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

Maria Felice Brizzi, MD; Luisa Formato, MD; Patrizia Dentelli, PhD; Arturo Rosso; Marzia Pavan, PhD; Giovanni Garbarino; Marco Pegoraro, MD; Giovanni Camussi, MD; Luigi Pegoraro, MD

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 ■ smooth muscle cells ■ cytokines ■ signal transduction

Macrophages and T lymphocytes are considered to be essential in the development and progression of atherosclerotic lesions. T lymphocytes are known to play a key, yet incompletely defined, role in such a significant pathological condition. The pathogenesis of this disease has been extensively studied and can be traced to the intimal event of macrophages and lymphocytes infiltrating the arterial wall, presumably secondary to endothelial dysfunction. Moreover, T lymphocytes and macrophages are particularly abundant at the leading edge of the lesion, or the “shoulder” region, a side where the atherosclerotic plaques are more vulnerable and tend to rupture. This observation highlights the critical role of the T cell in this process and sustains the proposal that T cell–derived cytokines implicated in the regulation of collagen synthesis may be involved in weakening the fibrous cap. Despite connective tissue remodeling, atherosclerosis is a consequence of proliferation and directed migration of vascular smooth muscle cells (SMCs). Numerous observations suggest that, in the lesions, SMCs show a synthetic phenotype, which could have profound effects on the capacity of the lesion to respond to various agonists. SMCs in the synthetic phenotype can respond to platelet-derived growth factor (PDGF) or to other possible growth stimulators and can produce a vast array of cytokines and growth factors, including transforming growth factor-\( \beta \), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF). In particular, the expression of VEGF has been detected in atherosclerotic lesions, suggesting a possible physiopathological significance in the progression of atherosclerosis.

We recently demonstrated that the hemopoietic growth factor interleukin-3 (IL-3), which is produced predominantly by the CD4 subset of activated T lymphocytes infiltrating the inflammatory tissue, is able to induce endothelial cell proliferation and in vivo new vessel formation. CD4- and CD8-positive lymphocytes are present in the atherosclerotic...
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 antiserum 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 carotid arteries 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′-GCCGCTCTGCCTGCTGCTCA-3′), 5′-CCGGAAATTCATTCG- CAGTCA-3′), CD3 γ-chain (5′-CATCCCTGCTATCA- TTTCCTATT-3′), 5′-GGCACTGCTGACATCTGC- TGAA-3′), and IL-2R α-chain (5′-TTATCATTTCGCTGG GGCCAGATGGTTTAC-3′), 5′-AGATAGAAGGAGAGACAGAGGCACGGTC-CA-3′) and subjected to Southern blot analysis. A 204-bp VEGF cDNA fragment was generated from human SMCs as described by Williams et al. and Northern blot analysis was performed by standard methods.

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. RNA 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 5, IL-3 induced chemotaxis of primary SMCs, and...
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 PD9805912 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 approximately 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 hematopoietic 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 recep-

---

**Table:**

<table>
<thead>
<tr>
<th>Hours of Culture</th>
<th>Control</th>
<th>IL-3</th>
<th>PDGF</th>
<th>bFGF</th>
<th>IL-3 + PD98059</th>
<th>bFGF + PD98059</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>3.1</td>
<td>5.3</td>
<td>4.2</td>
<td>8.9</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>48</td>
<td>3.5</td>
<td>17.3</td>
<td>10.2</td>
<td>19.8</td>
<td>4.8</td>
<td>5.1</td>
</tr>
</tbody>
</table>

SMCs resuspended at 4 × 10⁴/mL seeded in triplicate culture chambers. After 24 hours, the medium was replaced with MEM D-Val containing 0.1% BSA, and the cells were incubated for different times in medium alone or with IL-3 (20 ng/mL), PDGF (50 ng/mL), bFGF (20 ng/mL), or in the presence of PD98059 (35 μmol/L). The results represent the means of 3 individual experiments.

---

**Figure 6.** Autoradiographic picture of SMCs pulse-labeled with IL-3, PDGF-BB, and bFGF. SMCs were detached, resuspended in MEM D-val medium supplemented with 15% BCS, seeded in culture chamber, and allowed to adhere. After 24 hours, medium was replaced with MEM D-val containing 0.1% BSA, and cells were incubated for 48 hours without (A) or with IL-3 (20 ng/mL) (B), PDGF-BB (50 ng/mL) (C), bFGF (10 ng/mL) (D), PD98059 plus IL-3 (E), or PD98059 plus bFGF (F). Proportion of cells in DNA synthesis was evaluated as previously described.6

**Figure 7.** VEGF gene transcription in SMCs treated with IL-3. RNA was isolated from unstimulated or IL-3 (20 ng/mL)– or PDGF-BB (20 ng/mL)–stimulated SMCs. Northern blot analysis was performed according to standard methods. Filter was hybridized with a DNA probe corresponding to a 204-bp cDNA VEGF fragment (top) or β-actin cDNA (bottom).
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 vasculature remodeling. One current hypothesis of neovascularization in atherosclerosis is that the new, small vessels arise from adventitial vasa vasorum. Several factors that stimulate angiogenesis exist 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.

Acknowledgments
This work was supported by grants from the Italian Association for Cancer Research and from the Ministero dell’Università e della Ricerca Scientifica e Tecnologica to Drs Pegoraro and Camussi.

References


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

Maria Felice Brizzi, Luisa Formato, Patrizia Dentelli, Arturo Rosso, Marzia Pavan, Giovanni Garbarino, Marco Pegoraro, Giovanni Camussi and Luigi Pegoraro

_Circulation_. 2001;103:549-554
doi: 10.1161/01.CIR.103.4.549

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/103/4/549

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/