**Research Paper**

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**Cell–cell interaction of macrophages and vascular smooth muscle cells in the synthesis of leukotriene B₄**

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**Introduction**

The generation of leukotrienes (LTs) by lipoxygenase catalysed reactions is associated with a wide range of cell types that are involved in both physiological and pathological events.¹ The transcellular metabolism of lipoxygenase derived metabolites has recently been reported not only to amplify the level of eicosanoids within a local milieu but also to stimulate the generation of biologically active metabolites with functions different from those in their original cells. For example, the presence of erythrocytes can greatly induce LTB₄ formation from neutrophil-derived LTA₄; co-incubation of endothelial cells,² smooth muscle cells³ or platelets⁴–⁸ with neutrophils, can result in transcellular LTC₄ synthesis. Perfusion of blood free rabbit lung and isolated pulmonary artery with neutrophils induced an overall LTB₄, LTC₄ and LTD₄ release.⁹ The transfer of unstable metabolic intermediates has been considered to be a biochemical basis for these phenomena.³–⁵,10

Macrophages are well known to play a central role in the modulation of inflammatory and immune processes, as well as in tissue injury and repair.¹¹ Macrophages have 5-lipoxygenase activity that inserts molecular oxygen into arachidonic acid to form an unstable peroxide 5-HPETE leading to the generation of 5-HETE, or through the epoxide intermediate, LTA₄ to the generation of LTB₄ by hydrolase; or conjugation with glutathione by glutathione-S-transferase to form the cysteine LTC₄, D₄ and E₄.¹²,¹³ Recently, many studies have indicated that macrophages are a potent source of arachidonate metabolites generated via lipoxygenase pathways.¹⁴⁻¹⁵ In vivo, SMC and macrophages have been noted to interact at the site of thrombosis, vessel injury and inflammation by secreting interleukin-1 (IL-1), tumour necrosis factor (TNF), prostaglandins (PG) and leukotrienes.¹⁶⁻¹⁸ This investigation was designed to study whether or not a shift in the metabolic profile of arachidonate products generated by activated macrophages or vascular SMC could be induced under circumstances that may be related more closely to those operative inflammatory reactions in vivo.

**Materials and Methods**

Reagents: Human recombinant IL-1β and TNFα were obtained fromSigma (Saint Quentin Fallavier, France). Labelled ³H-AA and radioactive standards were from Amersham (Aylesbury, UK). Synthetic

**Key words:** Cell–cell interaction, Interleukin-1, Leukotriene, Macrophage, Smooth muscle cell, Tumour necrosis factor
LTA₄ methyl ester from Sigma was hydrolysed to yield the free acid according to the methods described by Maycock. Octadecyl silica Sep-Pak cartridges were obtained from Millipore/Waters (Les Ulis, France). Media, sera and reagents for cell cultures were obtained, if not further specified, from Gibco (Paisley, UK). All solvents used in chromatographic systems were of HPLC grade.

Vascular SMC culture: Vascular SMC cells were obtained by dissociation of rat abdominal aorta with 0.05% EDTA and 0.1% trypsin in HAM F10 medium. During the first 2 weeks, the cells were cultured at 37°C, with 5% CO₂ in HAM F10 medium supplemented with 20% foetal calf serum. When a monolayer was obtained, the cells were removed by trypsin-EDTA (0.05–0.02%) and cultured in 25 ml flasks in HAM F10 medium, supplemented with 10% foetal calf serum and 1% penicillin-streptomycin. Culture SMC grew in a 'hill and valley' formation. Cells up to the 25th passage were used for the experiments.

Alveolar macrophage isolation and culture: Respiratory disease-free 125 to 150 g female Wistar rats were housed under pathogen-free condition. Rats were anaesthetized with i.p. sodium pentobarbital and lungs were exercised and washed as described previously. Bronchoalveolar lavage cells were 96% AM by microscopic examination of cyt centrifuge preparations stained with a modified Wright–Giemsa stain (Diff-Qick, American Scientific Products, IL). Bronchoalveolar cells (5 x 10⁶) suspended in 10 ml of M199 were plated in 25 ml flasks and cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. After 2 h, nonadherent cells were removed by washing twice with the medium. Cell monolayers were then cultured in M199 containing 10% heat inactivated newborn calf serum before experimental incubation.

³H-arachidonic acid metabolism in macrophages and SMC: Mono nuclear vascular SMC cells (5 x 10⁶) or macrophages (5 x 10⁶) were plated in a fresh culture medium. The cells were incubated with 1 µM ³H-AA for 18 h and then the medium was harvested. The cells were washed three times with PBS containing 0.25% fatty acid free bovine serum albumin (BSA) to eliminate non-incorporated AA. These cells were covered with 7 ml of serum free medium. IL-1β (5 units/ml) and TNFα (10 units/ml) were added and the cells were incubated at 37°C for 24 h. Then the culture medium was harvested and centrifuged. The supernatants were stored at −80°C for eicosanoid assay.

Co-culture of macrophages and SMC: Biosynthesis of LTB₄ during cell–cell interaction was studied by incubating fixed concentrations of ³H-AA labelled SMC with various numbers of alveolar macrophages. For co-culture experiments, ³H-labelled SMC (5 x 10⁶ cells) were further incubated with macrophages in the presence of IL-1β (5 units/ml) and TNFα (10 units/ml) at 37°C for 24 h following the ratios of SMC to macrophages: SMC alone, 100:1, 10:1, 1:1, 1:10, 1:100, macrophages alone, and macrophages alone but in the presence of IL-1β and TNF. After the incubation, the supernatants were collected, centrifuged and stored at −80°C until eicosanoid assay.

Transformation of LTA₄ by macrophages: Macrophages (1 x 10⁶ cells) in 35 mm wells were allowed to equilibrate for 5 min at 37°C in 1 ml HBSS/BSA and then incubated at 37°C for 15 min with LTA₄ at concentrations described in the figure legends. At the end of the incubation, 1 ml of ice-cold methanol was added and the medium was harvested, centrifuged and kept at −20°C for analysis by RIA for LTB₄.

HPLC analysis: The HPLC system utilized was from Waters associated (Milford, MA) using two pumps (6000A and 510) coupled to a Model 680 gradient controller. UV absorbance of the eluate was monitored using two serial detectors. Radioactivity was determined by mixing 1 ml of the effluent with 5–10 ml of Atomlight (NEN) and counting in a liquid scintillating counter.

The procedure used for the precolumn extraction/RP–HPLC of the supernatant was similar to that described previously, with the six-port valve in the 'load' position. The sample, diluted to a total volume of 10 ml, was applied to a C₁₈ reverse-phase guard column (Nucleosil C₁₈, 10 µm, 10 mm, Macherey Nagel) located in the sample loop of the six-port switching valve (Rheodyne) which had been equilibrated with solvent A (2.5 mM H₃PO₄ in 15% methanol). A 3 µm filter was placed between the outlet of the pump and the six-port valve. The precolumn was then washed with 8 ml of solvent A. The AA metabolites, remaining on the precolumn, were injected by turning the six-port valve to the 'inject' position onto a Spherisorb ODS2 (5 µm, 4.6 x 150 mm) column (phase separation). RP–HPLC was carried out using a mobile phase consisting of a non-linear gradient starting with 30% solvent B (water/acetic acid 0.05, v/v, buffered to pH 5.7 with ammonium hydroxide) leading to 100% solvent C (65% acetonitrile–35% methanol) with the following program: 0–10 min, linear to 65%; 10–35 min, linear to 100% C; 35–55 min, linear to 50% C and isocratic until 70 min. The flow rate was 1 ml/min. Under these conditions, retention times (in min) of eicosanoids were as follows: 20-COOH-LTB₄, 5–6; 20-OF-LTB₄, 11.5; LTC₄, 18; Δ₂-trans-LTB₄, 21.5; LTB₄, 22.5; 11-HETE, 23.5; 15-HETE, 24.5; 12-HETE, 38.5; 5-HETE, 40; arachidonic acid, 57.1.
Radioimmunoassay of LTB₄: The samples, acidified to pH 3.5, were extracted with Waters Sep-Pak C₈ (Milford, MA). Eicosanoids were eluted with methanol (5 ml). The solvent was pooled and evaporated to dryness under nitrogen. The residue was then dissolved in PBS for RIA assay. The detection limit of the radioimmunoassay for LTB₄ was 4 pg/ml.

Results

Induction of HETE synthesis in SMC by IL-1 and TNF: Adherent rat vascular SMC, prelabelled with ³H-AA, were exposed to IL-1β (5 units/ml) or TNFα (10 units/ml) for 24 h. Following this incubation, the media were collected and then analysed by HPLC as described in Materials and Methods. As shown in Fig. 1, both IL-1β and TNFα induced a significant increase of lipoxygenase metabolites. These compounds eluted from an RP–HPLC column with retention times that corresponded to 15- and 5-HETE, and represented 10.9 ± 2.1% and 3.8 ± 0.9%, respectively, of the total radioactivity applied to the column. However, neither IL-1 nor TNF induced LTB₄ production. IL-1β or TNFα induced lipoxygenase metabolite formation in a dose-dependent manner (Fig. 1). The release of HETEs in response to IL-1β or TNFα, reached a plateau of release at concentrations above 100 units/ml. In comparison to the control, the amounts of HETEs in SMC treated with IL-1β or TNF were enhanced 6-fold and 4-fold, respectively. In all cases, the release of HETEs in response to IL-1β was significantly greater than that induced by TNFα (Fig. 2).

The response to IL-1 or TNF was also time-dependent having a lag phase of 8 h before significant generation of HETEs in both cases (Fig. 2).

The simultaneous addition of IL-1β and TNFα stimulated HETE release to a greater extent than did either agent alone (Fig. 2). The effect was additive and the sum of the HETEs released by both IL-1β and TNF always exceeded the amount of HETEs generated after separate addition of IL-1β or TNFα to SMC.

In addition, preincubation of SMC with NDGA (10⁻⁵ M) inhibited recovery of HETEs induced by IL-1β and TNFα (90% and 95% inhibition for IL-1 and TNF, respectively). Metyrapone (10⁻⁶ to 10⁻⁴ M), a cytochrome P450 inhibitor, did not modify the recovery of monohydroxylated compounds. The protein synthesis inhibitors cycloheximide (10⁻⁴ M) and actinomycin (10⁻⁵ M) also abolished HETEs production induced by IL-1 and TNF. In contrast, pretreatment of cells with aspirin (10⁻⁶ to 10⁻³ M) inhibited the synthesis of cyclooxygenase metabolites (20 to 90%) and slightly increased HETEs recovery (Table 1).

In contrast to the augmentation of AA metabolites in SMC, incubation of macrophages with IL-1β (5 units/ml) or TNFα (5 units/ml) did not result in significant synthesis of LTB₄ (2.7 ± 0.5 pmol/10⁶ cells) although the levels obtained from stimulated cells were higher than those obtained from unstimulated cells (2.5 ± 0.4 pmol/10⁶ cells).

Incubation of macrophages with LTA₄: In order to synthesize LTB₄ from the small amounts of LTA₄ generated from adjacent cells, macrophages must be

| Sample       | Monohydroxylated compounds (dpm/10⁶ cells) |
|--------------|-------------------------------------------|
|              | IL-1 | TNF | IL-1 + TNF |
| Control      | 1201 ± 215 | 570 ± 127 | 2370 ± 469 |
| Aspirin      | 1375 ± 271 | 613 ± 136 | 2643 ± 437 |
| NDGA         | 126 ± 21 | 103 ± 23 | 129 ± 47 |
| Metyrapone   | 1172 ± 175 | 535 ± 139 | 2145 ± 513 |
| Cycloheximide| 225 ± 57 | 175 ± 27 | 375 ± 53 |
| Actinomycin  | 269 ± 36 | 196 ± 36 | 257 ± 27 |

Adherent smooth muscle cells (5 × 10⁶ cells) were incubated with IL-1β (5 units/ml) or/and TNF (10 units/ml) for 24 h after preincubation with aspirin (10⁻⁶ M), NDGA (10⁻⁵ M), cycloheximide (10⁻⁴ M) and actinomycin (10⁻⁴ M) for 1 h. The whole incubation mixture was extracted and analysed by HPLC as described in Materials and Methods. Results are expressed as mean ± S.E.M. from five experiments.

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capable of converting exogenous supplies of LTA₄ into LTB₄. The ability of macrophages to utilize exogenous LTA₄ was examined by incubating macrophages (1x10⁶ cells) with exogenously added LTA₄. As shown in Fig. 3, macrophages were able to transform, in dose-dependent manner, extremely low concentrations of LTA₄ into LTB₄ but the amount of LTB₄ formed from the exogenous LTA₄ (1 to 10,000 pmol) in the absence of AM but in the presence of HBSS/BSA remained very low (5.6 to 10 pmol, as shown in Fig. 3). This finding suggested that transcellular synthesis of LTB₄ could occur when very limited amounts of LTA₄ were available to these cells.

Transcellular LTB₄ synthesis in the co-culture of macrophages and SMC: Biosynthesis of LTB₄ during cell–cell interaction was studied using co-incubation of SMC and macrophages in the presence of IL-1β or TNFα. As shown in Figs 4 and 5, incubation of SMC with various ratios of macrophages (100:1, 10:1, 1:1, 1:10, 1:00 and 1:100, SMC alone) resulted in significant amounts of LTB₄ in the supernatants. LTB₄ levels were 14.7±2.5, 34.9±6.7, 17.8±4.3, 12.6±2.9, 6.7±2.7 and 2.4±0.8 pmol/10⁶ cells respectively. In addition, the preincubation of SMC with NDGA (10 μM), cycloheximide (10 μM), and actinomycin (10 μM) abolished LTB₄ production induced by IL-1 and TNF, when co-incubated with macrophages (Table 2). However, no LTB₄ was found when IL-1- and TNF-treated SMC were co-incubated with NDGA pretreated macrophages.

Discussion

In this study, the transcellular synthesis of LTB₄ during cell–cell interaction between IL-1β and TNFα activated vascular SMC and macrophages has been

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Table 2. Effect of aspirin, NDGA, cycloheximide and actinomycin on LTB₄ formation in co-culture of macrophages with IL-1 and TNF stimulated rat vascular smooth muscle cells

| Sample          | LTB₄ (pmol/10⁶ cells) |
|-----------------|----------------------|
| IL-1            | 34.9±6.7             |
| Aspirin + IL-1  | 37.2±5.9             |
| NDGA + IL-1     | 4.7±1.7              |
| Cycloheximide + IL-1 | 4.9±2.5            |
| Actinomycin + IL-1 | 4.7±2.1             |

Adherent smooth muscle cells (5x10⁶ cells) were incubated with 5x10⁶ macrophages in the presence of IL-1β (5 units/ml) and TNF (10 units/ml) for 24 h after preincubation with aspirin (10⁻⁶ M), NDGA (10⁻⁶ M), cycloheximide (10⁻⁴ M) and actinomycin (10⁻⁴ M) for 1 h. The whole incubation mixture was extracted and analysed by HPLC as described in Materials and Methods. Results are expressed as mean ± S.E.M. from five experiments.
demonstrated for the first time. The incubation of individual SMC with IL-1β or TNFα did not produce LTβ. Neither IL-1β or TNFα induced significant LTβ production in macrophages incubated at 37°C for 24 h. However, using co-culture of SMC with various ratios of macrophages in the presence of IL-1β and TNFα, it was found that levels of LTβ were greatly increased compared with macrophages alone.

In accordance with previous reports on rabbit chondrocytes,20 rheumatoid synovial cells,21 and human glomerular mesangial cells,22 both IL-1β and TNFα induced a dose- and time-dependent formation of eicosanoids. The amount of HETE was significantly greater than the additive effects of the two cytokines alone. The mechanism by which IL-1β and TNFα activated the HETEs synthesis is not known. Many reports have indicated that IL-1β and TNFα cause an up-regulation of phospholipase A₂ and cyclooxygenase-2, which, unlike cyclooxygenase-1, metabolize arachidonic acid not only to prostaglandins but also to a significant extent to 11-, 15- and 5-HETE.23,24 The increase of HETE synthesis in SMC might also be due to the increase of enzyme protein synthesis, such as cyclooxygenase and/or phospholipase A₂ in IL-1β or TNFα stimulated cells because cycloheximide, a protein inhibitor, inhibited HETE increase induced by IL-1β and TNFα in SMC, but the mechanism remains to be elucidated.

The synthesis of LTβ involves a complex series of reactions within macrophages. A limiting factor for LTβ biosynthesis was found to be the availability of the unstable intermediate, LTA₄.6 However, the generation of LTA₄ depends upon activation of 5-lipoxygenase as well as the presence of its substrate, arachidonic acid.25 The production of LTs by human polymorphonuclear leukocytes has been known to be significantly influenced by adjacent cells possessing distinctly different metabolic properties.2,3 In this study, macrophages transformed very low concentrations of exogenous LTA₄ into LTβ, suggesting that macrophages could metabolize LTA₄ into biologically active LTβ. The co-culture of SMC with various ratios of macrophages greatly increased LTβ synthesis when compared with very low levels of LTβ produced by individual cells. The highest augmentation in LTβ was obtained when the ratio of SMC to macrophages was 10:1. Because IL-1β and TNFα did not induce a significant LTβ production either in SMC alone or in macrophages alone, and because the pretreatment of SMC cells and macrophages with the lipooxygenase inhibitor NDGA and protein synthesis inhibitors, blocked LTβ formation, it could be concluded that cell-cell interaction could be responsible for this augmentation, i.e. macrophages utilizing SMC-derived LTA₄ or its precursor, 5-HPETE for its synthesis of LTβ during co-incubation of SMC and macrophages. According to the cell cooperation classification of Marcus, LTB₄ biosynthesis could come from the interaction of type IA (cells can share a common precursor synthesized by different cells).24

However, the mechanism of LTβ transcellular synthesis is certainly more complex. Irvine25 has demonstrated that arachidonic acid availability is a major limiting factor for LTs synthesis. Furthermore, several studies have suggested that a transfer of arachidonic acid takes place in in vitro cell–cell cooperation such as during platelet–neutrophil interaction.8,10,25,26 For example, Palmantier and Borgeat7 have presented evidence that thrombin activated platelets offer free arachidonic acid to neutrophils that are utilized for LTβ synthesis. Antoine et al.8 have also found that neutrophils can utilize platelet-derived arachidonic acid for the formation of the 5-lipoxygenase product.4 Therefore, the possibility that other mechanisms are responsible for LTβ synthesis cannot be excluded from the present data.

The transcellular synthesis of LTβ, between vascular SMC and macrophages may have an important physiological and pathophysiological significance. The lipoxygenase products of AA metabolism are involved in various aspects of inflammation and atherosclerotic lesions.27 For example, the intimal accumulation of smooth muscle cells in rabbit carotid arteries, an early stage of atherosclerosis, is inhibited by dexamethasone, which prevents formation of both cyclooxygenase and lipoxygenase metabolites, but is not inhibited by nonsteroidal anti-inflammatory drugs such as indomethacin.28,29 The transcellular LTβ synthesis could be involved in the genesis of atherosclerosis and other vascular diseases because of its action of initiating vascular inflammation, promoting vascular constriction and proliferation as well as platelet aggregation.

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