

Interleukin 1 receptor antagonist in human epidermis and cultured keratinocytes

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Interleukin 1 (IL-1), present in high amounts in normal human skin without any sign of inflammation, suggests a complex mechanism by which its bioactivity is regulated. The specific receptor antagonist of IL-1 (IL-1ra) was analyzed in human skin, sweat and cultured keratinocytes. Extracts of both skin and cultured keratinocytes blocked the binding of [¹²⁵I]IL-1 to its receptor whereas sweat did not. The inhibitory activity was cell-associated, was not secreted by cultured keratinocytes, and IL-1ra mRNA was identified in these cells. There was an inverse relationship between the level of IL-1ra and that of IL-1 α and β since extracts of differentiating keratinocytes (DK) and higher IL-1ra levels and expressed more mRNA for IL-1ra than non-differentiated keratinocytes (NDK), whereas NDK contained 4 times more IL-1 α and β proteins than DK. This association of cell differentiation with a shift in agonist/antagonist ratio might be related to important autocrine or paracrine functions of IL-1 in normal and inflamed human skin.

Keratinocyte; Human epidermis; Interleukin-1; Interleukin-1 receptor antagonist

1. INTRODUCTION

IL-1 α and β are 17.5 kDa polypeptides which play an important role in immunological and inflammatory reactions as well as in the body's response to microbial invasion [1]. Initially described as a monocyte product, IL-1 is synthesized by a wide variety of cells including keratinocytes [2,3]. High levels of biologically active IL-1 were found in normal unstimulated human epidermis and human sweat [4,5]. The functional and physiological relevance of this IL-1 pool is still unclear. Immunohistological studies suggest a link between epidermal IL-1 and the differentiation process of keratinocytes [6]. Cultured keratinocytes contain mRNA for both forms of IL-1 but the functional activity both in cultured cells and normal human epidermis depends essentially on IL-1 α [7]. Translation of IL-1 β mRNA as an inactive product (31 kDa IL-1 β), which can not be cleaved to the mature form has been recently reported, and deficiency of a protease necessary to process IL-1 β might be important to avoid persistent inflammation [8]. However, high amounts of biologically active IL-1 α are also present in normal human epidermis after extraction and in sweat without any sign of inflammation. This suggests that IL-1 activity in human epidermis might be regulated by specific inhibitors. During the last few years interferences with IL-1 biological activities have been de-

scribed [9]. A specific inhibitor of IL-1 acting as receptor antagonist (IL-1ra) has been originally isolated from the urine of patients with monocytic leukemia and in the supernatants of stimulated human monocyte cultures [10–12]. In this study, we demonstrate the presence of IL-1ra in normal human epidermis and in extracts of cultured human keratinocytes and show that the expression of IL-1ra is higher in more differentiated cells. In contrast, IL-1ra was not detected in human sweat.

2. MATERIALS AND METHODS

2.1. Preparation of specimens and culture conditions

Normal human skin was obtained from abdominal plastic surgery. The epidermis was separated from the dermis by heating whole skin for 1 min at 56°C in 0.9% sodium chloride as described [6]. Human sweat was collected from patients with spontaneous hand hyperhidrosis as described [5]. Human keratinocytes from foreskin, obtained as described by Rheinwald and Green [13], were cultured in a mixture of Dulbecco-Vogt and Ham's F12 (3:1) medium supplemented with 10% fetal calf serum (FCS) (Seromed, Berlin, Germany), insulin (0.4 μ g/ml), adenine (24 μ g/ml), transferrin (6 μ g/ml), triiodothyronine (2 ng/ml), and epidermal growth factor (10 ng/ml). Keratinocytes were grown in this medium at normal Ca²⁺ (1.3 mM) concentration until confluency (10 days) and then with low Ca²⁺ (0.03 mM) during the next 2 or 3 days. With this latter medium, the more differentiated cells (subsequently quoted as differentiating keratinocytes, DK) are released after gentle shaking and the less differentiated cells (subsequently quoted as non-differentiated keratinocytes, NDK) remain attached to the culture dish. Supernatants were collected by centrifugation and DK washed 3 times with phosphate-buffered saline (PBS) (pH 7.2) before being stored at -70°C as dry pellet. NDK were washed 3 times with PBS by scraping with a rubber policeman in PBS containing 20 μ M EDTA and 5 μ M isoacetamide, and stored at -70°C until use. Human skin and keratinocytes were homogenized on ice 2 times for 1 min using a polytron and sonicated 3 times for 15 s in PBS containing

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20 μ M EDTA and 5 μ M iodoacetamide. After centrifugation at 10 000 $\times g$ supernatants were used for total protein measurement (Lowry), IL-1 α and β enzyme-immunoassay and IL-1 binding assay.

2.2. IL-1 α and IL-1 β determination

Content of IL-1 α and β in samples before and after IL-1 immuno-depletion was measured by enzyme-immunoassay (EIA) as previously described [14]. IL-1 was removed by immunoaffinity gels specific to IL-1 α or IL-1 β according to the manufacturer's method (Endogen Inc; Boston, MA).

2.3. IL-1ra determination

IL-1ra was measured by receptor binding assay [11] after depleting specimens of IL-1 α and IL-1 β which can interfere with the determination. Human recombinant IL-1 α (hrIL-1 α), radioiodinated by the chloramine T method (specific activity 3 to 5 $\times 10^5$ cpm/ng), was used for binding to the murine thymoma subline EL4-6.1 as described elsewhere [11,15]. Binding inhibition (%) was calculated as follows:

$$1 - \frac{[(\text{cpm in the presence of samples} - \text{cpm nonspecific binding}) / (\text{cpm of total binding} - \text{cpm non-specific binding})] \times 100}{}.$$

Total binding, non-specific and total radioactivity added were approximately 1080, 80 and 10 000 cpm, respectively.

2.4. RNA extraction and Northern blot analysis

Cell pellets were frozen (-80°C) in 4.5 M guanidium thiocyanate and 0.2 M β -mercaptoethanol, and total RNA was purified as described previously [16]. Total RNA (10 μ g) was denatured with 2% formaldehyde, electrophoresed on 1.2% agarose gel and stained with ethidium bromide (0.5 μ g/ml) to assess RNA integrity. After transfer to a nylon membrane (Biodyne, Pall, Ultrafine Filtration Corp, Glencore, NY, USA), the filters were baked for 2 h at 80°C and prehybridized for 6 h at 58°C with 200 $\mu\text{l}/\text{cm}^2$ of the hybridization mixture containing 50% (v/v) deionized formamide, 0.8 M NaCl, 2 mM EDTA, 0.1% SDS, 2.5 \times Denhardt's solution and 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm. The filters were then hybridized overnight at 58°C with 100 $\mu\text{l}/\text{cm}^2$ of fresh hybridization mixture containing the IL-1 α or IL-1 β RNA anti-sense probe labelled with ^{32}P or with a 210-bp fragment of IL-1ra DNA labelled by multi-priming system kit (Boehringer Mannheim, Germany) with [α - ^{32}P]dATP. The hybridized membrane was washed to a stringency of 0.1 \times SSC, 0.1% SDS for 30 min at 70°C . Membranes were exposed to Kodak XAR Film at -70°C for 24 h. The IL-1 α cDNA was a 1764-bp clone inserted into the *Hind*III site of pUC8. A 500-bp fragment isolated between *Pst*I-*Hind*III was subcloned in pSP64. The IL-1 β cDNA was a 1200 bp clone inserted in the *Pst*I site of pAT 153. A 570-bp fragment isolated between *Hind*II-*Hind*III was subcloned in pSP65. The probe used to detect the IL-1ra mRNA was a 210-bp fragment coding for the COOH-terminal half of the protein and recognized the common region coding for sIL-1ra and icIL-1ra (gift of S. Eisenberg, Synergen, Boulder) [17].

3. RESULTS

3.1. Content of IL-1 α and β and Northern blot analysis of IL-1 mRNA

As previously described [5,8] a high level of IL-1 α and low amount of IL-1 β were observed in extracts of normal human epidermis and in sweat (Table I). In cultured keratinocytes, the amount of cell-associated IL-1 β was greater than in human skin extract (Table I). IL-1 α and IL-1 β levels in keratinocyte supernatants were below the limit of detection of the assay (<10 pg/ml). These data confirm that human epidermal cells contain a high level of IL-1 (mainly IL-1 α), but secrete only little amounts of this cytokine [19]. Moreover, IL-1 α and β

concentrations depend upon the stage of cell differentiation, as 4 times more IL-1 α or β was found in NDK than in DK. This result contrasts with that of Mitzutani et al. [8] who observed comparable levels of IL-1 α and β in normal cultured keratinocytes under standard (1.5 mM) and low (0.15 mM) calcium concentrations. However, these investigators used other culture conditions and immunoassays to evaluate both forms of IL-1.

Northern blot analysis identified a stronger signal for IL-1 α mRNA in NDK as compared to DK (Fig. 1). The same observations applied to the steady-state level of IL-1 β mRNA whose signal was however less intense than that of IL-1 α mRNA. Therefore, these results are in accordance with the protein values showing more IL-1 in NDK than in DK.

3.2. Detection of IL-1ra biological activity

To specifically measure IL-1ra by the receptor binding assay, each lysate was immuno-depleted of IL-1 α and β . The specific binding of [^{125}I]IL-1 α to EL4-6.1 cells was significantly inhibited (75%) by human epidermis extract and by cultured human keratinocyte extract (84–100%). The extraction buffer did not modify the binding assay (Table II). The amount of IL-1 α and β remaining in the extracts after depletion was less than the concentration of hrIL-1 α and hrIL-1 β which affect the binding of [^{125}I]IL-1 α [20]. Contrary to monocytes/macrophages, keratinocytes do not appear to secrete IL-1ra since we could not detect significant ($<20\%$) inhibition in the supernatants of cultured keratinocytes. The % inhibition obtained for the sample of sweat was also insignificant.

3.3. Dose-response of IL-1 inhibitory activity in human epidermis, NDK and DK

As shown in Table II, the inhibitor activity at 1/20 dilution was higher in DK (100%) than in NDK (84.1%). Dilution curves were performed on specimens depleted of IL-1 α and IL-1 β showing that binding of [^{125}I]IL-1 α was inhibited in a dose-dependent fashion by human epidermis, NDK and DK extracts (Fig. 2).

Table I

Content of IL-1 α and β in epidermis, sweat and cultured human keratinocytes (EIA)

Specimen	IL-1 α (ng/mg prot.)	IL-1 β (ng/mg prot.)
Human epidermis extract	25.76	0.06
Human sweat	10.43*	0.10*
NDK extract	34.09	5.84
DK extract	7.36	1.69
Supernatant	0.16*	$<0.01^*$

Human epidermis, sweat, extracts of non-differentiated keratinocytes (NDK) or differentiating keratinocytes (DK) and supernatants of cultured keratinocytes were analyzed for their content of IL-1 α and IL-1 β by EIA. Values are normalized to ng protein or ml*.

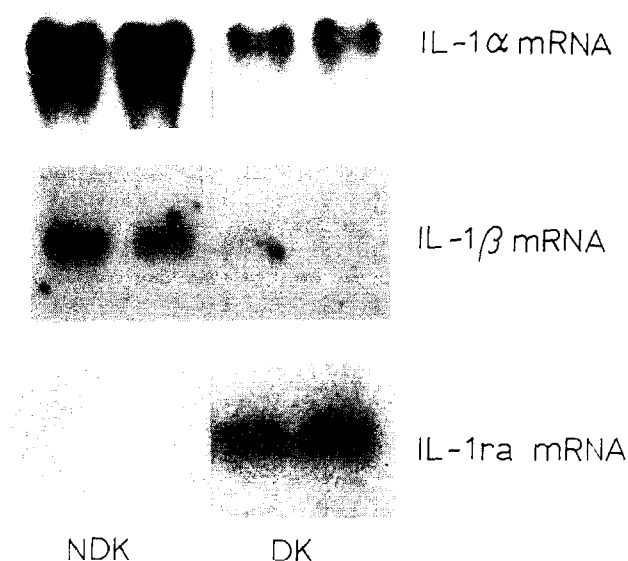


Fig. 1. IL-1 α , IL-1 β and IL-1ra mRNAs in non-differentiated (NDK) are differentiating (DK) cultured human keratinocytes. Results are shown in duplicate for each mRNA.

Furthermore, DK extract contained more IL-1ra than extract of NK. In contrast, the reverse was observed for IL-1 α and IL-1 β concentrations which were lower in DK.

Table II

IL-1ra (IL-1 receptor binding assay), IL-1 α and IL-1 β (EIA) in human epidermis, sweat and keratinocyte extracts

Specimen (1/20 dilution)	IL-1 α (ng/ml)	IL-1 β (ng/ml)	Inhibition (%)
Buffer	NT	NT	0
Epidermis extract			
undepleted	8.69	0.02	100
depleted	<0.01	<0.01	75
Sweat			
undepleted	0.52	<0.01	18.24
depleted	<0.01	<0.01	7.09
NDK extract			
undepleted	5.37	0.80	100
depleted	0.03	0.02	84.1
DK extract			
undepleted	1.43	0.33	100
depleted	0.08	<0.01	100
Supernatant			
undepleted	<0.01	<0.01	12.2
depleted	<0.01	<0.01	14.19

Specimens in Table II were tested before and after depletion of IL-1 α and IL-1 β for inhibitory activity in the IL-1 receptor assay. IL-1 α and IL-1 β were measured by EIA. NT: non-tested.

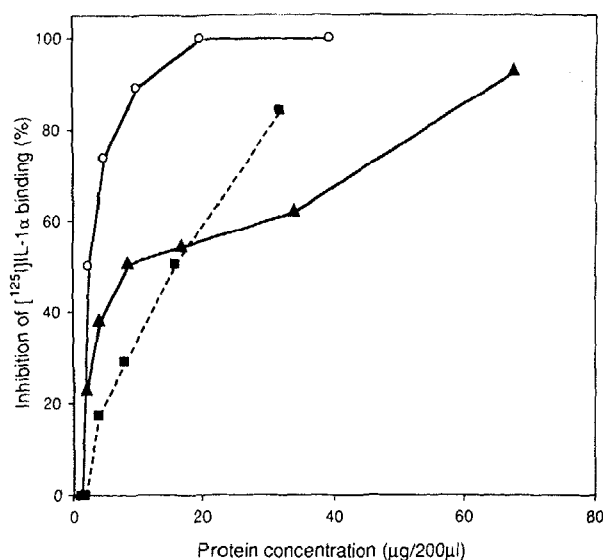


Fig. 2. IL-1ra in human epidermis and keratinocyte extracts measured by IL-1 receptor binding assay. EL4-6.1 cells (1×10^6) were incubated for 1 h at 4°C with serial dilutions of epidermis (Δ) NDK (\blacksquare) or DK (\circ) extracts. [125 I]IL-1 α was then added (10 000 cpm/assay) for 4 h at 4°C. Non-specific binding was determined in the presence of an excess of cold hrIL-1 α and total binding of [125 I]IL-1 α was determined in the absence of cold hrIL-1 α .

3.4. IL-1ra mRNA in differentiated keratinocytes

To establish that IL-1ra biological activity is consistent with the level of IL-1ra mRNA expressions, IL-1ra mRNA was analyzed in DK and NDK. IL-1ra mRNA expression was clearly detected in DK whereas no signal was identified in NDK (Fig. 1). This is in accordance with the above findings on the IL-1ra protein showing that DK contained more inhibitory activity than NDK.

4. DISCUSSION

We describe the presence of IL-1ra using the specific inhibition test at the receptor level and the presence of IL-1 mRNA in human epidermis and cultured keratinocytes. There was an inverse relationship between the level of IL-1ra and the amount of IL-1 α or β since DK extract had higher IL-1ra and mRNA level than NDK and the reverse was found for IL-1 α or β . Of interest, our data obtained with keratinocytes are similar to those obtained in other cell types since IL-1 production decreases during monocyte differentiation whereas IL-1ra expression increases during the same process [20,21]. In addition, there is evidence that human macrophages at terminal stage and obtained from bronchoalveolar lavage or synovial fluids produce higher amounts of IL-1ra than blood monocytes and less IL-1 α or β [22,23]. Besides the IL-1ra which is secreted (sIL-1ra) from cells another IL-1ra has been identified recently which remains intracellular (icIL-1ra) [24].

Both are products of same gene, but icIL-1ra does not contain the leader sequence necessary for secretion. sIL-1ra is principally produced by monocytes, whereas cIL-1ra is found only in epithelial cells [24]. Our studies in addition clearly demonstrate by the receptor binding assay that the biological inhibitory action occurs at the receptor level. The presence of intracellular IL-1ra in normal human epidermis and in keratinocyte extracts of unstimulated epidermal cells could explain why a high level of IL-1 can also be present in normal skin without evidence of inflammation. The ratio of IL-1 and IL-1ra concentrations may be important for the regulation of keratinocyte differentiation and inflammatory processes, and an imbalance between these two products may lead to deleterious effects.

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