Interleukin-3 Stimulates Migration and Proliferation of Vascular Smooth Muscle Cells
A Potential Role in Atherogenesis

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Background—Cytokines released by activated T lymphocytes are key regulators of chronic inflammatory response, including atherosclerosis. The aim of this study was to investigate the presence of interleukin-3 (IL-3) in lymphocytes infiltrating the atherosclerotic plaque and the effect of this cytokine on primary vascular smooth muscle cells (SMCs).

Methods and Results—Twenty atherosclerotic carotid arterial specimens and 5 early atherosclerotic lesions from the internal carotid were manually minced to fragments, and T lymphocytes infiltrating the atherosclerotic plaque were isolated on solid-phase anti-CD3 polystyrene plates. Southern blot analysis demonstrated that in all samples, lymphocytes expressed IL-3 and IL-2 receptor \( \alpha \)-chain transcripts, indicating that in this context, the activated T lymphocytes may release IL-3. We further analyzed the expression of the IL-3 receptor and the biological effects exerted by the ligand on vascular SMCs. \( \beta \)-IL-3–transducing subunit was detected both on cultured SMCs and on endothelial cells and SMCs within atheroma. The analysis of the IL-3–induced biological effects demonstrated that it was able to trigger both mitogenic and motogenic signals. Moreover, we demonstrated that the addition of PD98059, a known inhibitor of the MAP–extracellular signaling-regulated/MAP kinase pathway, completely inhibited IL-3–mediated MAP kinase activation and IL-3–induced migration and proliferation. Finally, IL-3 was found to stimulate vascular endothelial growth factor (VEGF) gene transcription.

Conclusions—IL-3, expressed by activated T lymphocytes infiltrating early and advanced atherosclerotic plaques, may sustain the atherosclerotic process either directly, by activating SMC migration and proliferation, or indirectly, via VEGF production. (Circulation. 2001;103:549-554.)

Key Words: atherosclerosis n smooth muscle cells n cytokines n signal transduction
lesions at all stages of the process. Therefore, the aim of the present study was to analyze the role of IL-3 in the atherosclerotic process by evaluating the expression of IL-3 in plaque-infiltrating lymphocytes (PILs) and the ability of this cytokine to exert biological effects on SMCs.

Methods

Reagents

Medium D-val (MEM D-val) was purchased from Gibco. Bovine calf serum (BCS) (endotoxin tested) was obtained from Hyclone. Type I collagen was from UBI. Solid-phase anti-CD3 polystyrene plates were from Ortho Pharmaceuticals. hIL-3 was a gift from Sandoz Pharma Ltd, Basel, Switzerland. PDGF-BB and bFGF were from Euro Clone. Human VEGF165 was from R&D Systems. Endotoxin contamination of hIL-3 preparation was tested by the limulus amoebocyte assay. Polyclonal rabbit anti–IL-3 receptor (IL-3R β-subunit) antisera was prepared as previously described. Rabbit polyclonal antiserum to IL-3 was from Genzyme. Phospho-p44/42 mitogen-activated protein (MAP) kinase was from New England BioLabs.

Plaque-Infiltrating Lymphocytes

Twenty-five atherosclerotic plaques were obtained from human carotid arteries at endarterectomy. Early lesions from internal carotids were obtained from subjects who died of noncardiovascular causes. PILs were isolated as described. Peripheral T lymphocytes were obtained as previously described.

Reverse Transcription, Polymerase Chain Reaction, and Southern and Northern Blot Analysis

Cytoplasmic RNAs were isolated from T lymphocytes and from cells recovered from a nonatherosclerotic specimen. After DNase digestion, RNA was subjected to reverse transcription–polymerase chain reaction (RT-PCR) with specific primers: IL-3 (5'-GCCGGTCCTGCTCCTGCTCCA-3'), CD3 γ-chain (5'-TTATCATTTCGTGGTGAGATG-3'), and IL-2R α-chain (5'-TTATCATTTCGTGGTGAGATG-3'). Peripheral blood lymphocytes (Figure 1A, lane 1) were assayed for the expression of IL-3. The results reported in lane 2 of Figure 1A and lane 13 of Figure 1B demonstrated the absence of IL-3 and CD3 γ-chain (289 bp), IL2R α-chain (391 bp), and β-actin (250 bp). Southern blot analysis was performed with gibbon IL-3 (A), human CD3 γ-chain (B), and human IL-2R α-chain (C) and β-actin (D) cDNA probes. IL-3 producing gibbon MLA cell line was used as control (lane 1).

Human Vascular SMC Culture and Migration

Human SMCs were isolated from umbilical cord and identified as described. Migration of SMCs was performed in Boyden’s chambers as described.

Immunoprecipitation, Immunoblotting, and Proliferation Assay

Serum-starved SMCs stimulated with IL-3 or PDGF-BB or unstimulated were subjected to immunoprecipitation and processed for immunoblotting as previously described. The proliferative activity of SMCs was assayed as previously described by determination of the fraction of cells in the S phase.

Immunofluorescence Microscopy

Specimens fixed and embedded in paraffin were deparaffinized and stained with the polyclonal rabbit anti–IL-3R β-subunit antiserum preadsorbed or not with the immunized synthetic peptide.

Results

PILs Express the mRNA for IL-3

We evaluated whether lymphocytes infiltrating the advanced atheromatous plaques (lesions types IV and V, containing macrophages and lymphocytes, data not shown) and early lesions (fatty streaks) were able to express the IL-3 transcript. From PILs was subjected to RT-PCR and Southern blot analysis. The results (not shown) demonstrated that in all samples, the PILs examined expressed the IL-3 transcript. In Figure 1A, lane 10, representative samples of advanced lesions and 5 samples of fatty-streak lesions are reported. The presence of T lymphocytes in the cell preparation was confirmed by the expression of CD3 γ-chain (Figure 1B) and their activation by the expression of the IL-2R α-chain (Figure 1C). Peripheral blood lymphocytes (Figure 1A, lane 13) recovered from the patient corresponding to lane 12 were also assayed for the expression of IL-3. The results reported in Figure 1 demonstrated that although they were activated, as shown by the expression of the IL-2R α-chain and CD40L (data not shown), peripheral T lymphocytes did not express IL-3. Moreover, the data reported in lane 2 of Figure 1A and 1B demonstrated the absence of IL-3 and CD3 γ-chain mRNA, respectively, in cells recovered from a normal arterial specimen, indicating the absence of inflammatory cells infiltrating the nonatherosclerotic specimen.
SMCs Express the β-Subunit of the IL-3R

Cell lysates immunoprecipitated with the anti–β-subunit antiserum or with a preimmune serum were subjected to SDS-PAGE, and the filter was immunoblotted with the same antiserum. As shown in Figure 2, the anti–β-subunit antiserum was able to recognize the IL-3R β-subunit both in endothelial cells (used as positive control) and in SMCs immunoprecipitated with the same antiserum. Moreover, in experiments that are not presented, we found that 24 hours of treatment with IL-3 (20 ng/mL) was able to upregulate ~60% of the level of the receptor transcript in SMCs.

To extend the analysis of the IL-3R expression, in the context of the atheromatous tissue, sections of advanced atherosclerotic arterial specimens were subjected to immunofluorescence analysis using an anti–IL-3R β-subunit antiserum. The results shown in Figure 3 demonstrate that both endothelial cells (A) and SMCs from media (B) and from neointima (C) express the IL-3R β-subunit.

IL-3 Triggers Erk1/Erk2 MAP Kinase Activation and Induces SMC Migration via This Signaling Pathway

Erk1/Erk2 MAP kinase activation was investigated to assess the specificity of IL-3 receptor engagement by the ligand. Cell extracts from unstimulated or IL-3–stimulated SMCs were subjected to SDS-PAGE, and the filter was immunoblotted with the anti–phospho-Erk1/Erk2 MAP kinase antibody. The results presented in Figure 4 demonstrate that IL-3 retained the ability to induce Erk1/Erk2 MAP kinase activation, which peaked after 10 minutes of IL-3 stimulation.

To extend the analysis of the role played by IL-3 in SMCs, we investigated its chemotactic properties. As shown in Figure 5A, IL-3 induced chemotaxis of primary SMCs, and

![Figure 3](https://example.com/figure3.png)

**Figure 3.** SMCs and endothelial cells within human atheroma express IL-3R β-subunit. Arterial specimens were subjected to immunofluorescence microscopy with an anti–IL-3R β-subunit antiserum. Specificity was provided by experiments in which anti–IL-3R β-subunit antiserum was adsorbed with immunized synthetic peptide (D). Arrows indicate endothelial cells (A), SMCs from media (B), and SMCs from thickened intima (C).

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Erk1/Erk2 MAP kinase activation in response to IL-3. Lysed proteins from untreated (0) or IL-3–treated SMCs were subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose filter. Filter was probed with anti-Erk1 MAP kinase antibody (top) and reprobed with anti-Erk1 MAP kinase antibody (bottom). Three individual experiments were performed with similar results.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Erk1/Erk2 MAP kinases regulate IL-3–induced SMC migration. A, Migration of SMCs. SMC migration induced by vehicle alone (control), PDGF-BB (20 ng/mL), IL-3 (20 ng/mL), IL-3 preincubated with anti–IL-3 antiserum (40 μg/mL), IL-3 heat-inactivated, IL-3 plus PD98059 (35 μmol/L), VEGF (20 ng/mL), VEGF preincubated with anti-VEGF antibody (40 μg/mL), VEGF plus PD98059, and IL-3 preincubated with anti-VEGF antibody. Migration assay was performed as described. Numbers are mean ± SEM of cells counted per 10 fields (×200) of 5 individual experiments. ANOVA with Newman-Keuls multiple-comparison test was performed: control vs experimental groups: *P < 0.05; IL-3 vs IL-3 plus anti–IL-3 antiserum, IL-3 vs IL-3 heat-inactivated, IL-3 vs IL-3 plus PD98059, IL-3 vs IL-3 plus anti-VEGF, VEGF vs VEGF plus anti-VEGF, and VEGF vs VEGF plus PD98059: #P < 0.05. B, Inhibition of IL-3–induced Erk1/Erk2 MAP kinase phosphorylation in cells treated with PD98059. SMCs were either untreated (−) or treated with IL-3 (20 ng/mL, for 15 minutes) (+) alone or in combination with PD98059. Lysed proteins were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose filter. Filter was probed (IB) with an anti–phospho-Erk1/Erk2 MAP kinase antibody (top) and reprobed with an anti-Erk1 MAP kinase antibody (bottom). Three individual experiments were performed with similar results.
preincubation with an anti–IL-3 antiserum almost completely abolished this effect. Similarly, heat-inactivated IL-3 did not stimulate SMC migration. The extent of the migratory response observed at a concentration of 20 ng/mL IL-3 (a dose that was found to induce maximal migratory effect, data not shown) was much more effective than 10 ng/mL of VEGF but less effective than the PDGF-BB (20 ng/mL) used as a positive control.

The involvement of MAP kinase in SMC migration has been reported. We therefore evaluated the role of this signaling pathway in mediating IL-3–induced SMC migration. As shown in Figure 5A, the Erk1/Erk2 MAP kinase inhibitor PD98059 drastically reduced both IL-3– and VEGF-induced SMC migration. Similar results were obtained for the biochemical effect exerted by IL-3 on MAP kinase activation (Figure 5B).

**IL-3 Triggers SMC DNA Synthesis and VEGF Gene Transcription**

The effect of IL-3 on SMC DNA synthesis was studied. In our system (data not shown), 20 ng/mL of IL-3 yielded maximal response. As reported in the Table and shown in Figure 6, 48 hours of IL-3 treatment led to an increase of cells in S phase of ≈5-fold above control. Similar results were obtained when SMCs were stimulated with bFGF (positive control), whereas a 3-fold increase was observed on PDGF-BB stimulation. Moreover, PD98059 completely abrogated the IL-3– and bFGF-mediated DNA synthesis, indicating that the activation of the Erk1/Erk2 MAP kinase pathway is a downstream event in IL-3– and bFGF-induced DNA synthesis (Table).

At the site of injury, SMCs are the major cellular source of VEGF. We therefore evaluated the ability of IL-3 to trigger VEGF gene transcription. Indeed, IL-3, like PDGF-BB, was able to induce VEGF gene transcription in SMCs (Figure 7). A role of newly produced VEGF in mediating IL-3–induced SMC migration can be postulated; however, the finding that the anti-VEGF antiserum only partially reduced (≈29%) the chemotactic effect of IL-3 points to a direct effect of IL-3.

**Discussion**

In the present study, we demonstrate that activated T cells infiltrating the advanced atherosclerotic lesions express the transcript of IL-3. Several studies indicate that the hemopoietic growth factor IL-3 can also act on endothelial cells by inducing the expression of adhesion molecules such as E and P selectins, which, in turn, promote the adhesion of T cells. Indeed, such adhesion molecules, which act as receptor...
tors for glycoconjugates, are associated with migration of inflammatory cells across the endothelium. Therefore, the expression of IL-3 transcript by activated PILs sustains the possibility that the released cytokine could amplify the recruitment of inflammatory cells promoted by other soluble factors in the context of the atherosclerotic lesion.

T lymphocytes infiltrating the advanced human atherosclerotic plaques are in a state of immunological activation. Our finding that IL-3 is also expressed in early lesions indicates that, not only in advanced human atheroma but also at the beginning of the atherosclerotic process, T cells are in an activated state and may contribute, by elaborating soluble mediators, to lesion evolution.

It has been shown that microenvironment may play a role in modulating T-cell cytokine profile. Our observation that PILs, but not activated peripheral blood T lymphocytes, did express the IL-3 transcript is consistent with this concept. According to the profile of cytokine produced, T cells may thus regulate important processes associated with plaque formation, such as macrophage activation, SMC growth, synthesis of extracellular matrix component, and synthesis of matrix metalloproteinases.

SMC migration and proliferation into the intima after vascular injury are triggered and regulated by multiple factors. The mechanisms involved in this regulation depend on phosphorylation of cellular proteins, such as kinases. Among these kinases, MAP kinases appear to be nearly universal. In the present study, we demonstrate that in SMCs, IL-3 is able to trigger the activation of the Erk1/Erk2 MAP kinase pathway, which is known to integrate signals involved in cell growth. Indeed, we demonstrated that the MAP kinase inhibitor PD98059 completely abrogated the entry into S phase of both IL-3- and bFGF-stimulated SMCs, indicating that IL-3- and bFGF-induced DNA synthesis is strictly dependent on the Erk1/Erk2 MAP kinase pathway. This observation is also supported by our unpublished data demonstrating that PD98059 was also able to block the expression of the cell cycle-specific cyclin D1 in SMCs stimulated with IL-3.

Migration of SMCs from tunica media to intima is mediated by growth factors, such as PDGF-BB and bFGF, synthesized and released by cells of the vessel wall. Our results demonstrate that IL-3 not only stimulates SMC proliferation but also can act as a SMC chemotactant. These findings are strengthened by the detection of the IL-3R β-subunit in primary SMCs from media and thickened intima. Moreover, the finding that PD98059 also abrogates IL-3-mediated SMC migration indicates that not only cell growth but also cell migration is strictly dependent on Erk1/Erk2 MAP kinase pathway activation. Cell migration depends on the remodeling of cytoskeletal proteins such as actin and myosin. In this context, it has been reported that activation of MAP kinases can increase cell migration via the phosphorylation of myosin and F-actin. Therefore, the finding that both DNA synthesis and cell migration triggered by IL-3 depend on the MAP kinase pathway sustains the possibility that the Erk1/Erk2 MAP kinases are involved in the activation of discrete substrates, leading to either cell migration or cell proliferation.

In conditions such as atherosclerosis or thrombosis, intimal angiogenesis occurs as part of an adaptive change recently addressed as vasculara remodelin. One current hypothesis of neovascularization in atherosclerosis is that the new, small vessels arise from adventitial vasa vasorum. Several factors that stimulate angiogenesis both in vitro and in vivo, including VEGF, have been detected in atherosclerotic lesions. Moreover, recent data demonstrate that VEGF receptors are expressed on SMCs, which, in response to the ligand, acquire the ability to migrate. Because both endothelial cells and SMCs are capable of synthesizing VEGF at sites of angiogenesis, it can be speculated that autocrine and paracrine actions contribute to SMC activation during neangiogenesis. Our finding that IL-3 was able to induce the expression of VEGF by SMCs and that the anti-VEGF antibody only partially reduced the IL-3-mediated motogenesis sustains the possibility that the produced VEGF may be primarily involved in driving neangiogenesis.

Earlier work indicates that vessels, during their development, are dependent on exogenous survival factors. Recent data suggest that the association of forming vessels with SMCs/mural cells marks the end of this period of growth factor dependence and mediates vessel stabilization and maturation. Data presented here demonstrating the ability of IL-3 to induce both VEGF expression and SMC migration and proliferation provide new insights to understand the cellular and the molecular bases of new vessel formation and maturation. Moreover, our study provides evidence for a potential role of IL-3 in the atherosclerotic process.

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