Elastin-Derived Peptides Induce a T-Helper Type 1 Polarization of Human Blood Lymphocytes

Romain Debret, Frank Antonicelli, Aurore Theill, William Hornebeck, Philippe Bernard, Moncef Guenounou, Richard Le Naour

Objective—Increased level of elastin-derived peptides (EDPs) is observed in the serum of patients with manifestations of arterial diseases. We here investigated whether EDPs might exert, at systemic level, a regulatory role for the T-helper type 1 (Th-1)/Th-2 cellular immune response by human peripheral blood lymphocytes (PBLs) expressing the spliced-galactosidase (S-gal)–elastin receptor.

Methods and Results—Using flow cytometry and Western blot analysis, we demonstrated that EDPs led to an activation of the S-gal-elastin receptor associated with cytokine production on PBLs and CD4+ T cell subpopulations. The constitutive expression of the S-gal–elastin receptor at the surface of human PBLs was upregulated at the mRNA (RT-PCR) and protein (ELISA) levels on cell activation. In nonactivated and phytohemagglutinin-activated conditions, expressions of the predominant Th-2 cytokine interleukin-5 (IL-5) and IL-10 were reduced, whereas those of the major Th-1 cytokines interferon-γ and IL-2 were enhanced by EDPs. Furthermore, we evidenced that EDPs could not only potentiate the IL-12–induced Th-1 profile but also could reverse the Th-2 (over Th-1) profile induced by IL-4. Finally, Th-1 cytokine upregulation was associated to an increased activator protein-1 DNA binding and enhanced pro–matrix metalloproteinase-9 secretion.

Conclusions—This study highlights the importance of EDPs as stimuli for Th-1 differentiation, whether T cells are in an inactivated state or already orientated toward a Th-1 (IL-12) or Th-2 (IL-4) response. (Arterioscler Thromb Vase Biol. 2005;25:1353-1358.)

Key Words: elastin peptides ■ T lymphocytes ■ cytokines ■ Th-1/Th-2 ■ MMP-9 ■ AP-1

Elastic fibers provide elasticity to tissues such as lung, skin, and large blood vessels.1-3 Elastin represents the main amorphous component of those fibers that proteolysis is catalyzed by proteases as serine elastases4 and matrix metalloproteinases (MMPs) such as MMP-2, MMP-9, MMP-7, and MMP-12.5-7 Excessive expression of those elastinolytic MMPs is hallmark of atherosclerosis and abdominal aortic aneurysm (AAA).8-14 However, the physiopathological relevance of these elastase-increased expression is not clear. Indeed, intense elastic fiber fragmentation and increased level of circulating elastin-derived peptides (EDPs) has been consistently observed mainly in AAA.15-17 EDPs display a large panel of biological effects largely mediated through their interactions with a receptor complex including a 67-kDa elastin-binding protein (EBP)18,19 identified as an enzymatically inactive spliced variant of human β-galactosidase.20,21 EDPs exhibit chemotactic activity for monocytes, fibroblasts, and tumor cells,22-24 regulate cell proliferation in normal and pathological conditions,25 and provide control of vascular tone.26 Elastin peptides have also been shown to induce elastase production by human phytohemagglutinin (PHA)-activated T lymphocytes27 and to promote cell death of human activated T cells expressing the elastin receptor.28

As documented, in human atherosclerotic diseases, T lymphocytes are orientated toward T-helper type 1 (Th-1) cells, which produce interleukin-2 (IL-2), interferon-γ (IFN-γ), IL-12, IL-15, and IL-18 cytokines.29,30 On the contrary, in AAA, Th-2 immune responses predominate locally, although a systemic increase of IFN-γ, the major cytokine associated with Th-1 phenotype, has been reported.31 Indeed, Th-2 cytokines such as IL-4, IL-5, and IL-10 are highly expressed in AAA,30 and recently, Th-2–predominant immune response in human AAA development was confirmed through the induction by Th-2–inflammatory environment of aneurysms in a murine model of aortic allograft.32 We thus investigated the influence of EDPs in T-helper orientation and evidenced that interaction of EDPs with the 67-kDa spliced-galactosidase (S-gal)–elastin receptor expressed on human peripheral blood lymphocytes (PBLs) induced a functional differentiation or shift toward Th-1 phenotype, which was accompanied by an increased pro–MMP-9.
Methods

Human PBLs Isolation and Culture

Human PBLs were obtained by countercurrent centrifugal elutriation, followed by density-gradient centrifugation from heparinized venous blood of healthy donors. Isolated PBLs (10^6 cells/mL) were cultured in a serum-starved RPMI-1640 medium supplemented with l-glutamine (300 μg/mL), penicillin (100 U/mL), and streptomycin (50 μg/mL; Invitrogen), and treated or not with 10 μg/mL soluble elastin peptides obtained as described previously.33 Four hours after incubation at 37°C in 5% CO₂, cells were collected for intracellular cytokine staining. To detect intracytoplasmic cytokines, the secretion inhibitor Brefeldin A (10 μg/mL; Sigma) was added to the culture medium. In some experiments, PBLs were activated with 10 μg/mL PHA (Sigma) at the time of EDP treatment and then incubated for 24 hours. Cell viability was ≥90% when cell culture supernatants were collected and cells lysed for cytokine analysis. Pretreatment of PBLs with 10 mmol/L lactose (3 hours) or 10 μg/mL anti-EBP antibody (1 hour) was used to define the specificity of EDP effects.

Western Blot Analysis

Protein extracts from human PBLs were resolved by electrophoresis as described previously.34 Blots were developed with either a purified rabbit antibody raised against a 14-aa sequence specific of the S-gal (Neosystem) or an anti-phospho-Erk1/2 (Cell Signaling) or a total anti-Erk1/2 (R & D Systems). After incubation with anti-rabbit horseradish peroxidase–labeled IgG (Cell Signaling), immune complexes were detected with the enhanced luminescence system according to manufacturer instructions (Perkin-Elmer).

Flow Cytometry Analysis of EBP and Intracellular Cytokine Staining

All labeled antibodies were purchased from BD PharMingen. 10⁶ nonpermeabilized PBLs were incubated with anti-CD3 alone or associated with anti-CD4 or anti-EBP for 30 minutes at 4°C in the dark. In some experiments, after centrifugation (500 g for 5 minutes), cell pellets were treated for cytokine staining as described previously.35 A 3-color flow cytometric analysis was performed with a fluorescence-activated cell sorter (FACS; Calibur Instrument; BD Biosciences). Human CD4⁺ T lymphocytes from healthy donors were initially gated on the PBL population based on forward and side light-scattering properties, and thereafter on the presence of the surface markers CD3 and CD4. Analysis was performed on a logarithmic scale using CellQuest software (BD Biosciences).

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from human PBLs with Trizol reagent (Invitrogen). Precipitated RNA was reverse-transcribed with oligo-dT as the first-strand cDNA primer and Moloney murine leukemia virus reverse transcriptase superscript (Life Technologies). After reverse transcription, the cDNA product was amplified by PCR as described previously.36 RT-PCR was performed with specific primers for IFN-γ, IL-2, 18S RNA, and β-galactosidase (Invitrogen). The ethidium bromide–stained cDNA bands were analyzed from 1.5% agarose gels under UV light (Gel Doc 2000; Bio-Rad).

Enzyme-Linked Immunosorbent Assay

Quantification of cytokine protein levels was performed using commercially available high-sensitivity ELISA kit according to manufacturer instructions (R & D Systems).

Electrophoretic Mobility Assay

EMSA was performed to appreciate DNA binding using an activator protein-1 (AP-1) consensus sequence (Promega).37

Gelatin Zymography

Samples were run in nonreducing Laemmli buffer and separated through a 10% SDS-PAGE containing 1 mg/mL gelatin as substrate. MMP gelatinolytic activities were evidenced as white zones of lysis as described previously.38

Statistical Analysis

Statistical analysis was performed using either Student’s t test or Wilcoxon test as appropriate. P values of <0.05 were considered statistically significant.

Results

EDPs Influence Cytokines Production by Human T Cells

We first investigated whether human PBLs isolated by countercurrent centrifugal elutriation from healthy donors were able to express the S-gal–elastin receptor. Western blot analysis of proteins extracted from human PBLs using antibody directed against a specific sequence of S-gal protein corresponding to the 67-kDa EBP (ie, S-gal) subunit of the elastin receptor revealed the constitutive presence of this S-gal–elastin receptor in human PBLs. Antibody specificity was performed with a protein extract from fibroblastic cells, which was analyzed simultaneously to human PBLs. B, Intracellular expression of cytokine by T cells from healthy donors. B, Intracellular expression of cytokine by T cells from healthy donors. B, Intracellular expression of cytokine by T cells from healthy donors.

Figure 1. Cytokine regulation during interaction of elastin peptides with PBLs expressing elastin receptor. A, Western blot and flow cytometry analysis of constitutive expression of S-gal–elastin receptor on human PBLs. B, Intracellular expression of cytokine by elastin peptide–treated PBLs in the presence or absence of anti-EBP antibodies. All data are representative of 3 independent experiments. FITC indicates fluorescein isothiocyanate; SSC-H, side scatter; FSC-H, forward scatter.
PBL proteins (Figure 1A). Presence of this receptor at the cell surface was confirmed by flow cytometry analysis on nonpermeabilized cells directly with an anti-EBP antibody and indirectly by competition between fluorescein isothiocyanate–labeled elastin and anti-EBP antibody (Figure 1A).

To analyze the EDP/S-gal interaction–mediated influence on immunomodulatory properties of human T cells, intracellular Th-1 (IFN-γ) and Th-2 (IL-5 and IL-10) cytokine expression was determined by flow cytometry analysis before and after EDP treatment. Untreated PBL cells expressed detectable basal levels of IL-5, IL-10, and IFN-γ (Figure 1B, red curves). On stimulation with EDPs at a concentration of 10 μg/mL, a concentration corresponding to an average level of elastin peptides in circulating blood,39 and that did not alter cell viability as investigated by the trypan blue exclusion method (data not shown) and as described by others,28 IL-5 and IL-10 levels were reduced, whereas IFN-γ production was enhanced (Figure 1B, green curves versus red curves). In setting with our former results (Figure 1A), modulation of cytokine expression on EDP stimulation was abolished with an anti–S-gal pretreatment (Figure 1B, purple curves). To define whether the effects observed on PBLs treated with EDPs on Th-1/Th-2 cytokine production was correlated to an orientation toward a Th-1/Th-2 immune response within PBL population, the same experiments were conducted on a CD3+CD4+ gated population (Figure 1, available online at http://atvb.ahajournals.org). On EDP stimulation, IL-5, IL-10, and IFN-γ levels (Figure 1A, green curves versus red curves) were affected similarly to the PBL population (Figure 1B). Statistical analysis of cytokine production expressed as a mean intensity of fluorescence shows that EDP stimulation led to a significant decrease of Th-2 cytokine (IL-5 and IL-10) secretion, whereas Th-1 cytokine (IFN-γ) was enhanced (Figure 1B).

Specificity of ligand–receptor interaction was further demonstrated on CD4+ T cells using a pretreatment with 10 mmol/L lactose, known to induce the shedding of S-gal from the cell surface, before elastin peptide activation.40 Treatment with lactose alone did not affect cytokine production (data not shown). However, lactose pretreatment abolished the down and upregulatory effects of EDPs on the expression of IL-5, IL-10, and IFN-γ, respectively (Figure 1B).

**EDPs Favor a Th-1 Profile in Human Lymphocytes**

To provide further evidence that EDPs may act as critical mediators to promote or maximize a Th-1 cytokine profile, we used the mitogenic compound PHA (10 μg/mL) for PBL activation, which was characterized by: (1) the presence of the CD69 glycoprotein, an activation inducer molecule that is highly expressed on the surface of T lymphocytes 24 hours after PHA stimulation41 (data not shown); and (2) by IL-2 expression on activated lymphocytes was associated to an enhanced Th-1 cytokine profile such as IFN-γ and IL-2 expression at the protein and mRNA levels (Figures 2B and 3). On the contrary, EDPs antagonized the PHA effect on IL-5 and IL-10 expressions (Figure 2B).

**Figure 2.** Regulation of intracellular cytokine expression by elastin peptides in PHA-activated PBLs. A, Expression of S-gal–elastin receptor (Western blot, left) and S-gal mRNA (RT-PCR, right) in PHA-activated PBLs. All results are representative of 3 independent experiments. B, Intracellular cytokine expression in PHA-activated PBLs treated or not with elastin peptides. All results are representative of 3 independent experiments.

EDPs favor a Th-1 profile in PHA-activated PBLs compared with untreated cells. RT-PCR experiments confirmed the increased S-gal mRNA expression in PHA-activated human PBLs (Figure 2A). Increased elastin receptor expression on activated lymphocytes was associated to an enhanced IFN-γ and IL-2 expression on EDP stimulation observed at the protein and mRNA levels (Figures 2B and 3). On the contrary, EDPs antagonized the PHA effect on IL-5 and IL-10 expressions (Figure 2B).

Similarly, as found using nonactivated lymphocytes, EDP effects were receptor-dependent in PHA-activated lymphocytes. Exposure of cells to 10 mmol/L lactose totally abolished the effect of elastin peptides on IFN-γ and IL-2 expression at the mRNA and protein levels (Figure 3). Therefore, these results demonstrate that the interaction of elastin peptides with S-gal is critically involved in the upregulation of Th-1 cytokine such as IL-2 and IFN-γ in nonactivated and activated human PBLs.

To further evidence that EDPs maximize or promote Th-1 over Th-2 cytokine profile, we used preorientated lymphocytes with IL-12 (Th-1 orientation) or IL-4 (Th-2 orientation) cytokine, as shown in Figures II and III (available online at http://atvb.ahajournals.org). Indeed, Th-1 T-lymphocyte sub-
population characterized by IL-5 and IL-10 low profiles and IL-2 and IFN-γ high profiles was reinforced by EDP cotreatment (Figure II, purple curves versus green curves), whereas a shift toward Th-1 T lymphocytes was demonstrated by a reduction of IL-5 and IL-10 production, which paralleled and increased IL-2 and IFN-γ production in Th-2–preorientated T lymphocytes cotreated with EDPs (Figure III, purple curves versus green curves).

EDPs Increase Extracellular Signal-Regulated Kinase 1/2, AP-1, and MMP-9 Activities

Interaction of EDPs with their receptor was further analyzed by activation of the extracellular signal-regulated kinase (ERK) pathway (Figure 4A). Concomitantly, we demonstrated that downstream to the ERK pathway activation, stimulation by EDPs also led to a dose-dependent enhancement of the AP-1 DNA binding (Figure 4B), which is thought to play a major role in coordinating transcription of the IFN-γ gene and was demonstrated to be required for optimal IL-2 gene transcription. Interestingly, a single AP-1 element, which binds members of the AP-1 transcription factor family, is found in the promoter region of each inducible MMP gene. Because MMP-2 and MMP-9 are 2 of the most abundant elastinolytic proteases secreted at the site of arterial tissue damage, we decided to investigate the role of EDPs in the regulation of MMP-2 (pro–MMP-2) and MMP-9 (pro–MMP-9) expression. Figure 4C shows that the gelatinolytic activity of the latent pro–MMP-9 is constitutively expressed in nonactivated PBLs and increased 24 hours after incubation of cells with 10 μg/mL elastin peptides. In contrast, pro–MMP-2 gelatinolytic activity remained undetectable under whatever activation conditions used (Figure 4C). Pro–MMP-9 activity was also increased in response to 10 μg/mL PHA activation as compared with nonactivated cells. In such conditions, elastin peptides still enhanced pro–MMP-9 secretion (Figure 4C). A similar effect of EDPs on pro–MMP-9 secretion was also observed in orientated Th-1 (IL-12) or Th-2 (IL-4) lymphocytes (Figure 4C).

Discussion

We initially hypothesized that the elevated level of EDPs in the serum of patients with manifestations of arterial diseases might contribute to a T-helper phenotype polarization of human PBLs. In this setting, we found that EDPs led to activation of the S-gal–elastin receptor associated with cytokine production on PBLs and CD4+ T cell subpopulations. The constitutive expression of the S-gal–elastin receptor at the surface of human PBLs was upregulated at the mRNA and protein levels on cell activation. Previous studies described the presence of the S-gal–elastin receptor on isolated T lymphocytes from human tonsils or atherosclerotic plaques. However, to our knowledge, the presence of S-gal–elastin receptor at the surface of nonactivated and activated human PBLs (circulating lymphocytes) had not been convincingly established.

Suppression of the regulatory effects of EDPs on cytokine expression in the presence of lactose or anti–S-gal antibody demonstrated the critical role of the interaction between EDPs and S-gal in functional differentiation or shift of the systemic T-cell response toward Th-1 phenotype. On stimulation with EDPs, the levels of the predominant Th-2 cytokines IL-5 and IL-10 were reduced, whereas the major Th-1 cytokines IFN-γ and IL-2 were enhanced in nonactivated or PHA-activated T cells. Th-1 phenotype polarization of PBLs was also demonstrated using either insoluble fibrous elastin or the specific peptide sequence Val-Gly-Val-Ala-Pro-Gly, several-fold repeated in human tropoelastin (data not shown). Overall, our data add further insight into the well-documented biological effects of elastin peptides, as members of matrikines family, demonstrating their involvement in cytokine regulation. More generally, it is now emerging that matrix components might regulate T-cell response, as demonstrated with osteopontin, an early matricellular protein of type-1 cell-mediated immunity. In addition, we found that the ability of EDPs to direct the T-lymphocyte population toward a Th-1 profile was still effective when T cells were IL-12 Th-1 preorientated. The physiopathological relevance of EDP-mediated Th-1 orientation of the T-cell response could be illustrated during atherosclerosis, in which a Th-1 immune response predominates, although elastolysis was not as prominent as in aneurysms. Strikingly, EDPs were also shown to reverse the Th-2 (over Th-1) profile induced by IL-4. Recently, Schönbeck et al and Shimizu et al demonstrated that the dichotomy between IFN-γ– or IL-4–dominated cytokine environments plays a crucial part in AAA formations. Importantly, any change toward a Th-2 microenvironment might influence arterial lesions toward aneurysm development. To that
respect, our findings would suggest that somewhat paradoxically, EDPs could act as protective agents. Nevertheless, one might also consider that EDPs markedly increased levels of pro–MMP-9 after S-gal activation. Indeed, S-gal occupancy by EDPs led to ERK1/2 and AP-1 DNA-binding activation, key actors in coordinating MMPs and IFN-γ or IL-2 transcription. Consequently, that might contribute to positive feedback mechanism through exacerbation of MMP-9 production because IFN-γ and IL-2 were reported to stimulate the release of MMP-9 by various cell types. Thus, considering that: (1) MMP-9, as a zymogen form, could hydrolyze macromolecular substrates such as type IV collagen; and (2) MMP-9 displays a wide specificity, an upregulation of active MMP-9 production by EDP-activated PBLs would in turn promote destabilization and complication of atherosclerotic plaques or facilitate aortic aneurysm development as described previously.

These observations highlight a dual potential contribution of EDP-activated lymphocytes in the development of AAA and atherosclerosis and provide convincing evidence that physiopathological evolution of these 2 arterial pathologies might depend on the delicate balance between cytokines and MMPs produced by EDP-activated lymphocytes. Probably, initiation of an early inflammatory response within the damaged aortic wall orients the proper biological effects of elastin peptides. It needs to be considered that insoluble elastin is a very long-lived protein (half life \(70\) years), and its degradation (EDP production) probably constitutes a strong signal to modulate the inflammatory response by either preventing atherosclerotic development by reversing Th-2 profile or amplifying the Th-1 profile associated to atherosclerosis. To that respect, EDP production will display either beneficial or detrimental effects. Also, EDPs, either directly or indirectly (through IFN-γ or IL-2), were shown here to enhance MMP-9 expression by lymphocyte. Excessive MMP production has been observed in AAA and atherosclerosis, suggesting that upregulation of MMP-9 expression mediated by EDPs, when present at site of injury, would be detrimental to aneurysmal and atherosclerosis progression. Besides, because MMP-9 was described to display potent elastolytic activity, an amplification mechanism might be locally generated. As a whole, our data further illustrate the critical importance of elastolysis in the progression of arterial diseases.

Acknowledgments
This work was supported by funds provided from Ligue de la Marne (France) and the Region Champagne Ardenne (France). The authors thank Dr P. Nguyen and the Etablissement de Transfusion Sanguine Nord-Est (France) for providing the PBL samples.

References


Elastin-Derived Peptides Induce a T-Helper Type 1 Polarization of Human Blood Lymphocytes

Romain Debret, Frank Antonicelli, Aurore Theill, William Hornebeck, Philippe Bernard, Moncef Guenounou and Richard Le Naour

Arterioscler Thromb Vasc Biol. 2005;25:1353-1358; originally published online April 28, 2005;
doi: 10.1161/01.ATV.0000168412.50855.9f
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/7/1353

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/04/28/01.ATV.0000168412.50855.9f.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology 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 Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Untreated cells
EDP treated cells
Isotypic control

Figure I

A

CD3+CD4+
T lymphocytes

Relative fluorescence intensity

B

Cytokine expression in CD4+ T cells
(Mean Intensity of Fluorescence)
**Figure II**

- **Axes:**
  - **X-axis:** FSC-H
  - **Y-axis:** SSC-H

- **Legend:**
  - Isotypic control
  - Untreated cells
  - IL-12 treated cells
  - IL-12 and EDP treated cells

- **Graphs:**
  - **IL-5**
  - **IL-10**
  - **IFN-γ**
  - **IL-2**

- **Relative fluorescence intensity**

- **Cell Count**
Figure III

- Isotypic control
- Untreated cells
- IL-4 treated cells
- IL-4 and EDP treated cells

Relative fluorescence intensity

Cell Count

Relative fluorescence intensity

IL-5

IL-10

IFN-γ

IL-2
**Figure I.** Contribution of elastin receptor on cytokine synthesis by elastin peptides-treated CD4$^+$ T cells. A, Consecutive steps for acquisition of CD3$^+$CD4$^+$ lymphocytes from PBL and intracellular expression of cytokine by elastin peptides-treated CD4$^+$ T cells. Data are representative of three independent experiments. B, Consequences of pre-incubation of PBL with lactose on regulatory effects of elastin peptides on the intracellular cytokine expression by CD4$^+$ T cells. Data are mean intensity of fluorescence ± SEM; * p<0.05.

**Figure II.** Regulation of intracellular cytokine expression in IL-12-activated PBL treated or not with elastin peptides. All results are representative of three independent experiments.

**Figure III.** Regulation of intracellular cytokine expression in IL-4-activated PBL treated or not with elastin peptides. All results are representative of three independent experiments.