial sections of a calcified carotid plaque showing ELC mRNA expression by medial and intimal SMC. **A:** Dark-field photomicrograph showing *in situ* hybridization for ELC using the specific antisense probe. **B:** Nonhybridizing ELC sense strand. **C:** Immunohistochemical staining for SMC α -actin. **D:** Bright-field view of **A** showing tissue morphology. **E:** High power bright field photomicrograph of an area of the specimen indicated by the square in **D**, showing cells expressing ELC mRNA in an α -actin-stained area of the lesion. Area of calcification can be seen in bottom right section of each photomicrograph (asterisk). Bar, 200 μ m (**A**, **B**, **C**, and **D**); 50 μ m (**E**).

probed with the antisense strand compared with the levels obtained with the nonhybridizing sense strand.

The presence of chemokines in atherosclerosis and related conditions is well documented, suggesting an important role for these molecules in the pathogenesis of the disease. MCP-1, MCP-4, and RANTES have all been found to be expressed by plaque macrophages.^{11,13,27} In addition, other cell types have been found to express these chemokines: MCP-1 by SMC and MCP-4 by endothelial cells of the vasa vasorum.^{11,13} Chemokine expression is also associated with abdominal aortic aneurysms and transplant-associated accelerated atherosclerosis.^{28,29} In this study, we have found ELC mRNA to be expressed in atherosclerotic lesions by both intimal SMC and macrophages. Both cell types in plaques are known to be responsible for local production of a wide range of growth factors and cytokines.⁵ Furthermore, our data also indicate strong expression of ELC mRNA in the medial SMC of lesions that are highly calcified. The ability of SMC to express ELC in carotid endarterectomy speci-

mens suggests a pro-inflammatory role for these cells in human atherosclerosis. Indeed, we found that ELC mRNA could be up-regulated in human aortic SMC by $\text{INF-}\gamma$ and $\text{TNF-}\alpha$ but not by serum alone. Several other *in vitro* studies have shown that pro-inflammatory cytokine treatment of SMC results in increased levels of expression/secretion of CC chemokines, eg, human vascular SMC treated with $\text{IL-1-}\alpha$ or $\text{TNF-}\alpha$ secrete increased amounts of IL-8, MCP-1, and RANTES,³⁰ whereas human airway SMC can express RANTES mRNA and protein in response to treatment with $\text{TNF-}\alpha$ alone and to a greater extent with $\text{TNF-}\alpha$ in combination with $\text{INF-}\gamma$.³¹ These inflammatory cytokines are known to be expressed in atherosclerotic plaques, $\text{INF-}\gamma$ by T cells,³ and $\text{TNF-}\alpha$ by macrophages.²⁷ As indicated above, we found that ELC mRNA was also expressed by macrophages in atherosclerotic lesions. ELC was independently identified as MIP-3 β and found to be expressed in an activated monocyte cDNA library.¹⁹ We found expression of ELC mRNA by monocytes activated by LPS but not in naive mono-

Figure 5. Mammary artery serial sections hybridized with the antisense and sense ELC probe. **A:** High power photomicrograph showing *in situ* hybridization using the antisense strand. **B:** Nonhybridizing sense strand. The adventitia is shown underneath the medial SMC in this figure. Bar, 50 μ m.

cytes, further suggesting a role for ELC in the pro-inflammatory processes occurring during atherogenesis.

Our studies revealed PARC mRNA to show a more restricted pattern of expression in atherosclerotic plaques, localizing with CD68⁺ macrophages. Of all the stimulated/unstimulated cells we examined *in vitro*, PARC mRNA expression was confined to monocyte-derived dendritic cells and LPS-activated monocytes. Previously, Adema et al.¹⁷ found DC-CK1/PARC mRNA to be expressed in monocyte-derived dendritic cells *in vitro* but in

contrast to our findings, not to be expressed by LPS activated monocytes. Our *in situ* hybridization showed mRNA expression for PARC in CD68⁺ macrophages in line with our *in vitro* findings. We did find sparse CD1a⁺ dendritic cells in carotid endarterectomy specimens (results not shown) but these cells were not associated with PARC mRNA expression. The presence of CD1a⁺ dendritic cells in atherosclerotic plaques has been reported in the literature.^{32,33} However, it has not yet been clarified as to whether these cells play a role in antigen presentation during atherosclerosis. It is thought that destruction of these cells may be involved in the calcification process through release of the calcium binding protein S-100.³³ In agreement with our data, Hieshima et al.¹⁶ found that PARC mRNA could be induced in monocytes differentiated with LPS and in U937 cells stimulated with PMA. Also, *in situ* studies in lung sections showed high PARC expression in a subset of alveolar macrophages that stained positive for EBM11/CD68.¹⁶ The expression pattern for PARC mRNA by macrophages in human atherosclerosis strongly suggests a pro-inflammatory role for this chemokine in the disease.

The target cell type for both PARC and ELC is the lymphocyte.^{16–18} The receptor for ELC is EB1 (Epstein-Barr virus (EBV)-induced gene 1),^{34,35} which is known to be expressed on T and B cells.³⁵ Although the receptor for PARC has not yet been identified, saturation studies have shown that human blood lymphocytes express a single class of high affinity receptors for PARC.¹⁶ T cells are present throughout atherosclerotic lesion development,² and many are in an activated state.³ The presence

Figure 6. Detection of PARC and ELC mRNA in human cells *in vitro*. Each panel contains molecular weight markers (M) and appropriate control plasmid DNA for PARC and ELC (C). Lane 1, CD1a⁺ monocyte derived dendritic cells; lane 2, peripheral blood derived CD4⁺ dendritic cells; lane 3, peripheral blood derived monocytes; lane 4, LPS-stimulated monocytes; lane 5, quiescent human SMC; lane 6, SMC after 7 hours stimulation with TNF- α and INF- γ , and lane 7, SMC after 24 hours stimulation with TNF- α and INF- γ . Molecular weight markers (Sigma) are 2000, 1500, 1000, 750, 500, 300, 150, and 50 bp.

of these two T cell-specific chemokines, PARC and ELC, in plaques strongly suggests an active recruitment of this cell type into lesions rather than a nonspecific "trapping" of T cells in atherosclerotic plaques. Indeed, unlike other chemokines known to attract T cells, such as RANTES, MIP-1 α , and IL-8, PARC shows a specificity for attracting naive resting T cells.¹⁷ Atherosclerotic plaques contain all of the molecular components required for antigen presentation to CD4⁺ T cells. Expression of MHC (major histocompatibility complex) molecules have not only been found on macrophages in human lesions but also on SMC and endothelial cells.³⁶ There are still conflicting reports in the literature on the role of T cells in atherosclerosis. A study has shown that total lymphocyte deficiency in the ApoE^{-/-} mouse, achieved by deletion of the recombinase activator gene 2, has no effect on the extent of aortic atherosclerosis in these mice.³⁷ However, this study does not rule out the involvement of T cells at different time points or stages of the disease. It is also important to note that lesions of atherosclerotic mice do not rupture. Therefore, a study of this type would not address the modulatory effects that T cells exert on other plaque cell types, which may have an important effect on overall plaque stability. For example, it is known that T cell-secreted pro-inflammatory cytokines are capable of inducing metalloproteinase expression by macrophages.³⁶ The CD40 receptor and CD40L (ligand) are important co-stimulatory factors in antigen presentation and autoimmunity in addition to T cell and macrophage activation. Both receptor and ligand have also been detected on all cell types in atherosclerotic plaques.³⁸ A recent study has shown that treatment of the LDL receptor knockout mouse with an antibody against CD40L results in a reduction in the size of aortic lesions and their lipid content and also reduces the number of macrophages and T cells in plaques.³⁹ These data provide strong evidence for a specific immune response occurring during atherosclerosis. A pivotal role for the chemokines PARC and ELC can easily be envisaged during the sequence of events occurring prior to and after antigen presentation. PARC may be used by plaque macrophages to attract naive T cells, which after recognition of MHC molecule/peptide presented by activated macrophages and/or SMC results in induction of a primary immune response. ELC may participate in a similar fashion, although recent reports have shown further functions for this chemokine. In addition to its chemotactic properties, ELC has been shown to be capable of inducing adhesion of circulating lymphocytes to intercellular adhesion molecule-1 and induce the arrest of rolling cells under physiological shear,⁴⁰ suggesting a role for ELC in T cell-endothelial cell recognition.

In conclusion, the data presented in this report suggest a role for PARC and ELC in the recruitment of T cells into atherosclerotic lesions. Through paracrine interactions, T cells could perpetuate their infiltration into the plaque through local secretion of inflammatory cytokines capable of up-regulating the production of the lymphocyte specific chemokines, PARC and ELC, in neighboring cells.

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