Function and survival of dendritic cells depend on endothelin-1 and endothelin receptor autocrine loops

Georgi Guruli, Beth R. Pflug, Stefana Pecher, Valeria Makarenkova, Michael R. Shurin, and Joel B. Nelson

The biologic effects of endothelin-1 (ET-1) are not limited to its potent vasoconstricting activity. The endothelin receptors, ET\textsubscript{A} and ET\textsubscript{B}, have differential tissue and functional distributions. Here we showed that dendritic cells (DCs), the major antigen-presenting cells in the adaptive limb of the immune system, produce large amounts of ET-1 and significantly increase the expression of endothelin receptors upon maturation. Selective blockade of the ET\textsubscript{A} receptor significantly reduced expression of the mature DC marker CD83, decreased the production of the immunostimulatory cytokine interleukin-12, down-regulated DC ability to stimulate T cells, and promoted DC apoptosis. Selective ET\textsubscript{B} receptor blockade, on the other hand, resulted in increased expression of CD83 and improved DC survival. Therefore, ET-1/ET\textsubscript{A}/ET\textsubscript{B} autocrine/paracrine loops on DCs appear to be essential for the normal maturation and function of human DCs, presenting a unique target for immunomodulatory therapies. (Blood. 2004;104:2107-2115)

© 2004 by The American Society of Hematology

Introduction

Endothelin-1 (ET-1), the most potent vasoconstrictor known, was first described in 1988.\textsuperscript{1} The endothelin family also includes endothelin-2, endothelin-3, and the sarafotoxins. The endothelins are widely distributed in tissues, produced by endothelial cells and many epithelial cell types.\textsuperscript{2-5} Under normal conditions, ET-1 apparently maintains vascular tone and exerts its action in a variety of tissues and organs.\textsuperscript{6-9} Recently, through other investigations of endothelin’s pleiotropic actions, ET-1 has emerged as an important peptide in a host of biologic functions, including development, cellular proliferation, apoptosis, and cancer.\textsuperscript{10-12} Endothelin-1 sequence is highly conservative among all mammalian species and most higher vertebrates, emphasizing its central role in fundamental biologic processes. There are 2 endothelin receptors, endothelin receptor A (ET\textsubscript{A}) and endothelin receptor B (ET\textsubscript{B}), that have been identified in different mammalian tissues. They are 63% identical in amino acid sequence, but are encoded by distinct genes located in the chromosomes 4 (ET\textsubscript{A}) and 13 (ET\textsubscript{B}).\textsuperscript{13-15} Endothelin receptors are distributed in a variety of cells and tissues in different proportions, suggesting potentially opposite regulatory functions. Endothelin receptor expression is up-regulated by ischemia,\textsuperscript{16} cyclosporin,\textsuperscript{17} and interleukin-1β (IL-1β).\textsuperscript{18}

Production of endothelin or endothelin receptor knock-out mice allowed the evaluation of the role and biologic significance of different members of the endothelin family, ET-1 or ET\textsubscript{A} receptor-deficient mice had abnormalities of craniofacial tissue derived from the first pharyngeal arch. Changes were incompatible with life, and mice died of respiratory failure at birth.\textsuperscript{19} On the other hand, it appears that the ET\textsubscript{B} receptor is essential in the development of neural crest-derived cell lineages, and its deficiency produces pigmentary disorders and aganglionic megacolon, resembling human Hirschsprung disease.\textsuperscript{20-22} Because of obvious ET-1 involvement in the vasoconstriction, a number of endothelin receptor antagonists have been developed to block ET-1 interaction with its receptors, both to study the specific results of ET-1 actions and for possible therapeutic use.\textsuperscript{23,24}

One fundamental biologic process to which ET-1 has not yet been definitely linked is the development of immune responses and particularly an immune function mediated by the most effective antigen-presenting cells, dendritic cells (DCs). First described in 1973,\textsuperscript{25} these cells present as immature DCs in nonlymphoid tissue with the capacity to recognize, take up, and process antigen. After acquiring antigen, they migrate to lymph nodes and, as mature DCs, present the antigen to T cells, stimulating their proliferation and differentiation. Thus, as antigen-presenting cells, DCs play a crucial role in the development of specific immune responses and DC stimulation or suppression can effectively alter immune reactions.

ET-1 is produced by many cell types, including macrophages,\textsuperscript{30} another type of antigen-presenting cell. ET-1 affects macrophage function, promoting tumor necrosis factor α (TNF-α) production through its action on the ET\textsubscript{A} receptor,\textsuperscript{31} thus demonstrating proinflammatory function. However, there are currently no data on the relationship between ET-1 and DCs. Since ET-1 has almost ubiquitous presence in both lymphoid and nonlymphoid tissues, it is reasonable to hypothesize that it may affect DC function. The aim of this study was to examine if the endothelin axis (including ET-1 and both endothelin receptors) influences DC function, and through them, the immune response. We have demonstrated that DCs produce large amounts of ET-1 upon maturation, with concomitant up-regulation of both endothelin receptors. Functional studies showed that the modification of the endothelin axis has a profound effect on the development, function, and survival of
mature DCs. To the best of our knowledge, this is the first demonstration that DCs produce ET-1, as well as express ET receptors, and that the endothelin axis can modify DC function, with a subsequent effect on immune response.

Materials and methods

Cells

DCs were generated from CD14+ monocytes obtained from human peripheral blood of healthy volunteers, as described earlier. After gradient separation on HistoPrep medium (1.077 g/mL; Sigma, St Louis, MO), adherent cells were isolated from peripheral blood mononuclear cells on plastic (37°C, one hour) and cultured in 6-well plates in complete medium, supplemented with 1000 U/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and 1000 U/mL rhIL-4 (Cito G&M, Wexford, PA). Nonadherent DCs were harvested 7 to 8 days later and examined by flow cytometry. Cultured cells were CD14+, CD3-, CD20+, HLA-DR+, CD80+ CD86+ CD40+ and CD1a+, suggesting a common phenotype of monocyte-derived DCs. DC maturation was induced either by TNF-α, IL-1β, 50 ng/mL leupeptin; pH 7.7 at 37°C (American Peptide, Sunnyvale, CA), ET A receptor antagonists ABT-627 (Abbott Labs, Chicago, IL), and Q-B-121 (American Peptide), and ET B receptor antagonist A-192621 (Abbott Labs) were added to DCs at a final concentration of 10–7 M during the last 48 hours of culture.

ET-1 ELISA

The ET-1 enzyme-linked immunosorbent assay (ELISA) system (Biorad; Amersham Biosciences, Piscataway, NJ) was used for determination of ET-1 concentrations in cell-free supernatants. In every assay, samples and standards were run in duplicates and read at 450-nm wavelength on a Benchmark microplate reader (Bio-Rad Labs, Hercules, CA). ET-1 concentrations were normalized based on cell counts and determined by computer software–generated interpolation from the standard curve.

Immunohistochemistry

DCs were harvested and placed on microscope slides using a Cytospin centrifuge (Shandon Lipshaw, Pittsburgh, PA). Samples were air-dried overnight and fixed in ice-cold acetone. Slides were washed in phosphate-buffered saline (PBS) and incubated for one hour at room temperature with the ET A or ET B antibodies (Abbott Labs) at a dilution of 1:250. Further staining with secondary antibodies and development was performed using Vectastain ABC system (Vector Labs, Burlingame, CA), following the manufacturer’s recommendations. Negative controls included staining with irrelevant isotype-matched antibodies. Semiquantitative assessment of slides was performed by 2 authors (G.G. and M.R.S.) independently to evaluate the intensity of staining.

Radioreceptor assay

DCs were grown in 6-well plates and collected on day 7. Cells were centrifuged at 200g for 7 minutes. Pellets were resuspended in 20 mL “cold” buffer (50 mM Tris [tris(hydroxymethyl)aminomethane]-HCl, 1 μg/mL peptatin, and 1 μg/mL leupeptin; pH 7.7 at 4°C) and homogenized. Resulting suspensions were centrifuged at 60 000g for 30 minutes at 4°C. Pellets were resuspended in “warm” buffer (50 mM Tris-HCl, 1 μg/mL pepstatin, 1 μg/mL leupeptin; pH 7.7 at 37°C), kept at 37°C for 40 minutes, and then centrifuged at 60 000g for 30 minutes at 20°C. Pellets were kept at −80°C until used. To define the dissociation constant (KD) and the binding maximum (Bmax), duplicate aliquots (2 × 125 μL) of membrane suspensions were incubated with increasing concentrations (0.98 to 500 nM) of nonradioactive ET-1, and corresponding 125I–ET-1 (200 Ci/mmol [74 Tbq/mmol]; Amersham Biosciences) was added with increasing concentrations (3.9 to 2000 PM) as well. For the competitive binding studies, membrane suspensions were incubated with increasing concentrations (0 to 5 μM) of nonradioactive ET-1, ABT-627 and A-192621, and a fixed concentration (100 pM) of 125I–ET-1. Incubation was carried out for 2 hours on ice. Next, membrane suspensions were pipetted onto 25-mm diameter glass microfiber filters (GM/C filters; Whatman, Kent, United Kingdom) under the vacuum suction, and filters were washed with 10 mL cold (4°C) Hanks buffered saline solution. Filters were placed in the 5-mL vials, and the radioactivity was determined on Wallac 1470 gamma counter (PerkinElmer Life Sciences, Turku, Finland). Obtained results (in cpm) were converted to femtomole per milligram of protein using web-based radioactivity calculator software from GraphPad (San Diego, CA).

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Cells were lysed using RNA-Beep (1 mL per 5 × 106 cells), and the total RNA was extracted as per the manufacturer’s (Tel-Test, Friendswood, TX) recommendations. The amount of RNA was determined by a spectrophotometric reading at 260 nm. Synthesis of cDNA from 3 μg total RNA was carried out using Superscript Preamplification System (GIBCO BRL, Grand Island, NY) with oligo(dT) primers. Amplification of cDNA was performed with 35 cycles of PCR in a 50-μL mixture with Taq DNA Polymerase (GeneChoice, Frederick, MD) and primers at a final concentration of 0.2 μM. To confirm the quality of cDNA for PCR in every case, human β-actin was amplified using β-actin cDNA primers (5’-CCACGGCACACGGCGTGAT and 5’-TCAACATGACTCTGGTG- CAT, 262-bp product). For endothelin receptors, the following primers were used: forward, 5’-CAGTGTTGAGTTGTAAC and reverse, 5’-GGGATCACTGACCACTAG, for human ET A receptor (366-bp PCR product); forward, 5’-TCAACAGGGTGTTGTCCTG and reverse, 5’-ACTGATAGCCACCACTT, for human ET B receptor (530-bp PCR product). Each reaction cycle consisted of 1 minute at 93°C (denaturation), 1 minute at 56°C (annealing), and 2 minutes at 72°C (extension). Products of PCR were electrophoresed on 1.2% agarose gel and stained with ethidium bromide (Bio-Rad Labs) for visualization on Molecular Imager FX (Bio-Rad Labs). Obtained PCR products were sequenced using ABI model 3100 Automated DNA Sequencers by the University of Pittsburgh sequencing facility to confirm the specificity.

Gene microarray

For gene array analyses, DCs were obtained from 2 healthy volunteers and divided into 4 groups: unstimulated DCs, DCs stimulated with S aureus during the last 48 hours of culture, and DCs stimulated with S aureus and treated either with ABT-627 or A-192621 (ETA and ETB receptor antagonists, respectively) for the last 48 hours of culture. DCs were collected and snap frozen. Affymetrix chip analysis (Santa Clara, CA) was performed as described earlier. Briefly, total RNA was extracted from DCs using Trizol and Qiagen RNeasy kit (Qiagen, Valencia, CA), following the manufacturer’s recommendations. Total RNA (approximately 8 μg) was used for the synthesis of first-strand cDNA with Gibco BRL Superscript II system and T7-(dT)24 primer (5’-GGGCCAGTGAA TTGTAA TACGACTCACTA TA -3’). The second-strand cDNA synthesis was carried out at 16°C by adding Escherichia coli (E coli) DNA ligase, E coli DNA polymerase I, and Rnasenase in the reaction. The cDNA was purified through phenol/chloroform and ethanol precipitation. Double-stranded DNA was then converted into cRNA using in vitro transcription (IVT) and biotinylated nucleotides with the MEGAscript system (Ambion, Austin, TX) according to the manufacturer’s recommendations.

The IVT product was cleaned using Qiagen RNeasy columns, and 15 μg cRNA was fragmented at 95°C for 35 minutes. The fragmented cRNA was hybridized with pre-equilibrated Affymetrix chips at 45°C for 14 to 16 hours (human genome U133A set was used, containing more than 22 000 genes and expression sequence tags). The hybridizations were then washed and stained with streptavidin-phycocerythrin (SAPE) according to the manufacturer’s (Affymetrix) recommendation. The hybridized gene chips were scanned using Agilent ChipScanner (Affymetrix) to detect hybridization signals.

For analysis, hybridization data from text files were imported to a Microsoft Excel spreadsheet (Seattle, WA). To determine gene products with a significant increase of expression, we analyzed all data sets for at
least 3-fold increase in signal intensity. Obtained data were normalized using the ratio of signal intensity derived from GAPDH (glyceraldehydes-3-phosphate dehydrogenase) in comparing sets. The level of significance was set at a probability of .05.

Mixed leukocyte reaction
Allogeneic CD3+ T cells were generated using human T-cell enrichment columns (R&D Systems, Minneapolis, MN). Isolated T cells were placed in the round-bottom 96-well plates, 3 × 10^5 cells per well. DCs were stimulated either with TNF-α or S aureus, and treated with either ET-1, ABT-627, A-192621, or BQ-123 for 48 hours. DCs were then washed, resuspended in complete medium, counted using trypan blue, and added to T cells at different ratios in triplicates. The starting number of live (trypan blue negative) DCs was the same in each group. After 72 hours of incubation at 37°C, radioactive thymidine (³H-TdR; New England Nuclear, Boston, MA) was added to the DC/T-cell mixture, 1 μCi (0.037 MBq) per well. T-cell proliferation was measured by the ³H-TdR incorporation in 16 hours. Cells were harvested onto GF/C glass fiber filter paper with a semiautomated microharvester, and ³H-TdR incorporation was determined by liquid scintillation spectroscopy and expressed as cpm.

IL-12 ELISA
DuoSet ELISA development kit calibrated for human IL-12 p70 (R&D Systems) was used to determine IL-12 concentration in DC supernatants, according to the manufacturer’s recommendations. DCs were counted using trypan blue before the experiment and supernatants were collected. Samples and standards were run as duplicates in every assay, and were read at 450 nm wavelength in reference to 540 nm. IL-12 concentrations were determined by computer software–generated interpolation from the standard curve and normalized based on cell counts (per million live cells).

Assessment of apoptosis
Annexin V assay. DCs were collected and double stained with fluorescein isothiocyanate–conjugated Annexin V (PharMingen, San Diego, CA) and propidium iodide (Sigma). Annexin V was added according to the manufacturer’s recommendations. Propidium iodide was used at a final concentration of 10 μg/mL. Samples were analyzed by the FACScan flow cytometer with LYSYS II software package (Becton Dickinson, San Jose, CA).

Cell death ELISA. DCs were collected on day 8, after the 48-hour treatment with dexamethasone (10⁻⁶ M) and with or without ET receptor inhibitors, and counted. Samples (30 000 cells) were assayed for death using an ELISA kit according to the manufacturer’s specifications (Roche Diagnostics, Indianapolis, IN). This kit uses monoclonal antibodies directed against DNA and histones in a quantitative sandwich–enzyme–based format. The amount of histone-associated DNA fragments in the cell lysates was determined by a spectrophotometric reading at 405-nm wavelength. Untreated cells were used as controls.

Statistical analysis
For a single comparison of 2 groups the Student t test was used (SigmaStat Statistical Software; SPSS, Chicago, IL). If data distribution was not normal, Mann-Whitney rank sum test was used. For all analysis, the level of significance was set at a probability of .05 to be considered significant. Data are presented as the mean ± standard error of the mean (SEM). All experiments were repeated at least 3 times.

Results
ET-1 production by human DCs
In the immature state, DCs secreted little or no ET-1, as was determined by ELISA (Figure 1). Following the stimulation with either TNF-α or S aureus for 48 hours, ET-1 production by DCs increased significantly (Figure 1). ET-1 concentration in the culture medium from immature DCs was 15.3 ± 6.3 pg/mL per 10⁶ cells, whereas TNF-α–induced DC maturation resulted in a 10-fold increase in ET-1 levels in culture supernatants (170.5 ± 36.9 pg/mL per 10⁶ cells, P = .004). Similarly, ET-1 concentrations increased 36-fold (559.9 ± 172.3 pg/mL per 10⁶ cells, P = .006) when DCs were stimulated with S aureus.

Expression of endothelin receptors on human DCs
On unstimulated DCs, expression of the endothelin receptors ET A and ET B was also low, whereas stimulated DCs displayed a large increase in ET A and ET B expression (Figure 2) as was assessed by immunostaining using specific ET A and ET B receptor antibodies. We performed semiquantitative evaluation of the slides. The staining of unstimulated DCs was graded as +/+ with both ET A and ET B staining, whereas the staining of stimulated DCs was graded as + ++/+++ for ET A receptors and + +/+++ for ET B receptors. These findings were confirmed by radioceptor assay using ⁱ²⁵I–ET-1 (Figure 3). During saturation studies, receptor density on mature DCs was more than 2.5-fold higher than on immature DCs: Bmax was 760.3 ± 162.6 fmol/mg protein versus 285.8 ± 86.5 fmol/mg protein, respectively (P = .02), whereas binding affinity was the same on mature and immature DCs (Kd was 1.81 ± 0.6 nM versus 1.78 ± 0.9 nM, respectively). Competitive binding assays with ABT-627, a highly selective ET A receptor antagonist, and A-192621, a highly selective ET B receptor antagonist, were carried out to determine relative density of each receptor. Total binding was 25.29 ± 1.17 fmol/mg protein in the presence of 0.1 nM ¹²⁵I–ET-1 alone. ET A receptor density was calculated in the presence of 10⁻⁶ M A-192621 (16.78 ± 8.1 fmol/mg protein), and ET B receptor density was assessed in the presence of 10⁻⁶ M ABT-627 (9.75 ± 1.82 fmol/mg protein). Obtained results suggested approximately a 2:1 ratio of ET A to ET B receptors on mature DCs.

Effect of endothelin axis on DC maturation
Endothelin receptor inhibitors have been used to determine if there was a functional significance in the up-regulation of ET-1 production and endothelin receptor expression on mature DCs. Selective blockade of the ET A receptor with either ABT-627 or BQ-123, ET A receptor antagonists, administered together with the DC maturation
stimuli (TNF-α or S aureus) significantly reduced the expression of CD83 on DCs (from 34.71 ± 3.17 to 19.16 ± 1.13, P < .001) (Figure 4). However, blocking of ETB receptors with A-192621 induced an increase in CD83 expression (from 34.71 ± 3.17 to 48.18 ± 3.32, P = .014) on DCs. The expression of other DC markers (CD40, CD80, CD1a, CD86, and HLA-DR) was not significantly affected by ET receptor antagonists, though changes were noticeable in some cases (Table 2). Interestingly, the blockade of both ETα and ETβ receptors on stimulated DCs resulted in a statistically significant drop in the CD83 expression (23.99 ± 2.57%, P < .05) in comparison with mature DCs. It seems that in normal conditions, ET-1-ETα receptor interaction is the more prominent part of the endothelin axis. The addition of exogenous ET-1 at high concentrations (10−7 M) to cultures of immature or mature DCs had no significant effect on the expression of CD83. The administration of ET receptor antagonists (ABT-627, A-192621, BQ-123) to immature DCs without the addition of stimulation factors did not change CD83 expression on DCs. These data indicate that endogenous ET-1 production by DCs might adequately stimulate the expression of CD83 on DCs.

Regulation of DC ability to activate T cells by ET receptor antagonists

The functional consequence of DC maturation is commonly measured by their ability to stimulate T-cell proliferation in a mixed leukocyte reaction (MLR) assay. In MLRs (Figure 5A), as expected, mature DCs induced a significantly higher level of T-cell

Table 1. Gene expression changes in DCs after activation with Staphylococcus aureus

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Fold change expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased expression</td>
<td></td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>12.55</td>
</tr>
<tr>
<td>ETα receptor</td>
<td>6.24</td>
</tr>
<tr>
<td>ETβ receptor</td>
<td>10.50</td>
</tr>
<tr>
<td>Endothelin-converting enzyme 1 (ECE 1)</td>
<td>4.36</td>
</tr>
<tr>
<td>Decreased expression</td>
<td></td>
</tr>
<tr>
<td>Endothelin-2</td>
<td>4.76</td>
</tr>
<tr>
<td>Endothelin-3</td>
<td>3.84</td>
</tr>
</tbody>
</table>

48.18 ± 3.32, P = .014) on DCs. The expression of other DC markers (CD40, CD80, CD1a, CD86, and HLA-DR) was not significantly affected by ET receptor antagonists, though changes were noticeable in some cases (Table 2). Interestingly, the blockade of both ETα and ETβ receptors on stimulated DCs resulted in a statistically significant drop in the CD83 expression (23.99 ± 2.57%, P < .05) in comparison with mature DCs. It seems that in normal conditions, ET-1-ETα receptor interaction is the more prominent part of the endothelin axis. The addition of exogenous ET-1 at high concentrations (10−7 M) to cultures of immature or mature DCs had no significant effect on the expression of CD83. The administration of ET receptor antagonists (ABT-627, A-192621, BQ-123) to immature DCs without the addition of stimulation factors did not change CD83 expression on DCs. These data indicate that endogenous ET-1 production by DCs might adequately stimulate the expression of CD83 on DCs.
proliferation compared with immature DCs. ET$_A$ receptor blockade on mature DCs with either ABT-627 or BQ-123 resulted in a statistically significant reduction of their ability to induce T-cell proliferation ($P < .05$ at DC:T-cell ratios ranging from 1:2187 to 1:9), whereas ET$_B$ receptor blockade on DCs with A-192621 had no effect on antigen-presenting capacity of DCs. The addition of exogenous ET-1 to mature DCs had no significant effect on MLR results, since mature DCs produce high levels of ET-1 by themselves (Figure 1). Similarly, ET-1 or endothelin receptor antagonists did not change antigen-presenting activity of immature DCs.

**Effect of ET-1 on IL-12 production by DCs**

Production of the IL-12 by DCs has an important regulatory function on the host immune response, particularly by regulating T helper 1 (Th1)/Th2 balance. Treatment of mature DCs with ET$_A$ receptor antagonist ABT-627 significantly reduced $S$ aureus-induced IL-12 production from 584.8 ± 152.2 pmol/mL per 10$^6$ cells to 179.8 ± 77.4 pmol/mL per 10$^6$ cells ($P < .05$) (Figure 5B). However, neither exogenous ET-1 nor ET$_B$ receptor antagonist A-192621 had any significant effect on the induction of IL-12 secretion by mature DCs.

**Modulation of DC survival by endothelin axis**

In many cell types, ET-1 is both a mitogenic and an antiapoptotic factor primarily binding to the ET$_A$ receptor. Using either dexamethasone ($10^{-6}$ M) or serum starvation to induce apoptosis in DCs, we have shown that blockade of ET$_A$ receptors by ABT-627 or BQ-123 resulted in increased levels of DC apoptosis, as measured by both Annexin V binding ($P < .05$) and the cell death ELISA assay ($P = .002$) (Figure 6A and 6B, respectively). Interestingly, blockade of ET$_B$ receptors on DCs with A-192621 led to an increased resistance of DCs to the dexamethasone-induced apoptosis, as assessed by Annexin V binding assay ($P = .002$) and cell death ELISA ($P < .001$).

**Regulation of gene expression in DCs by endothelin axis**

We have analyzed gene expression in DCs using human genome U133A set from Affymetrix. Comparison of gene expression was first made between immature DCs and mature DCs stimulated with $S$ aureus. Next, we analyzed changes in gene expression in mature DCs after blocking ET$_A$ or ET$_B$ receptors with selective antagonists ABT-627 and A-192621, respectively. Signal intensity is the GeneChip (Affymetrix) parameter indicating the level of gene expression. Fold change of the same gene was obtained by comparing signal intensity between different sets of the DCs from the same individual. The results of these studies are presented in Tables 1, 3, and 4. As shown, blocking of ET$_A$ receptors on DCs led to the increased expression of many proapoptotic genes, including genes for several caspases. In addition, down-regulation of the genes involved in the generation of type I immune response (interferon, IL-12, major histocompatibility complex [MHC] class II molecules) was also seen after ET$_A$ receptor blockade on DCs. In contrast, ET$_B$ receptor blockade on DCs was associated with the down-regulation of proapoptotic genes, for instance, serine/threonine kinase 17a, bcl2-like protein 11, and others. Thus, the analysis of gene expression on DCs confirmed our data demonstrating the
up-regulation of ET-1 and endothelin receptors on DCs during maturation, and the influence of endothelin axis on the function and survival of mature DCs.

Discussion

After being discovered as a potent vasoconstrictor, ET-1 was later demonstrated to possess a wide range of pleiotropic functions as well.11,12,25,40 This includes the regulation of basic cell functions such as cell survival, proliferation, and matrix remodeling. Endothelin-1 action is mediated by the ET_A and ET_B receptors.13-15 While so-called “positive” ET-1 actions (vasoconstriction, improved survival, mitogenic action) are mediated primarily through the ET_A receptors, the activation of the ET_B receptors counteracts these activities in many cases, such as inducing vasodilation through nitric oxide (NO) release.41

We have shown here that DCs produce large amounts of ET-1 after stimulation with either TNF-α/H9251 or inactivated S aureus, with concomitant increase in the expression of ET_A and ET_B receptors. The range of ET-1 production by DCs after their stimulation with S aureus is wide, but the coefficients of variation (standard deviation/mean) were similar in both stimulated groups (0.53 for TNF-α/H9251 and 0.62 for S aureus), indicating that variability is consistent across treatments. The wide ET-1 concentration range with S aureus treatment may be explained by the nonspecificity of its action and individual response of DCs derived from different individuals.

Table 3. Gene expression changes in mature DCs after treatment with ABT-627, an endothelin A receptor antagonist

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Fold change expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased expression</td>
<td></td>
</tr>
<tr>
<td>Caspase-5</td>
<td>19.60</td>
</tr>
<tr>
<td>IL-10</td>
<td>6.25</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>5.20</td>
</tr>
<tr>
<td>PS3 regulated apoptosis inducing protein mRNA</td>
<td>5.03</td>
</tr>
<tr>
<td>Death receptor 3</td>
<td>3.90</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>3.89</td>
</tr>
<tr>
<td>Apoptotic protease activating factor 1 (ARAF 1)</td>
<td>3.57</td>
</tr>
<tr>
<td>Caspase recruitment domain protein 10 mRNA</td>
<td>3.28</td>
</tr>
<tr>
<td>Decreased expression</td>
<td></td>
</tr>
<tr>
<td>Interferon-α1</td>
<td>19.79</td>
</tr>
<tr>
<td>Mature T-cell proliferation 1 protein mRNA</td>
<td>6.50</td>
</tr>
<tr>
<td>IL-12 A</td>
<td>4.28</td>
</tr>
<tr>
<td>IL-12 receptor</td>
<td>4.01</td>
</tr>
<tr>
<td>MHC class II</td>
<td>3.72</td>
</tr>
</tbody>
</table>

Table 4. Gene expression changes in mature DCs after the treatment with A192621, an endothelin B receptor antagonist

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Fold change expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased expression</td>
<td></td>
</tr>
<tr>
<td>IL-15</td>
<td>19.20</td>
</tr>
<tr>
<td>Interferon-ω8</td>
<td>11.76</td>
</tr>
<tr>
<td>IL-12 receptor α</td>
<td>6.80</td>
</tr>
<tr>
<td>Interferon-ω F</td>
<td>5.18</td>
</tr>
<tr>
<td>IL-2 receptor α</td>
<td>3.14</td>
</tr>
<tr>
<td>Decreased expression</td>
<td></td>
</tr>
<tr>
<td>Bcl2-like 11 (apoptosis facilitator) protein mRNA</td>
<td>6.29</td>
</tr>
<tr>
<td>Programmed cell death 4 protein mRNA</td>
<td>4.39</td>
</tr>
<tr>
<td>Serinethreonine kinase 17α (apoptosis inducing)</td>
<td>4.02</td>
</tr>
<tr>
<td>Caspase-10d</td>
<td>3.40</td>
</tr>
</tbody>
</table>
volunteers. The mechanisms of ET-1 production from DCs upon stimulation will be the subject of future studies. Addition of an exogenous ET-1 to immature or mature DC cultures had no significant effect on DC function. Immature DCs express low levels of endothelin receptors, apparently insufficient to significantly impact their function following the ET-1 binding. On the other hand, mature DCs had high numbers of endothelin receptors and appear to be sufficiently stimulated with the DC-derived endogenous ET-1, explaining why the addition of exogenous ET-1 to DC culture did not significantly change DC phenotype or function. The large increase in both ligand (ET-1) and receptor (ETα, ETβ) expression occurring with DC maturation indicates the potential for a functional autocrine loop: DCs produce abundant ET-1 that binds to greater numbers of endothelin receptors. Blockade of the endothelin receptors had a profound effect on mature DCs. We observed a significant decline in the expression of CD83 with the blockade of ETα receptors. It is known that CD83 is the marker of mature DCs and that CD83+ cells are the most effective stimulators in allogeneic MLRs.42 Recent studies also demonstrated that CD83 is not just a DC marker, but probably has immunostimulatory as well as regulatory effects.43 and it is possible that decreased expression of CD83 by itself might affect DC function. We have observed some changes in the expression of other costimulatory molecules as well (Table 2). Although these differences were not statistically significant, they might have induced biologic effects. In addition, it is possible that proapoptotic features of the ETα receptor antagonists have contributed to the observed effects as well.

Another DC function affected by ETα receptor blockade was IL-12 production. IL-12 increases proliferation and activity of natural killer and T cells, and plays a central role in promoting type 1 T helper cell (TH1) responses. Its production by antigen-presenting cells seems to be essential for host defense against intracellular microbial infections and control of malignancy.44–47 and alterations in its production may affect the competence of the immune system. Our data demonstrated significant decrease in the IL-12 production by mature DCs with the blockade of the ETα receptor, suggesting the involvement of the ET-1/ETα loop in the generation of TH1 responses.

ET-1 is a known survival factor for many cell types, acting mainly through the ETα receptor.1,11,38,40,48,49 We observed a decreased resistance of mature DCs to apoptotic stimuli (high-dose dexamethasone or serum starvation) when ETα receptor was blocked, meaning that ETα receptor activation is protective and antiapoptotic in DCs. The role of the ETβ receptor so far is not well studied. In general, it is believed that ETβ receptor activation leads to cell differentiation and apoptosis.50 but in some cell types ETβ was found to promote cell survival.51,52 In our experiments, ETβ receptor blockade in the presence of agonist (endothelial ET-1) resulted in increased resistance of DCs to apoptotic stimuli. Thus, ETβ receptor activation on DCs seems to promote their apoptosis, and might play an important role in abolishing the specific immune responses.

Based on the loss of function associated with the administration of ETα receptor antagonists, it appears that ETα receptor stimulation improves DC survival, and mediates antigen-presenting T-cell–stimulating and IL-12–producing features of DCs. These alterations in DCs are consistent with an immunostimulating role of ETα. These findings were further supported by gene array studies, demonstrating the up-regulation of proapoptotic genes associated with the ETα receptor blockade and down-regulation of proinflammatory cytokines (Table 3). Gene array data obviously need to be confirmed with additional experiments, but changes in the expression levels of several genes in pathways involving apoptotic and immunostimulatory factors warrant further studies.

We have shown here that the blockade of ETα receptors improved survival and increased CD83 expression on DCs. It seems that observed ETα-mediated effects on DCs are the opposite of those mediated by the ETβ receptor. This pattern of cell regulation was also seen in other endothelin-driven functions. The results of gene array experiments also demonstrated decreased expression of several proapoptotic genes (Table 4) in DCs after the blockade of ETα receptors. These divergent effects of ETα on DC function provide 2 means for potential immunomodulation, depending on receptor subtype targeted: blocking of ETα receptors may be immunosuppressive, whereas blocking of ETβ receptors may be stimulatory for DCs.

The exact mechanisms of ET-1 action on DCs still need to be elucidated. Although the outcome of some experiments may be thought to be the result of increased DC apoptosis due to ETα receptor blockade (for example, CD83 expression), in most cases experiments were performed with the same number of live cells (MLR), or results have been normalized according to the number of counted live cells (IL-12 production). Still, due to apparent enhanced apoptosis of DCs with ETα receptor blockade, this possibility needs to be always kept in mind, with some monitoring of the number of live DCs before the start of experiment.

Another possible mechanism of ET influence on DCs worth exploring is the involvement of NO. As we have mentioned, ETβ receptor activation was found to increase NO synthesis in endothelial cells.41 Other studies also demonstrated the change in NO level with the stimulation or blockade of different ET receptors, with ETα receptor activation or ETβ receptor blockade leading mostly to the decreased NO levels,53–54 while ETβ receptor stimulation was accompanied with increased NO synthesis.55 NO was also found to interfere with the ET-1/ETα–mediated increased cell proliferation.56 Regarding DCs, it is known that NO may be involved in DC apoptosis57,58 and may have a role in DC-mediated T-cell proliferation.59,60 Further experiments should clarify the role of NO in ET-1–induced DC modifications.

Administration of ETα receptor antagonist in rats during the experimental renal transplantation improved graft survival.61,62 which was mainly attributed to the probable vasodilating features of the ETα receptor antagonists. It is possible, however, that improved graft survival was due to the ETα receptor blockade on DCs, accompanied by the suppression of their T-cell–stimulating capacity. In other studies, administration of BQ-123, an ETα receptor antagonist, inhibited eosinophil and mononuclear cell migration, due to the inhibition of CD4+ and CD8T lymphocytes.53 We failed to identify endothelin receptors on T cells by immunohistochemical staining (data not shown), leading us to speculate that the inhibition of T cells might be mediated by DC suppression due to blockade of ETα receptors. However, this hypothesis requires further experimental confirmation.

In conclusion, our data showed for the first time that human DCs produce ET-1 and express functional ETα and ETβ receptors, which are up-regulated during DC maturation. We demonstrated that ET-1 production and ETα activation play an important role in the activity of human DCs. The observed ability of ETα receptor antagonists to interfere with the behavior of DCs and suppression of DC-mediated immune response should be further explored, both experimentally and clinically, including many areas of possible DC-based therapies, ranging from cancer to transplantation.64,69 Likewise, the counter-regulatory activities of ET-1/ETα and ET-1/ ETβ autocrine or paracrine loops might provide a new means to modulate DC function, such as increase in cell survival, cytokine production, and T-cell activation. The wide availability of selective ET receptor antagonists should accelerate these investigations.
References


34. Bonham CA, Lu L, Li Y, Hoffman RA, Simmons RL, Thomson AW. Nitric oxide production by...


Function and survival of dendritic cells depend on endothelin-1 and endothelin receptor autocrine loops

Georgi Guruli, Beth R. Pflug, Stefana Pecher, Valeria Makarenkova, Michael R. Shurin and Joel B. Nelson