Interleukin-15 is not a Constitutive Cytokine in the Epidermis, but is Inducible in Culture or Inflammatory Conditions

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The regulation in the skin of interleukin-15 (IL-15), a potent modulator of T-cell-mediated immune responses, is not fully understood. We investigated the levels of IL-15 and its mRNA produced by epidermal and cultured keratinocytes and found that normal keratinocytes did not constitutively express IL-15 in the epidermis, but in culture began to produce the cytokine. Some epidermal keratinocytes expressed IL-15 in inflammatory conditions associated with infiltration of neutrophils and eosinophils. IL-15 was detected only in the cell lysates, not in the supernatants of cultured keratinocytes. Dexamethasone ($10^{-5} – 10^{-6}$ M) markedly inhibited IL-15 mRNA expression by normal and transformed keratinocytes in a range of pharmacological concentrations. IFN-γ (200 and 400 U/ml) slightly increased the IL-15 message level in a squamous cell carcinoma cell line, HSC-5, in a dose-dependent fashion, whereas no significant change was observed in cultured normal human keratinocytes. Our data indicate that IL-15 is not a constitutive cytokine in epidermal keratinocytes but is inducible. *Key words: IL-15; keratinocyte; interferon-γ; dexamethasone; human squamous cell carcinoma; Northern blotting.*

Interleukin-15 (IL-15) is a newly described cytokine that was first characterized from the culture supernatants of a simian kidney epithelial cell line (1). Although IL-15 has no homology with IL-2, it does share the ability to bind to the β and γ-chains of the IL-2 receptor complex and to stimulate the proliferation of mitogen-activated T-cells and induce natural killer (NK) cell activation (1–3). Northern blotting analysis has demonstrated that IL-15 mRNA is expressed by a variety of cell types including placenta, skeletal muscle, kidney, lung, heart, lipopolysaccaride (LPS)-activated monocytes, fibroblasts and keratinocytes (1, 4–6). Resting and phytohaemagglutinin (PHA)-activated T-cells expressed no detectable IL-15 mRNA.

IL-15 was reported to play a pathological role in the development of inflammatory diseases such as rheumatoid arthritis and sarcoidosis by attracting T-cells into the inflammatory sites with subsequent activation of the T-cells (7, 8). Furthermore, IL-15 was found to augment the proliferation of murine γδT-cells in experimental salmonella infection (9).

Although IL-15 message was expressed by various cell types, it has been difficult to demonstrate mature IL-15 in the supernatants. Mohamadzadeh et al. (10) reported that ultraviolet B radiation up-regulated the expression of IL-15 in human skin, showing by Western blotting analysis that keratinocytes can secrete IL-15 to the culture supernatants. Recently, IL-15 expression was demonstrated in normal epidermis by immuno-staining using an antibody to IL-15 (6). Keratinocyte-derived IL-15 may therefore be an important factor in the generation of cutaneous inflammatory responses, although little is known about its regulation and expression pattern in the epidermis.

To determine whether keratinocytes are a major source of IL-15, we investigated the expression pattern of keratinocyte-derived IL-15, and the production levels of IL-15 mRNA under various culture conditions. Unlike previous reports, we observed that normal epidermal keratinocytes did not constitutively contain IL-15 nor IL-15 mRNA, and both were detected in inflammatory epidermis and cultured keratinocytes.

MATERIALS AND METHODS

Cells and culture study

A human squamous cell carcinoma cell line, HSC-5, provided by Professor S. Kondo (Department of Dermatology, Yamagata University School of Medicine), was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% inactivated foetal calf serum (FCS). When cultured cells reached 80–90% confluence, the medium was changed to serum-free DMEM containing one of each of the following agents: medium alone, rIFN-γ (200 and 400 U/ml) (R & D Systems, Minneapolis, MN, USA), $1\alpha,25$-dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$) (10^{-6} M) (Teikoku Pharmaceutical Co. Kagawa, Japan), cyclosporin A (1 μg/ml), or dexamethasone ($10^{-5} – 10^{-7}$ M) (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Twenty-four hours after culture, the cells were washed twice with phosphate-buffer saline (PBS) and used for mRNA analysis.

Normal human keratinocytes (NHKs) were obtained from the foreskin tissue of newborns, and cultured with keratinocyte growth medium (KGM) (Clonetics Co., San Diego, CA, USA) supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 5 μg/ml insulin and 0.5 μg/ml hydrocortisone at 37°C in a moist atmosphere containing 5% CO_2. Almost-confluent NHKs were cultured for a further 24 h with freshly prepared KGM with or without 200 U/ml rIFN-r or 10^{-6} M dexamethasone.

Control cell types included human T-lymphotrophic virus type I (HTLV-I)-infected T-cell lines (16T, 35T, MH-1, and KS-2), peripheral blood mononuclear cells stimulated with PHA (5 μg/ml), T-cells freshly isolated with CD3-coated immunobeads (Dynal, Oslo, Norway) and normal epidermal sheets obtained by dispase.

Normal skin specimens were obtained after surgical removal of benign skin tumours from 3 patients, and 12 biopsy specimens were obtained from patients with inflammatory skin diseases, including 4 patients with bullous pemphigoid, 3 with vasculitis, 2 with erythroderma, 2 with lupus erythematosus, and 1 with subcorneal pustular dermatosis.

Immunostaining for IL-15

Biopsy specimens and cultured keratinocytes were pre-incubated with 10% normal rabbit serum, and reacted with a monoclonal antibody to IL-15 (clone; M115, IgG1, Genzyme, Cambridge, USA) or with control murine IgG1. The tissue sections were reacted with biotin-labelled rabbit anti-murine IgG, and then with streptavidin–horse radish peroxidase. Colour development was performed with 4-chloro-1-naphthol.
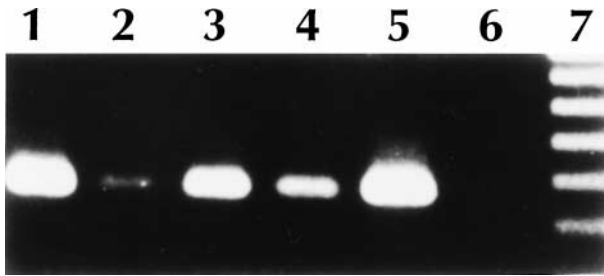
Fig. 1. IL-15 mRNA expression by various cell types. Lanes 1–4, HTLV-1-infected cell lines designated 16T, 35T, MH-1, and KS-2, respectively; lane 5, cultured keratinocytes; lane 6, no template DNA; lane 7, DNA marker.

Samples	IL-15 level (pg/ml)
NHK supernatants (almost confluent in 28 cm^2 dish)	<10
NHK lysates[a] (almost confluent in 28 cm^2 dish)	59[b]
Peripheral blood mononuclear cells (1×10^6 cells)	<10
Monocyte culture supernatants (1×10^6 cells)	<10
ATL cell culture supernatants (5×10^5 cells)	<10

[a]The volume of the lysate was adjusted to the original volume of culture supernatant with culture medium.
[b] The mean value of three wells for ELISA.

Enzyme-linked immunosorbent assay (ELISA)

IL-15 levels were assayed in culture supernatants and cell lysates using an IL-15-specific ELISA (Genzyme, Cambridge, USA). The cell lysates were extracted from cultured keratinocytes with Tris-HCl, 0.5% NP40, pH 7.4 containing 1mM phenylmethyl-sulphonylfluoride and 5mM N-ethylmaleimide, and adjusted to the original volume of culture medium with 50 mM Tris-HCl buffer.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

For RT-PCR, mRNA was prepared from normal epidermal sheets, cultured keratinocytes and HSC-5 cells, and reverse-transcribed to cDNA using Micro-FastTrack™ mRNA isolation and cDNA

synthesis kits (Invitrogen, San Diego, CA, USA). The following primer sets were used for this study: IL-15 (5), (sense) 5′CAA GTT ATT TCA CTT GAG TCC GGA G3′, (antisense) 5′TTC TAA GAG TTC ATC TGA TCC AAG G 3′; γ-actin, (sense) 5′TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA 3′, (antisense) 5′ CTA GAA GCA TTT GCG GTG GAC GAT GGG 3′. The PCR amplification was carried out in 50 μl reaction mixture containing 10 mmol/L Tris-HCl (pH 8.0), 1.5 mmol/L MgCl$_2$, 50 mmol/L KCl, 0.1 mg/ml gelatin, 200 μmol/L each of dATP, dGTP, dCTP, dTTP, 50 pmol/L of each primer, 1.25 units Taq polymerase (Takara, Tokyo, Japan). The reaction was first processed at 94°C for 5 min, 62°C for 5 min, and then was subjected to 35-cycle amplification. Each cycle includes DNA extension at 72°C for 90 s, denaturation at 94°C for
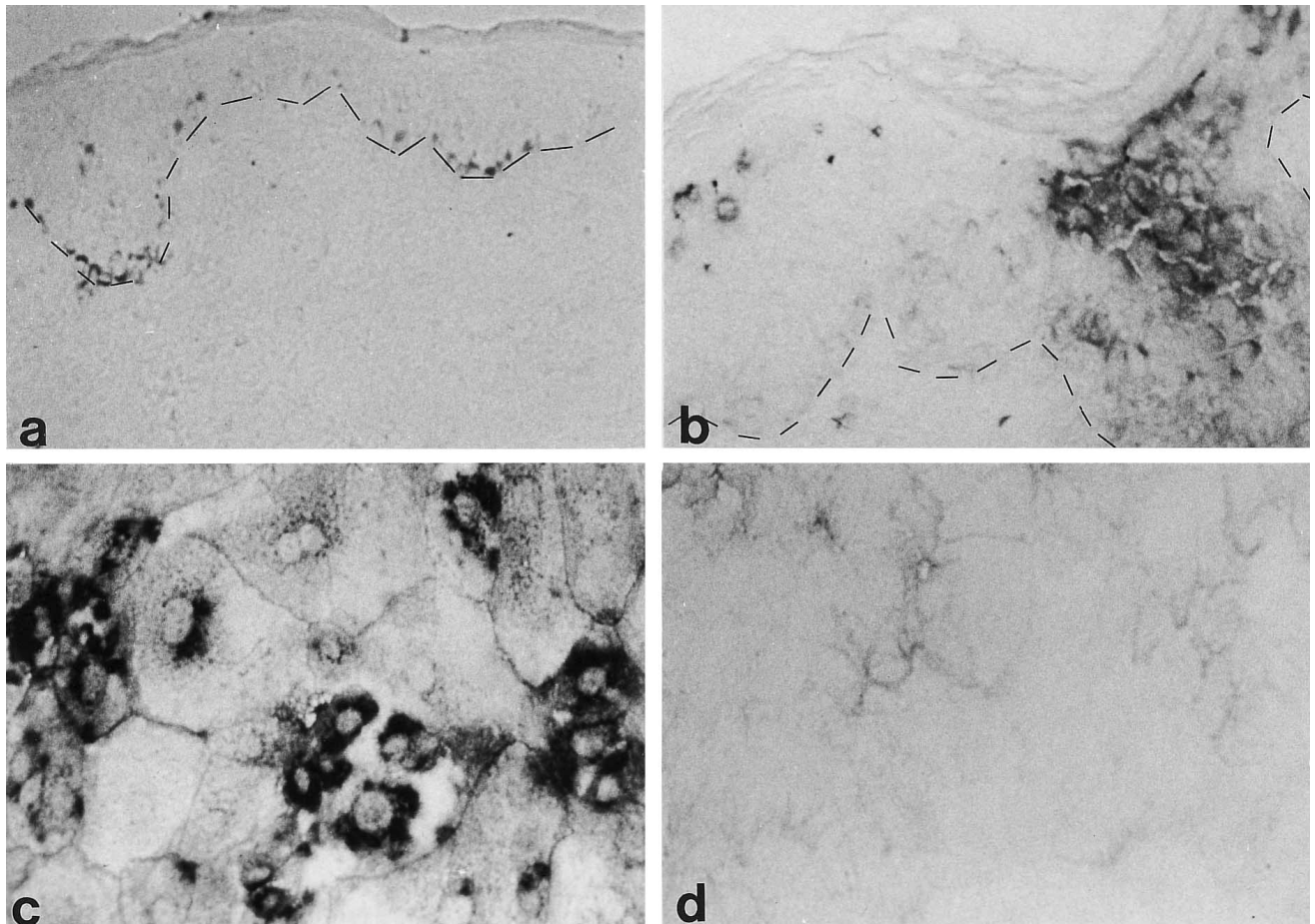

Fig 2. Immunostaining for IL-15. (*a*) Normal epidermis is negative for IL-15, excluding non-specific staining for basal melanins. (*b*) Some keratinocytes in the bullous pemphigoid lesion are positive. (*c*) Dense perinuclear staining of IL-15 in the cultured NHKs, and (*d*) no specific staining with IgG1 control antibody in cultured NHKs.

45 s and annealing at 62°C for 45 s. IL-15 and β-actin probes were prepared by incorporation of digoxigenin-labelled 11-dUTP during PCR amplification (Boehringer Mannheim, Mannheim, Germany). PCR products and probes were analysed on 2% agarose gel and visualized by staining with ethidium bromide.

Northern blotting analysis

For Northern blotting analysis, total RNA was extracted from approximately 10^7 cells using the guanidium thiocyanate-phenol-chloroform extraction method, and Poly(A)+ mRNA was further isolated with oligotex-30 (Super) (Takara, Tokyo, Japan). Two μg mRNA or 20 μg total RNA was electrophoresed on a 1% formaldehyde-containing gel and then transferred to nylon membrane filter. The blot was hybridized with digoxigenin-labelled IL-15 probe at 42°C overnight, and signals were detected using alkaline phosphatase-labelled anti-digoxigenin antibody and chemiluminescence kit (Boehringer Mannheim, Mannheim, Germany). After the IL-15 probe was removed, the membrane was reused for detection of β-actin mRNA. The density of each mRNA was measured using NIH Image 1.55 software, and the expression of IL-15 mRNA was valued by comparison with the internal positive signals for β-actin mRNA.

RESULTS

Expression pattern of IL-15 and IL-15 mRNA by epidermal and cultured keratinocytes

A screening test by RT-PCR demonstrated that many cell types including ATL cell line cells, cultured normal human keratino-cytes, and a squamous carcinoma cell line (HSC-5) constantly expressed IL-15 mRNA (Fig. 1). The IL-15 message was negative or very weak in mRNA samples obtained from T-cells freshly isolated with CD3-coating immunobeads and epidermal sheets.

IL-15 antigens were negative in the epidermis of three normal tissue sections (Fig. 2a). Of the 12 inflammatory skin biopsy specimens, only 2 patients, including 1 of 4 patients with bullous pemphigoid and 1 patient with subcorneal pustular dermatosis, showed IL-15-positive keratinocytes in the epidermis (Fig. 2b). In contrast, approximately 30% of cultured keratinocytes exhibited cytoplasmic staining for IL-15 antigens (Fig 2c). Addition of IFN-γ (400 U/mL) and dexamethasone (10^{-6} M) to the culture did not significantly alter the staining pattern and the percentage of IL-15-positive cells.

IL-15 levels in the culture supernatants and cell lysates

IL-15 levels were below the sensitivity limit of the procedure (10 pg/ml) in the culture supernatants of normal human keratino-cytes (almost confluent in a 28 cm^2 dish), whereas the cell lysates contained IL-15 at a mean level of 59 pg/ml (Table I). No detectable level of IL-15 was present in the supernatants of SCC cell line cells (HSC-5) cultured with or without 400 U/ml of IFN-γ, peripheral blood mononuclear cells (1×10^6 cells/ml), adherent cells (1×10^6 cells/ml), and ATL cell line cells (5×10^5 cells/ml) including 16T, 35T, MH-1 and KS-2.
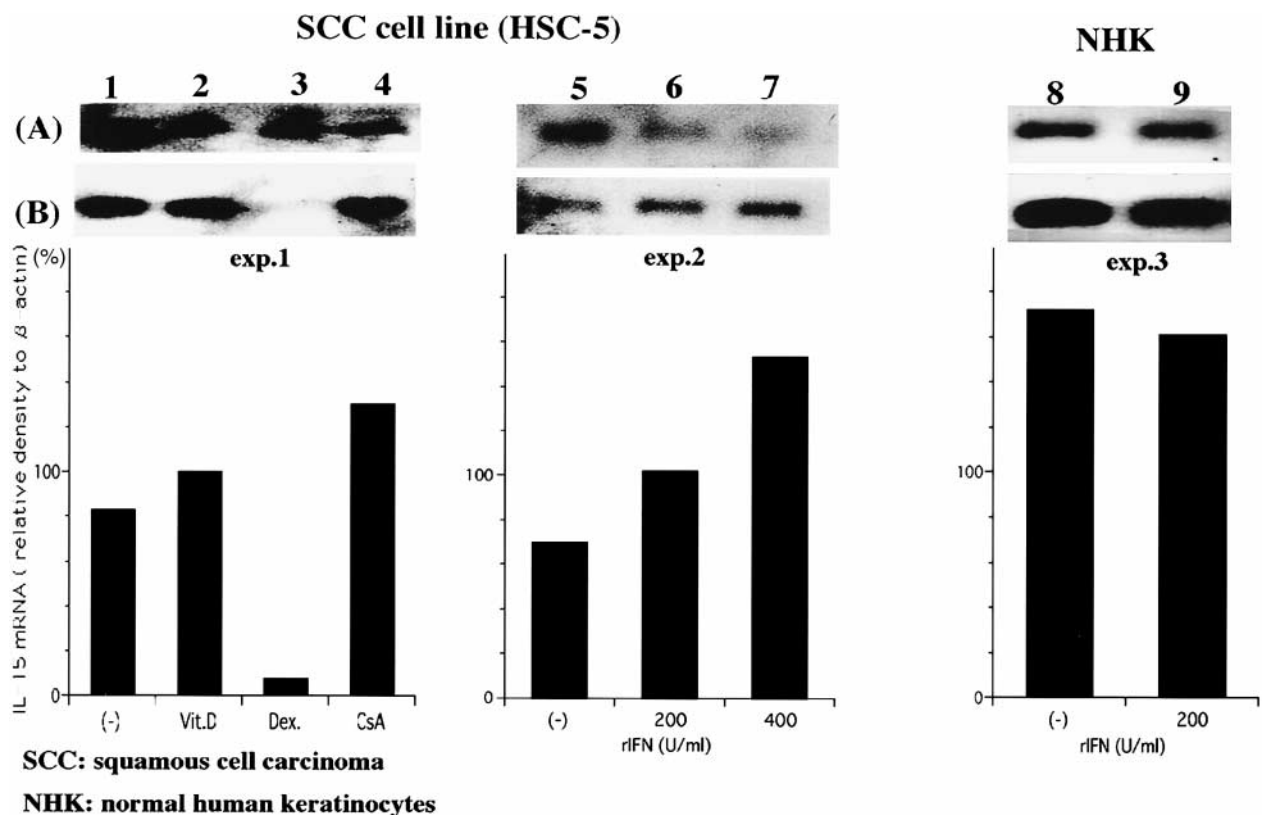

Fig. 3. IL-15 mRNA expression by keratinocytes in various culture conditions. (*Left panel*): dexamethasone markedly inhibits the IL-15 mRNA expression by HSC-5. Lane 1, untreated; lane 2, treated with 1,25(OH)$_2$D$_3$ (10^{-6} M); lane 3, dexamethasone (10^{-6} M) and lane 4, cyclosporin A (1 μg/ml). (A) β-actin, and (B) IL-15 mRNA. Twenty μg of total RNA in each lane. (*Middle panel*): rIFN-γ slightly increases the IL-15 mRNA expression by a SCC cell line, HSC-5. (A) β-actin, and (B) IL-15 mRNA. Lane 5, untreated; lane 6, treated with rIFN-γ (200 U/ml) and lane 7, rIFN-γ (400 U/ml). Two μg of mRNA in each lane. (*Right panel*): no significant effect of rIFN-γ (200 U/ml) on IL-15 mRNA expression by cultured NHKs. (A) β-actin, and (B) IL-15 mRNA. Lane 8, untreated and lane 9, rIFN-γ (200 U/ml). Two μg of mRNA in each lane.

*Northern blotting for IL-15 mRNA expression by
keratinocytes in various culture conditions*

Northern blotting analysis demonstrated that both HSC-5 and cultured normal human keratinocytes expressed IL-15 message. Dexamethasone markedly decreased IL-15 mRNA expression by both normal and cell line keratinocytes at a concentration of $10^{-5}-10^{-6}$ M (Fig. 3) after a 24 h culture. On treatment with rIFN-γ (200 and 400 U/ml), the message level was slightly increased in HSC-5 cells in a dose-dependent fashion, whereas no significant change was observed in normal human keratinocytes treated with 200 U/ml of rIFN-γ. Neither 1,25(OH)$_2$D$_3$ nor cyclosporin A altered the IL-15 mRNA level significantly.

DISCUSSION

In this study it was demonstrated that normal epidermal keratinocytes did not constitutively express IL-15, but cultured keratinocytes began to produce the cytokine. IL-15 was also expressed by some epidermal keratinocytes in inflammatory conditions associated with infiltration of neutrophils and eosinophils. Therefore, IL-15 expression might be induced by mediators released from the infiltrating cells *in vivo*. Our data indicate that IL-15 is not a constitutive cytokine produced by epidermal keratinocytes but is inducible.

Previous publications have reported that IL-15 message is expressed by a variety of cell types (1, 4–6). However, it has been difficult to detect IL-15 in the supernatants of most cells expressing the mRNA. A recent report described that a leading peptide of IL-15 containing 48 amino acids can inhibit the secretion of IL-15 molecules (11). A few cell types such as a simian kidney epithelial cell line (1) and an HTLV-1-infected T-cell line (6) were found to secrete mature IL-15, but it is debated whether keratinocytes can secrete IL-15 (6, 10, 11). We examined IL-15 levels in the supernatants and cell lysates of cultured keratinocytes separately, and detected IL-15 only in the cell lysates. Although it is possible that a small amount of IL-15 was secreted by keratinocytes, it is evident that IL-15 was deposited mainly in the cytoplasm. The release of the cytosolic molecules by cell death or the formation of membrane-bound IL-15 is essential, for this is when its biological properties are exhibited.

Although ultraviolet B irradiation was known to enhance IL-15 transcription (10), little is known about other stimulants. A previous report described the stimulatory effect of IFN-γ on IL-15 mRNA production by human foetal retinal pigment epithelial cells (12). We demonstrated that rIFN-γ slightly increased the IL-15 message level of HSC-5 in a dose-dependent fashion, whereas no significant change was observed in normal human keratinocytes. Therefore, the effect of IFN-γ may differ with cell type or differentiation state. Glucocorticosteroids have previously been reported to inhibit the production of IL-1 and IL-6 by keratinocytes (13, 14). Our results demonstrate that IL-15 mRNA expression is markedly inhibited by dexamethasone in the range of $10^{-5}-10^{-6}$ M in both normal and transformed keratinocytes. However, no apparent difference was observed in the percentages of IL-15-positive keratinocytes in culture when they were treated with dexamethasone or rIFN-γ, as determined by immunostaining. This discrepancy may imply different mechanisms in transcriptional and translational regulation of IL-15.

Our study demonstrated that IL-15 is an inducible cytokine deposited in the cytoplasm of cultured keratinocytes and in some epidermal keratinocytes in inflammatory conditions. The biological properties and regulation mechanisms of cytosolic or membrane-bound IL-15 remain to be elucidated.

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