A novel bioactive 31-amino acid ET-1 peptide stimulates eosinophil recruitment and increases the levels of eotaxin and IL-5

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Abstract. Objective and design: Investigation of the role of a novel inflammatory mediator 31-amino acid endothelin-1 [ET-1(1-31)], a major ET derivative in granulocytes, in eosinophil recruitment after its subcutaneous administration to mice.

Methods: Various ET-1 derivatives (100 pmol), with or without ET receptor antagonists (200 pmol), were administered subcutaneously to mice, and then the eosinophil migration into and chemokine levels in the injected loci were analyzed.

Results: ET-1(1-31) and a 21-amino acid endothelin-1 (ET-1), but not big ET-1, induced eosinophil migration into the injected loci with a peak after administration for 12 h, and increased the levels of eotaxin and interleukin-5 with peaks at 6 and 24 h, respectively. These effects of ET-1(1-31) and ET-1 were significantly inhibited by an ETA receptor antagonist, BQ-123, but not by an ETB receptor antagonist, BQ-788.

Conclusion: Novel bioactive ET-1(1-31) induces local eosinophil recruitment, and increases in eotaxin and interleukin-5 through an ETA or ETA-like receptor.

Key words: Endothelin – Eosinophil – Eotaxin – IL-5

Introduction

The endothelins (ETs), a family of 21-residue peptides, were first isolated from the culture medium of porcine endothelial cells and were shown to be vasoconstrictors [1, 2]. Three forms of the peptides have been characterized, termed ET-1, -2, and -3. Recent studies indicated that ETs are found in various cell types [3], and have a variety of physiological and pathological functions, not only in smooth-muscle constriction [4, 5] but also as inflammatory mediators [6–9]. In asthmatic patients, elevated levels of immunoreactive ET in bronchoalveolar lavage fluid [10, 11] and the airway epithelium [12], and infiltration of eosinophils and neutrophils into the bronchioles and lungs have been reported, suggesting a pathophysiologic role for ET in allergic inflammation.

Recently, the bioactive ET family has expanded: novel, smooth muscle-constricting 31-amino acid ETs, which are generated from big ETs through specific cleavage of the Tyr10-Gly11 bond by human mast cell chymase or other chymotrypsin-type proteases in various cells, have been found by our group [13, 14], and Hanson et al. [15]. Among the ET derivatives in human neutrophils, ET-1(1-31) is the predominant bioactive peptide [16], and exhibits chemotactic activity toward human neutrophils and monocytes in vitro [9]. The activity of ET-1(1-31) is not the consequence of conversion to the corresponding 21-amino acid ET-1 by phosphoramidon-sensitive ET-converting enzymes or metalloendopeptidases [13, 14, 17–19]. Although pharmacological analyses of the effects of ETs(1-31) on vascular and tracheal smooth muscle constriction [13, 17], calcium signaling [18, 19], and the chemotactic effects in vitro on human leukocytes [9] have been performed, the effect of ET-1(1-31) on the infiltration of eosinophils, one of the hallmarks of loci of allergic inflammation, has not been examined so far.

To better understand the contribution of ET-1(1-31) to infiltration of eosinophils, in this study we analyzed the effects of ET-1(1-31) and related ET derivatives on eosinophil recruitment and the levels of chemokines, such as eotaxin, interleukin-5 (IL-5), and RANTES (regulated on activation, normal T cells expressed and secreted), in the loci after the subcutaneous administration of ET derivatives. We also analyzed the effects of selective ET(1) receptor antagonist BQ-123 and ET(2) receptor antagonist BQ-788 on these biological functions of ET-1(1-31) and ET-1, and discussed the mechanism of action of ET-1(1-31).

Materials and methods

Reagents

Human ET-1, ET-1(1-31), big ET-1 and phosphoramidon were purchased from the Peptide Institute (Osaka, Japan). BQ-123 [cyclo-(D-
Trp-D-Asp(ONa)-Pro-D-Val-Leu) and BQ-788(N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-MeLeu-D-Trp(COOMe)-D-Nle-Ona) were obtained from Calbiochem Novabiochem (La Jolla, CA), and ELISA kits for mouse eotaxin, RANTES and IL-5 were purchased from TECHNE Corp. (Minneapolis, MN), and one for ET-1(1-31) from Immunobiological Laboratories (Gumma, Japan). All other reagents were commercial products of the highest grade available.

**Animals**

Male BALB/c (7-week-old), C3H/HeN (8-week-old), and IL-5 transgenic C3H/HeN (8-week-old) mice were used. The animals had free access to food and water, and were kept in a room with controlled temperature (23 ± 2°C) and lighting. All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain [20].

**Experimental design, staining of tissue eosinophils and counting**

ET-1, ET-1(1-31), or big ET-1 (1-200 pmol), with or without an inhibitor of ET-converting enzyme, phosphoramidon (1 nmol), and BQ-123 or BQ-788 (200 pmol) in 100 μl of saline were injected subcutaneously into back skin shaved with an electric razor 3 days beforehand. Control animals received the same volume of sterile saline. Animals were killed at 1, 6, 12, 24 and 36 h after injection, and an about 8–10 mm diameter section of injected locus skin was removed with a surgical knife. The tissues were fixed in 10% phosphate-buffered formalin, pH 7.2, for 1–2 days at 4°C, and then embedded in paraffin. To detect eosinophils, 3 μm sections were stained by the modified lunar method [21], and then the numbers of migrating eosinophils were determined in 10 randomly selected high power fields (HPF; × 400).

**Determination of the levels of tissue ET-1(1-31) and cytokines**

At 0.5, 1, 3, 6, 12 and 24 h after subcutaneous injection of ET derivatives with or without phosphoramidon or the ET receptor antagonists, animals were killed and then an about 1 g section of injected locus skin was obtained. The change in the level of injected ET-1(1-31) in the skin was determined after acid extraction of it by means of a specific enzyme-linked immunosorbent assay (ELISA), as described previously [22]. To determine the levels of chemokines for eosinophils, such as eotaxin, IL-5 and RANTES, 1 g samples were homogenized in 3 ml of phosphate-buffered saline containing 1 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride and 1 μM leupeptin on ice. Each tissue extract was then centrifuged at 12,000 g for 30 min at 4°C and this supernatant was collected. Control animals received the same volume of saline. This assay selectively recognizes mouse eotaxin, IL-5 and RANTES, with limits of detection of less than 3 pg/ml, 7 pg/ml and 2 pg/ml, respectively. Protein concentrations were determined with bicinchoninic acid protein assay reagent [23] using bovine serum albumin as a standard.

**Statistical analysis**

Statistical significance was determined by means of the unpaired t-test for comparisons between the stimulated groups and the control groups and comparisons between the ET-1-treated groups and the ET-1(1-31)-treated groups using Statview 4.0 software. A value of $P < 0.05$ was accepted as significant.

**Results**

*ET-1(1-31), but not big ET-1, induces eosinophil migration similar to ET-1*

Initial studies were performed to obtain information on the effects of ET-1 and ET-1(1-31) on eosinophil migration and the concentration-responses of ET-1 derivatives. As shown in Fig. 1A, eosinophil migration was detected explicitly in the injected loci at 6 h after subcutaneous administration of ET-1(1-31) or ET-1 at the dose of 100 pmol in BALB/c mice, reached a peak at 12 h, and then slowly decreased, although the numbers of eosinophils in the loci were maintained at high levels at 36 h after injection. The time courses of eosinophil migration evoked by ET-1 and ET-1(1-31) were similar, but the effect of ET-1 on eosinophil recruitment at 12 and 24 h was significantly stronger than that of ET-1(1-31). There was no eosinophil accumulation in control mice. We