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Modulation of the IL-1 cytokine network in keratinocytes by intracellular IL-1α and IL-1 receptor antagonist

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SUMMARY

The IL-1 cytokine network in epidermal cells was studied *in vitro*, using the spontaneously transformed HaCAT human keratinocyte line. Intracellular (ic) IL-1α and IL-1 receptor antagonist protein (IL-1Ra) following cell lysis were readily identified assayed using a capture ELISA; whereas in culture supernatants IL-1Ra was not detected, and IL-1α was present at only very low levels. Confluent cultures of HaCAT cells were shown to provide optimal conditions for the study, since confluence increased the icIL-1Ra : IL-1α ratio to a level as seen *in vivo*, which was independent of Ca^{2+} concentration in the culture medium. The IL-1Ra extracted from HaCAT cell lysates was functionally active, as demonstrated in the mouse thymocyte co-proliferation assay which could be blocked using a rabbit anti-IL-1Ra antibody. Transforming growth factor-beta (TGF-β1) stimulated a dose-dependent increase in HaCAT cell IL-1α without changing IL-1Ra concentration, with a resultant reduction in the icIL-1Ra : IL-1α ratio from 320 : 1 to 100 : 1. Similarly, TGF-α, interferon-gamma (IFN-γ), IL-6, and tumour necrosis factor-alpha (TNF-α) substantially increased HaCAT cell IL-1α, but had no effect on the IL-1Ra, with a concomitant reduction in the icIL-1Ra : IL-1α ratio. In contrast to their effects on monocytes, IL-4 and IL-10 at biologically active levels had no effect on IL-1α, IL-1Ra or the icIL-1Ra : IL-1α ratio in confluent HaCAT cells. Hydrocortisone reduced IL-1α to below the limit of sensitivity of the ELISA, and induced a small increase in IL-1Ra of questionable biological significance. Thus, regulation of the IL-1 cytokine network in keratinocytes involves modulation of icIL–1α rather than of icIL-1Ra levels, and is markedly different from that noted in monocytes.

Keywords IL-1 IL-1 receptor antagonist keratinocytes

INTRODUCTION

IL-1 is a proinflammatory cytokine constitutively synthesized by keratinocytes, which induces inflammation when injected into human dermis [1]. IL-1 has therefore been postulated to play a fundamental role in the pathophysiology of many inflammatory skin diseases. For this reason, there has been considerable interest in defining regulatory pathways which control the IL-1 cytokine network. The three ligands which bind the type I and type II IL-1 receptors consist of two agonists, IL-1α and IL-1β, and one antagonist, IL-1 receptor antagonist (IL-1Ra). These ligands are products of related genes and share 19–30% amino acid homology [2]. IL-1 bioactivity in the skin is predominantly cell-associated, and is confined to IL-1α in extracts of cultured keratinocytes and keratome biopsies of normal skin [3]. IL–1β is present only as a 31-kD latent peptide, as keratinocytes lack IL-1 convertase which is required to cleave latent IL-1β peptide into the 17-kD active form [4]. IL-1Ra was originally purified from supernatants of IgG-stimulated monocytes [5]. Secretory (sIL-1Ra) and intracellular (icIL-1Ra) isoforms of IL-1Ra have subsequently been identified, as alternative spliced products of a single gene. Keratinocytes make only the intracellular form of IL-1Ra which is not secreted, since icIL-1Ra lacks the required leader sequence for transport into the Golgi apparatus. However, this icIL-1Ra is released together with IL-1α on disruption of keratinocyte membranes [5,6]. Thus the net proinflammatory effect of the IL-1 cytokine network in diseased or damaged skin reflects the balance between the concentrations of IL-1α, its receptors, and IL-1Ra. Although IL-1Ra, IL-1α and IL-1β have a similar affinity for type I and type II IL-1 receptors, IL-1Ra must be present in excess to block signal transduction following exposure to IL-1, as only a small number of the target cell IL-1 receptors need to bind ligand to elicit a maximal response [2]. The ratio of IL-1Ra : IL-1α required to block signal transduction is in the range of 10 : 1

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to 500:1, depending on the number of IL-1 receptors on the target cell, which is in turn a reflection of the target cell type and its activation state [7]. The importance of assessing the IL-1Ra:IL-1α ratio, rather than just the levels of the individual cytokines in isolation, is illustrated by recent studies on psoriasis. We and others [8,9] have documented reduced levels of IL-1Ra in lesional as opposed to involved skin from psoriasis patients. However, despite the reduction in keratinocyte IL-1Ra in psoriatic plaques, it has been reported that the IL-1Ra:IL-1α ratio in keratome skin biopsies is actually increased from 120:1 in normal skin to 1076:1 in psoriatic plaques, due to a diminution of IL-1α levels [9].

Recent *in vitro* studies have investigated the effects of differentiation, and of a number of immunoregulatory cytokines, on keratinocyte synthesis of IL-1α and IL-1Ra [10,11]. Under the culture conditions used for these studies, however, keratinocyte IL-1Ra:IL-1α ratios varied within the range 10:1 to 25:1, compared with the 120:1 ratio reported for normal skin *in vivo* [9]. We therefore sought to develop a model which would allow us to investigate regulation of the IL-1 regulatory network under more physiological conditions, using an *in vitro* keratinocyte culture system which more closely mimics the *in vivo* situation. We have used this model to study the effects on keratinocyte production of IL-1α and IL-1Ra of: the proinflammatory cytokines tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and IL-6; the cytokines transforming growth-beta (TGF-β) and IL-4, which are known to induce sIL-1Ra in monocytes [12,13]; IL-10, which has a variety of inhibitory actions on T cells, monocyte/macrophages; and murine Langerhans cells, [14] and the anti-inflammatory agent hydrocortisone.

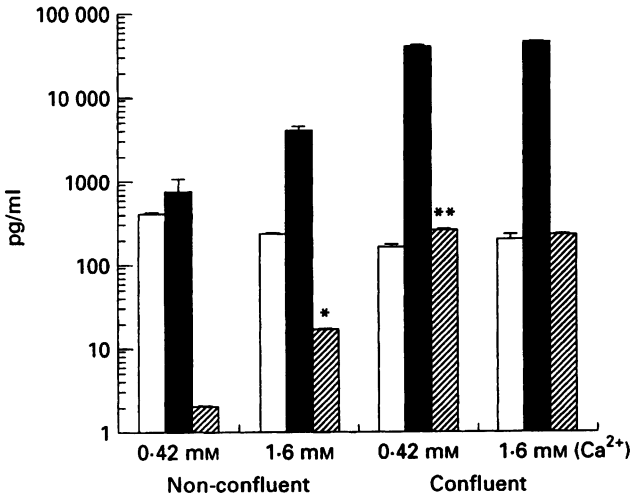

Fig. 1. The effect of extracellular Ca^{2+} concentration and cell confluence on HaCAT cell-associated IL-1α and IL-1Ra levels. HaCAT cells were cultured in medium containing either low (0·4 mM) or high (1·6 mM) levels of Ca^{2+}. Intracellular IL-1α and IL-1Ra levels (pg/ml) were determined in both confluent and non-confluent cells. Extracellular Ca^{2+} concentration in the non-confluent cell cultures significantly increased the IL-1Ra:IL-1α ratio (*$P < 0.02$). This ratio was further increased if the cells were grown to confluency (**$P < 0.002$), but was not further modulated by extracellular Ca^{2+} concentration in these cell cultures. □, icIL-1α; ■, icIL-1Ra; ▨, icIL-1Ra:IL-1α ratio.

MATERIALS AND METHODS

Cell culture and test reagents

HaCAT cells (the gift of Professor N. Fusenig, Institute für Zell- und Tumourbiologie, Deutsches Kresforschungszentrum, Heidelburg, Germany), a spontaneously transformed human keratinocyte cell line with relatively normal differentiation [15], were cultured in six-well plates in either RPMI (ICN Flow, Costa Mesa, CA), or Dulbecco's modified Eagle's medium (DMEM; ICN Flow), containing 5% heat-inactivated fetal calf serum (FCS; Gibco, Paisley, UK). Cells were grown to confluence, washed with fresh medium, and cultured for 24 h in medium with or without the following test reagents added at varying concentrations: recombinant human TGF-β1, TGF-α and IFN-γ (a gift from Genentech Inc., San Francisco, CA); recombinant human IL-1α (a gift from Hoffman-La Roche, Nutley, NJ); recombinant human TNF-α (a gift from Genentech Inc.); IL-4 (a gift from Sandoz, Basel, Switzerland); IL-10 (a gift from DNAX, Palo Alto, CA); and hydrocortisone succinate (Upjohn, Crawley, UK). HaCAT cell supernatants were centrifuged at 400 *g* for 10 min at 4°C and stored at −20°C before analysis. The HaCAT cell monolayers were washed three times with Hanks' balanced salt solution (HBSS; Sigma, Poole, UK), removed from the plate with a rubber policeman, and suspended in HBSS before being lysed by three cycles of freezing and thawing. The resulting suspensions were centrifuged at 400 *g* for 10 min, and supernatants containing soluble intracellular protein were stored at −20°C before assay.

ELISA assays

IL-1α and IL-1Ra were assayed using the following capture ELISA protocol. Nunc immunosorb plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of a 15 μg/ml solution in PBS pH 7·4 (Sigma) of ILA9-H18 monoclonal anti-recombinant human IL-1α (a gift from Syntex, Palo Alto, CA), or with 100 μl of a 1·6 μg/ml solution in PBS of I4

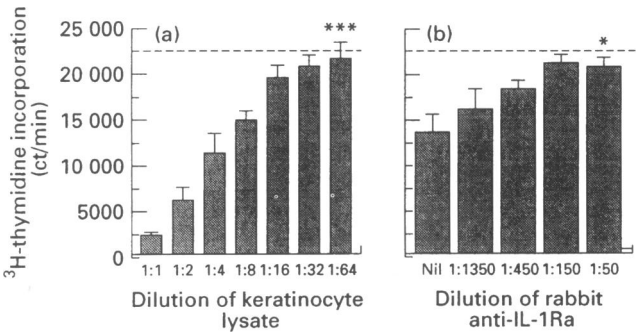

Fig. 2. HaCAT cell lysate inhibits recombinant IL-1α activity in the murine thymocyte co-mitogenic proliferation assay. HaCAT cell lysate dilutions were incubated in the presence of 20 U/ml human IL-1α, and proliferation was measured in the mouse co-mitogenic thymocyte assay (a). The proliferation of these cells in the presence of 20 U/ml human IL-1α alone is indicated by the dashed line, which was inhibited in a dose-dependent manner by HaCAT cell lysate, and reached baseline proliferation (IL-1α alone) at 1:64 dilution (**$P < 0.001$) compared with 1:1 dilution of keratinocyte lysate (a). This inhibition was reversed by rabbit anti-IL-1Ra antibody, which reached significance (*$P < 0.05$) at 1:50 dilution (b).

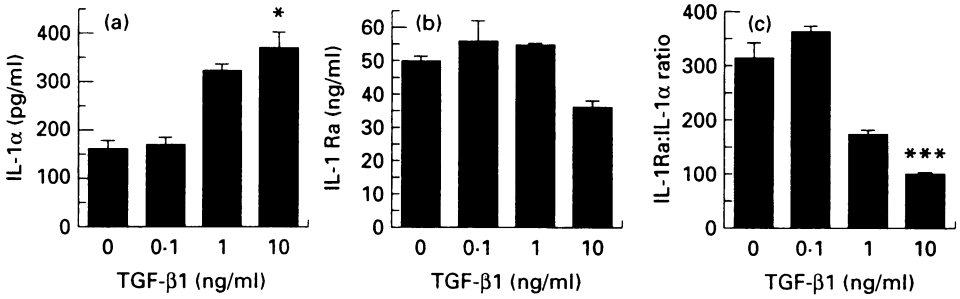

Fig. 3. Transforming growth factor-beta (TGF-β) increases icIL-1α levels in HaCAT cells. Confluent HaCAT cells were cultured in the presence or absence of TGF-β (0–10 ng/ml), and intracellular IL-1α and IL-1Ra levels determined. TGF-β (10 ng/ml) significantly (*$P < 0{\cdot}02$) increased IL-1α levels (a), but had no significant effect on IL-1Ra levels (b). The cumulative effect of TGF-β (c) was to significantly (***$P < 0{\cdot}001$) reduce the IL-1Ra : IL-1α ratio.

monoclonal anti-IL-1Ra (a gift from Upjohn, Kalamazoo, MI). Plates were washed three times with PBS containing 0·05% Tween 20 (PBS–T) and blocked with 2% bovine serum albumin (BSA; Sigma) in PBS for 2 h at 37°C. After washing with PBS–T, 50 μl of samples, or recombinant human IL-1α standard (a gift from Hoffman La Roche), or recombinant human IL-1Ra standard (a gift from Upjohn) were added to the plates.

For the IL-α assay, plates were then incubated with 50 μl of a 2 μg/ml solution of biotinylated ILA8-H12 monoclonal anti-recombinant human IL-1α (a gift from Syntex) diluted in 0·5% BSA in PBS–T at 37°C for 2 h. Plates were washed with PBS–T, before incubation with 1/3000 streptavidin horseradish peroxidase (HRP) complex (Amersham, Aylesbury, UK) for 1 h at 37°C. After washing five times with PBS–T, the ELISA was developed with *o*-phenylenediamine dihydrochloride (OPD; Sigma) in citrate buffer pH 5·6 with hydrogen peroxide, and stopped with 4 N sulphuric acid before reading at 492 nm. This ELISA was sensitive to 90 pg/ml.

For the IL-1Ra assay, plates were then incubated with 1/3000 affinity-purified rabbit anti-recombinant human IL-1Ra (generated 'in house') diluted in 0·5% BSA in PBS–T for 2 h at 37°C. After washing with PBS–T, plates were incubated with 1/3000 affinity-purified goat anti-rabbit IgG $F(ab')_2$ fragments conjugated to HRP (Jackson Laboratories Inc., Palo Alto, CA) for 1 h at 37°C. Plates were washed five times with PBS–T and developed with OPD and read as above. This ELISA was sensitive to 1 ng/ml of IL-1Ra. There was no cross-reaction with IL-1α or IL-1β at 100 μg/ml.

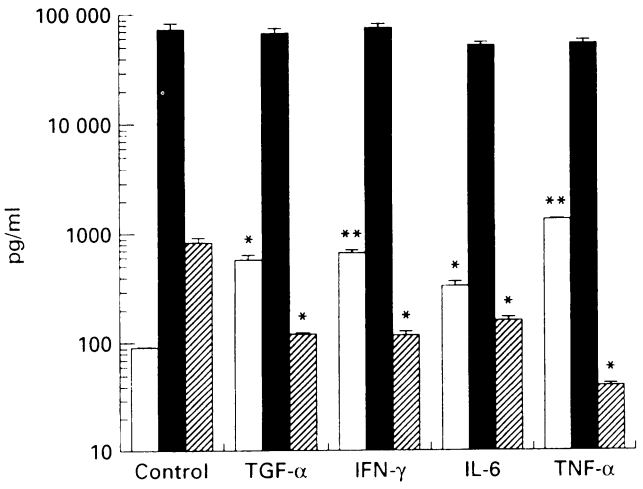

Fig. 4. Modulation of the IL-1Ra : IL-1α ratio (▨) by cytokines. Confluent HaCAT cells were cultured in the presence or absence of a range of different cytokines (10 ng/ml) which display differential effects on keratinocytes. IL-1α levels were increased by all cytokines transforming growth factor-alpha (TGF-α; *$P < 0{\cdot}05$), IFN-γ (**$P < 0{\cdot}002$), IL-6 (*$P < 0{\cdot}05$), and tumour necrosis factor-alpha (TNF-α; **$P < 0{\cdot}02$), whereas no significant effect was observed on icIL-1Ra levels. The cumulative effect of these cytokines on the IL-1Ra : IL-1α ratio was a significant (*$P < 0{\cdot}05$) reduction. □, icIL-1α; ■, icIL-1Ra.

IL-1 bioassay

IL-1 bioactivity of samples was assessed in the thymocyte proliferation assay. Thymocytes from 3-week-old male CBA mice were washed three times in RPMI with 10% FCS. Cells (10^6/well) were seeded in wells of a 96-well plate (Life Technologies, Paisley, UK) in RPMI with 10% FCS and 2×10^{-5} M β_2-mercaptoethanol (β_2-ME; Sigma) and 2·5 μg/ml phytohaemagglutinin (PHA; Difco Labs, Detroit, MI). Sample or standard recombinant human IL-1α (100 μl) was added to each well, and the cells were incubated at 37°C in 5% CO_2 for 54 h, before pulsing with 1 μCi ^{3}H-thymidine (Amersham) for 16 h. ^{3}H-thymidine incorporation was assayed using a scintillation counter. Experiments were carried out in triplicate, and are expressed as ± s.e.m. Statistical analysis of differences between groups was done with the paired Student's *t*-test. Rabbit polyclonal anti-IL-1Ra was used as a specificity control to neutralize IL-1 bioactivity.

RESULTS

Effect of Ca^{2+} and HaCAT cell density on intracellular IL-1Ra:IL-1α ratio

Soluble IL-1Ra was not detected, and soluble IL-1α was detected at only a very low level, in culture supernatants of HaCAT cells. The results of varying cell density, and Ca^{2+} concentration in the culture medium, on the icIL-1Ra:IL-1α ratio in cultures of HaCAT cells are summarized in Fig. 1. In non-confluent cultures of HaCAT cells, the extracellular Ca^{2+} concentration in the culture medium had a significant

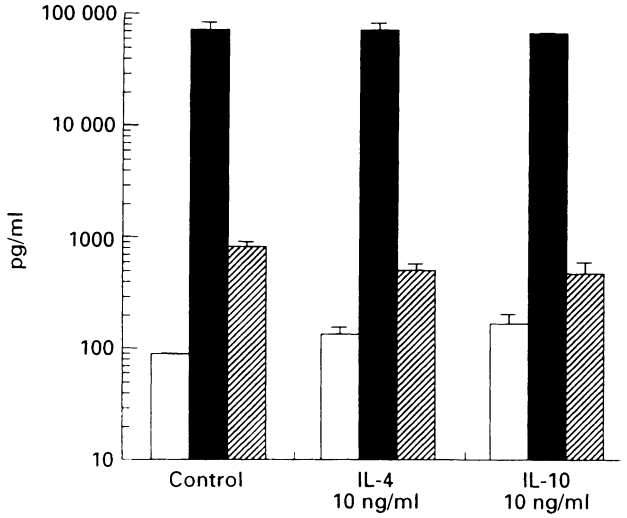

Fig. 5. IL-4 or IL-10 do not modulate the IL-1Ra : IL-1α ratio (▨). Confluent HaCAT cells were cultured in the presence or absence of IL-4 (10 ng/ml) or IL-10 (10 ng/ml). Neither of these cytokines modulated the icIL-1α or IL-1Ra levels. □, icIL-1α; ■, icIL-1Ra.

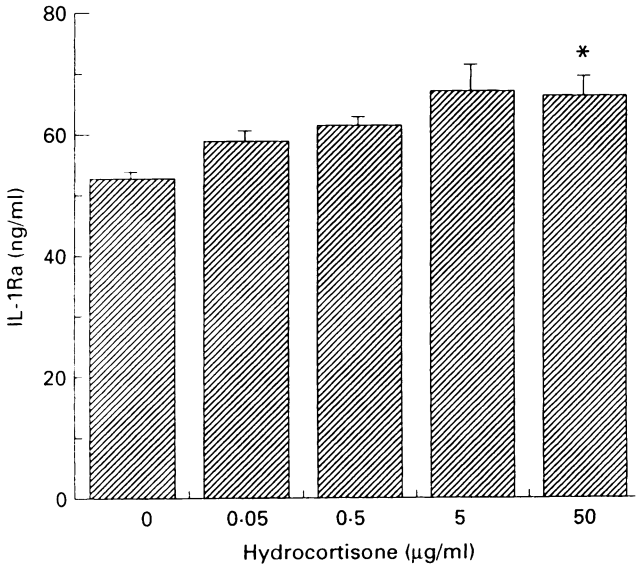

Fig. 6. Hydrocortisone increases icIL-1Ra levels. Confluent HaCAT cells were grown in the presence of increasing amounts of hydrocortisone (0–50 μg/ml). At 50 μg/ml a significant increase ($P < 0{\cdot}05$) in IL-1Ra levels was observed compared with control.

($P < 0{\cdot}02$) effect on the icIL-1Ra : IL-1α ratio, since this ratio was 1·9 ($\pm$ 0·22) : 1 when the cells were grown in 0·42 mM Ca^{2+} in RPMI FCS, and increased to 16 $\pm$ 1·95 when cells were grown in 1·6 mM Ca^{2+} in DMEM–FCS. Confluence increased the icIL-1Ra:IL-1α ratio to 248 $\pm$ 31 in 0·42 mM Ca^{2+} RPMI–FCS ($P < 0{\cdot}002$), but was not increased further (221 $\pm$ 28) in these confluent cultures in the presence of 1·6 mM Ca^{2+} DMEM–FCS. No effect of Ca^{2+} concentration on cell number was observed in either confluent or non-confluent cultures. Further experiments were therefore performed using confluent HaCAT cells grown in 0·42 mM Ca^{2+} RPMI–FCS.

Detection of functional activity of IL-1Ra

The functional activity of the IL-1Ra detected by ELISA in lysates of confluent HaCAT cells was demonstrated in the murine thymocyte co-proliferation assay, since the lysates blocked IL-1α (20 U/ml) bioactivity in a dose-dependent manner (Fig. 2a). Preincubation of confluent HaCAT cell lysates with increasing concentrations of rabbit anti-IL-1Ra blocked the inhibition by HaCAT cell lysates (1 : 4 dilution) of IL-1α (20 U/ml) bioactivity in the thymocyte co-proliferation assay (Fig. 2b), confirming that the inhibitory effect of lysates from HaCAT cells is the result of their IL-1Ra content, and not due to the presence of other potential inhibitors of thymocyte proliferation, such as TGF-β or soluble IL-1 receptors.

Effects of immunomodulatory cytokines on HaCAT cell icIL-1Ra : IL-1α ratio

TGF-β1 stimulated a dose-dependent increase in HaCAT cell IL-1α from the baseline control value of 160 $\pm$ 20 pg/ml up to 366 $\pm$ 34 pg/ml ($P < 0{\cdot}02$; Fig. 3a), without causing a significant change in IL-1Ra levels (Fig. 3b). There was a resultant dose-dependent reduction in the icIL-1Ra : IL-1α ratio in HaCAT cells from 320 : 1 in controls to 100:1 in cultures to which TGF-β1 had been added ($P < 0{\cdot}001$; Fig. 3c). Similarly, TGF-α, IFN-γ, IL-6 and TNF-α substantially increased HaCAT cell IL-1α, had no effect on the IL-1Ra, with a resultant reduction in the icIL-1Ra : IL-1α ratio (Fig. 4). In contrast to their effects on monocytes, IL-4 and IL-10 at 10 ng/ml (optimal stimulation doses in other systems) had no effect on IL-1α, IL-1Ra or the icIL-Ra : IL-1α ratio in confluent HaCAT cells (Fig. 5).

Effect of hydrocortisone on HaCAT cell icIL-1Ra : IL-1α ratio

Hydrocortisone induced a dose-related increase in icIL-1Ra in confluent HaCAT cells (Fig. 6). Although this was seen in several experiments, and reached statistical significance ($P < 0{\cdot}05$), the effect was small and the biological relevance of this phenomenon is uncertain. The increase in IL-1Ra was associated with an increase in the IL-1Ra : IL-1α ratio. This is not illustrated, because the IL-1α concentration fell below the limit of sensitivity of the IL-1α ELISA.

DISCUSSION

We have studied the regulation of keratinocyte synthesis of IL-1α and its specific antagonist icIL-1Ra using an *in vitro* model which reproduces the icIL-1Ra : IL-1α ratio seen in normal skin. We have shown that, despite optimal calcium concentration in the medium, in subconfluent cultures of the HaCAT spontaneously transformed keratinocyte cell line, the icIL-1Ra : IL-1α ratio does not rise above 16. By contrast, confluent cultures of HaCAT cells produce less IL-1α and have a higher icIL-1Ra : IL-1α ratio (248 : 1), which is of a similar order of magnitude to that reported for normal skin *in vivo* [9]. Differentiating keratinocytes, as found in confluent cultures, have previously been reported to contain higher IL-1Ra levels, and non-differentiated keratinocytes higher IL-1α levels [6]. Using confluent cultures of HaCAT cells, we found that TGF-α, IFN-γ, IL-6 and TNF-α markedly reduced the icIL-1Ra:IL-1α ratio

by increasing IL-1α, without affecting icIL-1Ra. This contrasts with a recent report that IL-6 and IFN-γ have no significant effect on IL-1α, and that TNF-α induces keratinocyte icIL-1Ra [11], but is consistent with a report that TGF-α increases keratinocyte IL-1α mRNA [16]. The reason for the discrepancy between our findings and those of Kutsch *et al.* [11] is not clear, but may be explicable on the basis of technical factors. Neonatal foreskin keratinocytes, rather than HaCAT cells, were used in the latter study, and it is not clear whether or not their cultures were confluent.

TGF-β1 is a pleitropic cytokine constitutively synthesized by keratinocytes, which is present in the intercellular space of lesional psoriatic epidermis [17], in epidermis overlying basal cell carcinomas [18] and in retinoid treated epidermis [19]. TGF-β inhibits keratinocyte proliferation [20], and has a range of immunosuppressive actions, including: inhibition of T cell, B cell and monocyte proliferation and effector function [21,22]; inhibition of antigen presentation by cultured but not fresh Langerhans cells [23]; and reduction in dermal microvascular endothelial cell adhesiveness for peripheral blood mononuclear cells (PBMC) [24]. However, TGF-β also has proinflammatory properties, in that it is chemotactic for monocytes, neutrophils and T cells both *in vitro* and *in vivo*, and may play a role in cell recruitment at the onset of inflammation [25–30]. In addition, TGF-β is a critical inflammatory mediator in streptococcal cell wall-induced arthritis in rats [25]. Treatment of confluent HaCAT cells with TGF-β1 caused an increase in IL-1α and a reduction in icIL-1Ra:IL-1α ratio similar to that produced by the proinflammatory cytokines TNF-α and IFN-γ and greater than that due to IL-6 and TGF-α. This was unexpected, because TGF-β inhibits IL-1 production by PBMC in response to lipopolysaccharide, and induces monocyte production of sIL-1Ra [12]. Our finding highlights the potential of TGF-β to play a proinflammatory role in human epidermis.

IL-10 is a recently described cytokine which inhibits the effector functions of T cells, natural killer cells, monocytes and macrophages. Murine keratinocytes make IL-10 mRNA in response to application of hapten to skin [31] and to ultraviolet light irradiation [32]. Thus IL-10 may be an important immunomodulatory cytokine in mouse epidermis. At the time of writing, IL-10 production by human monocytes and T cells is well documented, but synthesis by human keratinocytes has not been reported. IL-4 is produced by Th2 and Th0 T cell subsets and mast cells on activation [33]; it inhibits Th1 and stimulates Th2 responses [34], and may play an important role in the regulation of T cell-mediated inflammatory skin disease. As IL-10 and IL-4 are known to stimulate sIL-1Ra synthesis by monocytes [13,35], the finding that these cytokines did not induce icIL-1Ra over a wide dose range in confluent HaCAT cells is further evidence that keratinocytes and monocytes use different promoter sequences to control IL-1Ra synthesis (A. Leonard, personal communication).

Hydrocortisone produced a small increase in icIL-1Ra and a fall in icIL-1α levels in HaCAT cells. This finding is consistent with reports that glucocorticosteroids inhibit keratinocyte IL-1 protein and mRNA production [16,36], and result in production of an antagonist of IL-1 [36].

We have shown [35] that lysates of confluent HaCAT cells contain a substantial excess of icIL-1Ra over icIL-1α, and that this icIL-1Ra inhibited IL-1α bioactivity. Levels of icIL-1Ra were not altered by any of the cytokines investigated. By contrast, TGF-α, IFN-γ, IL-6, TNF-α and TGF-β1 all increased the icIL-1α. It seems that regulation of overall activity of the IL-1 cytokine network in confluent keratinocytes depends on modulation of IL-1α rather than of IL-1Ra levels. Cell-associated membrane-bound IL-1α may have functional activity which escapes inhibition by icIL-1Ra [37–39] or it may be secreted at a low level. It is likely that keratinocytes synthesize and release increased quantities of IL-1α during epidermal inflammation, in part in response to the above cytokines. Despite the fact that it is present in large excess intracellularly, IL-1Ra is not secreted, and therefore has no extracellular activity in normal skin. The physiological role of icIL-1Ra in keratinocytes is yet to be fully established. Under normal circumstances it may function purely to inhibit intracellular effects of IL-1. However, should keratinocyte necrosis occur, large quantities of IL-1Ra will be released. This will block IL-1 receptors on keratinocytes, Langerhans cells and infiltrating cells, and may thus serve to limit damage mediated by the effector arm of the immune system.

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