Leukotriene A_4 Hydrolase in Human Skin

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The biochemical properties and immunohistochemical localization of leukotriene (LT) A_4 hydrolase were investigated in human skin. The activity of LTA_4 hydrolase, which catalyzes the hydrolysis of LTA_4 to LTB_4, the most chemotactic compound known, was detected in the 100,000 $\times g$ supernatant of homogenates of human epidermis and a transformed epidermal cell line (HSC-1). No significant LTA_4 hydrolase activity was detected in human whole skin or dermis. The enzymatic properties of LTA_4 hydrolase isolated from human keratinocytes and peripheral leukocytes were similar. Their activities were inhibited by bestatin and captopril, and they were completely absorbed by anti-human LTA_4 hydrolase antibody. By immunoblotting analysis using this antibody, LTA_4 hydrolase was detected as a 70-kDa protein in human epidermis and HSC-1 and was found to be similar to the enzyme detected in peripheral mononuclear leukocytes. In human dermis, LTA_4 hydrolase was barely detected by Western blotting. On the other hand, LTA_4 hydrolase was demonstrated in the cytoplasm of keratinocytes in the epidermis, and in fibroblasts, infiltrating and endothelial cells in the dermis of normal human skin by immunohistochemical analysis using the immunoperoxidase method. These results suggest that LTB_4 can be generated from LTA_4 by LTA_4 hydrolase in keratinocytes as well as fibroblasts, infiltrating and endothelial cells in the dermis of human skin. *J Invest Dermatol 102:253–257, 1994*

Leukotriene (LT) B_4 is one of the most potent mediators of leukocyte chemotaxis and smooth muscle contraction [1,2]. LTB_4, through binding to receptors, stimulates neutrophil activities such as chemokinesis, aggregation, degranulation, adhesion to endothelium, etc. Intradermal injection of LTB_4 causes local edema and vasoconstriction in human skin [3,4]. Furthermore, keratinocytes, which possess specific binding sites for LTB_4, may be a target for its proliferative action [5–7]. LTB_4 can also induce pigmentation in human melanocytes [8,9]. Therefore, LTB_4 has been suspected to play an important role in the pathogenesis of various inflammatory and allergic diseases, including skin diseases such as psoriasis or atopic dermatitis [10–13].

LTB_4 is formed from LTA_4, which is a pivotal intermediate in the biosynthesis of other leukotrienes [14,15]. LTA_4 is formed from 5-HPETE, a 5-lipoxygenase product of arachidonic acid. Conversion of LTA_4 to LTB_4 is catalyzed by LTA_4 hydrolase, which is the rate-limiting enzyme for LTB_4 biosynthesis. LTA_4 hydrolase, reported to be ubiquitously distributed [16,17], is found in large amounts in blood cells, such as human leukocytes [18], human erythrocytes [19], and rat neutrophils [20]. Guinea pig lung and liver and human liver [21,22] and blood plasma [23] also possess this enzyme. Recently, LTA_4 hydrolase has been recognized as a Zn^{++}-metallohydrolase possessing intrinsic amino-peptidase activity [24–26], and its enzyme activity is inhibited by bestatin or captopril, which are inhibitors of aminopeptidase [25,26]. Human LTA_4 hydrolase cDNA has been cloned and expressed in *Escherichia coli* as an active fusion protein [27]. Although it has been reported that human skin can synthesize LTB_4, LTC_4, and LTD_4 from arachidonic acid [28,29], the details of LTB_4 biosynthesis in the epidermis have not been definitely established. To clarify this issue, we investigated the enzymatic properties and immunohistochemical localization of LTA_4 hydrolase in human skin. Such studies should provide a better understanding of the pathophysiologic role of LTB_4 and may identify new targets for pharmacologic manipulation, thus leading to new strategies for the treatment of various skin diseases.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LT, leukotriene.

MATERIALS AND METHODS

Materials Methyl ester of LTA_4 was supplied by Ono Pharmaceutical Co. (Osaka, Japan). LTB_4 and prostaglandin (PG) B_2 were purchased from Cayman Chemical Co. (Ann Arbor, MI). Bestatin [(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine] and captopril [(2S)-1-(3-mercapto-2-methyl-propionyl)-L-proline] were supplied by Sigma (St. Louis, MO). Eicosapentaenoic acid (EPA) was obtained from Mochida Pharmaceutical Co. (Tokyo, Japan). Docosahexaenoic acid (DHA) was obtained from Sagami Chemical Research Center (Sagamihara, Japan). Antibody against human LTA_4 hydrolase antibody [17] was supplied by Prof. T. Shimizu (Second Department of Biochemistry, Faculty of Medicine, University of Tokyo). Vectastain avidin-biotin–peroxidase complex kit was obtained from Vector Laboratories (Burlingame, CA). Polymorphonuclear leukocytes and mononuclear leukocytes were separated from heparinized human peripheral blood by the method of Ferrante *et al* [30]. Normal human skin was obtained from surgical specimens, and then epidermis was isolated from dermis by a sequence of trypsinization and separation. Primary cultured human keratinocytes from neonatal foreskins were purchased from Clonetics Co. (San Diego, CA). Transformed human keratinocyte cell line HSC-1, originally derived from human squamous cell carcinoma, was supplied by Prof. S. Kondo (Department of Dermatology, Yamagata University School of Medicine, Japan) [31]. All other reagents were of analytical grade and used without further purification.

Assay of LTA_4 Hydrolase LTA_4 hydrolase activity was measured as described previously [17,32]. The lithium salt of LTA_4 was prepared from the methyl ester of LTA_4 by saponification in a mixture of tetrahydrofuran and lithium hydroxide in water. After evaporation under a stream of nitrogen, LTA_4 was dissolved in an ethanol-water mixture. The concentration of LTA_4 was calculated from UV absorption at 280 nm ($\varepsilon = 40,000$ [32]). The

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standard reaction mixture (50 μl) contained the enzyme in 0.1 M Tris-HCl buffer (pH 7.8). After preincubation at 37°C for 1 min, LTA_4 (an ethanol-water solution, containing some lithium hydroxide) was added to a final concentration of 80 μM. After a 3 min incubation at 37°C, 100 μl of stopping solution [acetonitrile/methanol/acetic acid (150/50/3, v/v) containing 0.3 nmol of PGB_2 as an internal standard] was added. The mixture was kept at −20°C for 30 min and then centrifuged at 10,000 × *g* for 5 min at 4°C. A 120-μl aliquot of the supernatant was added to 20 μl of 0.35% ethylenediaminetetraacetic acid (EDTA), and 50 μl of this solution (140 μl) was injected onto a reverse-phase high-performance liquid chromatograph (HPLC) (655A-11, Hitachi, Tokyo, Japan). The column (TSK ODS-80TM, 5 μm, Toyosoda, Tokyo, Japan, 0.46 × 15 cm) was eluted with solvent (acetonitrile/methanol/water/acetic acid (300/100/300/0.6 v/v) containing 0.05% EDTA) at a flow rate of 1 ml/min at a room temperature. The absorbance at 270 nm was monitored, and the amount of LTB_4 formed was calculated from the peak-area ratio LTB_4/PGB_2 by using a data-processing system (D-2000, Hitachi, Tokyo, Japan). For each enzyme source, three separate incubations were carried out with different volumes of enzyme solutions. Linear correlations of the amounts of LTB_4 formed versus the amounts of enzyme added were obtained. Specific activities were expressed as nmol of LTB_4/mg of protein/min, or pmol/cell number/min. Protein concentrations were determined by the method of Lowry *et al* [33] using bovine serum albumin as a standard.

Immunochemical Study In immunotitration analyses, the 100,000 × *g* supernatants of HSC-1 cells or human peripheral leukocytes (approximately 80% polymorphonuclear leukocytes, 20% mononuclear leukocytes) were incubated overnight at 4°C with various amounts of antibody against human LTA_4 hydrolase [17]. After incubation with each antibody, the reaction mixture was centrifuged at 10,000 × *g* for 10 min, and the resulting supernatant was recovered for the determination of LTA_4 hydrolase activity.

Western Blotting Analysis The 100,000 × *g* supernatants of various human skin preparations were precipitated with 0.2 ml of 20% trichloroacetic acid and washed three times with 0.2 ml of the same solution. The precipitates were solubilized with 0.1 ml of 1 N NaOH, and their protein contents were measured by the method of Lowry *et al* [33]. Samples (10 μg protein) were subjected to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli [34]. Proteins separated in the gels were transferred to nitrocellulose paper and processed for Western blotting [35] using anti-human LTA_4 hydrolase antibody.

Immunohistochemical Staining Normal human skin was excised, cut into small blocks, and fixed in 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 15 h at 4°C, followed by successive washings with 10, 15, and 20% (w/v) sucrose in 10 mM sodium phosphate-buffered saline (PBS) (pH 7.4) as described previously [17]. Tissue blocks were placed in OCT compound and frozen in dry ice/acetone. Tissue blocks were then sliced into 6-μm sections in a cryostat and mounted on ovalbumin-coated glass slides. After being immersed in 5% normal goat serum for 1 h at a room temperature, sections were stained by the method of Hsu *et al* [36] using anti-human LTA_4 hydrolase antiserum (diluted 1/500 in PBS) as the first antibody, biotinylated anti-rabbit IgG antibody as the second labeled antibody, and a soluble avidin-biotin-complex (Vectastain ABC Kit). The control sections were stained with nonimmunized rabbit serum as the first reagent. Sections were then reacted with a diaminobenzidine-hydrogen peroxide solution for 30 min.

RESULTS

LTA_4 Hydrolase Activity in Human Skin LTA_4 hydrolase activity was found in the supernatant fraction (100,000 × *g*, 1 h) in normal human epidermis and transformed cultured keratinocytes (HSC-1 cells) (Table I). Human epidermis showed significant but low LTA_4 hydrolase activity (0.16 ± 0.03 nmol/min/mg protein), when compared to guinea pig lung (0.81 ± 0.21 nmol/min/mg protein) or small intestine (0.85 ± 0.23 nmol/min/mg protein) (data not shown). In contrast, human whole skin and dermis showed no significant LTA_4 hydrolase activity (data not shown). HSC-1 cells had a higher enzyme activity of LTA_4 hydrolase per 10^6 cells than peripheral leukocytes (Table I). It is concluded that epidermal keratinocytes themselves possess LTA_4 hydrolase because HSC-1 cells showed LTA_4 hydrolase activity.

Inhibition of LTA_4 Hydrolase Activity by Various Compounds Inhibition of LTA_4 hydrolase in supernatants (100,000 × *g*, 1 h) of HSC-1 cells by various compounds was examined. Bestatin at 50 μM and captopril at 200 μM reduced the formation of LTB_4 by 50% (Fig 1). In contrast, EPA and DHA did not inhibit the production of LTB_4. Similar degrees of inhibition were observed with these compounds when LTA_4 hydrolase was isolated from human peripheral leukocytes (data not shown).

Table I. LTA_4 Hydrolase Activity in Human Skin and Peripheral Leukocytes[a]

Tissue	Specific Activity (pmol/min/10^6 cells)	Specific Activity (nmol/min/mg protein)
Normal epidermis		0.16 ± 0.03
HSC-1 cells	27.6 ± 5.4	0.11 ± 0.02
Polymorphonuclear leukocytes	17.5 ± 3.5	0.21 ± 0.04
Mononuclear leukocytes	11.4 ± 2.9	0.12 ± 0.03

[a] Values are expressed as the mean ± SD of three experiments. Enzyme preparation procedures (100,000 × *g*, 1 h, supernatant) and the LTA_4 hydrolase assay are described in *Materials and Methods.*

Immunochemical Properties of LTA_4 Hydrolase in Human Skin In immunotitration analyses with the antibody against LTA_4 hydrolase, the activities in HSC-1 cells and human peripheral leukocytes showed identical titration curves (Fig 2). An excess of anti-LTA_4 hydrolase antibody completely eliminated enzyme activity, whereas a nonimmunized IgG had no effect.

Immunoblotting Analysis of LTA_4 Hydrolase in Human Skin With immunoblotting analysis, LTA_4 hydrolase in normal human mononuclear leukocytes (Fig 3, *lane 1*), transformed human epidermal cell line (HSC-1) (Fig 3, *lane 2*), human primary cultured keratinocytes (Fig 3, *lane 3*) and normal human epidermis (Fig 3, *lane 4*) were precipitated as a 70-kDa protein. LTA_4 hydrolase was not detected by Western blotting in human whole skin (Fig 3, *lane 5*) and the dermis (Fig 3, *lane 6*). As for human polymorphonuclear leukocytes the protein band at 70-kDa and a few low-molecular-weight protein bands were visible (data not shown). The extra bands may be due to proteolytic degradation of the enzyme in polymorphonuclear leukocytes.

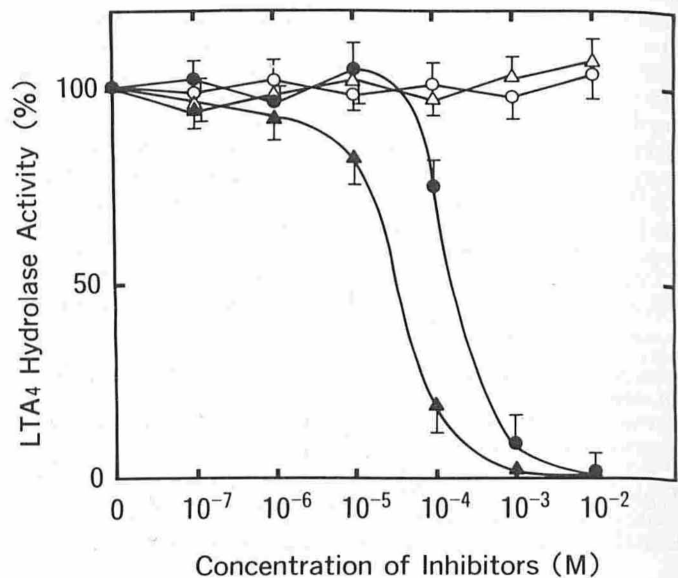

Figure 1. Inhibition of LTA_4 hydrolase in supernatants (100,000 × *g*, 1 h) of HSC-1 cells by various compounds. LTA_4 hydrolase activity, which was assayed as described in *Materials and Methods,* was inhibited by increasing concentrations of bestatin (▲) and captopril (●). In contrast, EPA (○) and DHA (△) showed no effect on the production of LTA_4. Each point represents the mean ± SE of measurements performed in triplicate.

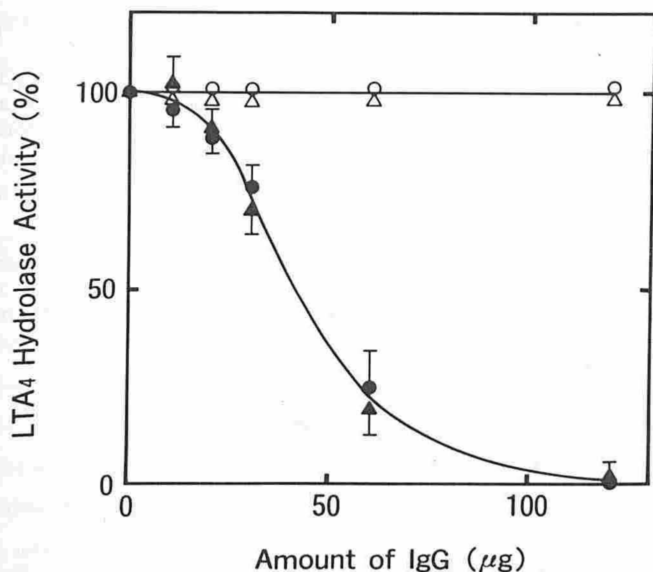

Figure 2. Immunotitration analyses of LTA$_4$ hydrolase activity in supernatants (100,000 × *g*, 1 h) of HSC-1 cells and human peripheral leukocytes using an antibody against human LTA$_4$ hydrolase. After incubation of supernatants containing a given activity of LTA$_4$ hydrolase and various amounts of antibody at 4°C overnight, the residual activity was determined as described in *Materials and Methods.* The percentage of remaining activity relative to that before incubation is shown for HSC-1 (▲) and leukocytes (●) incubated with anti-LTA$_4$ hydrolase IgG, and HSC-1 (△) and leukocytes (○) incubated with nonimmunized IgG. Each point represents the mean ± SE of measurements performed in triplicate.

Immunohistochemical Distribution of LTA$_4$ Hydrolase in Human Skin LTA$_4$ hydrolase was demonstrated immunohistochemically in the cytoplasm of epidermal cells (Fig 4*A*). In the dermis, fibroblasts *(arrows)*, infiltrating cells, and endothelial cells *(arrowheads)* also stained positively (Fig 4*B*). No specific immunostaining was observed upon incubation with nonimmunized rabbit serum (Fig 4*C*).

DISCUSSION

LTB$_4$, which promotes leukocyte chemotaxis and smooth muscle contraction, is found in significant amounts in lesional skin of psoriatic patients and in the skin and serum of atopic dermatitis patients [10–13]. Therefore, LTB$_4$ is suspected to play a significant role as a mediator in the pathogenesis of atopic dermatitis, psoriasis, and other inflammatory and allergic skin diseases. LTB$_4$ is formed from LTA$_4$, which is a pivotal intermediate in the biosynthesis of other leukotrienes [14,15]. Despite a number of investigations, the actual cellular origin of LTB$_4$ in skin lesions has not been definitively established. We tried to answer this question by focusing on human skin LTA$_4$ hydrolase, the enzyme that catalyzes the conversion of LTA$_4$ to LTB$_4$. In our study, we repeated each experiment at least three times, and obtained the same results each time. Based on our results with immunoabsorption (Fig 2) and immunoblotting (Fig 3), human epidermal keratinocytes clearly showed LTA$_4$ hydrolase activity; the LTA$_4$ hydrolase was also shown to be identical to the enzyme found in peripheral leukocytes. The immunoabsorption test (Fig 2) also demonstrated that the conversion of LTA$_4$ to LTB$_4$ is catalyzed only by LTA$_4$ hydrolase. Some authors claim that epidermal cells can generate LTB$_4$ from arachidonic acid [28,29]. However, these studies are hampered by the problem of cellular heterogeneity because preparations of keratinocytes obtained from human or animals usually contain Langerhans cells or melanocytes. In previous studies [37,38], we found that rat epidermis possesses potent PGD synthetase activity, but none was detected in keratinocytes. An immunohistochemical study demonstrated that Langerhans cells in epidermis, but not in keratinocytes, showed active PGD synthetase [37]. To confirm that keratinocytes can synthesize LTB$_4$ from arachidonic acid requires a homogenous preparation of keratinocytes. Some authors claim that pure keratinocytes have weak but significant 5-lipoxygenase activity for the generation of LTA$_4$ from arachidonic acid [28,29]. In contrast, Solá *et al* [39] and Iversen *et al* [40] recently reported that pure keratinocytes cannot produce LTB$_4$ from arachidonic acid. We did not determine if human keratinocytes have 5-lipoxygenase activity for the generation of LTA$_4$ from arachidonic acid. Recently, several reports have suggested that some kinds of cells lack 5-lipoxygenase activity but that they possess LTA$_4$ hydrolase activity [41–44]. In these cells, LTB$_4$ can be converted from LTA$_4$ by LTA$_4$ hydrolase but LTA$_4$, a substrate, must be transferred from other cells such as neutrophils, which show 5-lipoxygenase activity. For example, LTA$_4$ is transferred from neutrophils or macrophages to lymphocytes, endothelial cells, or erythrocytes [19,41–43] for subsequent generation of LTB$_4$. In the skin, Solá *et al* [39] and Iversen *et al* [40] suggest that keratinocytes generate LTB$_4$ using LTA$_4$, which is obtained from neutrophils in the dermis. Although we did not specifically address this question, our study has confirmed that human keratinocytes possess LTA$_4$ hydrolase although the source of LTA$_4$ is not known.

From immunohistochemical studies, keratinocytes, endothelial cells, and fibroblasts all contain LTA$_4$ hydrolase (Fig 4). These results agree with reports that LTA$_4$ hydrolase shows a ubiquitous distribution in various organs of guinea pigs [16,17]. Furthermore, it has been demonstrated that endothelial cells [41] and fibroblasts [43] as well as leukocytes have active LTA$_4$ hydrolases. However, neither the enzyme assay nor Western blotting analysis could reveal the enzyme activity in the total dermis. LTA$_4$ hydrolase in these

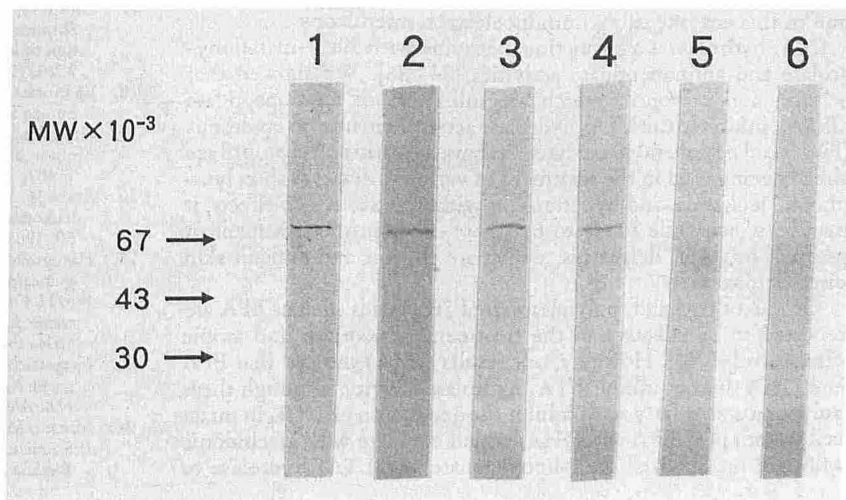

Figure 3. Immunoblot analysis using anti-LTA$_4$ hydrolase IgG. Supernatants (100,000 × *g*, 1 h; 10 μg protein in each lane) of various human skin preparations were separated on 12% SDS–polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. Then proteins were immunostained using anti-LTA$_4$ hydrolase IgG. *Lane 1,* human peripheral mononuclear cells; *lane 2,* HSC-1 cells; *lane 3,* human primary cultured epidermal cells; *lane 4,* normal human epidermis; *lane 5,* normal human whole skin; and *lane 6,* normal human dermis.

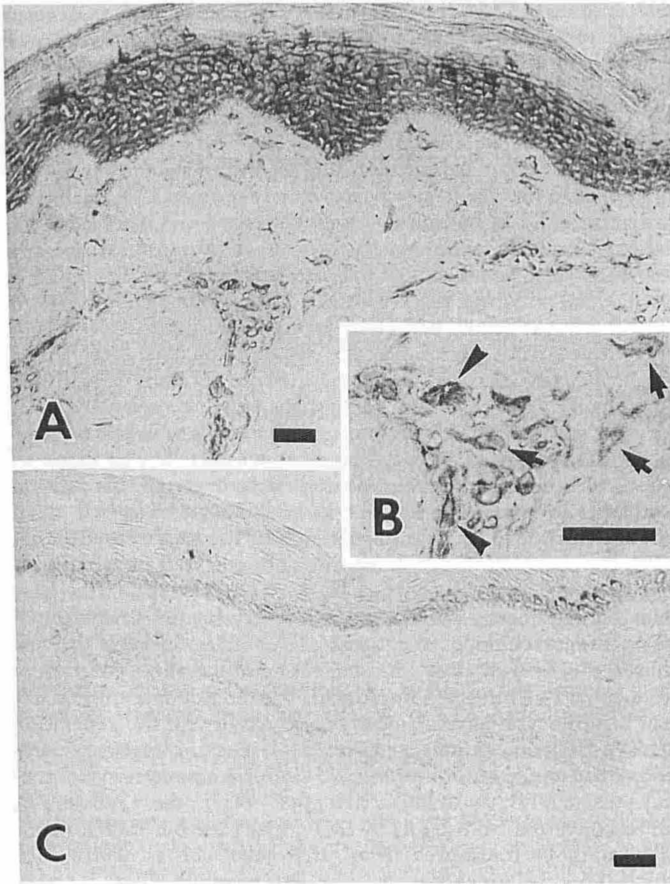

Figure 4. Immunohistochemical distribution of LTA_4 hydrolase in normal human skin as revealed with an immunoperoxidase method using antiserum against human LTA_4 hydrolase. *A)* Cytoplasmic distribution of LTA_4 hydrolase was demonstrated in the epidermal keratinocytes. *B)* In the dermis, infiltrating cells, fibroblasts *(arrows)* and endothelial cells *(arrowheads)* were stained. *C)* No specific immunostaining was observed after incubation with nonimmunized rabbit serum. *Bar,* 10 μm.

cells may participate only in a small portion in total dermis, and the enzyme activity would be below the limit of detection of our measurements. If more sensitive measurements were possible, we would expect to detect LTA_4 hydrolase activity. Furthermore, enzyme activity of LTA_4 hydrolase was not detected in primary cultured human keratinocytes in our experiment. This was due to the small sample size, which made the activity below the limit of detection. Currently, we are trying to more precisely determine the localization of this enzyme using immunoelectron microscopy.

LTA_4 hydrolase is a bifunctional enzyme with Zn^{++}-metallohydrolase and aminopeptidase activities [24–26]. We showed that bestatin and captopril, which are inhibitors of aminopeptidase [25,26], inhibited the LTA_4 hydrolase activities in human epidermis (Fig 1) and peripheral leukocytes. Because bestatin and captopril are already being used in the treatment of various diseases such as lymphoma, leukemia, and hypertension without serious side effects, it may be worthwhile to try to use these drugs in the treatment of psoriasis or atopic dermatitis, which are chronic and difficult skin diseases to treat.

On the other hand, polyunsaturated fatty acids such as EPA are reported to be effective in the treatment of psoriasis and atopic dermatitis [45,46]. However, our results (Fig 1) showed that EPA and DHA did not inhibit LTA_4 hydrolase activity, although these polyunsaturated fatty acids inhibit the generation of LTB_4 in intact cell system [47]. EPA and DHA, which compete with arachidonic acid, are metabolized by 5-lipoxygenase and LTA_4 hydrolase to LTB_5, which has much less biologic activity than LTB_4 [48,49]. Therefore, the results shown in Fig 1 are quite reasonable.

In preliminary studies we have found LTA_4 hydrolase activity is increased in the peripheral leukocytes of some patients with psoriasis or atopic dermatitis. Therefore, a useful therapy may be to control the LTA_4 hydrolase activity in patients with psoriasis or atopic dermatitis.

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