

Chemokine- and cytokine-induced expression of endothelin 1 and endothelin-converting enzyme 1 in endothelial cells

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Background: Endothelin 1 (ET-1) is a product of endothelial and many other cell types that possesses a wide range of actions, including vasoconstriction, bronchoconstriction, and mitogenic activity on smooth muscle cells and fibroblasts. ET-1 release and expression is induced in several disease conditions associated with inflammation and cellular injury.

Objective: The purpose of this study is to determine the effects of α -chemokines (IL-8 and melanoma growth-stimulating activator), β -chemokines (monocyte chemoattractant protein 1, macrophage inflammatory protein 1 α [MIP-1 α], MIP-1 β , and RANTES), and proinflammatory cytokines (IL-1 β , TNF- α , and IFN- γ) on the expression of both ET-1 and endothelin-converting enzyme 1 (ECE-1) by human umbilical vein endothelial cells.

Methods: Subconfluent monolayers of human umbilical vein endothelial cells were incubated with each chemokine individually for 24 hours or with a mixture (cytomix) of TNF- α , IL-1 β , and IFN- γ for 6 and 24 hours.

Results: Incubation with the α -chemokines melanoma growth-stimulating activity and IL-8 did not significantly increase ET-1 and ECE-1 messenger (m)RNA expression and had no effect on ET-1 release. Monocyte chemoattractant protein 1 exerted the most potent increase in ET-1 and ECE-1 mRNA and ET-1 release among all chemokines studied ($P < .05$). MIP-1 α and RANTES exerted a moderate, but significant, increase on the ET system ($P < .05$). The cytomix resulted in a significant increase in ET-1 and ECE-1 mRNA expression ($P < .05$). **Conclusion:** These data demonstrate that, like cytokines, chemokines can induce endothelial ET-1 and ECE-1 in vitro and suggest a possible role for these inflammatory mediators in the induction of the ET system in inflammatory and vascular diseases. (J Allergy Clin Immunol 2000;105:333-8.)

Key words: Endothelin, endothelin-converting enzyme 1, endothelial cells, Northern blot, messenger RNA, chemokines, cytokines, radioimmunoassay, human

Abbreviations used

EC:	Endothelial cell
ECE:	Endothelin-converting enzyme
ET:	Endothelin
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
HUVEC:	Human umbilical vein endothelial cell
MCP:	Monocyte chemoattractant protein
MGSA:	Melanoma growth-stimulating activity
MIP:	Macrophage inflammatory protein

The synthetic state of the vascular endothelium is prone to changes in blood flow and to insults by inflammatory cells, which often result in increased adhesion, transendothelial migration, subsequent chemotactic movement, and remodeling of vessel wall or extravascular tissue. One of the numerous mediators released by inflammatory cells are chemokines. The latter have been divided into 4 subfamilies, α (or CXC), β (or CC), γ (or C), and δ (or CX₃C), according to their amino acid sequences. The most extensively studied are members of the α - and β -chemokines. Although members of the α -family are predominantly chemotactic for neutrophils, members of the β -family are chemotactic for monocytes, lymphocytes, and eosinophils. Beside their chemotactic properties, α - and β -chemokines have been shown to possess other stimulatory activities, including induction of numerous inflammatory and growth mediators and regulation of the expression of cell adhesion molecules on the cell surface.¹⁻⁵ Despite the well-established effects of chemokines on several cell types, their effects on the expression, release, or both of endothelial-derived substances remain to be elucidated. Endothelin 1 (ET-1) is a potent vasoconstrictor peptide produced by vascular endothelium with a wide range of actions, including induction of monocyte chemoattractant protein 1 (MCP-1) and IL-8.^{6,7} ET-1 is first synthesized as a precursor, the pre-pro-ET, which is processed in pro-ET or big ET by proteases.^{8,9} The endothelin-converting enzyme (ECE) catalyzes the specific cleavage of the biologically inactive big ET to produce the active ET.¹⁰ ETs constitute a family of 3 peptide isoforms (ET-1, ET-2, and ET-3) of 21 amino acids each, but only ET-1 is synthesized by endothelial cells (ECs) in response to different stimuli.¹¹ Among them, TNF- α and IL-1 β were recently demonstrated to cause a concentration-dependent increase in

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ET-1 release by bovine aortic ECs.¹² ET acts on 2 types of receptors: the ET-A receptor, located mainly on smooth muscle cells, and the ET-B receptor, expressed mainly on ECs.^{13,14} Besides vasoconstriction, induction of endothelial ET-1 can cause cellular proliferation and enhance vascular permeability.^{15,16} ET-1 can also modulate the activation of inflammatory cells,¹⁶ and it induces the accumulation of leukocytes in pulmonary circulation.¹⁷ Moreover, ET-1 upregulates the expression of intercellular cell adhesion molecule and vascular cell adhesion molecule, and it induces the expression of E-selectin on ECs.¹⁸ The aim of the present study was to determine the effects of chemokines (IL-8 and melanoma growth-stimulating activity [MGSA]/Gro α for the α -family; MCP-1, macrophage inflammatory protein 1 α [MIP-1 α], MIP-1 β , and RANTES for the β -family) on the synthesis of ET-1 and ECE-1 by human umbilical vein endothelial cells (HUVECs). We also examined the effects of inflammatory cytokines (ie, TNF- α , IL-1 β , and IFN- γ) on the same system.

METHODS

EC culture

HUVECs were purchased from Clonetics (San Diego, Calif) in the second passage and cultured in 100-mm culture plates by using EC growth medium (Clonetics), which contained epidermal growth factor (10 ng/mL), hydrocortisone (1 μ g/mL), FBS (2%), gentamicin (50 μ g/mL), and amphotericin B (50 ng/mL) and was supplemented with bovine brain extract (3 mg/mL) and heparin (10 μ g/mL), at 37°C in an atmosphere of 5% CO₂ and 95% air. HUVECs that were immortalized by transfection of E6E7 human papilloma virus were also used. Cells (5×10^5 cells/plate) of 4 to 6 passages were used for experiments and incubated with cytokines or chemokines in 6 mL of medium in the absence of hydrocortisone.

Cytokine and chemokine treatment

HUVECs were incubated with the mixture of TNF- α (10 ng/mL), IL-1 β (20 U/mL), and IFN- γ (100 U/mL) for 6 and 24 hours. Stimulation of cells by chemokines was carried out by using IL-8, MGSA, MCP-1, MIP-1 α , MIP-1 β , and RANTES individually in a concentration of 100 ng/mL for 24 hours. Our previous investigations showed that incubation with 100 ng/mL of the above chemokines for 24 hours resulted in the highest level of ET-1 induction; we therefore used this constant dose throughout the study (unpublished observations). All cytokines and chemokines were obtained from DNAX (Paolo Alto, Calif).

Northern blot analysis

HUVECs from normal and transfected cell lines were homogenized, and total RNA was extracted by using the phenol-chloroform method.¹⁹ The RNA samples were electrophoresed in 1% agarose gel containing 5% formaldehyde and transferred to Hybond-N membrane (Amersham, Oakville, Ontario, Canada). Membranes were hybridized with ³²P-labeled ET-1 and ECE-1 complementary (c)DNA probes and exposed for autoradiography with an intensifying screen. The membranes were hybridized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe for standardization.

Reverse-phase HPLC of endothelin peptides

Samples were injected into a C₁₈ reverse-phase HPLC column (Ultrasphere ODS 5 μ m, 4.8 $\times$ 260 mm; Beckman) that was equili-

brated with 18% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid and maintained at 40°C. The column was eluted at a flow rate of 1 mL/min with an 18% to 30% linear gradient of acetonitrile in 0.1% trifluoroacetic acid over 12 minutes, followed by a 30% to 36% linear gradient over an additional 12 minutes. Peptides were detected by UV absorbency at 210 nm. Synthetic ET-1 (1-21), big ET-1 (1-38), and big ET-1 (22-38) were eluted at 23, 21, and 8.5 minutes, respectively. To confirm chemical authenticity of immunoreactivity in culture supernatants, the conditioned medium was pre-extracted with a Sep-Pack C₁₈ cartridge before HPLC, as described elsewhere.²⁰ HPLC fractions (0.5 mL) were collected and evaporated to dryness under vacuum. Dried residue from each fraction was reconstituted in an enzyme immunoassay buffer and subjected to big and mature ET-1 assay.²⁰ We consistently recovered greater than 90% of the immunoreactive big and mature ET-1 contained in the original medium as single peaks eluted at 21 and 23 minutes, respectively. No other immunoreactive peaks were detected.

Statistical analysis

The results are shown as mean $\pm$ SEM percentage increases in messenger (m)RNA expression, and ET-1 release was expressed in picomoles. Comparison between the different chemokines and baseline measurement was done by using the Kolmogorov-Smirnov 2-sample test. Probability (*P*) values of less than .05 were considered significant.

RESULTS

Effect of α - and β -chemokines on ET-1 release and mRNA expression

All the chemokines were incubated with the ECs under the same conditions, and the results of 4 sets of experiments are shown in Figs 1 and 2. The densitometric measurements of ET-1 mRNA in chemokine-treated HUVECs shown in Fig 2, A, were normalized to GAPDH. The α -chemokines did not induce a significant increase in ET expression or release. MGSA induced an 82% increase in ET-1 mRNA expression. IL-8 had little effect on ET-1 mRNA expression by HUVECs (14% increase, Fig 2, A). The β -chemokine MCP-1 exerted the most potent increase (*P* < .05) in ET-1 mRNA expression among all chemokines included in this study. MIP-1 α and RANTES significantly increased endothelial ET-1 mRNA expression (*P* < .05). MIP-1 β induced a slight increase in ET-1 mRNA expression.

ET-1 release was assessed by reverse-phase HPLC in the supernatant of EC cultures, and the results are shown in Fig 1. ET-1 was spontaneously released by HUVECs in a time-dependent manner (176.0 and 285.2 pmol/L after 6 and 24 hours of incubation, respectively). The α -chemokines IL-8 and MGSA did not induce ET-1 release. In contrast, MCP-1 induced a significant increase in ET-1 release in the cultured medium (*P* < .05). MIP-1 β , MIP-1 α , and RANTES had little or no effect on ET-1 release.

Effects of α - and β -chemokines on ECE-1 mRNA expression

The densitometric measurements of ECE-1 mRNA in chemokine-treated HUVECs (Fig 3) were normalized to

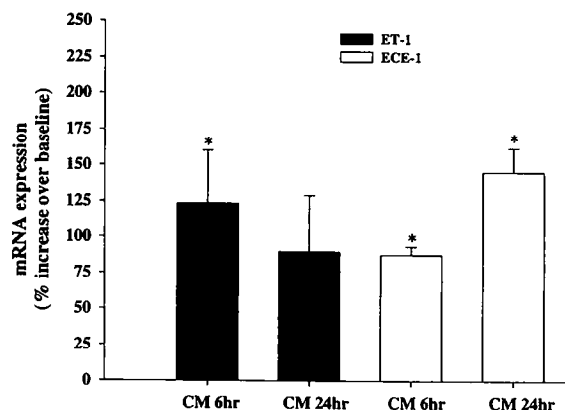


FIG 1. Effects of chemokines on the release of ET-1 by HUVECs as shown by reverse-phase HPLC. Co, Control; CM, cytomix. * $P < .05$ for chemokines versus controls at 24 hours.

GAPDH (Fig 2, B). Although both α -chemokines induced a small increase in endothelial ECE-1 mRNA expression, only IL-8 reached statistical significance ($P < .05$; Fig 2, B). Among the β -chemokines investigated, MCP-1 was still the most potent stimulant of ECE-1 mRNA expression by HUVECs. RANTES and MIP-1 β induced a small but significant increase in ECE-1 expression (Fig 2, B). MIP-1 α -induced ECE-1 mRNA expression did not reach statistical significance.

Effect of the cytomix on ET-1 and ECE-1 mRNA expression

ET-1 and ECE-1 mRNA were constitutively expressed by HUVECs, as shown in Fig 1. ET-1 mRNA appeared as a 2.3-kb fragment. ECE-1, a 4.7-kb mRNA, is expressed abundantly, whereas, as expected, a 3.1-kb fragment is shown in a smaller quantity. The densitometric measurements of ET-1 and ECE-1 mRNA (4.7 Kb) in cytokine-treated HUVECs were normalized to GAPDH (Fig 4). The cytomix significantly increased ET-1 mRNA expression by HUVECs ($P < .05$, Figs 1 and 4) and appeared to be effective as well on ECE-1 mRNA expression by HUVECs ($P < .05$, Figs 1 and 4).

DISCUSSION

In this study we have determined the in vitro effects of chemokines and cytokines on the mRNA expression of both ET-1 and its converting enzyme by HUVECs. We have shown that expression of ET-1 and ECE-1 mRNA in HUVECs was increased after incubation with α - and β -chemokines. The latter had similar effects on ET-1 release in the cultured media of ECs. We have also shown that a mixture of the 3 proinflammatory cytokines TNF- α , IL-1 β , and IFN- γ was not only able to increase the mRNA expression of ET-1 but also that of ECE-1. The study provides several novel findings, the most significant of which is a stimulatory role for chemokines on the vascular endothelium. To our knowledge, the current report is the first to demonstrate chemokine activation of

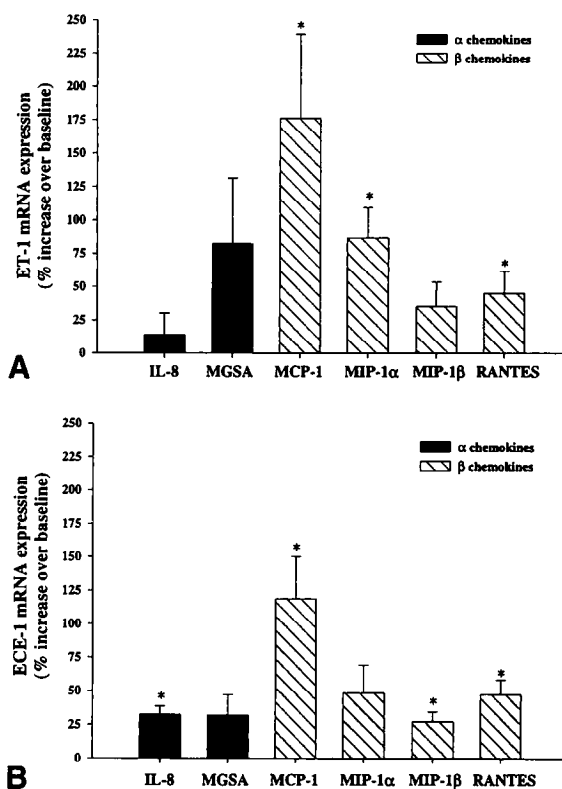


FIG 2. The densitometric measurements of ET-1 (A) and ECE-1 (B) mRNA in α - or β -chemokine-treated cells were normalized to GAPDH. The results are shown as mean $\pm$ SEM percentage increases in mRNA expression compared with control unstimulated cells. The reading of 0 for the y-axis represents baseline measurement. * $P < .05$.

ECs. Such a finding suggests a role for these mediators in the induction of the ET system in vascular and inflammatory diseases.

In the present report we have investigated the effects of α -chemokines (IL-8 and MGSA/Gro α) and β -chemokines (MCP-1, MIP-1 α , MIP-1 β , and RANTES) on ET-1 and ECE-1 mRNA expression by ECs. The effects of the α - and β -chemokines on ET-1 mRNA expression and release were superposable for all chemokines except MGSA. The latter induced an 82% increase in ET-1 mRNA expression but only a 32% increase in ECE-1 accompanied by no effect on ET-1 release. Lack of its effect on ET-1 release may be explained by its failure to induce a significant increase in ECE-1 expression. MCP-1 was the most potent molecule to induce endothelial ET-1 production. These results suggest the involvement of 2 different pathways for chemokines in the EC activation for ET-1. Of particular interest, a preferential action was noted for the 2 structurally close molecules MIP-1 α and MIP-1 β ; although MIP-1 α induced a moderate increase of the ET-1 mRNA expression and release and ECE-1 expression, MIP-1 β exerted a relatively weaker stimulatory effect on these molecules. Such differences between the activities of MIP-1 α and MIP-1 β have been previously reported for

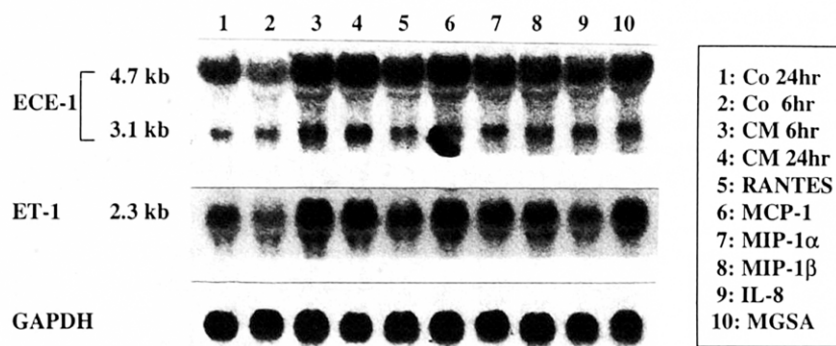


FIG 3. Northern blot analysis of HUVECs showing ECE-1, ET-1, and GAPDH mRNA in control (*Co*) and in cytotoxic (*CM*) or chemokine-treated cells. The cytotoxic comprised TNF- α (10 ng/mL), IL-1 β (20 U/mL), and IFN- γ (100 U/mL).

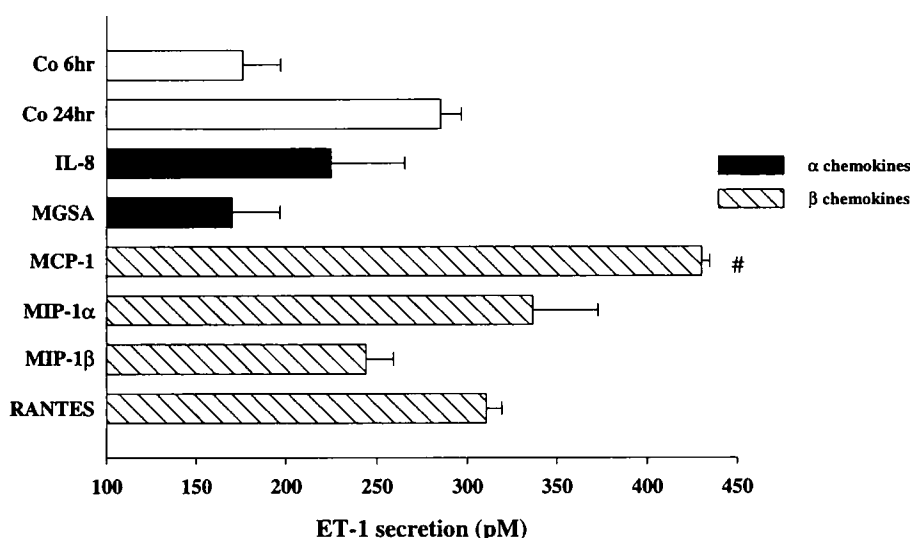


FIG 4. The densitometric measurements of ET-1 and ECE-1 mRNA in cytotoxic-treated HUVECs were normalized to GAPDH. The reading of 0 for the y-axis represents baseline measurement. * $P < .05$ for cytokine treatment after 6 and 24 hours versus baseline measurement. *Co*, Control.

chemotaxis.²¹ It is easy to understand why α - and β -chemokines allowed 2 different observations because they are active on distinct receptors (CXCR and CCR, respectively). Some of the CC chemokine receptors were shown to be shared by several chemokines (CCR-1 and CCR-5 for MIP-1 α , MIP-1 β , and RANTES), whereas others were reported to be more specific (CCR-2 for MCPs). Further investigations are required to clarify which CC chemokine receptor is involved in our system to explain the predominant action of MCP-1 among the β -chemokine family.

ET-1 and chemokines are released from ECs into the bloodstream, where their chemotactic action allows leukocytes to migrate through vessel walls. In addition to their chemotactic properties, chemokines also contribute to the development of inflammatory processes.^{22,23} Furthermore, MCP-1, MIP-1 α , or RANTES modulate the expression of adhesion molecules and induce the release of several mediators by neutrophils²⁴ and activated eosinophils.²⁵ As previously mentioned, β -chemokines

were demonstrated to be preferentially effective on monocytes, lymphocytes, and eosinophils, which constitute the main cell population in a variety of diseases. Interestingly, the ET system is thought to play an important role in diseases associated with induction of these inflammatory cells and adhesion molecules, mainly asthma^{16,26} and atherosclerosis.²⁷ Consequently, the preferential activation of ECs by β -chemokines for the ET-1 production is also of particular interest in the context of these diseases.

Induction by common mediators of both ET-1 and ECE-1 expression in the same cell population is probably of significant importance for biologic activities of ET-1. In a recent study we demonstrated induction of both ET-1 and ECE-1 in endothelial, epithelial, and inflammatory cells of lungs of patients with active-stage idiopathic pulmonary fibrosis.²⁸ ECs, like other cells, are an important source of some chemokines (IL-8, MGSA, MCP-1, and RANTES), which are synthesized in response to inflammatory mediators, such as TNF- α , IL-1, and IFN- γ .²⁹⁻³¹

We suggest that these chemokines exert an autocrine or paracrine amplification process for the ET-1 and ECE-1 mRNA expression, particularly MCP-1, which seemed to be the most potent chemokine among those investigated in our study. Interestingly, previous reports have shown induction of MCP-1 in ECs in response to ET-1 stimulation⁷ and its upregulation in several disease conditions associated with ET induction.³² These findings suggest that expression of ET-1 and chemokines may be interrelated in both endothelial and also other cell types.

The effects of cytomix on ET-1 release and expression observed in this study were not unexpected because individual cytokines in the cytomix have previously been shown to induce ET-1.^{12,16} What is interesting is the finding that these same cytokines have similar effects on ECE-1 expression by ECs. Indeed, we observed a constitutive expression of ECE-1, which was induced after cytomix activation. We have previously shown constitutive expression of ECE-1 in bovine aortic ECs and its wide distribution in most organs.³³ More recently, we reported significant induction of the enzyme in patients with chronic rhinitis³⁴ and idiopathic pulmonary fibrosis,²⁸ two diseases associated with increased ET-1 expression. Moreover, we reported increased ET-1 and ECE-1 mRNA expression in human bronchial epithelial cells after induction with the same cytokines used in the present study.²⁸ This is consistent with the current findings and suggests that inflammatory cytokines induce similar effects on ET-1 and its converting enzyme in different cell types. The mechanism of ET-1 or ECE-1 induction in response to these cytokines remains to be elucidated.

In summary, our data demonstrate for the first time that chemokines can activate ECs in vitro. This is evident by chemokine activation of 2 endothelium-derived substances with potent actions on the vessel homeostasis and structure. Evidently MCP-1 exerted the strongest effects on ET and ECE-1 expression; however, this does not rule out possible stimulatory or inhibitory effects for other chemokines on other EC-derived factors. In addition, we demonstrated that, like ET-1, cytokine activation of ECs induces the expression of ECE-1. These findings provide new insight into the role of chemokines on the vascular endothelium and into the mechanism of ET-1 production by ECs.

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