Overexpression of Eotaxin and the CCR3 Receptor in Human Atherosclerosis

Using Genomic Technology to Identify a Potential Novel Pathway of Vascular Inflammation

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Background—Unstable atherosclerotic lesions typically have an abundant inflammatory cell infiltrate, including activated T cells, macrophages, and mast cells, which may decrease plaque stability. The pathophysiology of inflammatory cell recruitment and activation in the human atheroma is incompletely described.

Methods and Results—We hypothesized that differential gene expression with DNA microarray technology would identify new genes that may participate in vascular inflammation. RNA isolated from cultured human aortic smooth muscle cells treated with tumor necrosis factor-α (TNF-α) was examined with a DNA microarray with 8600 genes. This experiment and subsequent Northern analyses demonstrated marked increases in steady-state eotaxin mRNA (>20 fold), a chemokine initially described as a chemotactic factor for eosinophils. Because eosinophils are rarely present in human atherosclerosis, we then studied tissue samples from 7 normal and 14 atherosclerotic arteries. Immunohistochemical analysis demonstrated overexpression of eotaxin protein and its receptor, CCR3, in the human atheroma, with negligible expression in normal vessels. Eotaxin was predominantly located in smooth muscle cells. The CCR3 receptor was localized primarily to macrophage-rich regions as defined by immunopositivity for CD 68; a minority of mast cells also demonstrated immunopositivity for the CCR3 receptor.

Conclusions—Eotaxin and its receptor, CCR3, are overexpressed in human atherosclerosis, suggesting that eotaxin participates in vascular inflammation. These data demonstrate how genomic differential expression technology can identify novel genes that may participate in the stability of atherosclerotic lesions.

Key Words: atherosclerosis ■ genes ■ inflammation

Unstable atheromatous lesions have an abundant inflammatory infiltrate, including macrophages, T cells, and mast cells.1–4 Mediators produced by leukocytes may be critical determinants of plaque stability.4 Activated leukocytes secrete factors that may act in a paracrine fashion to promote matrix degradation by other cells.5 Furthermore, leukocytes may contribute directly to plaque degradation by secreting enzymes such as matrix metalloproteinases.6

Human atherosclerotic lesions have increased expression of the cytokine tumor necrosis factor-α (TNF-α).7 Potential functions of TNF-α include promoting macrophage and T-cell maturation and/or activation and inducing the production of other proinflammatory mediators.8 We hypothesized that treatment of human vascular smooth muscle cells with TNF-α would reveal novel genes or pathways that may participate in vascular inflammation. Using DNA microarray technology, we found a dramatic induction of eotaxin mRNA in cells treated with TNF-α. Because eotaxin has been identified as a chemotactic factor for eosinophils, and eosinophils are rarely observed in human atherosclerotic lesions, we explored this finding in human tissues. We report that some smooth muscle cells in human atheroma prominently express the eotaxin protein and that lesional macrophages and mast cells may express the CCR3 receptor, suggesting that eotaxin and its receptor contribute to inflammatory cell recruitment and activation in atheroma. These data demonstrate the potential of genomic technology, specifically DNA microarrays, to identify candidate genes that are expressed in human atherosclerotic lesions, providing novel investigational targets.

Methods

Tissue Acquisition and Cell Preparation

The protocol for this investigation was approved by the Brigham and Women’s Hospital Human Research Committee. Tissue samples of...
1 normal carotid artery and 6 normal aortic arch samples were harvested from heart transplantation donors at the time of surgery. Human atherosclerosis samples were obtained from excised tissue from carotid endarterectomy procedures. Samples were frozen in liquid nitrogen and stored at −70°C. Cells were prepared from explants from excess aortic tissue from the donor at the time of organ harvest for orthotopic heart transplantation. Vascular smooth muscle cells were maintained in Dulbecco’s modified essential medium, 10% FCS, and 1% penicillin/streptomycin sulfate. Vascular smooth muscle cells were maintained at 37°C, 5% CO2, up to passage 6 for experiments.

DNA Microarray Experiment
To identify differentially expressed genes potentially involved in unstable atherosclerosis, we used DNA microarray technology. Aortic vascular smooth muscle cells at passage 5 were incubated with serum-free medium for 48 hours. Cells were then exposed to TNF-α (10 ng/mL) or serum-free medium without TNF-α for 24 hours, and mRNA was prepared. The DNA microarray hybridization experiment was performed with the public domain UniGem 1.0 array (Incyte Inc), by methods previously described. The UniGem 1.0 array contains 8600 genes. Data were analyzed with the GemTools software package (Incyte Inc). The sensitivity of the assay was determined with the use of 2 independent assays. First, 200 ng of human RNA was labeled with either Cy3 or Cy5dCTP, mixed, and hybridized to an array. Fluorescent ratios were calculated for all called elements. These data demonstrated that when the same RNA is used for both fluorescent channels, 99% of elements of the UniGem 1.0 microarray give differential expression values within 2-fold. In a second series of experiments, RNA isolated from 2 unrelated cell lines was used. Comparison of these 2 RNAs over 3 separate hybridizations yielded an average correlation coefficient of r=0.97.

Northern Analysis
The cDNA for eotaxin was obtained through the IMAGE consortium, and the sequence of the clone was confirmed. To verify that eotaxin was induced by TNF-α (10 ng/mL), experimental conditions used for the microarray experiments were reproduced in cells from 2 separate patient sources. Total RNA was isolated by the guanidinium thiocyanate and acid phenol method. For Northern blotting, 15 μg of total RNA was loaded on a 1.0% agarose-formaldehyde gel (2.0 mol/L), transferred to a nylon membrane (Amersham Life Science), and UV cross-linked with a UV Stratalinker (Stratagene). The membrane was washed first with 2×SSC, 0.05% SDS solution for 30 to 40 minutes 3 times at room temperature and then with 0.1×SSC, 0.1% SDS solution with continuous shaking at 50°C for 40 minutes. The membrane was exposed to x-ray film at −80°C. Radiographs were scanned and analyzed with Optimas 6.0 software (Optimas Co).

ELISA Analysis
Smooth muscle cells were cultured in 6-well plates for 24 hours in serum, and then media was switched to serum-free media for 48 hours. In some samples, TNF-α (10 ng/mL) was added. Media was harvested and analyzed with commercially available ELISA assay for human eotaxin (R&D Systems).

Immunohistochemical Analysis
Immunostaining was performed by a modified avidin-biotin complex (ABC) technique. The IgG1 murine monoclonal antibodies included anti-eotaxin (Clone LS59 ZG6) (LeukoSite, Inc) used at 1:200, the muscle marker anti-α actin (clone HHF35) used at 1:50 (Enzo Diagnostics), anti-CCR3 (clone LS63 7B11) used at 1:200 (LeukoSite, Inc), the mast cell marker anti-trypase (clone AA1) (Dako Corp) used at 1:10, the macrophage marker anti-CD 68 (clones EBM11 and KP1) (Dako Corp) used at 1:100, and the endothelial cell marker anti-CD 31 (clone JDC70A) (Dako Corp) used at 1:30. Negative controls included substituting the primary antibody with the irrelevant murine IgG1, MOPC-21 (Sigma Chemical Co). For eotaxin, absorbing the primary antibody against 5 μg of recombinant human eotaxin (PeproTech) overnight at 4°C, followed by a second absorption with an additional 5 μg eotaxin peptide, was used as an additional negative control.

Frozen lung tissue sections were cut 5 μm thick on a cryostat, then brought to room temperature at the time of staining. All of the sections except those to be stained for eotaxin were fixed in acetone; the sections for eotaxin staining were fixed in 4% paraformaldehyde. Nonspecific immunoglobulin binding was blocked with 10% normal horse serum (GIBCO BRL). The primary antibodies, diluted as above in PBS with 2% BSA (the anti-eotaxin antibodies were diluted in 5% milk diluted in PBS), were applied to tissue sections overnight at 4°C in a humidified chamber. The slides were then incubated with biotinylated horse anti-mouse IgG (Vector Laboratories), diluted 1:200 in 5% powdered milk in PBS with 5 μL/mL normal human serum at 4°C for 2 hours. Endogenous peroxidase activity was quenched with methanol containing 0.3% hydrogen peroxide. ABC standard (Vector Laboratories) was made according to the manufacturer’s instructions, applied to sections, and incubated at room temperature for 1 hour. For eotaxin and CCR3 slides, additional amplification with tyramide (TSA ) (New England Nuclear Life Sciences) was performed. Biotinylated tyramide, diluted 1:50 in amplification buffer, was incubated for 6 minutes, 30 seconds at room temperature. This was followed by streptavidin horseradish peroxidase diluted 1:100 in PBS and incubated for 30 minutes at room temperature. Immunopositivity was visualized with the chromagen diaminobenzidine (0.025%) (Sigma Chemical Co). in PBS and 0.1% hydrogen peroxide. Immunostaining with the use of the negative controls proceeded as described for the primary antibodies. All sections were counterstained with 4% methyl green (Sigma Chemical Co).

The identity of cells expressing CCR3 was also determined by means of double staining. In brief, after blocking with 10% horse serum, anti-CCR3, diluted 1:50, was applied to sections overnight at 4°C. A donkey anti-murine IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc), diluted 1:200 in PBS, was then applied to sections and incubated at 4°C for 2 hours. The alkaline phosphatase was then visualized with the use of Vector red (Vector Laboratories), following the manufacturer’s instructions. The horse serum block was repeated, and biotinylated anti-CD 68 (clone EBM11) (Dako Corp), diluted 1:25, was applied and incubated for 45 minutes at room temperature. Endogenous peroxidases were blocked, and streptavidin horseradish peroxidase (Jackson ImmunoResearch), diluted 1:500, was applied and incubated at room temperature for 30 minutes. The CD 68 was then visualized with the use of the chromagen Vector SG (Vector Laboratories), following the manufacturer’s instructions.

Morphometry
Images at ×40 power were digitized with a CCD camera (Optronics) interfaced with image analysis software (Scionics). Adjacent but nonoverlapping images were obtained over the entire tissue. Images were loaded into a 1K by 1K buffer of the Optimas 6.0 image analysis system. A color threshold was established for immunopositivity, and the percentage of tissue expressing eotaxin or CCR3 was computed.

Statistical Analysis
Quantitative immunohistochemistry data were not distributed normally; the Kruskal-Wallis test was used. Differences were accepted as significant at a level of P<0.05. Results are reported as median with interquartile ranges.

Results
Northern Analysis
Among 8600 genes studied in the microarray experiment, the eotaxin gene was one of 3 genes demonstrating an increase in steady-state expression of >10-fold after TNF-α treatment; the other 2 genes were monocyte chemotactic protein-1 and superoxide dismutase. To confirm the DNA microarray finding that TNF-α induces eotaxin expression by human aortic smooth muscle cells, Northern analyses were performed. As shown in Figure 1, TNF-α markedly induced...
eotaxin expression (>20-fold). Similarly, TNF-α markedly induced expression of the other 2 genes—monocyte chemoattractant protein-1 and superoxide dismutase—identified by the microarray experiments (>20-fold for each, data not shown). An ELISA assay was used to quantitatively evaluate eotaxin protein expression. This analysis demonstrated marked induction of eotaxin protein expression by aortic smooth muscle cells after treatment with TNF-α, whereas untreated control cells did not show eotaxin protein expression (P<0.001) (Figure 1).

**Overexpression of Eotaxin**

Neither normal tissues nor atherosclerotic tissues had eosinophils by eosin staining. All atherosclerotic lesions demonstrated increased expression of the eotaxin protein; the median percentage of area that stained positive for eotaxin was 1.6% (interquartile range, 1.1% to 2.3%) (Figure 2). In contrast, the normal samples expressed negligible eotaxin (median 0.7%, interquartile range 0.2% to 0.7%, P<0.05 versus atherosclerotic specimens). No staining was observed after substitution of the primary antibody with an irrelevant IgG1 (MOPC-21, Figure 2C). In addition, eotaxin staining was blocked by preabsorbing the primary antibody against recombinant eotaxin (data not shown).

The identity of the cell types expressing eotaxin was evaluated by using serial sections immunostained with markers for smooth muscle and endothelial cells. In atheromatous samples, eotaxin was predominantly expressed in regions of smooth muscle cells as identified by the smooth muscle marker α-actin (Figure 3, A and B). Not all cells positive for α-actin stained for eotaxin; we noted a tendency for the smooth muscle cells that were positive for eotaxin to be further from the lumen.

**Overexpression of the Eotaxin Receptor CCR3**

CCR3 expression was significantly increased in the atheromatous vessel compared with the normal vessel (P<0.05) (Figure 4, A and B). The normal vessels demonstrated negligible CCR3 expression, consistently <1% of the wall area (median 0.2%, interquartile range 0.1% to 0.3%). CCR3 expression in atheromatous vessels comprised a median of 1.6% of the tissue area (interquartile range 1.1% to 2.5%, P<0.001 versus normal). No staining was observed in atheroma after substitution of the primary antibody with an irrelevant IgG1 (MOPC-21, Figure 4C). The identity of the cell types expressing CCR3 was evaluated by the use of serial sections immunostained with markers for macrophages and mast cells. In the atheromatous vessels, CCR3 expression predominantly localized with macrophages identified by...
staining with CD68 (Figure 5, A through F). Less than 10% of the cells expressing CCR3 also expressed the mast cell marker tryptase (Figure 6, A and B).

Discussion
The pathogenesis of atherosclerotic inflammation is incompletely defined. In this study, we used DNA microarray technology to identify genes with increased steady-state mRNA in smooth muscle cells exposed to TNF-α, a cytokine found in human atheroma. Exposure to TNF-α resulted in a >20-fold induction in 3 genes: eotaxin, superoxide dismutase, and monocyte chemotactic protein-1. These findings led to the demonstration of overexpression of eotaxin and its receptor, CCR3, in the inflammatory infiltrate of human atheroma. Our findings illustrate how genomic technology may provide novel insight into pathological processes. As is typical of the cellular components of the atheroma, the inflammatory infiltrates of our lesions did not contain significant numbers of eosinophils. Therefore, our data suggest that in atheroma, eotaxin has functions other than eosinophil recruitment and/or activation. Because the CCR3 receptor was predominantly expressed by macrophages, it is possible that eotaxin modulates macrophage function. Additionally, because the CCR3 receptor was also identified on mast cells, eotaxin may participate in mast cell activation and/or recruitment.

The recruitment of inflammatory cells occurs early in atheroma formation. One of the earliest histologically identified events associated with hyperlipidemia is the adherence of monocytes to endothelial cells with subsequent infiltration into the vessel wall. The processes of adhesion, attachment, and migration are initiated and regulated by chemotactic signals. Chemokines are small proteins that can both attract and activate leukocytes from the vessel lumen and may participate in cell recruitment into the atheromatous plaque. Interruption of normal chemotaxis pathways is associated with decreased atheroma size in mice. Mice lacking the gene for monocyte chemoattractant protein-1 or CCR2 are less prone to develop atherosclerosis.

Eotaxin is a C-C chemokine produced by several cell types and is a potent eosinophil chemoattractant and activator. However, recent studies suggest that eotaxin may be involved in regulation of cells other than eosinophils. Although eosinophils constitutively express CCR3, this receptor can be induced on several cell types including TH2 lymphocytes and ba-
sophils. In addition, CCR3 has been observed on macrophages, mast cells, neutrophils, and endothelial cells in endobronchial biopsies of the atopic asthmatic lung. Our data suggest a possible role for eotaxin in noneosinophilic inflammatory processes. The possibility that eotaxin contributes to noneosinophilic immune responses is also supported by descriptions of eotaxin null mice. These animals develop normally and are able to recruit eosinophils into the lung after ovalbumin sensitization.

This is the first report to our knowledge demonstrating the expression of eotaxin by human atheroma. However, eotaxin mRNA and protein have been examined in rat aortic smooth muscle. Using cold ischemia as a model for vascular injury, Chen and colleagues demonstrated an increase in eotaxin mRNA after 1 hour of cold exposure. We speculate that either eotaxin or CCR3-null mice might be resistant to atherogenesis.

Our data suggest that TNF-α expressed by activated human vascular smooth muscle cells may recruit and activate macrophages and mast cells through the CCR3 receptor. Increased eotaxin expression was identified in a minority of the smooth muscle cells present in the atheromatus vessels, suggesting that only activated cells expressed eotaxin protein. This interpretation is supported by the recent study by Ghaffar and colleagues, which demonstrated increased eotaxin expression in smooth muscle cultures after treatment with either TNF-α or interleukin-1β. Although we used TNF-α as an inflammatory stimulus in our in vitro experiments, it is important to note that several cytokines and growth factors may induce eotaxin expression. Therefore, we cannot exclude the possibility that factors other than TNF-α induce the synthesis of eotaxin by smooth muscle cells of the atheroma.

With continued understanding of the human genome, it is likely that the transcriptional profile of vascular smooth muscle cells may reveal additional, unanticipated genes that participate in vascular inflammation. In the new area of “functional genomics,” identification of differentially expressed genes by DNA microarray technology and other genomic technologies must be followed by crucial steps of establishing the presence of protein in individuals affected by the disease and, eventually, identifying the contribution of the protein to pathophysiological processes. By using the transcriptional profile of activated vascular smooth muscle cells, we have demonstrated an unexpected expression of mRNA and protein for eotaxin and protein for its receptor, CCR3, in human atheroma. The present results support the need for further studies of mice with targeted deletion of eotaxin or CCR3 or studies with pharmacological inhibitors of this pathway to clarify the role of this chemokine in atherogenesis.

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