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 Vasc Biol. 2005;25:1-6.)

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 phagocytic cells,27 which 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.11 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.12 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) antibody 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. 10^6 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 (500g for 5 minutes), cell pellets were treated for cytokine staining as described previously.13 A 3-color flow cytometric analysis was performed with a fluorescence-activated cell sorter (EACS; Caliber 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.14 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).15

Gelatin Zymography

Samples were run in nonreducing Laemmli buffer and separated through a 10% SDS-PAGE containing 1 mg/mL gelatin as substrate.
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, and that did not alter cell viability as investigated by the trypan blue exclusion method (data not shown) and as described by others, 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 PBLs on the expression level of the S-gal–elastin receptor. As seen in Figure 2A, protein blots probed with specific S-gal protein antibody showed a significant increase of the S-gal–elastin receptor in PHA-activated PBLs compared with untreated cells. RT-PCR experiments confirmed the increased S-gal mRNA expression in PHA-activated 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) cyto-
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.42 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.43,44,27 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.45 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 preoriented. 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.46 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.30,32 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 orientates 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 aneurismal 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.

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References


Figure 4. Regulation of Erk1/2 pathway, AP-1 DNA binding, and pro–MMP-9 secretion by elastin peptides in PBLs. A, Increase of Erk1/2 activation in nonactivated PBLs treated with elastin peptides. B, Increase of AP-1 binding in nonactivated PBLs treated with elastin peptides. C, Upregulation of pro–MMP-9 by elastin peptides (vs control [Ct]) in PBLs activated or not with PHA, IL-12, or IL-4. Pro–MMP-9 and pro–MMP-2 gelatinolytic activities were evidenced as white zones. All results are representative of 3 independent experiments.


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Figure I

A

![Flow cytometry plots showing relative fluorescence intensities for IL-5, IL-10, and IFN-γ in untreated EDP-treated cells and isotypic controls.](image)

B

![Bar graphs showing cytokine expression in CD4+ T cells.](image)
Figure III

Graphs showing relative fluorescence intensity for IFN-γ, IL-2, IL-5, IL-10, and IFN-γ for untreated cells, IL-4 treated cells, and IL-4 and EDP treated cells. The graphs are labeled with different colors to represent the cell counts.
**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.