Journal of Dermatological Science (2003) 33, 31–40

ELSEVIER

Journal of Dermatological SCIENCE

www.elsevier.com/locate/jdermsci

Up-regulation of interleukin-13 receptor α1 on human keratinocytes in the skin of psoriasis and atopic dermatitis

Jongkonnee Wongpiyabovorn[a,b], Hajime Suto[a,b], Hiroko Ushio[b,*], Kenji Izuhara[c], Koichi Mitsuishi[a], Shigaku Ikeda[a], Atsuhito Nakao[b], Ko Okumura[b,d], Hideoki Ogawa[a,b]

[a] *Department of Dermatology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*
[b] *Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*
[c] *Department of Biomolecular Sciences, Saga Medical School, 5-1-1, Nabeshima, Saga 849-8501, Japan*
[d] *Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*

Received 18 February 2003; received in revised form 2 June 2003; accepted 3 June 2003

KEYWORDS
Interleukin-13;
Interleukin-4;
IFN-γ;
Psoriasis;
Atopic dermatitis

Summary *Background:* Interleukin (IL)-13 is a pleiotropic cytokine, which shares many biological functions with IL-4. The receptor subunits of IL-13 consist of IL-4Rα, IL-13Rα1 and IL-13Rα2. The regulatory mechanisms of the IL-13Rα expression in the keratinocytes of certain skin disease have not been known. *Objective:* To clear the roles of IL-13 and the regulatory mechanisms of its receptor in atopic dermatitis (AD) and psoriasis. *Method:* The expression of IL-13Rα1 in the skin of AD and psoriasis was investigated by immunohistochemistry. The regulation of IL-13Rα mRNA in the skin and human primary keratinocyte (HPK) was investigated by quantitative PCR. The secretion of IL-6 and RANTES from HPK was measured by ELISA. *Results:* The expression of IL-13Rα1 was more prominent on the suprabasal keratinocytes in the skin of AD and striking increase of staining was observed on all layers of keratinocyte in the skin of psoriasis. The mRNA of IL-13Rα1, but not of IL-13Rα2 was overexpressed in both skin of AD and psoriasis. In vitro experiment using HPK demonstrated that IFN-γ, IL-13 but not IL-4 could up-regulate the mRNA expression of IL-13Rα1. In contrast, IL-13Rα2 mRNA expression was up-regulated by IFN-γ plus IL-4. Furthermore, the stimulation of HPK with IFN-γ plus IL-13 and/or IL-4 resulted in significant enhancement of IL-6 and RANTES secretion. *Conclusion:* These findings indicate that IL-4 and IL-13 have different regulatory effects on the expression of IL-13Rα1 and α2, and the overexpression of IL-13Rα1 may play some roles in the pathogenesis of chronic stage of AD or psoriasis.

*Corresponding author. Tel.: +81-3-5802-1591; fax: +81-3-3813-5512.
E-mail address: hushio@med.juntendo.ac.jp (H. Ushio).

0923-1811/03/$30.00
doi:10.1016/S0923-1811(03)00148-8

1. Introduction

Interleukin (IL)-13 is an immunoregulatory cytokine secreted predominantly by activated Th2 cells and mast cells [1,2]. IL-13 shares many functional properties with IL-4, including the induction of IgE synthesis, CD23 expression by B cells [3], the up-regulation of major histocompatibility complex class II on monocytes [4,5], and the induction of vascular cell adhesion molecule-1 on endothelial cells [6]. IL-13 exerts its actions via binding to the complex receptor consisting of IL-4Rα and at least two other cell surface proteins, IL-13Rα1 and IL-13Rα2 [7–10]. IL-13Rα1 itself, binds IL-13 with low affinity, but when paired with IL-4Rα, it binds IL-13 with high affinity and form functional receptor which signals [7,11]. Whereas, IL-13Rα2 that can bind IL-13 with high affinity, has been postulated to be a decoy receptor because of its short cytoplasmic tail without having any signaling motifs [12]. We have previously demonstrated that IL-13Rα1 is expressed on the keratinocyte in healthy skin [13]. But the physiological and pathological roles of this receptor in the function of keratinocyte are largely unknown.

Also the regulation of IL-13Rα1 and IL-13Rα2 expression has not been understood completely. It is reported that these receptor components are expressed on both haematopoietic (T, B lymphocyte, monocyte, mast cell, basophil) and non-haematopoietic cell (endothelial cell, keratinocyte, fibroblast, respiratory epithelium, heart muscle and hepatocyte) [13,14]. IL-4 increases IL-4Rα mRNA transcription in T cells [14], whereas IL-13 and IL-4 down-regulate IL-4Rα and IL-13Rα1 expressions on monocytes [14–16] but up-regulate the mRNA and the surface expression of IL-13Rα1 on B lymphocyte [17]. In addition, it is reported that IFN-γ up-regulates IL-13Rα2 surface expression on U937 cell line, A549 cell line and primary nasal epithelial cells [18]. Thus, it is possible that each cell has specific regulatory mechanisms of the expression of IL-13Rα1 and IL-13Rα2.

It is generally believed that IL-4Rα/IL-13Rα1 serves as a functional receptor complex for both IL-4 and IL-13 in non-haematopoietic cells, such as endothelial cell, fibroblast, respiratory epithelium, smooth muscle cell, all of which lack the common γ chain of IL-2 receptor (IL-2Rγ) [17,19]. Thus, the most of the biological effects and the signaling events elicited by IL-4 and IL-13 are overlapping. IL-13 utilizes Janus tyrosine kinase (JAK)-signal transducer and activation of transcription (STAT) pathways like IL-4. It has been demonstrated that Tyk2, Jak1, member of JAK family, and STAT6, STAT3, a member of the STAT family, are activated by IL-13 [20,21]. But recent accumulating evidence in the murine asthma model suggests that IL-13 can exert its biological function by acting directly on the bronchial epithelial cell or on the smooth muscle cell, independent of IL-4 and IgE synthesis [22,23]. However, there is not enough information how IL-13 exerts its specific biological function that is different from IL-4 via same subunits IL-4Rα/IL-13Rα1 in different cell types.

In this study, we first demonstrate that the expression of IL-13Rα1 is increased in the chronic inflammatory skin disease, such as atopic dermatitis (AD) or psoriasis in which the roles of IL-13 have been implicated [24–26]. Furthermore, we demonstrate the expression of each receptor, IL-13Rα1 and IL-13Rα2, on keratinocyte is differently regulated by IFN-γ, IL-13 and IL-4.

2. Materials and methods

2.1. Cell cultures

Human primary keratinocytes (HPKs) were prepared from human foreskin as previously described [27] and cultured in the serum-free Keratinocyte Growth Medium (Kurabo, Osaka, Japan) supplemented with 0.1 ng/ml human epidermal growth factor, 2 ml of bovine pituitary extract, 10 μg/ml Insulin, 0.5 μg/ml hydrocortisone and antibiotics. Cells were grown at 37 °C in 5% CO_2, two to three passages of the cells were used for all experiments. Human premyeloid erythroleukemia (TF-1) and human basophilic cell line (KU812) were cultured in the RPMI 1640 medium, supplemented with 10% heat inactivated fetal bovine serum (Sigma-Aldrich, Tokyo, Japan), 10 mM HEPES and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin). Human endothelial cell was grown as previously described [28].

2.2. Immunohistochemistry

Skin punch biopsies from the forehand of chronic AD ($n = 6$), psoriasis ($n = 6$) or the breast of normal ($n = 6$) skin were obtained with written informed consent under the approval of the ethical committee of the University. Chronicity of AD was diagnosed based on the standard criteria [29]. Frozen sections were made and fixed with cold acetone for 5 min. The specimens were probed with anti-human IL-13Rα1 (UU15) antibody [13] or isotype control, mouse IgG2aκ (DAKO JAPAN Co., Ltd, Kyoto, Japan) followed by incubation with biotin-conjugated rabbit anti-mouse IgG (DAKO JAPAN Co., Ltd) and then alkaline phosphatase-conju-

gated streptavidin (DAKO JAPAN Co., Ltd). The signal was detected by the alkaline phosphatase method according to the manufacturer's instruction (DAKO JAPAN Co., Ltd).

2.3. RT reaction and quantitative PCR

Total RNA was extracted using RNA STAT-60 (Teltest inc., Fiends wood, TX) according to the manufacturer's instruction from the stimulated primary keratinocytes or the 5-mm skin biopsies of chronic atopic, psoriatic skin lesions and normal skin of plastic surgery. Three μg of total RNA was reverse-transcribed using superscript II RT (Invitrogen Japan, Tokyo) and oligo dT primer (Invitrogen Japan, Tokyo). The specific pairs of primers used for detecting human IL-13Rα1, IL-13Rα2, IL-4Rα, common γ chain and GAPDH were 5′ CAG TGT AGC ACC AAT GAG AGT GAG 3′ and 5′ TCA GGT TTC ACA CGG GAA GTT A 3′ for IL-13Rα1; 5′ ATA CCT TTG GGA CCT ATT CC 3′ and 5′ TGA ACA TTT GGC CAT GAC TG 3′ for IL-13Rα2; 5′ GTC TGC AGA TGA GGACTA GGG G3′ and 5′ TAC TCT CAT GGG ATG TGGG CG 3′ for IL-4Rα; 5′ GAC AGG CCA CAC AGA TGC TA 3′ and 5′ GTT CAC TGT AGT CTG GCT GC 3′ for common γ chain; and 5′ ACC ACA GTC CAT GCC AT 3′ and 5′ ACC ACC CTG TTG CTG TA 3′ for GAPDH, respectively.

The competitive cDNA fragment for IL-13Rα1, IL-13Rα2 and GAPDH were constructed by deletion of a 51-bp fragment of the IL-13Rα1 target cDNA (386 bp) by cleaving between position 395 and 446 by NsiI; by deletion of a 141-bp fragment of the IL-13Rα2 target cDNA (428 bp) by cleaving between position 1101 and 1242 by BslI, and by deletion of a 106-bp fragment from GAPDH target cDNA (451 bp) by cleaving between position 711 and 816 by XcmI, respectively. The PCR reactions were carried out with 1 μl of cDNA as a template with specified primers and various amounts of competitors (0.1 fg/μl–100 pg/μl) using Ampli Taq Gold polymerase (Applied Biosystems, Branchburg, NJ). The PCR cycles were composed of the denature at 94 °C for 5 min and 45 cycles of 94 °C for 45 s, 60 °C for 1 min, 72 °C for 1 min for IL-13Rα1; 35 cycles of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 2 min for IL-13Rα2: 35 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min for IL-4Rα and common γ chain; and 27 cycles of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 2 min for GAPDH. The 8 μl of each PCR product was run on a 2% agarose gel, stained with 2 μg/ml of Ethidium Bromide solution for 30 min and quantified with a Storm Fluor Imager (Molecular Dynamics, Inc, Uppsala Sweden). Data was normalized according to the GAPDH expression and expressed as the ratio of each α chain of mRNA to GAPDH mRNA. Semi-quantitative PCR was performed using normalized amount of cDNA according to the GAPDH expression and specific primers without competitors. PCR product was analyzed as described above using a Storm Fluor Imager after staining with EtBr and expressed as the value of EtBr volume.

2.4. Measurement of cytokine concentrations

Human keratinocytes (1×10^5 cell/ml) in serum-free Keratinocyte Growth Medium were stimulated with 100 ng/ml of recombinant human IFN-γ (Genzyme Techne, Minneapolis, MN) alone or combination with 100 ng/ml of recombinant human IL-13 and IL-4 (PeproTech EC Ltd, London, England) for 48 h. The levels of IL-6 and RANTES in the supernatant were measured by ELISA kit (Genzyme Tech Corp). The viability of the cells under each condition was more than 98% and there were no significant differences in the number of viable cells under each condition after 48 h incubation. The experiments were performed four times using different keratinocyte preparations. Results were shown as pg/10^6 cell $\pm$S.D.

2.5. Statistical analysis

Statistical analysis was performed using the Student t-test. Values of $P \leq 0.05$ were considered as significant.

3. Results

3.1. Immunohistochemical detection of IL-13Rα1 in the skin of AD and psoriasis

Since we have previously demonstrated that the keratinocyte was a main cell expressing IL-13Rα1 in the skin [13], we examined the expression of this receptor in the chronic inflammatory skin of AD and psoriasis patients. The distribution of IL-13Rα1 in normal skin was consistent with the previous results, in that, mostly lower layer of keratinocytes expressed IL-13Rα1 (Fig. 1a and b). There were some positive staining cells in the dermis, which had mast cell like futures (Fig. 1a and b). When we performed immunohistochemical staining on the skin of chronic AD, positive results were observed in the upper, more differentiated keratinocytes (Fig. 1d and e). Also positive staining was observed among the infiltrating cells in the dermis. Even though we have not specified phenotype of these

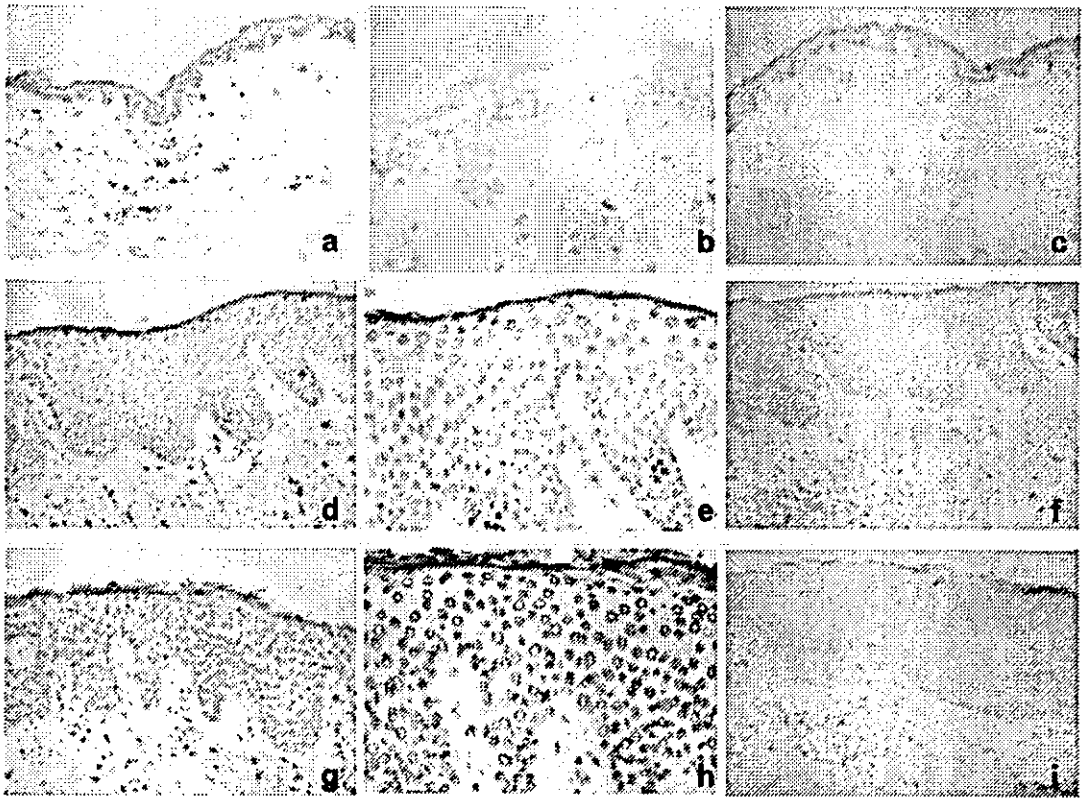

Fig. 1 Immunohistochemical detection of IL-13Rα1 in the skin of AD and psoriasis. Immunohistochemical staining was performed on the skin specimens from normal (a–c), AD (d–f) or psoriasis patients (g–i), using anti-IL-13Rα1 (UU15) or isotype control, mouse IgG2a antibody (c, f and i), as described in Method. The signal development was done using alkaline phosphatase method according to the manufacture's instruction. Nuclear staining was done by methyl green. Data shown is one representative result from several experiments that had similar results. (b, e, h: 120 × , a, c, d, f, g, i: 60 ×).

cells, the size and shape showed mast cell- and monocyte-like features (Fig. 1a, b, d and e). No clear differences were observed in the staining of IL-13Rα1 in the dermis of healthy and AD patients except that there were increased numbers of mast cell-like, positive-stained cells in the AD lesion. Striking increase of positive staining of IL-13Rα1 was observed in all stages of keratinocytes from the psoriatic skin (Fig. 1g and h) and the staining intensity was higher in the skin of psoriasis than that of normal or AD. No positive staining was observed in all specimens stained with isotype control, mouse IgG2a (Fig. 1c, f and i).

3.2. The expression of IL-13Rα1 and IL-13Rα2 mRNA in the skin of chronic AD and psoriasis

The mRNA expressions of IL-13Rα1 and IL-13Rα2 in the skin biopsies from chronic AD and psoriasis were analyzed by quantitative PCR. IL-13Rα1, but not IL-13Rα2 mRNA expression, was significantly increased in the chronic skin lesion of AD and psoriasis compared to the normal skin (Fig. 2a and b). The result, the increment of IL-13Rα1 mRNA was much higher in the skin of psoriasis than AD, was consisted with the immunohistochemical data. We aware of that we can not completely exclude the possibility that the increment of IL-13Rα1 mRNA expression we observed was due to the increase of infiltrating cells in the dermis but not due to the expression of this receptor on keratinocyte, since we used whole skin for the isolation of RNA. But immunohistochemical data and the differences in the expression of IL-13Rα1 mRNA between AD and psoriasis (both have almost same amount of infiltrating cells) strongly support that the keratinocyte is a major cell contributing to the increment of IL-13Rα1 mRNA expression in AD and psoriasis. Since the high affinity IL-13 receptor consists of heterodimeric unit of IL-13Rα and IL-4Rα, we also measured the levels of IL-4Rα mRNA

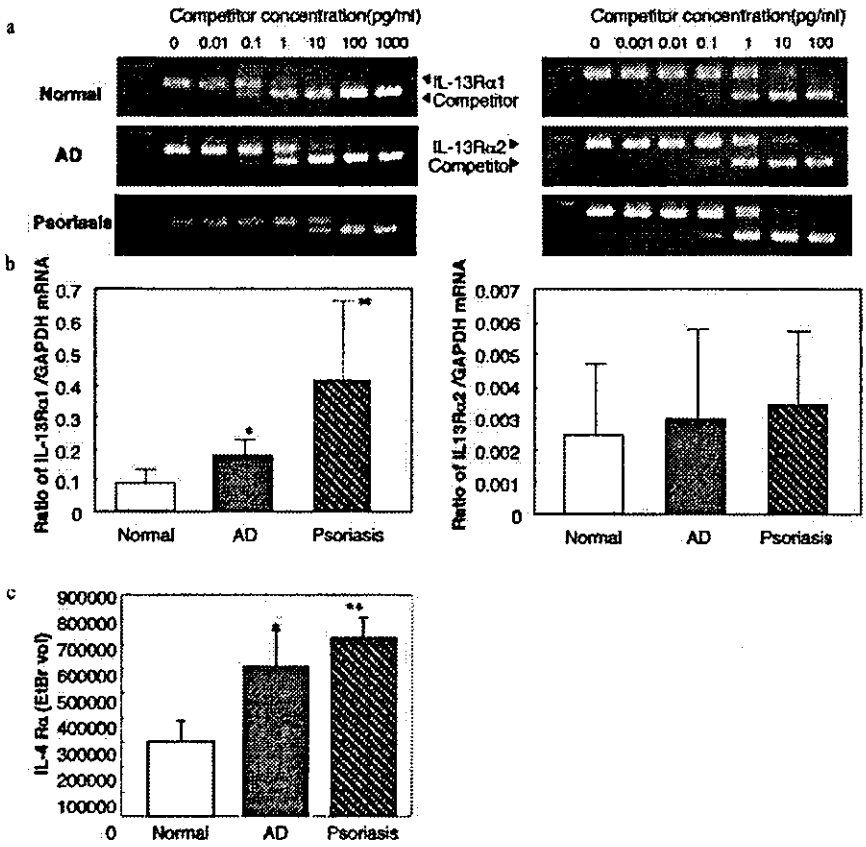

Fig. 2 The expression of IL-13Rα1 and IL-13Rα2 mRNA in the skin of chronic AD and psoriasis. Total RNA was isolated from the skin specimens of normal, AD and psoriasis, and quantitative PCR was performed as described in Section 2. The amount of cDNA was normalized according to the expression of GAPDH. Representative result from each skin sample were shown in (a) upper; normal, middle; AD and lower; psoriasis. The quantitation of each band was done using image analyzing soft and showed as the ratio of mRNA of IL-13Rα1 to GAPDH (left) or IL-13Rα2 to GAPDH (right) (b). The expression of IL-4Rα mRNA was measured semi-quantitatively as described in Section 2 (C). Data shown are mean ± S.D. of six experiments conducted with different skin preparations from different patients. $^{**}P < 0.01$, $^{*}P < 0.05$, significantly different from the mean value of the normal skin.

by semi-quantitative PCR. Consisted with previous report [25,35,38], IL-4Rα was significantly increased in the chronic skin lesion of AD and psoriasis compared to the normal skin (Fig. 2c).

3.3. Modulation of IL-13Rα1 and IL-13Rα2 mRNA expression in primary human keratinocyte by IFN-γ, IL-4 and IL-13

Having results that IL-13Rα1 was overexpressed on human keratinocyte with the chronic skin disease (Fig. 2a and b), next we investigated how the expression of IL-13Rα1 and α2 were regulated in the keratinocytes. Since IL-13, IL-4 or IFN-γ have been reported to have regulatory role in IL-13 receptor expression on both haematopoietic and non-haematopoietic cells, we first examined the effects of these cytokines on the expression of IL-13Rα1 and IL-13Rα2 mRNA using HPKs in vitro. As shown in Fig. 3a, IL-13 or IFN-γ alone significantly enhanced the IL-13Rα1 mRNA expression. In contrast, the same concentration of IL-4 had no effect on the IL-13Rα1 mRNA expression. Furthermore, simultaneous addition of IL-4 and IL-13 abolished this effect of IL-13. Combined stimulation of IL-4 with IFN-γ suppressed, but not significantly, the IL-13Rα1 mRNA expression which was up-regulated by IFN-γ alone. In contrast, as shown in Fig. 3b, IL-4 combined with IFN-γ significantly increased the IL-13Rα2 mRNA expression which was not observed by the combined stimulation with IFN-γ and IL-13. These results demonstrated that not only IL-13 or IL-4 could modulate their own receptor expressions, but also IFN-γ could regulate the IL-13Rα mRNA expression in primary human keratinocyte,

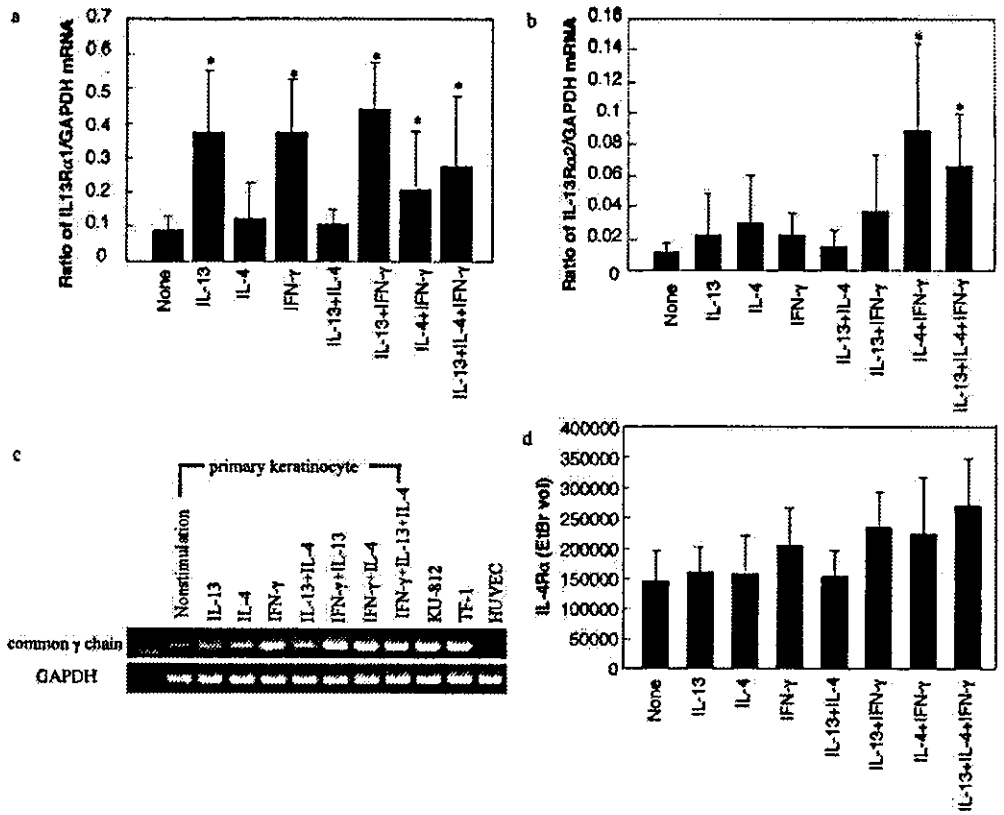

Fig. 3 Modulation of IL-13Rα1 and IL-13Rα2 mRNA expression in primary human keratinocyte by IFN-γ, IL-4 and IL-13. Primary human keratinocytes were stimulated with IFN-γ (100 ng/ml) alone or combination of either IL-13 (100 ng/ml) or IL-4 (100 ng/ml) for 48 h and total RNA was isolated and proceed for quantitative PCR of IL-13Rα1 and α2 or for semi-quantitative PCR for IL-4Rα. The amount of cDNA was adjusted according to the expression of GAPDH and the data was showed as the ratio of mRNA of IL-13Rα1 to GAPDH (a) or IL-13Rα2 to GAPDH (b). Data shown were mean ± S.D. of four experiments conducted with different preparation of keratinocytes. (c) The mRNA expression of common γ-chain and (d) IL-4Rα was detected as described in Section 2. Human premyeloid erythroleukemia (TF-1), human basophilic cell line (KU812) and human umbilical endothelial cell (HUVEC) were used as positive or negative control, respectively. *$P < 0.05$, significantly different from the mean value of unstimulated keratinocytes.

and the mRNA expressions of IL-13Rα1 and IL-13Rα2 were differently regulated by IL-4 and IL-13.

To address the possibility that the different effects of IL-13 and IL-4 on the expression of each IL-13R component may come from that IL-4 signals through type I IL-4R consisting of IL-4Rα1 and common γ chain, we investigated the expression of common γ chain in HPK. Semi-quantitative PCR revealed that, the mRNA for the common γ chain was slightly expressed in the resting HPK and the expression was enhanced by IFN-γ, comparable to the levels of other haematopoietic cells, such as KU812 or TF-1 (Fig. 3c). Same condition of PCR did not reveal the detectable expression of the mRNA of common γ chain in the human endothelial cells as has been reported (Fig. 3c) [6]. But, no detectable protein for the common γ chain was confirmed by FACS analysis even in the IFN-γ-stimulated keratinocytes (Data not shown). Also we tried to confirm the increased surface expression of the IL-13Rα1 and IL-13Rα2 on keratinocytes, there were no differences in the expression of these receptors upon stimulation with IFN-γ, IL-4, IL-13 analyzed by FACS (Data not shown). Semi-quantitative PCR for IL-4Rα mRNA revealed that its expression was seemed to increase upon stimulation with IFN-γ, and simultaneous stimulation with IL-4 and IL-13, but not significant. (Fig. 3d).

3.4. Inflammatory cytokine secretion from the primary keratinocytes stimulated with IL-4, IL-13 and IFN-γ

To examine the biological consequence of the enhanced expression of IL-13Rα1, we measured inflammatory cytokine production from keratino-

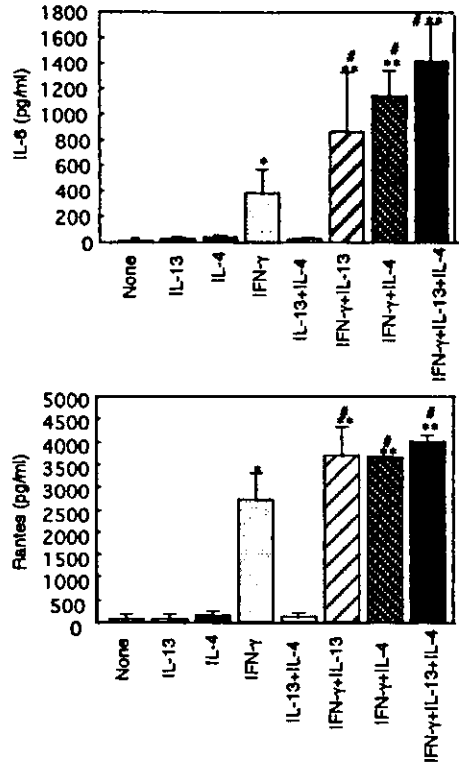

Fig. 4 Inflammatory cytokine secretion from the primary keratinocytes stimulated with IL-4, IL-13 and IFN-γ. Primary human keratinocytes were stimulated with IFN-γ (100 ng/ml) alone or combination of either IL-13 (100 ng/ml) or IL-4 (100 ng/ml) for 48 h and the levels of IL-6 and RANTES were measured by ELISA kit. Data shown are mean ±S.D. of four experiments conducted using different sources of keratinocytes. $^{**}P < 0.01$, $^{*}P < 0.05$, significantly different from the mean value of the unstimulated control. $\#P < 0.05$, significantly different from the mean value of the IFN-γ-stimulated cells.

cytes upon stimulation with these cytokines. As shown in Fig. 4, even though IL-4 or IL-13 alone could not induce secretion of IL-6 and RANTES from the HPKs 48 h after stimulation, the presence of IL-13 or IL-4 significantly enhanced the IFN-γ-stimulated secretion of these cytokines. IL-8 and IP-10, two cytokines involves in the psoriasis, were not produced by the primary keratinocyte stimulated by IL-4 or IL-13 alone. Also the IFN-γ-induced secretion of these cytokines was tend to increase but not significantly modified by the presence of IL-4 or IL-13 (Data not shown).

4. Discussion

The biological effects of IL-4 and IL-13 are thought to be mediated by a shared receptor composed of IL-4Rα and IL-13Rα1 chain, expressed on a variety of cell types. In this study, we examined the expression of IL-13Rα1 in the skin of chronic inflammatory disease and the regulation of IL-13Rα1, IL-13Rα2 mRNA expression in HPK.

We first demonstrated that there were differences in the expression patterns of IL-13Rα1 in the skin, especially epidermal layer of keratinocytes of normal, AD and psoriasis. Although the epidermal hyperplasia is a characteristics observed in both AD and psoriasis, the biochemical mechanisms or mediators responsible for this remain unclear. Recent reports have suggested that the signaling through the IL-20 receptor, which binds both IL-19 and IL-20, causes proliferation and aberrant differentiation of keratinocytes in the psoriasis [31,32]. Thus, it is tempting to hypothesis that different staining patterns of IL-13Rα1 observed in AD vs. psoriasis reflect the different mechanism of hyperplasia between them. Another possibility to explain this phenomenon may come from the different cytokine environment in AD and psoriasis. AD is the chronic inflammatory disease that has biphasic cytokine response, predominant Th2 cytokine such as IL-4, IL-5, IL-10 and IL-13 in acute phase, while Th1 cytokine, especially IFN-γ accompanied by Th2 cytokines in chronic phase [33]. Whereas, the predominant cytokine produced in psoriasis is reported to be IFN-γ, IL-15 and less prominent, IL-13 [34]. Consisted with our in vitro data in which IFN-γ appears to be a major cytokine for the induction of IL-13Rα1, the expression of IL-13Rα1 was much higher in the skin of psoriasis than AD. These phenomena may reflect the predominance of IFN-γ in the psoriasis compared to the AD that may have other inhibitory cytokine, such as IL-4. Cancino-Diaz et al. [35] have recently demonstrated that overexpression of the mRNA but underexpression of the protein of IL-13Rα1 in psoriatic keratinocyte. The clear discrepancy in the detection of IL-13Rα1 protein observed between them and us may come from the different antibody used for the detection of IL-13Rα1, since we have not get similar staining using commercially available antibody and same skin sample (Data not shown). Or because of we used frozen section instead of parafolmalized-paraffin section. We have observed different regulation of IL-13Rα1 or IL-13Rα2 mRNA in primary keratinocyte by IL-4 and IL-13 (Fig. 3). Since the signaling unit for the IL-4 and IL-13 on non-haematopoietic cell is though to be a heterodimer consisting of IL-4Rα and IL-13Rα1 subunit, we do expect that IL-4 and IL-13 have similar effect. To explain this phenomenon, we look for the IL-4 specific signaling unit, common γ chain. Even though we could detect the slight

mRNA expression for common γ chain in resting keratinocyte and the enhancement of the expression in keratinocyte with IFN-γ stimulation, no detectable surface protein was observed by FACS analysis in any conditions of keratinocytes. Thus, it is reasonable to think that IL-4 and IL-13 signal through the same receptor unit, IL-4Rα/IL-13Rα1, or keratinocytes express new signaling unit as has been reported in the myofibroblast (IL-13Rα1, IL-4Rα plus IL-13Rα2 or IL-13Rα1, IL-4Rα plus common γ chain) [36,37].

Consisted with previous reports [25,35,38], the expression of IL-4Rα mRNA was simultaneously overexpressed in the skin of patients with psoriasis and AD. Since the affinity difference of IL-4 and IL-13 to each subunit has been reported [39], the proper ratio of IL-13Rα1 to IL-4Rα may determine the responsibility of the cell to IL-4 or IL-13.

So far, we do not know how IL-4 and IL-13 has different biological effects on keratinocytes through the same receptor unit consists of IL-13Rα1/IL-4Rα. Recent experiment using HaCaT cell line has reported that IL-4 and IL-13 have different effect on the TNF-α-and IFN-γ-induced secretion of Thymus and activation-regulated chemokine and interferon-induced protein (IP)-10 [40]. Thus further experiment is required to elucidate the signaling unit shared or unshared by IL-4 and IL-13 in human keratinocytes.

The regulation of decoy receptor, IL-13Rα2 has been recently reported in different cells. IFN-γ stimulates the rapid mobilization of IL-13Rα2 from intracellular storage to the cell surface without protein synthesis and resulted in diminished IL-13 signaling in nasal epithelial cell, monocytic cell (U937) and lung carcinoma (A549) [18]. Other report using HaCaT showed that IL-4 and IL-13 itself induce the IL-13Rα2 mRNA by STAT6, ERK and p38 MAPK dependent manner [41]. In contrast to their data, our experiment revealed that IL-4 or IL-13 alone could not, but IFN-γ plus IL-4 could induce the IL-13Rα2 mRNA in primary keratinocyte. The difference between our data and their data may come from primary vs. immortalized cell line. Since several differences in the response have been reported between HaCaT and primary human keratinocytes [42].

Much has not been known about the functional significance of IL-13 on the HPK. There were several reports about the secretion of cytokines or chemokines using immortalized keratinocytes, A432, SVK14 and HaCaT but not in the primary keratinocytes [30,40]. Although the induction of CD60w has been demonstrated in primary keratinocytes by IL-4 and IL-13 and its suppression by IFN-γ [43], the biological significance of CD60w in psoriasis remains unclear. Recent report has suggested the subcutaneous administration of IL-4 improved the psoriasis by antagonizing the IFN-γ-producing T cells and reducing the concentration of IL-8 and IL-19 in the skin, two cytokines directly involved in psoriasis [31]. The target cell to this treatment is not clear. In addition to T cells, the keratinocyte, which express IL-4Rα/IL-13Rα1, may be a direct target to IL-4. It is interesting to know whether IL-13 has same effect or not.

Collectively we found that up-regulation of IL-13Rα1 in the skin of chronic inflammatory disease, such as AD or psoriasis and suggest the possibility that the expression of IL-13Rα1 and IL-13Rα2 is modulated by the IFN-γ, IL-13 or IL-4 or combination of these cytokines. We also found using primary human keratinocytes that regulatory mechanism of IL-13Rα by IL-13 and IL-4 was not the same. These results may help to explain complicated pathology observed in the chronic phase of AD or psoriasis and to understand the role of IL-13 in the function of keratinocyte, such as proliferation or differentiation or any other unknown function in physiological and pathological conditions.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology. Dr Wongpiyabovorn Jongkonnee was supported by grants from Japan International Cooperation Agency (JICA). The authors thank to Drs Kamolwat, P., Suga, Y., Hira, K., Hasegawa, T for providing us valuable clinical samples and Drs Nakano, N, Nakayama, K. and Ms Tokura, T. for technical assistances.

References

[1] Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, et al. Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. Nature 1993;362:248–50.

[2] Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. Science 1998;282:2258–60.

[3] Defrance T, Carayon P, Billian G, Guillemot JC, Minty A, Caput D, et al. Interleukin 13 is a B cell stimulating factor. J Exp Med 1994;179:135–43.

[4] Doherty TM, Kastelein R, Menon S, Andrade S, Coffman RL. Modulation of murine macrophage function by IL-13. J Immunol 1993;151:7151–60.

[5] de Waal Malefyt R, Figdor CG, Vries JE. Effects of interleukin 4 on monocyte functions: comparison to interleukin 13. Res Immunol 1993;144:629–33.

[6] Kotowicz K, Callard RE, Friedrich K, Matthews DJ, Klein N. Biological activity of IL-4 and IL-13 on human endothelial

cells: functional evidence that both cytokines act through the same receptor. Int Immunol 1996;8:1915–25.
[7] Hilton DJ, Zhang J-G, Metcalf D, Alexander WS, Nicola NA, Willson TA. Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. Proc Nat Acad Sci 1996;93:497–501.
[8] Obiri NI, Debinski W, Leonarud WJ, Puri RK. Receptor for interleukin 13. J Biol Chem 1995;270:8797–804.
[9] Orchansky PL, Kwan R, Lee F, Schradu JW. Characterization of the cytoplasmic domain of interleukin-13 receptor alpha. J Biol Chem 1999;275:20818–25.
[10] Miloux B, Laurent P, Bonnin O, Lupker J, Caput D, Vita N, et al. Cloning of the human IL-13R alpha1 chain and reconstitution with the IL-4R alpha of a functional IL-4/IL-13 receptor complex. FEBS Lett 1997;401:163–6.
[11] Zurawski SM, Chomarat P, Djossou O, Bidaud C, Mckenzie AN, Miossec P, et al. The primary binding subunit of the human interleukin-4 receptor is also a component of the interleukin 13 receptor. J Biol Chem 1995;270:13869–78.
[12] Donaldson DD, Whitters MJ, Fitz LJ, Neben TV, Finnerty H, Henderson SL, et al. The murine IL-13 receptor alpha2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha1. J Immunol 1998;161:2317–24.
[13] Akaiwa M, Yu B, Umeshita-Suyama R, Tera N, Suto H, Koga T, et al. Localization of human interleukin-13 receptor in non-haematopoietic cells. Cytokine 2001;13:75–84.
[14] Graber P, Gretener D, Herren S, Aubry JP, Elson G, Poudrier J, et al. The distribution of IL-13 receptor alpha1 expression on B cells, T cells and monocytes and its regulation by IL-13 and IL-4. Eur J Immunol 1998;28:4286–98.
[15] Hart PH, Bonder CS, Balogh J, Dickensheets HL, Vazquez N, Davies KV, et al. Diminished responses to IL-13 by human monocytes differentiated in vivo: role of the IL-13Rα1 chain and STAT6. Eur J Immunol 1999;29:2087–97.
[16] So EY, Park HH, Lee CE. IFN-γ and IFN-α posttranscriptionally down-regulate the IL-4-induced IL-4 receptor gene expression. J Immunol 2000;165:5472–9.
[17] Vita N, Lefort S, Laurent P, Caput D, Ferrara P. Characterization and comparison of the interleukin 13 receptor with the interleukin 4 receptor on several cell types. J Biol Chem 1995;270:3512–7.
[18] Daines MO, Hershey GK. A novel mechanism by which interferon-γ can regulate interleukin (IL)-13 responses. J Biol Chem 2002;277:10387–93.
[19] Palmer-Crocher RL, Hughes CC, Pober JS. IL-4 and IL-13 activate the JAK2 tyrosine kinase and STAT6 in cultured human vascular endothelial cells through a common pathway that does not involve the gamma c chain. J Clin Invest 1996;98:604–9.
[20] Izuhara K, Umeshita-Suyama R, Akaiwa M, Shirakawa T, Deichmann KA, Arima K, et al. Recent advances in understanding how interleukin-13 signals are involved in the pathogenesis of bronchial asthma. Arch Immunol Ther Exp 2000;48:502–12.
[21] Umeshita-Suyama R, Sugimoto R, Akaiwa M, Arima K, Yu B, Wada M, et al. Characterization of IL-4 and IL-13 signals dependent on the human IL-13 receptor alpha chain 1: redundancy of requirement of tyrosine residue for STA3 activation. Int Immunol 2000;12:1499–509.
[22] Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998;282:2261–3.
[23] Laporte JC, Moore PE, Baraldo S, Jouvin MH, Church TL, Schwartzman IN, et al. Direct effects of interleukin-13 on signaling pathways for physiological responses in cultured human airway smooth muscle cells. Am J Respir Crit Care Med 2001;164:141–8.
[24] Hamid Q, Naseer T, Minshall EM, Song YL, Boguniewicz M, Leung DY. In vivo expression of IL-12 and IL-13 in atopic dermatitis. J All Clin Immunol 1996;98:225–31.
[25] Taha RA, Leung DYM, Ghaffar O, Boguniewicz M, Hamid Q. In vivo expression of cytokine receptor mRNA in atopic dermatitis. J All Clin Immunol 1998;102:245–50.
[26] Skov L, Chan L, Fox DA, Lansen JK, Voorhees JJ, Cooper KD, et al. Lesional psoriatic T cell contain the capacity to induce a T cell activation molecule CDw60 on normal keratinocytes. Am J Pathol 1997;150:675–83.
[27] Barlow Y, Pye RJ. Keratinocyte culture. Method Mol Biol 1997;75:117–29.
[28] Moser R, Scheleiffenbaum B, Groscurth P, Fehr J. Interleukin 1 and tumor necrosis factor stimulate human vascular endothelial cells to promote transendothelial neutrophil passage. J Clin Invest 1989;83:444–55.
[29] Beltrani VS. The clinical spectrum of atopic dermatitis. J Allergy Clin Immunol 1999;104:S87–98.
[30] Wery-Zennaro S, Letourneur M, David M, Bertoglio J, Pierre J. Binding of IL-4 to the IL-13Rα1/IL-4Rα receptor complex leads to STAT3 phosphorylation but not to its nuclear translocation. FEBS Lett 1999;464:91–6.
[31] Ghoreschi K, Thomas P, Breit S, Dugas M, Mailhammer R, Van Eden W, et al. Interluekin-4 therapy of psoriasis induced Th2 responses and improves human autoimmune disease. Nature Med 2003;9:40–6.
[32] Blumberg H, Conklin D, Xu WF, Grossmann A, Brender T, Carollo S, et al. Interelukin-20: discovery, receptor identification and the role in epidermal function. Cell 2001;104:9–19.
[33] Pastore S, Mascia F, Giustizieri ML, Giannetti A, Girolomoni G. Pathogenetic mechanism of atopic dermatitis. Arch Immunol Ther Exp 2000;48:497–504.
[34] Nickoloff BJ, Bonish B, Huang BB, Porcelli SA. Characterization of a T cell line bearing natural killer receptors and capable of creating psoriasis in a SCID mouse model system. J Dermatol Sci 2000;24:212–25.
[35] Cancino-Diaz JC, Reyes-Maldonado E, Carmen A, Banuelos-Panuco CA, Jimenez-Zamudio L, Garcia-Latorre E. Interleukin-13 receptor in psoriatic keratinocytes: overexpression of the mRNA and underexpression of the protein. J Invest Dermatol 2002;119:1114–20.
[36] Joshi BH, Kawakami K, Leland P, Puri RK. Heterogeneity in interleukin-13 receptor expression and subunit structure in squamous cell carcinoma of head and neck: differential sensitivity to chimeric fusion proteins comprised of interleukin-13 and a mutated form of pseudomonas exotoxin. Clin Cancer Res 2002;8:1948–56.
[37] Doucet C, Giron-Michel J, Canonica GW, Azzarone B. Human lung myofibroblasts as effectors of the inflammatory process: the common receptor γ chain is induced by Th2 cytokines, and CD40 ligand is induced by lipopolysaccharide, thrombin and TNF-α. Eur J Immunol 2002;32:2437–49.
[38] Prens E, Hgmans J, Lien RC, Debets R, Van Joost T, Benner R. Increased expression of interleukin-4 receptors on psoriatic epidermal cells. Am J Pathol 1996;148:1493–502.
[39] Schnyder B, Lugli S, Feng N, Etter H, Lutz RA, Ryffel B, et al. Interleukin-4 (IL-4) and IL-13 bind to a shared heterodimeric complex on endothelial cells mediating vascular cell adhesion molecule-1 induction in the absence of the common gamma chain. Blood 1996;15:4286–95.
[40] Wakugawa M, Nakamura K, Akatsuka M, Nakagawa H, Tamaki K. Interferon-gamma-induced RANTES production by human keratinocytes is enhanced by IL-1β, TNF-α, IL-4

and IL-13 and is inhibited by dexamethasone and tacrolimus. Dermatology 2001;202:239–45.
[41] David M, Ford D, Bertoglio J, Maizel AL, Pierre J. Induction of the IL-13 receptor α2-chain by IL-4 and IL-13 in human keratinocyte: involvement of STAT6, ERK and p38 MAPK pathways. Oncogene 2001;20:6660–8.
[42] Matsuura H, Sakaue M, Subbaramaiah K, Kamitani H, Eling TE, Dannenberg AJ, et al. Regulation of cyclooxygenase-2 by interferon γ and transforming growth factor α in normal human epidermal kerationcyte and squamous carcinoma cell. J Biol Chem 1999;274:29138–48.
[43] Huang BB, Bonish BK, Chaturvedi V, Qin J-Z, Nickoloff BJ. Keratinocyte CDw60 expression is modulated by both a Th-1 type cytokine IFN-gamma and Th-2 cytokines IL-4 and IL-13: relevance to psoriasis. J Invest Dermatol 2001;116:305–12.

J. Biochem. 133, 607–614 (2003)
DOI: 10.1093/jb/mvg078

Molecular Cloning of a cDNA for the Human Phospholysine Phosphohistidine Inorganic Pyrophosphate Phosphatase

Fumiaki Yokoi*, Hiroyuki Hiraishi† and Kenji Izuhara
Department of Biochemistry, Saga Medical School, Nabeshima 5-1-1, Saga-shi, Saga 849-8501

Received December 8, 2002; accepted February 16, 2003

We previously reported the isolation from bovine liver of a novel 56-kDa inorganic pyrophosphatase named phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPPase). It is a unique enzyme that hydrolyzes not only oxygen-phosphorus bonds in inorganic pyrophosphate but also nitrogen-phosphorus bonds in phospholysine, phosphohistidine and imidodiphosphate *in vitro*. In this study, we determined the partial amino acid sequence of the purified bovine LHPPase. To investigate whether humans have the same enzyme, we isolated a cDNA clone from a HeLa cell cDNA library that encodes for the human homologue of LHPPase. Although its sequence does not include the consensus sequence of a typical inorganic pyrophosphatase, it does contain a similar sequence of the active site in other phosphatases such as protein-tyrosine phosphatase, dual-specific phosphatase and low molecular weight acid phosphatase. Human LHPPase was highly expressed in the liver and kidney, and moderately in the brain. The recombinant protein was produced in *E. coli*. Its ability to hydrolyze oxygen-phosphorus bonds and nitrogen-phosphorus bonds was confirmed. The enzymatic characteristics of this human protein were similar to those of purified bovine LHPPase. Thus, we concluded that the cDNA encoded the human counterpart of bovine LHPPase.

Key words: LHPP, lhpp, LHPPase, phosphatase, pyrophosphatase.

Abbreviations: LHPPase, phospholysine phosphohistidine inorganic pyrophosphate phosphatase; His-hLHPPase, His-Tag human LHPPase fusion protein; *p*-CMPS, *p*-chloromercuriphenyl sulfonic acid; PNP, imidodiphosphate; O-P, oxygen-phosphorus; N-P, nitrogen-phosphorus; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; PPase, inorganic pyrophosphatase; PNPase, imidodiphosphatase.

Inorganic pyrophosphate is produced as a byproduct in many biosynthetic reactions using nucleotide triphosphates in cells. Inorganic pyrophosphatase [EC 3.6.1.1] is an enzyme that hydrolyzes inorganic pyrophosphate (PP_i) to produce two molecules of orthophosphate (P_i) (*1*, *2*). Although the reaction is bidirectional, the equilibrium tends to produce orthophosphate. It is believed that this enzyme is coupled with the reactions producing inorganic pyrophosphate. Consequently, it facilitates reactions that produce orthophosphate and prevents the reactions from reversing (*3*). Inorganic pyrophosphatase exists in many organisms including animals, plants and bacteria. It is an essential enzyme for controlling the cellular level of inorganic pyrophosphate. Human inorganic pyrophosphatase is 94% identical to the bovine one (*4*, *5*). Inorganic pyrophosphatases are classified by their enzymatic characteristics. A mammalian cytosolic enzyme is a homodimer of 30- to 36-kDa subunits and requires Mg^{2+} for its full activity (*6–8*). Its hydrolytic activity on PPi is competitively inhibited by imidodiphosphate (PNP), an analog of PPi (*7–9*). On the other hand, a yeast enzyme hydrolyzes PNP into P_i and amidophosphate in a Mg^{2+}-dependent manner, although the hydrolytic rate of PNP is very low compared with that of PP_i (*10*).

We surveyed phosphatases that hydrolyze PNP in mammals and found a novel 56-kDa enzyme that is a homodimer in bovine liver (*11*). This enzyme hydrolyzes PNP into two molecules of Pi and ammonia. It also hydrolyzes PPi in the presence of Mg^{2+}. The main difference between this enzyme and the yeast enzyme is that the former works as a phosphoamidase (EC 3.9.1.1), while the latter hydrolyzes substrates having diphosphate bonds. We also reported that this enzyme hydrolyzes 3-phosphohistidine and 6-phospholysine in addition to PNP and PPi (*12*). We designated this novel inorganic pyrophosphatase as phospholysine phosphohistidine inorganic pyrophosphate phosphatase and abbreviated it as LHPPase (phospho-Lysine phospho-Histidine inorganic Pyrophosphate Phosphatase). In this study, we determined partial amino acid sequences of the purified bovine LHPPase.

To investigate if humans have the same enzyme, we isolated a human cDNA encoding the corresponding protein. To confirm that the cDNA clone codes for a functional enzyme, we measured the enzymatic activity of the protein to hydrolyze oxygen-phosphorus (O-P) bonds and nitrogen-phosphorus (N-P) bonds by the methods used for bovine LHPPase (*11*).

*To whom correspondence should be addressed at the present address: Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, 3614 Beckman Institute, MC-251, 405 North, Mathews Avenue, Urbana, IL 61801 USA. Tel: +1-217-244-3218, Fax: +1-217-244-1726, E-mail: yokoi@uiuc.edu
†Present address: Research Organization of Science and Engineering, Ritsumeikan University, Kusatsu, Shiga 525-8577

MATERIALS AND METHODS

Protein Sequencing of Bovine LHPPase—Bovine LHPPase was purified from the liver as previously described (*11*). The purified enzyme was applied to an Applied Biosystems model 477A/120A protein sequencer. It was also digested with V8 proteinase (Takara, Shiga) in 50 mM ammonium bicarbonate (pH 8.0). The digests were separated by 13% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*13*). The partial amino acid sequence of a peptide fragment (peptide 1) was determined using the protein sequencer. The purified enzyme was also *S*-carboxymethylated and digested with lysylendopeptidase (Wako Pure Chemical, Osaka). The peptide fragments were separated by reverse phase chromatography with TSK gel ODS-80Ts (4.6 × 150 mm) in a linear gradient of acetonitrile/1% TFA (9:1). A partial amino acid sequence of a second peptide fragment (peptide 2) was determined by using a Shimadzu PSQ-1 Systems protein sequencer in APRO science (Tokushima).

Cloning of a cDNA Encoding the Human Homologue—Based on the partial amino acid sequence of the bovine LHPPase, a nucleotide sequence (accession No. H75601) reported in the EST project (*14*) was selected as a probe to isolate a cDNA encoding a full-length human homologue. Two oligonucleotides, 5′-GGCCGAGCGCCATGGCACCGTGGG-3′ and 5′-CGTTAATCATGTTTTIAATAAGAA-3′, were designed as PCR primers. The DNA fragment was amplified by a polymerase chain reaction using a heat-stable DNA polymerase KOD dash (Toyobo, Osaka) and a human HeLa S3 5′-stretch plus cDNA library (Clontech, Tokyo) as a template. The amplified DNA fragment was ligated into a pT-Adv vector (Clontech). The DNA fragment of 425 bp was labeled with ^{32}P using random primers and used as a screening probe. A cDNA clone was isolated from the HeLa cells S3 cDNA library and subcloned into a plasmid Bluescript II SK M13+ (Stratagene, La Jolla, CA). The nucleotide sequence was determined by the dideoxy chain termination method with an Applied Biosystems model 373A stretch DNA sequencer (*15*). An online homology search of the DDBJ/EMBL/GeneBank database was made with the FASTA software program (*16*, *17*). The gene location was searched for in the EBI human genome database (*18*).

Northern Blot Analysis—The coding region was subcloned into a pT-Adv vector. The DNA fragment was obtained by digesting with *Eco*RI and labeled with ^{32}P by random primer labeling. Two kinds of commercial membranes (Human 12-lane MTN blot and human fetal MTN blot II, Clontech) were hybridized with the labeled probe in ExpressHyb Hybridization Solution (Clontech). They were washed according to the manufacturer's procedure. The signals were detected on Fuji RXU X-ray films (Fuji Film, Tokyo). Human 12-lane MTN blot and human fetal MTN blot II contained approximately 1 μg and 2 μg of polyA+ RNA per lane, respectively.

Preparation of His·Tag Human LHPPase Fusion Protein—The coding region was amplified with *Eco*RI sites at both ends by PCR and subcloned into pET30a plasmid (Novagen, Madison, WI). The His·Tag human LHPPase fusion protein (His-hLHPPase) was expressed in *Escherichia coli* BL21(DE3)pLysS and isolated by use of a Ni^{2+} chelating affinity column (His·Bind, Novagen) according to the manufacturer's instructions. The protein concentration was measured by the Bradford assay (Bio-Rad, Tokyo) (*19*). The fusion protein was dialyzed in 50 mM Tris-Cl (pH 6.5) containing 1 mM $MgCl_2$. The protein was mixed with an equal volume of glycerol and stored at –20°C for the enzyme assay.

Molecular Mass Estimation of Human LHPPase—The molecular mass of human LHPPase was estimated by SDS-PAGE. Five micrograms of the His-hLHPPase was dissolved in 50 mM Tris-Cl (pH6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol in the presence or absence of 100 mM dithiothreitol. Each sample was boiled for five minutes and subjected to electrophoresis on a 15% SDS–polyacrylamide gel (*13*). Proteins were stained with Coomassie Brilliant Blue R250. The molecular mass of human LHPPase was also estimated by gel filtration. Two hundred micrograms of the His-hLHPPase or standard proteins were applied on a Sephadex G-100 superfine column (0.9 × 54 cm, Amersham Pharmacia Biotech, Tokyo) equilibrated with 20 mM Tris-Cl (pH 7.5) and 0.15 M NaCl and fractionated to estimate the molecular mass. The fractionated fusion protein was detected by the Bradford assay (*19*). The molecular mass was estimated from the standard curves calculated by the method of least squares.

Measurement of Phosphatase Activity—Malachite green reagent was used to detect phosphate (*11*). Briefly, solution M was prepared as 1.5 mM Malachite green oxalate (Certistain, Merck, Tokyo) containing 2% polyvinyl alcohol 1500 (Wako Pure Chemical). Solution A was prepared as 40 mM hexaammonium heptamolybdate (Nacalai tesque, Kyoto) in 6 N HCl. Aliquots of solutions M and A were mixed to make the Malachite green reagent containing 0.2 mM Malachite green oxalate, 0.27% polyvinyl alcohol, 5.3 mM hexaammonium heptamolybdate in 0.8 N HCl. The solution was incubated at room temperature for more than 30 min after mixing. One or two ml of Malachite green reagent was added to 100 μl of the reaction mixture described as follows. Absorbance at 630 nm was measured 30 min after the addition of the reagent.

A 100 mM GTA buffer consisting of 33.3 mM 3,3-demethylglutaric acid, 33.3 mM Tris, and 33.3 mM 2-amino-2-methyl-1,3-propanediol was used as reaction buffer. Optimum pH was obtained in 100 μl of 100 mM GTA buffer (pH 4.0–10.0) containing 1 mM $MgCl_2$, 1 mM PP_i or 0.125 mM PNP, and 0.5 μg of the His-hLHPPase. The reaction was performed at 30°C for 30 min in duplicate. One milliliter of Malachite green reagent was added to each sample. The standard curve of phosphate was obtained with 100 μl of 0–300 μM sodium phosphate.

Inorganic pyrophosphatase (PPase) and imidodiphosphatase (PNPase) activities were measured as previously described (*11*). PPase activity was measured in 100 μl of reaction mixture containing 100 mM GTA buffer (pH 5.5), 1 mM $MgCl_2$, 0–4 mM PP_i and 1 μg of the fusion protein at 30°C for 30 min. One milliliter of Malachite green reagent was added. PNPase activity was measured in 100 μl of reaction mixture containing 100 mM GTA buffer (pH 7.0), 1 mM $MgCl_2$, 0–4 mM PNP, and 1 μg of the fusion protein. The reaction was performed in triplicate. Standard deviations were calculated by use of Excel (Microsoft, Tokyo).

```
AAGAACTTTAAAAATCACCTAGGTGTGGGCCGGGCACGGTGGCTAACGCCTGTAATCCCA    60
GCACTTTGAGATGCTGAGGCAGGTGGATCACGAGGTCAGGAGATCGAGACCATCCTGGAT   120
AACACGGAGAAACCCCGGCGGAGCTGAGGAGCAGGGCCGGGCGCCATGGCACCGTGGGGC   180
                          (*)                 M  A  P  W  G     (5)
AAGCGGCTGGCTGGCGTGCGCGGGGTGCTGCTTGACATCTCGGGCGTGCTGTACGACAGC   240
 K  R [L] A  G  V  R  G  V [L] L  D  I  S  G  V [L] Y  D  S    (25)
GGCGCGGGCGGCGGCACGGCCATCGCCGGCTCGGTGGAGGCGGTGGCCAGACTGAAGCGT   300
 G  A  G  G  G  T  A  I  A  G  S  V  E  A  V  A  R  L  K  R    (45)
TCCCGGCTGAAGGTGAGGTTCTGCACCAACGAGTCGCAGAAGTCCCGGGCAGAGCTGGTG   360
 S  R  L  K  V  R  F (C) T  N  E  S  Q (K)(S) R  A  E  L  V    (65)
GGGCAGCTTCAGAGGCTGGGATTTGACATCTCTGAGCAGGAGGTGACCGCCCCGGCACCA   420
 G  Q  L  Q  R  L  G  F  D  I  S  E  Q  E  V  T  A  P  A  P    (85)
GCTGCCTGCCAGATCCTGAAGGAGCGAGGCCTGCGACCATACCTGCTCATCCATGACGGA   480
 A  A  C  Q  I  L  K  E  R  G  L  R  P  Y  L  L  I  H  D  G   (105)
GTCCGCTCAGAATTTGATCAGATCGACACATCCAACCCAAACTGTGTGGTAATTGCAGAC   540
 V  R  S  E  F  D  Q  I  D  T  S  N  P  N  C  V  V  I  A  D   (125)
GCAGGAGAAAGCTTTTCTTATCAAAACATGAATAACGCCTTCCAGGTGCTCATGGAGCTG   600
 A  G  E  S  F  S  Y  Q  N  M  N  N  A  F  Q  V  L  M  E  L   (145)
GAAAAACCTGTGCTCATATCACTGGGAAAAGGGCGTTACTACAAGGAGACCTCTGGCCTG   660
 E  K  P  V  L  I  S  L  G  K  G  R  Y  Y  K  E  T  S  G  L   (165)
ATGCTGGACGTTGGTCCCTACATGAAGGCGCTTGAGTATGCCTGTGGCATCAAAGCCGAG   720
 M  L  D  V  G  P  Y  M  K  A  L  E  Y  A  C  G  I  K  A  E   (185)
GTGGTGGGGAAGCCTTCTCCTGAGTTTTTCAAGTCTGCCCTGCAAGCGATAGGAGTGGAA   780
 V  V  G  K  P  S  P  E  F  F  K  S  A  L  Q  A  I  G  V  E   (205)
GCCCACCAGGCCGTCATGATTGGGGACGATATCGTGGGCGACGTCGGCGGTGCCCAGCGG   840
 A  H  Q  A  V  M  I  G  D  D  I  V  G  D  V  G  G  A  Q  R   (225)
TGTGGAATGAGAGCGCTGCAGGTGCGCACCGGGAAGTTCAGGCCCAGTGACGAGCACCAT   900
(C) G  M  R  A  L  Q  V (R)(T) G  K  F  R  P  S  D  E  H  H   (245)
CCGGAAGTGAAGGCTGATGGGTACGTGGACAACCTCGCAGAGGCAGTGGACCTGCTGCTG   960
 P  E  V  K  A  D  G  Y  V  D  N  L  A  E  A  V  D  L  L  L   (265)
CAGCACGCCGACAAGTGATGGCCTCCTGGGAGAGCCCCGCCTCCTCCACCCCTGCCTCTC  1020
 Q  H  A  D  K  *                                             (270)
CTCCACCCCTGCCTCCCCTCCACCCCTGCCTCTCCTCCACCCGCCCAGGAGAGCCCCACC  1080
TCCTCCACCCCTGCCTCTCCTCCACCCCTGCCTCCCCTCCACCTGCCCCAGTGCCCAGAC  1140
CAACCAAGGCCCTGACAGCCCTGCCTTCTGCCCTCTGCCCTGCATGGGCAGGCATTTGTT  1200
CCCTACCTGGGTGGCCTGCTCCCCTGCCTGGGCCCTGACTTCAGCTCCCTGTAGTGAAGT  1260
CCAGGAGGGTGGGACAGGCCTGTCAGGCCTCTGGGAATCTCCCAAATCCCAGAACTCACC  1320
ACTCACCATGGGCCTTTAAATGCAGTAAACTCCACCTAACCAGATTCAGGGGCACTATGC  1380
CCACTGCCTCCTCTTCAGACTCTTTGCATTTCAGTGAAGAGCCTGGAAGAAACCCAGGGG  1440
CCTCCTATGCACAGATCTTGCAGCCCAGAACCAAGTCAGCCTCCCTGCGACTGCCCAGGC  1500
ACACTGCCCACCACCCCACCCCCGAAACAATGCCAGCCCGCTGCTTTTTCTATCCTCCCA  1560
GTCACCTTTGCAGACAAAGACCAGGGGCAGCTCCCGAGGGCACTGTGAAGGCTCCCATGC  1620
CACACAGTGAGAACTGTAGCCTCTGCGTCCAAGGCACACAGGGTACTTTCTGGACCCACT  1680
GCTGGACAGACTTGAAGGTGTCATGCCCGGTGTGTGCAGGAGGAAACTAACAGTTCAGTA  1740
AACTCTGCCTTGACCAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1800
AAAAAAAAAAAAAA                                                 1814
```

Fig. 1. **Nucleotide and derived amino acid sequence of a cDNA coding human LHPPase.** The stop codon is marked by the asterisk. The Kozak sequence in vertebrates is underlined. The amino acids in two putative phosphatase active site sequences are shown in circles. The three leucines in a leucine-zipper–like sequence are shown in squares.

```
hLHPPase   MAPWGKRLAGVRGVLLDISGVLYDSGAGGGTAIAGSVEAVARLKRSRLKV   (50)

hLHPPase   RFCTNESQKSRAELVGQLQRLGFDISEQEVTAPAPAACQILKERGLRPYL  (100)
                          ***** ** *********  **
Bp2                       RLGFDVSEGEVTAPAPAAXLIL

hLHPPase   LIEDGVRSEFDQIDTSNPNCVVIADAGESFSYQNMNNAFQVLMELEKPVL  (150)
                    ********** ** *
Bp1                 FDQIDTSNPNXVVDA

hLHPPase   ISLGKGRYYKETSGLMLDVGPYMKALEYACGIKAEVVGKPSPEFFKSALQ  (200)
hLHPPase   AIGVEAHQAVMIGDDIVGDVGGAQRCGMRALQVRTGKFRPSDEHHPEVKA  (250)
hLHPPase   DGYVDNLAEAVDLLLQHADK                                (270)
```

Fig. 2. **Comparison of the amino acid sequences of human and bovine LHPPase.** The determined partial amino acid sequences of bovine LHPPase (Bp1: peptide 1 and Bp2: peptide 2) are indicated under the corresponding human LHPPase sequence (hLHPPase). The amino acid number of human LHPPase is indicated in parentheses. Asterisks indicate identical residues.

PPase activity was also measured in 100 μl of reaction mixture containing 100 mM GTA buffer (pH 5.5), 1 mM $MgCl_2$, 0–10 μM *p*-chloromercuriphenyl sulfonic acid (*p*-CMPS), 1 mM PP_i, and 1 μg of the fusion protein, which was incubated at 30°C for 30 min before the addition of 1 ml of Malachite green reagent. PNPase activity was measured in 100 μl of reaction mixture containing 100 mM GTA buffer (pH 7.0), 1 mM $MgCl_2$, 0–10 μM *p*-CMPS, 0.1 mM PNP, and 0.5 μg of the fusion protein. The reaction mixtures in triplicate were incubated at 30°C for 30 min. Then 2 ml of Malachite green reagent was added.

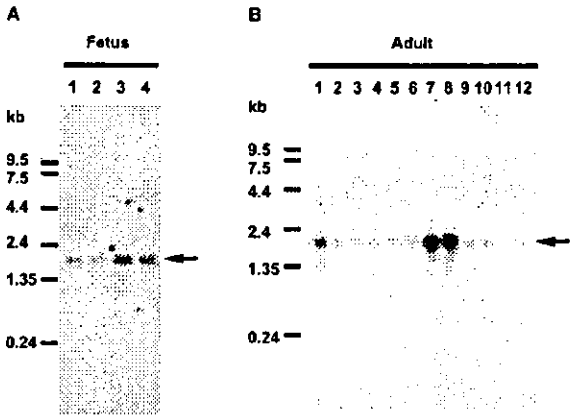

Fig. 3. **Expression of LHPPase in human tissues.** The expression of LHPPase was analyzed by Northern blotting using human fetal MTN blot II and human 12-lane MTN blot (Clontech). Arrows indicate human LHPPase mRNA. (A) Samples from fetal tissues. From left to right: (1) brain, (2) lung, (3) liver, and (4) kidney. (B) Samples from adult tissues. From left to right: (1) brain, (2) heart, (3) skeletal muscle, (4) colon (no mucosa), (5) thymus, (6) spleen, (7) kidney, (8) liver, (9) small intestine, (10) placenta, (11) lung, and (12) peripheral blood leukocyte.

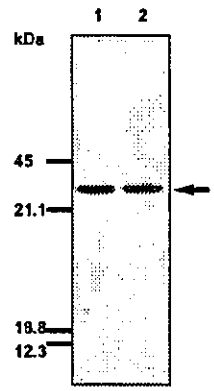

Fig. 4. **SDS-PAGE of the His-hLHPPase.** The migration of His-hLHPPase on SDS-PAGE in the absence (1) or presence (2) of the reducing reagent. The molecular mass was estimated from comparison with the migration of the standard proteins (45-kDa ovalbumin, 21.1-kDa trypsin inhibitor, 18.8-kDa myoglobin, 12.3-kDa cytochrome *c*). The arrow indicates His-hLHPPase.

RESULTS

Partial Amino Acid Sequence of Bovine LHPPase—To investigate whether humans have LHPPase, we analyzed the primary structure of the bovine LHPPase and used the data to isolate a cDNA encoding the human counterpart. Bovine LHPPase was purified from the liver as previously described (*11*) and subjected to amino acid sequencing. However, no substantial sequence was obtained, suggesting that the bovine LHPPase N-terminus was blocked. To obtain its internal sequence, the protein was digested with V8 protease and lysylendopeptidase. Two peptide fragments were isolated and their partial amino acid sequences were determined. A homology search indicated that the two peptides were similar to those encoded in cDNA clone H75601 analyzed in the human EST project (*14*).

A cDNA Encoding the Human Homologue—Although the homology search suggested that the cDNA encoded a human counterpart of the bovine enzyme, the human EST cDNA sequence was incomplete and too short to encode the full length of a corresponding human enzyme. Thus, we tried to isolate a cDNA clone that encodes a full-length human homologue. A cDNA clone encoding an insert of 1,814 bp was isolated from a cDNA library of HeLa cells (Fig. 1). The translation initiation site was predicted from the Kozak's sequence in vertebrates (*20, 21*). The cDNA contained an in-frame stop codon sequence between 976 bp and 978 bp. There was a stop codon (145–147 bp) upstream of the predicted in-frame initiation codon and no other intervening initiation codon. This indicated that the sequence upstream of the predicted initiation codon did not encode any amino acids. Thus, the open reading frame encoded 270 amino acids, and the molecular mass of the protein was predicted to be 29,192 Da. Because the sequence was similar to the partial amino acid sequences of the bovine LHPPase as shown in Fig. 2, we designated the gene as lhpp.

The human LHPPase sequence had no similarity to the consensus sequences of known inorganic pyrophosphatases (*22, 23*). Thus, we compared the human LHPPase sequence with that of other phosphatases. Protein-tyrosine phosphatase, dual-specific phosphatase and low molecular weight acid phosphatase are known to have a consensus sequence of $XCX_5RS(T)$ at their active sites (*24*). We compared the sequences around the five cysteine residues of human LHPPase with the consensus sequence and found two homologous regions, as shown in Fig. 1. There is a candidate sequence of XCX_5KS at residues 52–60, although lysine is substituted for arginine, another basic amino acid. Another candidate is XCX_7RT at residues 225–235, although there are seven amino acids between cysteine and threonine instead of five. Motif analysis also indicated that human LHPPase had a leucine-zipper–like sequence (*i.e.* three leucines accounted for every seventh residue at positions 8, 15, and 22).

Expression of Human LHPPase—LHPPase was originally purified from bovine liver, but its presence in other tissues has not been reported. We analyzed the expression of human LHPPase by Northern blotting. Although the mRNA was expressed at low level in other tested tissues, it was highly expressed in the liver and kidney, and moderately in the brain (Fig. 3). The expression patterns were the same in both fetal and adult tissues. The results indicated that LHPPase plays an important role in these tissues. The RNA detected in the Northern blot was 1.8 kb in length.

Subunit Interaction of Human LHPPase—Because the purified bovine enzyme is a homodimer, we analyzed the molecular mass of human LHPPase. We used the His-hLHPPase because it was possible to purify the proteins containing His-Tag sequence in one step by immobilized metal affinity chromatography. The His-hLHPPase was expressed in *E. coli* and purified on a Ni^{2+} chelating affinity column as a single band protein on SDS-PAGE (Fig. 4). The fusion protein was composed of 322 amino acids, and the molecular mass was predicted to be 34,900 Da.

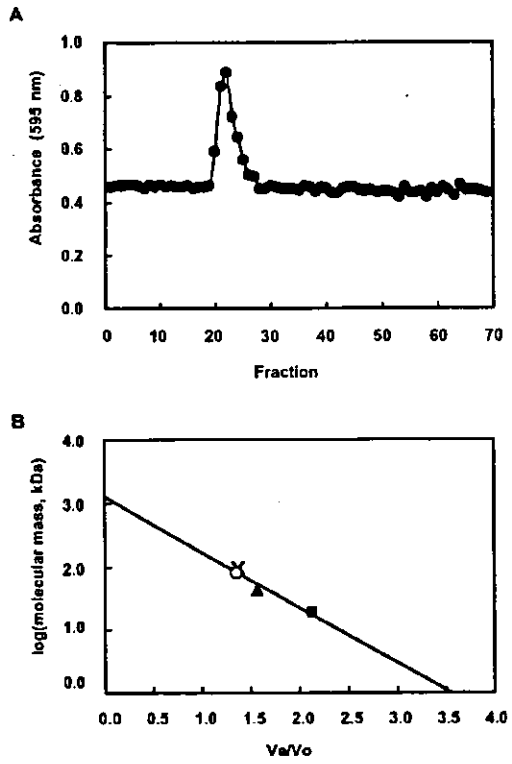

Fig. 5. **Gel filtration of the His-hLHPPase.** (A) Elution profile of the His-hLHPPase from a Sephadex G-100 superfine column. The fusion protein was detected by the Bradford assay at 595 nm. (B) Estimation of molecular mass. The molecular mass was estimated from the standard curves calculated by the method of least squares. Symbols are: open circles, His-hLHPPase; ×, 96-kDa hexokinase; solid triangles, 45-kDa ovalbumin; solid squares, 18.8-kDa myoglobin.

The migration of the fusion protein on SDS-PAGE was the same in the presence or absence of reducing reagents. The fusion protein was fractionated from Sephadex G-100 superfine gel and the molecular mass was estimated with standard proteins (Fig. 5). The fusion protein was eluted in the 80-kDa fraction, and its molecular mass was almost double the 35-kDa molecular mass of the fusion protein estimated from the sequence and migration on SDS-PAGE. These results suggest that the human LHPPase is also a homodimer, at least in this buffer condition, and the disulfide bonds are not used for LHPPase dimerization.

Optimal pH for the Phosphatase Activities—To confirm that the cDNA encodes an active enzyme, the phosphatase activities of the His-hLHPPase were assayed as previously described (*11*). The hydrolysis of nitrogen-phosphorus bonds in PNP and oxygen-phosphorus bonds in PPi by human LHPPase was tested through assays in various pH ranges of GTA buffers (Fig. 6). The optimal pH for the reaction was 7.0 and 5.5, respectively. Accordingly, the activity hydrolyzing PPi appeared to function in a lower pH range than the activity hydrolyzing PNP. These enzymatic characteristics were similar to those of the purified bovine enzyme. The enzyme activity

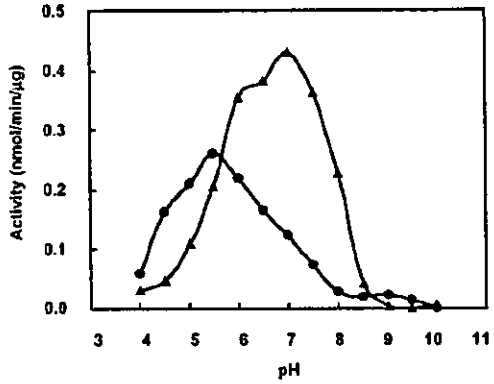

Fig. 6. **The pH curves of His-hLHPPase hydrolyzing PP_i and PNP.** Phosphatase activities of the His-hLHPPase to hydrolyze PP_i (solid circles) and PNP (solid triangles) were assayed under the indicated pH conditions (pH 4.0–10.0). The activities are shown as the released Pi (nmol)/reaction time (min) / enzyme (μg). The average values of duplicate experiments are shown.

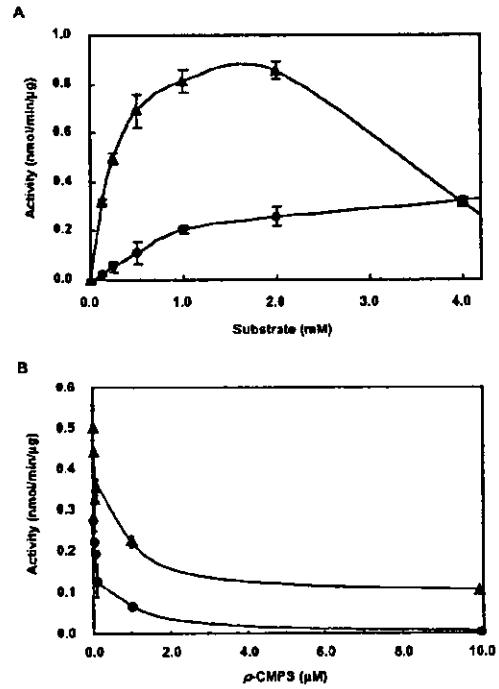

Fig. 7. **Phosphatase activities and the effect of *p*-CMPS.** (A) PPase (solid circles) and PNPase (solid triangles) activities of the His-hLHPPase were measured with PP_i and PNP as substrates, respectively. The activities are shown as the released P_i (nmol)/reaction time (min)/enzyme (μg). The standard deviations of triplicate experiments are shown in bars. (B) PPase (solid circles) and PNPase (solid triangles) activities of the His-hLHPPase were measured in 100 μl of reaction mixture with 0–10 μM *p*-CMPS. The standard deviations of triplicate experiments are shown in bars.

to hydrolyze nitrogen-phosphorus bonds was higher than that for oxygen-phosphorus bonds at the optimal pH.

Effect of a Thiol Reagent—The PPase and PNPase activities of the His-hLHPPase were each measured at their optimal pH (Fig. 7A). High concentration of PNP

inhibited the PNPase activity of human LHPPase. Similar substrate inhibition has been reported for the bovine enzyme (*12*). Because *p*-CMPS inhibited both PPase and PNPase activities of the purified bovine LHPPase, we analyzed the effect of this strong thiol reagent on the human LHPPase. Both PPase and PNPase activities were inhibited by *p*-CMPS as predicted (Fig. 7B). More than half of the full activity of PPase and PNPase were inhibited by 0.1 μM and 1 μM *p*-CMPS respectively. The results indicate that the hydrolytic activity on PPi was slightly more sensitive to *p*-CMPS than that on PNP. These enzymatic characteristics were similar to those of the purified bovine enzyme.

DISCUSSION

LHPPase was found as a novel inorganic pyrophosphatase in bovine liver in 1997 (*11*). Because the enzymatic characteristics of the purified bovine enzyme were unique, it was expected that LHPPase sequence would be quite different from those of typical inorganic pyrophosphatases. We confirmed this hypothesis by sequencing. To investigate whether humans have the same enzyme, we isolated a cDNA clone encoding the human homologue. We registered the human cDNA sequence as lhpp to DDBJ/EMBL/GeneBank database in 2000 (AB049629). After our registration, the human genome projects reported the draft sequence of the whole human genome in 2001 (*25*, *26*). In this paper, we detail the isolation of the human LHPPase cDNA and the enzymatic activities of its translated product. A homology search against the genome sequence database indicated that LHPPase is encoded by seven exons on chromosome 10 q26.13. The official gene name of lhpp is now identified as LHPP in the human genome database.

We suggested that human LHPPase is a dimer, but the disulfide bonds are not used for dimerization. There is a leucine-zipper–like sequence (three leucines at intervals of seven residues at positions 8, 15, and 22). This suggests a possible dimerization domain of LHPPase to form a homodimer. Mutation analysis of these residues will determine the dimerization domain.

The purified bovine enzyme has two different catalytic sites for hydrolysis of imidodiphosphate and *N*-phosphorylated amino acids, respectively (*12*). Two candidates for the active site may function to promote hydrolysis of the different substrates. The actual active site may be determined by point mutation analysis in further studies. A similar sequence was also found in phosphoarginine phosphatase purified from rat liver (*27–30*). Although human LHPPase hydrolyzes O-P bonds in inorganic pyrophosphate, it hydrolyzes N-P bonds more effectively. This suggests that another, more suitable substrate than inorganic pyrophosphate exists *in vivo*. One possibility is that LHPPase works as a protein phosphatase which hydrolyzes phospho-proteins containing phospholysine and/or phosphohistidine. The similarity of the putative active site to the consensus sequence of other phosphatases functioning as signal transduction components suggests that LHPPase may transduce signals in a novel signal transduction pathway.

It was suggested that the N-terminal amino acid of the bovine enzyme was blocked. In the case of a phosphoarginine phosphatase, the first methionine was removed and the second alanine was acetylated (*30*). LHPPase may also have such a modification. Tandem mass analysis of the modified N-terminal peptide fragment will determine the type of modification. Although the meaning of the N-terminal amino acid modification is not known at present, the N-terminus of LHPPase does not seem important for the enzymatic activity itself. In fact, the fusion protein containing a large extension at its N-terminal region of this protein has an activity to hydrolyze N-P and O-P bonds at the same level as that reported for the purified bovine enzyme.

The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank databases with an accession number of AB049629. We would like to express our special thanks to Professor emeritus Akira Kumon for his excellent teaching. We thank Drs. Minoru Tanaka, Eiji Majima, Tatsuya Wakasugi, Tsunehiro Mukai, Shuji Toda, Hajime Sugihara, Yuqing Li and Mr. Toshihiro Kondo for their technical advice, assistance and encouragement. We also thank Ms. Shizuko Furukawa for her secretarial assistance, and Dr. Shinichi Mitsui, Mr. Steve Lamos, Ms. Mai Tu Dang, Mr. Victor Campos, Ms. Mary Lindsey and Dr. Kazuhiko Arima for their critical reading of the manuscript.

REFERENCES

1. Josse, J. and Wong, S.C.K. (1971) Inorganic pyrophosphatase of *Escherichia coli* in *The Enzymes* (Boyer, P.B., eds.) Vol. IV, pp. 499–527, Academic Press, New York
2. Butler, L.G. (1971) Yeast and other Inorganic pyrophosphatase in *The Enzymes* (Boyer, P.B., eds.) Vol. IV, pp. 529–541, Academic Press, New York
3. Stadtman, E.R. (1973) Adenylyl transfer reactions in *The Enzymes* (Boyer, P.B., eds.) Vol. VIII, pp. 1–49, Academic Press, New York
4. Fairchild, T.A. and Patejunas, G. (1999) Cloning and expression profile of human inorganic pyrophosphatase. *Biochim. Biophys Acta* **1447**, 133–136
5. Yang, Z. and Wensel, T.G. (1992) Molecular cloning and functional expression of cDNA encoding a mammalian inorganic pyrophosphatase. *J. Biol. Chem.* **267**, 24641–24647
6. Yoshida, C., Shah, H., and Weinhouse, S. (1982) Purification and properties of inorganic pyrophosphatase of rat liver and hepatoma 3924A. *Cancer Res.* **42**, 3526–3531
7. Yang, Z. and Wensel, T.G. (1992) Inorganic pyrophosphatase from bovine retinal rod outer segments. *J. Biol. Chem.* **267**, 24634–24640
8. Felix, R. and Fleisch, H. (1975) Properties of inorganic pyrophosphatase of pig scapula cartilage. *Biochem. J.* **147**, 111–118
9. Smirnova, I.N., Kudryavtseva, N.A., Komissarenko, S.V., Tarusova, N.B., and Baykov, A.A. (1988) Diphosphonates are potent inhibitors of mammalian inorganic pyrophosphatase. *Arch. Biochem. Biophys.* **267**, 280–284
10. Smirnova, I.N., Baykov, A.A., and Avaeva, S.M. (1986) Studies on inorganic pyrophosphatase using imidodiphosphate as a substrate. *FEBS Lett.* **206**, 121–124
11. Hiraishi, H., Ohmagari, T., Otsuka, Y., Yokoi, F., and Kumon, A. (1997) Purification and characterization of hepatic inorganic pyrophosphatase hydrolyzing imidodiphosphate. *Arch. Biochem. Biophys.* **341**, 153–159
12. Hiraishi, H., Yokoi, F., and Kumon, A. (1998) 3-phosphohistidine and 6-phospholysine are substrates of a 56-kDa inorganic pyrophosphatase from bovine liver. *Arch. Biochem. Biophys.* **349**, 381–387
13. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685

14. Hillier, L.D., Lennon, G., Becker, M., Bonaldo, M.F., Chiapelli, B., Chissoe, S., Dietrich, N., DuBuque, T., Favello, A., Gish, W., Hawkins, M., Hultman, M., Kucaba, T., Lacy, M., Le, M., Le, N., Mardis, E., Moore, B., Morris, M., Parsons, J., Prange, C., Rifkin, L., Rohlfing, T., Schellenberg, K., Soares, M.B., Tan, F., Thierry-Meg, J., Trevaskis, E., Underwood, K., Wohldmann, P., Waterston, R., Wilson, R., and Marra, M. (1996) Generation and analysis of 280,000 human expressed sequence tags. *Genome Res.* **6**, 807–828
15. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA* **74**, 5463–5467
16. Bairoch, A., Bucher, P., and Hofmann, K. (1997) The PROSITE database, its status in 1997. *Nucleic Acids Res.* **25**, 217–221
17. Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Proc. Natl Acad. Sci. USA* **85**, 2444–2448
18. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402
19. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
20. Kozak, M. (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **266**, 19867–19870
21. Cavener, D.R. and Ray, S.C. (1991) Eukaryotic start and stop translation sites. *Nucleic Acids Res.* **19**, 3185–3192
22. Lahti, R., Kolakowski, L.F. Jr., Heinonen J., Vihinen, M., Pohjanoksa, K., and Cooperman, B.S. (1990) Conservation of functional residues between yeast and *E. coli* inorganic pyrophosphatases. *Biochim. Biophys Acta* **1038**, 338–345
23. Cooperman, B.S., Baykov, A.A., and Lahti, R. (1992) Evolutionary conservation of the active site of soluble inorganic pyrophosphatase. *Trends Biochem. Sci.* **17**, 262–266
24. Denu, J.M. and Dixon, J.E. (1995) A catalytic mechanism for the dual-specific phosphatases. *Proc. Natl Acad. Sci. USA* **92**, 5910–5914
25. Lander, E. S, Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J.P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J.C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R.H., Wilson, R.K., Hillier, L.W., McPherson, J.D., Marra, M.A., Mardis, E.R., Fulton, L.A., Chinwalla, A.T., Pepin, K.H., Gish, W.R., Chissoe, S.L., Wendl, M.C., Delehaunty, K.D., Miner, T.L., Delehaunty, A., Kramer, J.B., Cook, L.L., Fulton, R.S., Johnson, D.L., Minx, P.J., Clifton, S.W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J.F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R.A., Muzny, D.M., Scherer, S.E., Bouck, J.B., Sodergren, E.J., Worley, K.C., Rives, C.M., Gorrell, J.H., Metzker, M.L., Naylor, S.L., Kucherlapati, R.S., Nelson, D.L., Weinstock, G.M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Smith, D.R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H.M., Dubois, J., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R.W., Federspiel, N.A., Abola, A.P., Proctor, M.J., Myers, R.M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D.R., Olson, M.V., Kaul, R., Raymond, C., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G.A., Athanasiou, M., Schultz, R., Roe, B.A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W.R., de la Bastide, M., Dedhia, N., Blocker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J.A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D.G., Burge, C.B., Cerutti, L., Chen, H.C., Church, D., Clamp, M., Copley, R.R., Doerks, T., Eddy, S.R., Eichler, E.E., Furey, T.S., Galagan, J., Gilbert, J.G., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L.S., Jones, T.A., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W.J., Kitts, P., Koonin, E.V., Korf, I., Kulp, D., *Lancet*, D., Lowe, T.M., McLysaght, A., Mikkelsen, T., Moran, J.V., Mulder, N., Pollara, V.J., Ponting, C.P., Schuler, G., Schultz, J., Slater, G., Smit, A.F., Stupka, E., Szustakowski, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis , J., Wheeler, R., Williams, A., Wolf, Y.I., Wolfe, K.H., Yang, S.P., Yeh, R.F., Collins, F., Guyer, M.S., Peterson, J., Felsenfeld, A., Wetterstrand, K.A., Patrinos, A., Morgan, M.J., Szustakowki, J., de Jong, P., Catanese, J.J., Osoegawa, K., Shizuya, H., Choi, S., and Chen, Y.J. (2001) Initial sequencing and analysis of the human genome. *Nature* **409**, 860–921
26. Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A., Gocayne, J.D., Amanatides, P., Ballew, R.M., Huson, D.H., Wortman, J.R., Zhang, Q., Kodira, C.D., Zheng, X.H., Chen, L., Skupski, M., Subramanian, G., Thomas, P.D., Zhang, J., Gabor Miklos, G.L., Nelson, C., Broder, S., Clark, A.G., Nadeau, J., McKusick, V.A., Zinder, N., Levine, A.J., Roberts, R.J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di Francesco, V., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A.E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T.J., Higgins, M.E., Ji, R.R., Ke, Z., Ketchum, K.A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G.V., Milshina, N., Moore, H.M., Naik, A.K., Narayan, V.A., Neelam. B., Nusskern. D., Rusch, D.B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M.L., Curry, L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D., McCawley, S., McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y.H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N.N., Tse, S., Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J.F., Guigo, R., Campbell, M.J., Sjolander, K.V., Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T., Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y.H., Coyne, M., Dahlke, C., Mays, A., Dombroski, M., Donnelly, M., Ely, D., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., New-

man, M., Nguyen, T., Nguyen, N., Nodell, M., Pan, S., Peck, J., Peterson, M., Rowe, W., Sanders, R., Scott, J., Simpson, M., Smith, T., Sprague, A., Stockwell, T., Turner, R., Venter, E., Wang, M., Wen, M., Wu, D., Wu, M., Xia, A., Zandieh, A., and Zhu, X. (2001) The sequence of the human genome. *Science* **291**, 1304–1351

27. Kuba, M., Ohmori, H., and Kumon, A. (1992) Characterization of N^{ω}-phosphoarginine hydrolase from rat liver. *Eur. J. Biochem.* **208**, 747–752
28. Yokoyama, K., Ohmori, H., and Kumon, A. (1993) Isolation of N^{ω}-phosphoarginine hydrolase from rat liver and its physical properties. *J. Biochem.* **113**, 236–240
29. Kumon, A., Kodama, H., Kondo, M., Yokoi, F., and Hiraishi, H. (1996) N^{ω}-phosphoarginine phosphatase (17 kDa) and alkaline phosphatase as protein arginine phosphatases. *J. Biochem.* **119**, 719–724
30. Yokoi, F., Hamato, N., Kamei, K., Hara, S., Miyagi, M., Tsunasawa, S., Hiraishi, H., and Kumon, A. (1998) N^{ω}-phosphoarginine phosphatase activity of ubiquitin-conjugating enzymes, type UBC4A/10A and UBC2E. *Res. Commun. Biochem. Cell Mol. Biol.* **2**, 331–346

Clin Exp Allergy 2003; 33:684–687

Effect of lysed *Enterococcus faecalis* FK-23 (LFK) on allergen-induced peritoneal accumulation of eosinophils in mice

T. Shimada*‡, L. Cheng†‡, M. Ide*, S. Fukuda‡, T. Enomoto§ and T. Shirakawa‡

*Central Research Laboratories, Nichinichi Pharmaceutical Co. Ltd, Mie, Japan, †International Research Centre for Nasal Allergy, Nanjing Medical University and Department of Otorhinolaryngology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China, ‡Department of Health Promotion and Human Behaviour, Kyoto University Graduate School of Public Health, Kyoto, Japan and §Department of Otolaryngology, Japanese Red Cross Society Wakayama Medical Center, Wakayama, Japan

Summary

Background The interest in anti-allergy immunoregulation by lactic acid bacteria has been growing for the last few decades. There is some evidence to suggest that lysed *Enterococcus faecalis* FK-23 (LFK) could relieve the clinical symptoms of pollinosis. However, the mechanism responsible for this phenomenon remains unknown.

Objective To identify the effect of LFK, a lysozyme treated and heat-killed preparation from the lactic acid bacteria *Enterococcus faecalis* FK-23 strain, on allergen-induced eosinophil accumulation.

Methods BALB/c mice were sensitized with ragweed pollen extract, and peritoneal accumulation of eosinophils was induced. A total of 60 mg (0.5 mL) LFK was orally administered to the experimental mouse every day during 21 days of the sensitization period. In addition, LFK 4 mg, 25 mg and 60 mg (each 0.5 mL) were also orally administered to a mouse of each group every day for 21 days. Saline was fed in a dose of 0.5 mL/mouse per day for the same duration as a control.

Results Compared with control mice, LFK-treated mice exhibited decreased ragweed pollen allergen-induced peritoneal accumulation of eosinophils ($P = 0.013$), which showed a tendency to be in a dose-dependent fashion ($P = 0.14$).

Conclusion The results provide laboratory evidence of the role for LFK, a lactic acid bacteria preparation, in combating eosinophil accumulation.

Keywords allergy, *Enterococcus faecalis*, eosinophils, lactic acid bacteria

Submitted 12 April 2002; revised 22 November 2002; accepted 17 January 2003

Introduction

Recently, increasing research attention has been paid to a role of lactic acid bacteria (LAB) in anti-allergy immunoregulation [1]. Studies on lysed *Enterococcus faecalis* FK-23 (LFK) by our group have shown that it could inhibit active cutaneous anaphylaxis (ACA) in mice [2], and relieve clinical symptoms in patients with Japanese cedar pollinosis [3]. In a recent clinical study, we have observed that the number of peripheral blood eosinophils reduced significantly after LFK treatment in patients with perennial allergic rhinitis (unpublished data). In addition, FK23, a heat-killed preparation made from the same strain of *E. faecalis*, has been demonstrated to be capable of some activities important for maintenance of human health, such as anti-tumour [4, 5], immunomodulated [6, 7] and anti-hypertensive effects [8].

Eosinophils are believed to be key effecter cells in the pathogenesis of allergic disorders, and their recruitment into the upper or lower airways appears to be essential for the clinical manifestations of allergen inhalation. Given the perceived importance of eosinophils in allergic inflammation, they may be logical therapeutic targets [9]. The purpose of this experimental study was to identify the effect of LFK on allergen-induced eosinophil accumulation.

Correspondence: Takashi Shimada, Central Research Laboratories, Nichinichi Pharmaceutical Co. Ltd, 239–1 Tominaga Oyamada, Ayama-gun, Mie 518–1417, Japan. E-mail: labo@nichinichi-phar.co.jp

Materials and methods

Preparation of LFK

LFK was prepared as described previously [2]. Briefly, *E. faecalis* FK-23 was cultured for 24 h at 37 °C in a broth medium containing 2% glucose, 2% yeast extract, 2% meat extract and 4% K_2HPO_4. The cells were harvested by centrifugation, washed three times with distilled water and lysozyme treatment (added 1 mg/mL) at 37 °C for 2 h, and heated at 105 °C for 10 min, then lyophilized. The preparation is LFK.

Experimental animals

Female BALB/c mice were purchased from SLC Inc., Shizuoka, Japan. Five-week-old mice were used for all experiments. They were fed on pellet diet CE-2 (Clea Inc., Tokyo, Japan) and

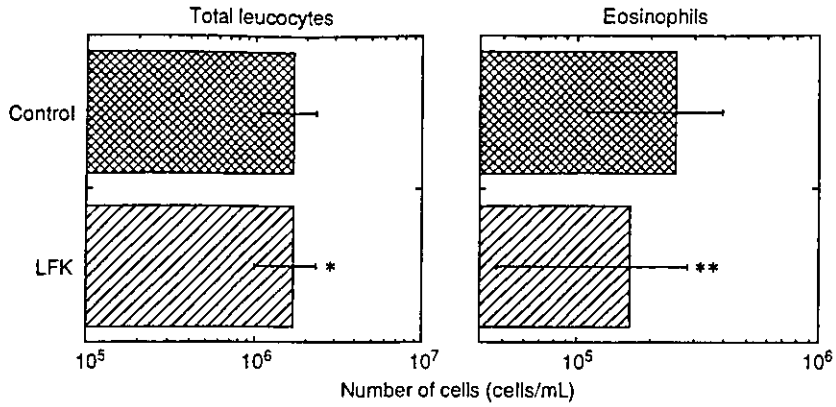

Fig. 1. Effect of LFK on peritoneal accumulation of total leucocytes and eosinophils in ragweed pollen allergen-sensitized mice. Each value represents mean ± SD. $^*P = 0.78$, $^{**}P = 0.013$, as compared with control.

received tap water that had been filtrated through a PF filter. All animals were housed in cages with a 12-h light/dark cycle. Temperature and humidity were controlled at 25.0 ± 1.0 °C and 55.0 ± 5.0%.

Allergen-induced cell accumulation

The animal model was prepared according to the procedure of Kaneko et al. [10]. BALB/c mice were sensitized with the commercially prepared ragweed pollen extract 1/100 w/v (Torii Pharmaceutical Co. Ltd, Tokyo, Japan); 0.1 mL of this dilution was injected subcutaneously on days 0 and 1, and 0.2 mL was injected subcutaneously on days 6, 8 and 14. The mice were challenged on day 20 by the intraperitoneal injection of 0.2 mL of the dilution of ragweed pollen extract. Peritoneal cells were harvested 24 h after challenge with 4 mL of phosphate-buffered saline containing 1.0% fetal calf serum and 5 U/mL heparin. An appropriate phosphate-buffered saline dilution of the infusion was added to Turk's solution, and the total number of blood cells was counted with a haemocytometer under a microscope. For this purpose, 50 μL of the peritoneal cell suspension (5×10^5 cells/mL) was smeared on a microscope slide after centrifugation. A differential cell count was carried out under a microscope after fixation and staining with May–Grunwald Giemsa dye.

Administration of LFK

LFK 60 mg (0.5 mL) was orally administered to the experimental mouse ($n = 29$) every day in LFK group, during 21 days of the sensitization period. Saline was fed in a dose of 0.5 mL/mouse per day for the same duration as a control ($n = 26$).

In addition, to explore the dose-dependent effect of LFK on allergen-induced peritoneal accumulation of eosinophils, LFK 4 mg, 25 mg and 60 mg (each 0.5 mL) were also orally administered to mice of each group ($n = 6$, respectively) every day for 21 days. The administration of saline for the control group ($n = 6$) was the same as above.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). The SPSS 10.0J was used for analysis. Statistical differences between the groups were determined by unpaired Student's *t*-test and one-way analysis of variance (ANOVA). Differences were considered significant if the *P*-value for the effect was less than 0.05.

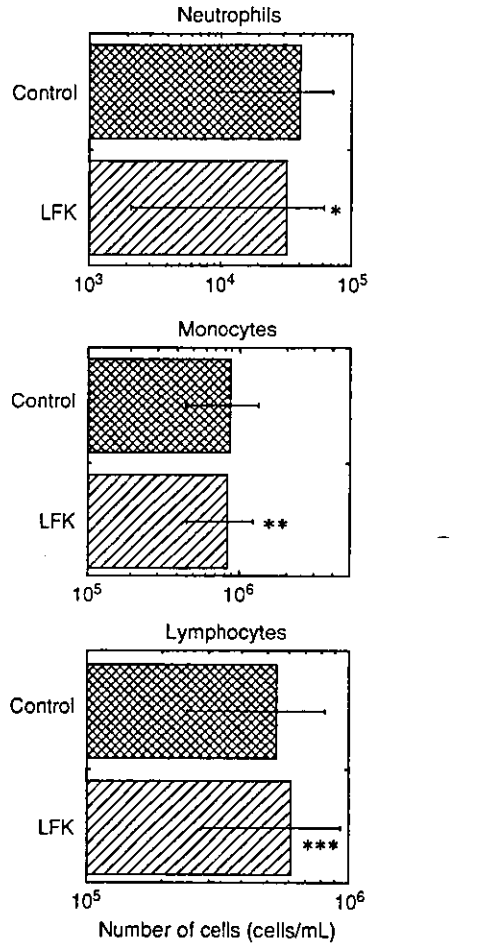

Fig. 2. Effect of LFK on peritoneal accumulation of neutrophils, monocytes and lymphocytes in ragweed pollen allergen-sensitized mice. Each value represents mean ± SD. $^*P = 0.31$, $^{**}P = 0.80$, $^{***}P = 0.37$, as compared with control.

Results

Effect of LFK on peritoneal accumulation of eosinophils in ragweed pollen allergen-sensitized mice

As shown in Fig. 1, the total number of accumulated cells in control and LFK groups were 1.69×10^6 and 1.64×10^6