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Interleukin 1 receptor antagonist in human epidermis and cultured keratinocytes

D. Gruaz-Chatellard[1], C. Baumberger[2], J.-H. Saurat[1] and J.-M. Dayer[2]

[1]*Clinic of Dermatology* and [2]*Division of Immunology and Allergy, Department of Medicine, Hôpital Cantonal Universitaire, 1211 Genève 4, Switzerland*

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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 [^{125}I]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-1α 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-1α 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-1α 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 synthetized 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-1α [7]. Translation of IL-1β mRNA as an inactive product (31 kDa IL-1β), which can not be cleaved to the mature form has been recently reported, and deficiency of a protease necessary to process IL-1β might be important to avoid persistent inflammation [8]. However, high amounts of biologically active IL-1α 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 described [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.

Correspondence address: J.-M. Dayer, Division of Immunology and Allergy, Dept. of Medicine, University Hospital, 1211 Geneva 4, Switzerland. Fax: (41) (22) 476486.

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 μg/ml), adenine (24 μg/ml), transferrin (6 μg/ml), triiodothyronine (2 ng/ml), and epidermal growth factor (10 ng/ml). Keratinocytes were grown in this medium at normal Ca^{2+} (1.3 mM) concentration until confluency (10 days) and then with low Ca^{2+} (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 μM EDTA and 5 μM iosoacetamide, 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

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

2.2. *IL-1α and IL-1β determination*

Content of IL-1α and β in samples before and after IL-1 immunodepletion was measured by enzyme-immunoassay (EIA) as previously described [14]. IL-1 was removed by immunoaffinity gels specific to IL-1α or IL-1β 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-1α and IL-1β which can interfere with the determination. Human recombinant IL-1α (hrIL-1α), radioiodinated by the chloramine T method (specific activity 3 to 5×10^5 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:

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

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 μg) was denatured with 2% formaldehyde, electrophoresed on 1.2% agarose gel and stained with ethidium bromide (0.5 μg/ml) to asses 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 μl/cm² of the hybridization mixture containing 50% (v/v) deionized formamide, 0.8 M NaCl, 2 mM EDTA, 0.1% SDS, 2.5 × Denhardt's solution and 100 μg/ml denatured salmon sperm. The filters were then hybridized overnight at 58°C with 100 μl/cm² of fresh hybridization mixture containing the IL-1α or IL-1β 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×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-1α cDNA was a 1764-bp clone inserted into the *Hin*dIII site of pUC8. A 500-bp fragment isolated between *Pst*I-*Hin*dIII was subcloned in pSP64. The IL-1β cDNA was a 1200 bp clone inserted in the PstI site of pAT 153. A 570-bp fragment isolated between *Hin*dII-*Hin*dIII 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-1α and β and Northern blot analysis of IL-1 mRNA*

As previously described [5,8] a high level of IL-1α and low amount of IL-1β were observed in extracts of normal human epidermis and in sweat (Table I). In cultured keratinocytes, the amount of cell-associated IL-1β was greater than in human skin extract (Table I). IL-1α and IL-1β 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-1α), but secrete only little amounts of this cytokine [19]. Moreover, IL-1α and β concentrations depend upon the stage of cell differentiation, as 4 times more IL-1α 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-1α 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-1α mRNA in NDK as compared to DK (Fig. 1). The same observations applied to the steady-state level of IL-1β mRNA whose signal was however less intense than that of IL-1α 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-1α and β. The specific binding of [^{125}I]IL-1α 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-1α and β remaining in the extracts after depletion was less than the concentration of hrIL-1α and hrIL-1β which affect the binding of [^{125}I]IL-1α [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-1α and IL-1β showing that binding of [^{125}I]IL-1α was inhibited in a dose-dependent fashion by human epidermis, NDK and DK extracts (Fig. 2).

Table I

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

Specimen	IL-1α (ng/mg prot.)	IL-1β (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-1α and IL-1β by EIA. Values are normalized to ng protein or ml*.

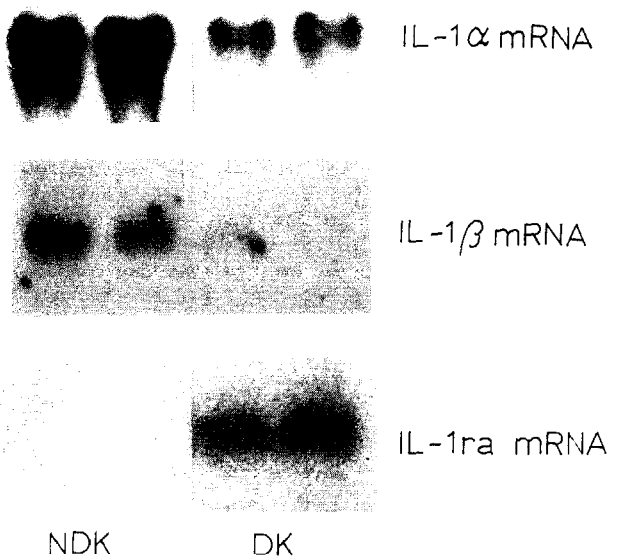

Fig. 1. IL-1α, IL-1β and IL-1ra mRNAs in non-differentiated (NDK) are differentiating (DK) cultured human keratinocytes. Results are shown in duplicate for each mRNA.

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

Table II

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

Specimen (1/20 dilution)	IL-1α (ng/ml)	IL-1β (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-1α and IL-1β for inhibitory activity in the IL-1 receptor assay. IL-1α and IL-1β were measured by EIA. NT: non-tested.

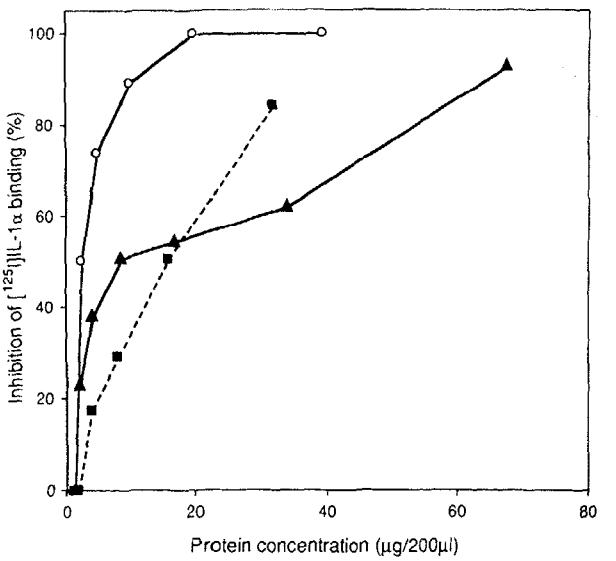

Fig. 2. IL-1ra in human epidermis and keratinocyte extracts measured by IL-1 receptor binding assay. EL4-6.1 cells (1×10⁶) were incubated for 1 h at 4°C with serial dilutions of epidermis (▲) NDK (■) or DK (○) extracts. [125I]IL-1α was then added (10 000 cpm/assay) for 4 h at 4°C. Non-specific binding was determined in the presence of an excess of cold hrIL-1α and total binding of [125I]IL-1α was determined in the absence of cold hrIL-1α.

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-1α or β since DK extract had higher IL-1ra and mRNA level than NDK and the reverse was found for IL-1α 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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