Leukotriene B₄ Stimulates Polymorphonuclear Leukocyte Adhesion to Cultured Vascular Endothelial Cells

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A**bstract.** Adhesion of polymorphonuclear leukocytes (PMN) to the endothelial lining of blood vessels is an essential component of the inflammatory response. We have examined the effects of various lipoxygenase metabolites of arachidonic acid on PMN adhesion to cultured vascular endothelial cells, using a quantitative monolayer adhesion assay. Our results indicated that leukotriene B₄ (LTB₄) could effectively stimulate PMN adhesion to endothelial cell surfaces, in contrast to the sulfidopeptide leukotrienes C₄, D₄, and E₄, and the monohydroxyacid lipoxygenase products of leukocytes and platelets, 5*S*-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid and 12*S*-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid, respectively. LTB₄-stimulation of PMN-endothelial adhesion did not appear to be dependent upon the generation of cyclooxygenase metabolites, nor was it inhibited by exogenous prostacyclin. Enhanced PMN adhesion was observed with endothelial cells that were cultured from different types of large vessels (arteries and veins) in several species. These findings suggest an important pathophysiologic

role for LTB₄ in regulating leukocyte-vessel wall interactions.

Introduction

Adhesion of polymorphonuclear leukocytes (PMN)¹ to vascular endothelium has long been recognized as a microscopic hallmark of the acute inflammatory response (1). This interaction appears to be an essential step in the translocation of PMN from the microcirculation into the extravascular space and their accumulation at sites of tissue injury. In larger vessels (both arteries and veins), PMN-endothelial adhesion also may play a role in the pathogenesis of vasculitis and thrombosis. Several naturally occurring and synthetic substances with chemotactic activity for PMN have been identified, including products of complement activation, *N*-formylmethionyl-peptides, and certain lipoxygenase-metabolites of arachidonate (e.g., 12*S*-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid [12-HETE], and 5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid [LTB₄]) (2, 3). Significant progress has also been made in characterizing receptors for chemotactic substances, as well as the biochemical coupling events and locomotor apparatus that are involved in PMN migration (4, 5). In contrast, the molecular and cellular processes that are involved in the adhesion of PMN to endothelial cell surfaces remain incompletely understood (1).

Recent in vitro studies from our laboratory (6, 7), employing various inhibitors of arachidonate metabolism, have provided indirect evidence that lipoxygenase-derived products may play a role in the regulation of PMN adhesion to cultured endothelial

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1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cell(s); HEC, human endothelial cell(s); 5-HETE, 5*S*-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 12-HETE, 12*S*-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid; LT, leukotriene; LTB₄, LTC₄, LTD₄, and LTE₄, leukotrienes B₄, C₄, D₄, and E₄; PMN, polymorphonuclear leukocytes(s); PGI₂, prostacyclin.

cells. In the experiments reported here, we have directly examined the effects of specific lipoxygenase products on the interaction of human PMN with vascular endothelial cells isolated from various anatomical sites in several species, using a quantitative *in vitro* assay.

Methods

Materials. Synthetic leukotrienes B₄, C₄, and D₄ were obtained from Dr. J. Rokach of Merck Frosst Canada, Inc., Pointe Claire-Dorval, Quebec. Prostacyclin (PGI₂) was obtained from the Upjohn Co., Kalamazoo, MI, and its potency was checked by platelet aggregometry. For the preparation of the 5-lipoxygenase metabolite 5S-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HETE), washed human PMN were incubated for 5 min at 37°C with sodium arachidonate (0.11 mM, NuChek Prep Inc., Elysian, MI) and calcium ionophore A23187 (20 μM, Calbiochem-Behring Corp., San Diego, CA). For the preparation of the 12-lipoxygenase metabolite, 12-HETE, human platelets were incubated for 20 min at 37°C with sodium arachidonate (50 μM). Products from each reaction were extracted and purified as previously described (8–10). Bovine albumin (Cohn fraction V) was obtained from Sigma Chemical Co., St. Louis, MO. Culture media and sera were obtained from M. A. Bioproducts, Walkersville, MD.

Cell cultures. Bovine aortic endothelial cells (BAEC) were cultured from calf thoracic aortae, as previously described (11), and a single strain (11-BAEC) was used at passages 10–30. Human endothelial cells (HEC) were isolated from umbilical cord veins (11) and several strains serially passaged in medium 199 with 20% fetal calf serum supplemented with endothelial cell growth supplement (50–100 μg/ml; this was obtained from Dr. T. Maciag, Meloy Laboratories Inc., Springfield, VA), and heparin (100 μg/ml; Sigma Chemical Co., porcine intestine). Cells of each type were replicate-plated on 12-mm round glass (Bellco Glass, Inc., Vineland, NJ) or 15-mm Thermanox plastic (Lux, Miles Laboratories, Inc., Naperville, IL) coverslips and allowed to grow to confluent densities.

Isolation and radiolabeling of leukocytes. Whole blood was collected by venipuncture from healthy volunteers into acid-citrate-dextrose (84:16) and processed, as previously described (7), to yield a washed suspension that consisted of >95% granulocytes by Wright-Giemsa staining with <1% platelet contamination by phase-contrast microscopy. Washed PMN were resuspended (10⁷/ml) in calcium-free Hank's balanced salt solution that contained 5 μCi/ml ¹¹¹In-oxine (Amersham Corp., Arlington Heights, IL), and incubated for 15 min at 22°C. The leukocytes were then centrifuged at 200 *g* for 2 min, resuspended in 1 mg/ml bovine albumin in Hank's balanced salt solution to remove unincorporated radioactivity, and finally suspended in assay buffer (Tyrode's solution, 2 mM Ca⁺⁺, 1 mM Mg⁺⁺, pH 7.4, supplemented with 1 mg/ml or 40 mg/ml bovine albumin) at a final concentration of 5 × 10³/μl. This labeling procedure yielded a specific activity of 4,000 cpm/10⁴ leukocytes and >95% cell viability by trypan blue exclusion. After treatment with 1 μM LTB₄ or 5 μM A23187 for 10–30 min at 37°C, <5% of the cell-associated radioactivity was released.

Leukocyte-monolayer adhesion assay. Adhesion of washed human PMN to cultured cells and blank (serum- or fibronectin-coated, 5 μg/cm²) coverslips was measured using a quantitative monolayer adhesion assay (6, 7). Test coverslips, in 16-mm plastic wells (Costar-24 multiwell plate, Costar, Cambridge, MA), were rinsed three times with assay buffer and incubated with 0.5 ml of radiolabeled PMN suspension for 10 min at 37°C under static conditions. Each coverslip was then subjected to a standardized wash procedure which reproducibly separated surface-bound from free leukocytes, thus providing an index of the

relative adhesivity of the interacting cell surfaces (6). A 10-min endpoint was selected because significant numbers of leukocytes migrate from the apical surface beneath the monolayer during longer (30–90 min) incubations.

Results

LTB₄ consistently stimulated human PMN adhesion to both bovine and human endothelial cell monolayers. Thus, in 37 consecutive experiments involving 18 different blood donors, when LTB₄ (1 μM in 40 mg/ml albumin-Tyrode's solution) was present during the adhesion assay, the number of adherent PMN/mm² BAEC surface was significantly increased (197 ± 7% basal, mean ± SEM, *P* < 0.0005, *t* test). Comparable LTB₄ stimulation of PMN adhesion also was observed with subcultured human umbilical vein endothelial cells (209 ± 21% basal, six experiments, *P* < 0.005). After LTB₄ treatment, the leukocytes adhering to both HEC and BAEC monolayers remained unclumped, but appeared more extensively spread than under basal conditions. Neither BAEC nor HEC monolayers showed microscopic evidence of cell detachment or overt injury under basal or LTB₄-stimulated conditions in the presence of 40 mg/ml albumin.

In experiments with BAEC monolayers, LTB₄-stimulated PMN adhesion was found to be dose related and sensitive to the protein content of the incubation medium (Fig. 1). At near physiologic albumin concentrations (40 mg/ml), stimulation was noted at >0.01 μM, whereas at lower albumin concentrations (1 mg/ml) the threshold was reduced to <0.01 nM and the magnitude of stimulation at 1 μM was increased several fold (Fig. 1, legend; Fig. 2). Enhanced sensitivity to LTB₄ was also noted when albumin was omitted from the assay buffer; however, under these conditions certain BAEC monolayers showed morphologic evidence of cytotoxicity which appeared to be PMN-dependent. LTB₄ stimulation of PMN adhesion to BAEC also occurred in Tyrode's buffer supplemented with human serum albumin (Cohn fraction V, Sigma Chemical Co.), fetal bovine serum, or highly purified fatty

Figure 1. Dose dependence of LTB₄ stimulation of PMN adhesion to cultured BAEC. Standard monolayer adhesion assays were performed in the presence of the indicated concentrations of LTB₄ in assay buffer that contained 1 mg/ml (●) or 40 mg/ml (○) albumin. Data are expressed as percentage of the maximal stimulated adhesion obtained with 1 μM LTB₄ (100% = 869 ± 62 PMN/mm², 15 experiments, and

224 ± 33 PMN/mm², 6 experiments, mean ± SEM, for 1 mg/ml and 40 mg/ml albumin, respectively). Each point represents the mean (±SEM) of triplicate determinations in several experiments.

Figure 2. Comparative effects of LTB₄ and the sulfidopeptide leukotrienes, LTC₄, LTD₄, and LTE₄, on PMN adhesion to cultured BAEC. Each bar represents percentage of stimulation of PMN adhesion [(stimulated-basal) ÷ basal × 100] that was observed with the indicated concentration of test substance in assay buffer that contained either 1 mg/ml or 40 mg/ml albumin (mean ± SEM of triplicate determinations in 4–14 experiments). BSA, bovine serum albumin.

acid free bovine albumin (BOVUMINAR Reagent Pure Powder, Armour Pharmaceutical Co., Tarrytown, NY).

In contrast to the marked stimulation observed with LTB₄, the sulfidopeptide leukotrienes LTC₄, LTD₄, and LTE₄ had little or no effect on human PMN adhesion to BAEC monolayers under various assay conditions (Fig. 2). Furthermore, the monohydroxyacid leukocyte product 5-HETE and the 12-lipoxygenase platelet product 12-HETE did not significantly enhance PMN adhesion to BAEC monolayers (108 ± 6% and 111 ± 12% basal, respectively, mean ± SEM, four experiments, 1 μM in 40 mg/ml albumin-Tyrod's), although both compounds did increase PMN adhesion to serum- or fibronectin-coated coverslips and induced spreading (data not shown).

To determine the possible influence of endogenous cyclooxygenase products, such as leukocyte-derived thromboxane A₂ or endothelial-derived PGI₂, on LTB₄-stimulated PMN-endothelial adhesion, PMN suspensions and BAEC monolayers were separately pretreated with 100 μM aspirin for 30 min before the addition of LTB₄. This treatment, which is sufficient to block the generation of cyclooxygenase products in the system (7), had no significant effect on LTB₄-stimulated adhesion (1 μM LTB₄, 390 ± 106% vs. 1 μM LTB₄ plus aspirin, 372 ± 46%; 1 nM LTB₄, 144 ± 27% vs. 1 nM LTB₄ plus aspirin, 155 ± 25%, percentage basal, mean ± SEM, three experiments, 1 mg/ml albumin). Furthermore, pretreatment of both cells with purified PGI₂ (1 μM, 5 min) and its inclusion in the assay did not block LTB₄-stimulated PMN-BAEC adhesion (1 μM LTB₄, 390 ± 106% vs. 1 μM LTB₄ plus PGI₂, 399 ± 98%; 1 nM LTB₄, 144 ± 27% vs. 1 nM LTB₄ plus PGI₂, 156 ± 46%, percentage basal, mean ± SEM, three experiments, 1 mg/ml albumin).

In addition to bovine aortic and passaged human umbilical vein endothelial cells, LTB₄ also stimulated PMN adhesion to other types of cultured endothelial cells including bovine vena cava, human and baboon saphenous vein, and primary and SV-40 transformed human umbilical vein (data not shown).

Discussion

The results obtained in our in vitro model system provide direct evidence that leukotriene B₄, a major product of the 5-lipoxygenase pathway in activated human polymorphonuclear leukocytes (3), can stimulate the adhesion of these cells to vascular endothelium. These findings thus support the in vivo microscopic observations of Dahlen et al. (12), who noted that exogenous LTB₄ enhanced the sticking of circulating leukocytes to the walls of postcapillary venules, and provide several mechanistic insights into the process of leukocyte-endothelial adhesion. First, LTB₄ stimulation of human PMN adhesion does not appear to be species or anatomical site specific (with regard to target cell), since comparable results were obtained with human, bovine, and baboon endothelial cells of arterial and venous origin. Second, the concentration of serum albumin in the assay system influences the dose dependence of enhanced PMN adhesion, as well as the occurrence of endothelial cell injury (13). Third, LTB₄ appears to be much more effective in stimulating PMN-endothelial adhesion than other leukocyte- or platelet-derived lipoxygenase products. Fourth, stimulation of PMN-endothelial adhesion by LTB₄ is not dependent upon the generation of cyclooxygenase products and is not inhibited by exogenous prostacyclin.

The selective effect of LTB₄ on leukocyte-endothelial adhesion, compared with the other monohydroxy- and dihydroxy-eicosatetraenoic acids tested, is especially noteworthy. Like LTB₄, the leukocyte-5-lipoxygenase product, 5-HETE, and the platelet 12-lipoxygenase product, 12-HETE, have been reported to have chemoattractant activity (2, 3). In addition, the preparations of 5-HETE and 12-HETE used in this study were active in stimulating PMN adherence to serum- or fibronectin-coated coverslips, yet neither compound significantly influenced PMN-adhesion to endothelial monolayers. The sulfidopeptide leukotrienes LTC₄ and LTD₄ have been reported to enhance PMN adherence to Sephadex G-25 in a manner similar to LTB₄ (14); yet, these compounds, as well as LTE₄, also were essentially nonstimulatory for endothelial adhesion. These results suggest that the mechanism of PMN adhesion to endothelial cell surfaces may be qualitatively different than that involved in PMN interactions with artificial surfaces (15); furthermore, LTB₄ may be playing a selective role, in contrast to other lipoxygenase products, in stimulating leukocyte-endothelial interactions.

As recently has been shown, leukotriene C₄ can stimulate PGI₂ production in human endothelial cells (16), and cyclooxygenase products, such as prostaglandin E₂, can inhibit the release of LTB₄ from activated PMN (17). Thus, there appears to be the potential for feedback regulation of LTB₄ generation at the vascular endothelial interface in an inflammatory site.

Our finding that PGI₂ does not inhibit LTB₄-stimulated PMN-endothelial adhesion thus may have special relevance for the initial interaction of an activated leukocyte with the vessel wall.

The biphasic LTB₄ dose-response curve seen with low (1 mg/ml) albumin (Fig. 1) suggests the possibility of two relevant receptors in this system. At present, there is no reported evidence for specific LTB₄ receptors on vascular endothelium, although both low and high affinity LTB₄ binding sites have been characterized in human polymorphonuclear leukocytes (18). Localized generation of LTB₄ by activated leukocytes and its binding by plasma proteins are potential factors influencing the effective concentration of this mediator at the vessel wall-blood interface.

In preliminary experiments, LTB₄ also stimulated PMN adhesion to cultured bovine aortic smooth muscle and human dermal fibroblasts (unpublished observations). However, the mechanism(s) involved in leukocyte adhesion to these non-polarized cell types (as well as to extracellular matrix components such as fibronectin) may well be different than with endothelium. Nonetheless, LTB₄ stimulation of these processes could be relevant to leukocyte-vessel wall interactions at sites of endothelial injury or myointimal hyperplasia *in vivo*.

In conclusion, our observations are consistent with the hypothesis (6, 7) that lipoxygenase-derived arachidonate metabolites are involved in the regulation of leukocyte-endothelial cell adhesion. Further studies will be required to characterize the relevant target cell receptor(s) as well as the relative contributions of leukotriene B₄ and other locally generated complex lipids (3, 19, 20) to this pathophysiologically important cell-cell interaction.

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Addendum

Since submission of this report, Hoover et al. (21) also have reported LTB₄ stimulation of human PMN adhesion to cultured bovine aortic endothelium.

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