Interleukin-1 Receptor Antagonist Production by Human Keratinocytes

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Human keratinocytes produce biologically active pro-IL-1α and inactive pro-IL-1β with most protein remaining intracellular. IL-1 receptor antagonist (IL-1ra) is a newly described member of the IL-1 family that is secreted by stimulated monocytes and binds competitively to IL-1 receptors without stimulating target cells. We examined the characteristics of IL-1ra production by cultured human keratinocytes. By ELISA, keratinocyte lysates contained 390 ng IL-1ra/mg total protein with little IL-1ra detected in supernatants. In contrast, monocytes produced 297 ng IL-1ra/mg total protein during 24 h of culture on adherent IgG with about half of the IL-1ra detected in supernatants. By Western blot analysis, keratinocyte IL-1ra was $\approx$20 kD in size and was slightly larger than recombinant monocyte IL-1ra. In contrast to monocytes, human keratinocyte IL-1ra was not secreted in 22-25-kD molecular weight glycosylated forms. Affinity-purified keratinocyte IL-1ra exhibited identical biologic activity to recombinant monocyte IL-1ra, each inhibiting IL-1-dependent augmentation of murine thymocyte proliferation to the same degree per amount of protein. An IL-1ra mRNA of 1.8 kb was detected by Northern blot analysis in RNA extracted from keratinocytes. In order to determine the effect of differentiation on IL-1 and IL-1ra production, human keratinocytes were cultured for 72 h in low (0.03 mM), medium (0.15 mM), or high (1.0 mM) -calcium concentrations. The absolute amounts of IL-1ra increased twofold and the ratio of IL-1ra to IL-1α in keratinocyte lysates increased from $\approx$12:1 to 25:1 during differentiation. These results indicate that keratinocytes constitutively produce large amounts of a biologically active intracellular variant of IL-1ra that increase with differentiation. IL-1ra released during keratinocyte damage may be important in modifying the inflammatory effects of IL-1α in human skin. *J Invest Dermatol 98:38-44, 1992*

Interleukin-1 receptor antagonist (IL-1ra) is a recently characterized member of the IL-1 family that is secreted by stimulated monocytes [1]. IL-1ra, IL-1α, and IL-1β bind to the IL-1 receptor with similar affinities. Because binding of IL-1ra to the IL-1 receptor does not produce detectable cell activation, IL-1ra functions as a competitive inhibitor of IL-1 [2]. IL-1ra is a 17.1-kD protein that has 26% amino acid sequence homology to IL-1β and 19% homology to IL-1α [3]. An epithelial form of IL-1ra has recently been described and differs from the mature form of monocyte IL-1ra in possessing seven more NH_2-terminal amino acids [4]. The absence of a signal peptide in synthesized epithelial IL-1ra would predict that this structural variant would remain intracellular (icIL-1ra), in contrast to secreted monocyte IL-1ra (sIL-1ra).

IL-1 has potent biologic effects within the cutaneous micro-environment. IL-1 stimulates fibroblasts to synthesize and release collagenase and prostaglandins, endothelial cells to up-regulate adhesion molecules, and keratinocytes to release a variety of cytokine mediators of inflammation [5-9]. Human skin and cultured keratinocytes produce both IL-1α and IL-1β, although epidermal IL-1 activity is completely blocked by antibodies to IL-1α alone [10,11]. The ratio of IL-1ra to IL-1α produced by keratinocytes may influence the relative inflammatory potential of IL-1 in intact skin.

In this study, we characterized the production of IL-1ra by human keratinocytes and examined the effects of differentiation on the relative production of IL-1α, IL-1β, and IL-1ra by keratinocytes.

MATERIALS AND METHODS

Cell Culture Neonatal foreskin keratinocytes and melanocytes were grown as previously described [12,13]. The keratinocyte growth medium (KGM, Clonetics, San Diego, CA) contained 0.1 ng/ml recombinant epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.15 mM calcium, 0.2% bovine pituitary extract, gentamicin, and amphotericin B. Fibroblasts were isolated and cultured from foreskin dermis as previously described [14]. All cultures were fed twice weekly and incubated in a humidified environment at 37°C and 5% CO_2. Third-passage keratinocytes, mela-

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Abbreviations:

EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
FCS: fetal calf serum
icIL-1ra: intracellular interleukin-1 receptor antagonist
IL-1: interleukin-1
IL-1α: interleukin-1 alpha
IL-1β: interleukin-1 beta
IL-1ra: interleukin-1 receptor antagonist
KGM: keratinocyte growth medium
NP-40: Nonidet P 40, nonionic detergent
PBS: phosphate-buffered saline
PHA: phytohemagglutinin
SDS: sodium dodecyl sulfate
sIL-1ra: secretory interleukin-1 receptor antagonist

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nocytes, and fibroblasts were used in all experiments. In some experiments, the third-passage human keratinocytes were plated on 6-well culture plates (Falcon, Lincoln Park, NJ) at 500,000 cells/well. After 24 h, the medium was changed to KGM with 0.03 mM, 0.15 mM, or 1.0 mM calcium at 2 ml/well and the cells were cultured for an additional 72 h.

45×10^6 fresh human monocytes were isolated from EDTA-anticoagulated blood of normal donors by centrifugation through lymphoprep gradients and allowed to adhere to 100-mm culture plates (Falcon) coated with IgG (Sigma, St. Louis, MO) to induce IL-1ra expression, as previously described [1,15]. Adherent monocytes were cultured in serum-free RPMI 1640 medium (GIBCO, Grand Island, NY) with 1 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin for 6 h for use on polyacrylamide gels, or in RPMI 1640 medium with 1% FCS (Irvine Scientific, Santa Ana, CA) for 24 h for use in ELISA.

Cell Lysis Supernatants (2 ml/well) were harvested and non-adherent cells were removed by centrifugation at $450 \times g$ for 5 min. Adherent cells were washed with sterile PBS and lysed by freeze-thaw three times in an equivalent volume of KGM (2 ml). Cell membranes were removed from the lysates by centrifuging at maximum speed in an Eppendorf microcentrifuge for 15 min. Supernatants and lysates were stored at −20°C until assayed. For SDS-polyacrylamide gels, cells were lysed in 1% NP-40 buffer (1% NP-40, 15 mM NaCl, 50 mM Tris, pH 8.0) for 30 min on ice.

IL-1ra ELISA This sandwich ELISA is described in detail elsewhere [16]. Briefly, the primary antibody used in this ELISA was polyclonal rabbit antibodies to recombinant IL-1ra obtained from the antiserum by affinity purification on an Affi-gel 15 column (Bio-Rad, Richmond, CA) with coupled recombinant IL-1ra (compliments of Dr. Robert C. Thompson, Synergen, Inc., Boulder, CO). Two hundred nanograms of the primary antibodies in 0.015 M $NaHCO_3$ buffer (pH 9.6) were added to each well of a 96-well ELISA plate (Costar, Van Nuys, CA). Nonspecific binding sites were blocked with bovine gamma globulin (Sigma), 5 mg/ml in PBS. The secondary antibody was the IgG fraction of the rabbit antiserum obtained by Protein A affinity chromatography (Pharmacia LKB Biotechnology, Piscataway, NJ) and biotinylated, utilizing a modification of a technique described by Guesdon et al [17], at a 1:60 dilution in PBS with 1 mg/ml bovine gamma globulin. Streptavidin-peroxidase at a 1:20,000 dilution in PBS/Tween was allowed to bind to the biotinylated secondary antibodies. The enzyme substrate was a solution of 1 mg/ml 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) (Sigma) and 0.005% hydrogen peroxide in McIlvain's buffer (pH 4.6). The colorimetric reaction was measured as the ratio of OD at wavelengths 490 nm/405 nm. The standard curve utilized recombinant IL-1ra. The IL-1ra ELISA was sensitive to 200 pg/ml and failed to detect recombinant IL-1α or IL-1β at 10 ng/ml.

IL-1α ELISA The primary antibody was a monoclonal antibody to IL-1α (compliments of Dr. Ann Berger, Upjohn Co., Kalamazoo, MI) at 200 ng/well. The secondary antibody was the biotinylated IgG fraction of a rabbit polyclonal antiserum to IL-1α at a 1:60 dilution in PBS with 1 mg/ml BSA (Sigma). The standard curve utilized recombinant IL-1α (compliments of Dr. Peter Lomedico, Hoffmann-La Roche, Nutley, NJ). The colorimetric reaction was the same as for the IL-1ra ELISA. The IL-1α ELISA was sensitive to 100 pg/ml.

IL-1β ELISA The primary antibody was a monoclonal antibody to IL-1β (Upjohn) at 200 ng/well. The secondary antibody was the biotinylated IgG fraction of a rabbit polyclonal antiserum to IL-1β at a 1:60 dilution in PBS/Tween with 1 mg/ml BSA. The colorimetric reaction was the same as for the IL-1ra ELISA. The standard curve utilized recombinant IL-1β (Synergen). The IL-1β ELISA was sensitive to 10 pg/ml.

Supernatant and lysate volumes were equal. ELISA results were expressed per ml of supernatant or lysate or per mg lysate total protein, as determined by a standard colorimetric assay (Bio-Rad). ELISA results were also expressed per cell number and per μg DNA. Cell number was determined by counting trypsinized cells in adjacent wells of the 6-well plates by a hemocytometer. Cellular DNA content was determined by a revised Burton DNA assay [18] from 3×10^6 cells plated on parallel T75 culture plates (Corning, Littleton, CO).

Western Blot Analysis of IL-1ra Protein in Lysates and Supernatants of Human Keratinocytes In order to determine the size distribution of keratinocyte supernatant IL-1ra protein, 2 ml of supernatant from 0.03 mM calcium-conditioned medium (≈6 ng IL-1ra) was precipitated in 10% trichloroacetic acid, washed with 100% ethanol, and dried by vacuum centrifugation. 0.5 ml of keratinocyte lysate was precipitated in a similar manner (≈20 ng IL-1ra by ELISA). The proteins were separated on 15% SDS-polyacrylamide gels under reducing conditions, then electrophoretically transferred to nitrocellulose paper. The primary antibody in the Western blot analysis was the affinity-purified rabbit polyclonal antibodies to IL-1ra at a 1:100 dilution in PBS with 1% milk, which were detected by biotinylated goat anti-rabbit secondary antibodies at a 1:2500 dilution in PBS with 1% milk. The reaction product was visualized using streptavidin-alkaline phosphatase conjugate at a 1:5000 dilution in PBS with 1% milk and a NBT (nitroblue tetrazolium) BCIP (5-bromo-4-chloro-3-indoyl phosphate) substrate (Protoblot System, Promega, Madison, WI) in developing buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5). Protein size was estimated by comparison to 17.1-kD recombinant monocyte IL-1ra.

Northern Blot Analysis of IL-1ra mRNA Total cellular RNA was extracted from cells by treatment with guanidine isothiocyanate [19] and cesium chloride gradient centrifugation [20]. The purified RNA was quantified by reading OD at 260 nm and was stored under ETOH at −20°C. Total cellular RNA was electrophoresed under denaturing conditions through a 1% agarose gel with 2% formaldehyde. 18S and 28S ribosomal RNA were determined by UV visualization after ethidium bromide staining and the RNA was transferred to nitrocellulose passively in 10 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate). A full-length cDNA probe for IL-1ra was provided by Dr. Stephen P. Eisenberg of Synergen and labeled by nick translation with alpha ^{32}P-labeled cytosine (Bethesda Research Laboratories, Bethesda, MD). Specific activity was 2.1×10^8 cpm/μg DNA. Blots were baked at 80°C under vacuum for 2 h with prehybridization (42°C), hybridization (42°C), and washing (63°C) performed in a standard manner [21]. Autoradiographs were exposed at −70°C under intensifying screens.

Affinity Purification of IL-1ra from Keratinocyte Lysates Affinity-purified rabbit polyclonal anti-IL-1ra antibodies were bound to Sepharose-protein A beads (Sigma), then covalently linked with dimethyl pimelimidate (Sigma) [22]. IL-1ra from keratinocyte lysates was allowed to bind to the column and then eluted with glycine buffer (pH 2.5) and collected in 1 ml fractions in neutralizing phosphate buffer (pH 8.0). Treatment of recombinant IL-1ra in a similar manner did not affect inhibitory activity in the mouse thymocyte proliferation assay. IL-1ra in each fraction was measured by ELISA and analyzed by Western blot. 1% FCS was added to the fraction with the highest yield of IL-1ra and this fraction was dialyzed against RPMI 1640 (molecular weight cutoff of dialysis tubing was 5000). The final concentration of affinity-purified keratinocyte IL-1ra was measured by ELISA.

Biologic Activity of Affinity-Purified Human Keratinocyte IL-1ra IL-1 biologic activity was determined by augmentation of PHA-induced murine thymocyte proliferation using cells from 5-

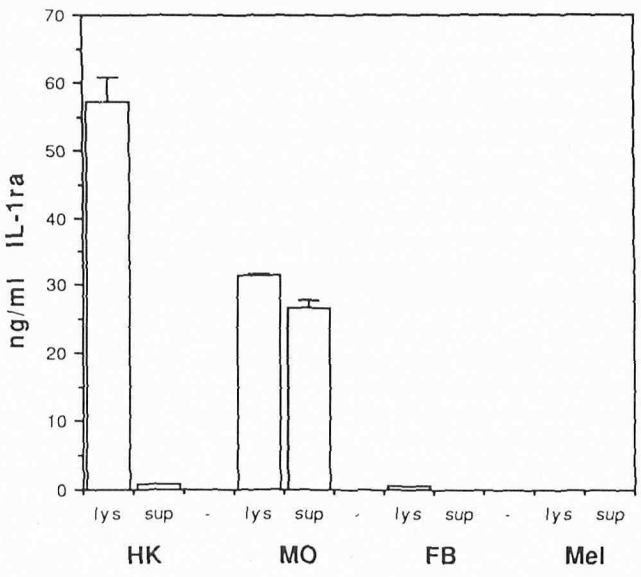

Figure 1. IL-1ra (ng/ml) in lysates (lys) and supernatants (sup) of human keratinocytes (HK), IgG-stimulated monocytes (MO), fibroblasts (FB), and melanocytes (Mel). The keratinocytes were cultured in KGM with 0.15 mM Ca^{++}. The data represent the mean ± SD of triplicate ELISA wells from one representative monocyte experiment and the mean ± SD of three experiments with keratinocyte, fibroblasts, and melanocytes.

to 8-week old C3H/HeJ mice [23,24]. After 72 h of culture at 37°C, 10 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) at 5 mg/ml was added to each well of the 96-well culture plates (Falcon). After 3 h, the crystals were dissolved in acid-alcohol and the OD was read on the ELISA reader at 630 nm [25]. A standard curve was generated for recombinant IL-1β; 100 pg/ml IL-1β was the amount in the middle of the steepest part of the curve. Increasing concentrations of recombinant monocyte IL-1ra or affinity-purified keratinocyte IL-1ra were added to 100 pg/ml IL-1β and tested for relative inhibitory activity in the murine thymocyte assay.

RESULTS

IL-1ra Production by Keratinocytes and Monocytes Cultured human keratinocytes constitutively produced large quantities of IL-1ra, which were detected primarily in cell lysates by a specific ELISA (Fig 1). In contrast, monocytes stimulated by IgG for 24 h produced approximately equal amounts of IL-1ra in lysates and supernatants. Fibroblasts and melanocytes produced negligible amounts of IL-1ra. When expressed per mg protein, keratinocytes produced a total (supernatants and lysates) of 320 ng IL-1ra/mg protein compared to 290 ng IL-1ra/mg protein by stimulated monocytes in 24 h. There was no difference in the amounts of IL-1ra produced by keratinocytes cultured for 72 h in KGM with and without hydrocortisone or with and without bovine pituitary extract (data not shown).

Northern Blot Analysis of Human Keratinocyte mRNA IL-1ra mRNA was identified by Northern blot analysis of total keratinocyte RNA (Fig 2). A radiolabeled full-length cDNA probe detected IL-1ra mRNA at 1.8 kb in human keratinocytes, the same size as monocyte IL-1ra mRNA. No IL-1ra mRNA was detected in fibroblast RNA.

Western Blot Analysis of IL-1ra Protein The predominant size forms of IL-1ra in monocyte supernatants are higher molecular weight (22–25-kD) forms, as determined by Western blot analysis [2,4]. Digestion with N-glycanase decreases the size of monocyte supernatant IL-1ra to 17 kD, identical to non-glycosylated recombinant monocyte IL-1ra [2]. Thus, monocyte IL-1ra is glycosylated during the process of secretion. To determine if keratinocyte IL-1ra is glycosylated or proteolytically processed during or after release into the conditioned medium, keratinocytes and lysates were analyzed by Western blot analysis. Two size forms of IL-1ra in keratinocyte lysates were identified; the predominant form was slightly larger than recombinant monocyte 17.1-kD IL-1ra (Fig 3). This observation is consistent with the presence of seven extra amino acids in keratinocyte IL-1ra. The other form of keratinocyte IL-1ra was slightly smaller. IL-1ra in keratinocyte supernatants had the same size distribution as in the lysates. Specifically, no larger (22–25-kD) glycosylated forms of IL-1ra were present in keratinocyte supernatants. Adding the proteinase inhibitors PMSF (phenylmethyl, 1-sulfonyl fluoride) (Sigma) and NEM (N-ethylmaleimide) (Sigma) at 1 mM to cultured cells prior to lysis did not change the distribution of keratinocyte IL-1ra size forms (data not shown).

IL-1ra Production and Keratinocyte Differentiation The effect of cell differentiation on IL-1ra production by keratinocytes was next examined. Keratinocytes were cultured for 72 h in KGM with 0.03 mM, 0.15 mM, and 1.0 mM calcium concentrations; human keratinocyte differentiation increases with increasing extracellular calcium concentration [26]. IL-1ra in keratinocyte lysates increased from 22.5 ± 3.0 to 57.3 ± 4.2 to 66.2 ± 3.2 ng/ml with increasing calcium concentrations (Fig 4). IL-1ra was detected in much smaller quantities in keratinocyte supernatants than in lysates (also see Fig 1) and was greatest at low calcium concentrations. When expressed per amount of total cellular protein (Table I), cell number, or DNA content (Table II), IL-1ra was still increased in lysates of differentiated cells. When cells were lysed in NP-40 buffer instead of by freeze-thaw, these differences persisted (data not shown).

IL-1α and IL-1β Production and Keratinocyte Differentiation IL-1α in cell lysates increased from 1.83 ± 0.16 to 2.31 ± 0.17 to 2.57 ± 0.19 ng/ml with increasing extracellular calcium

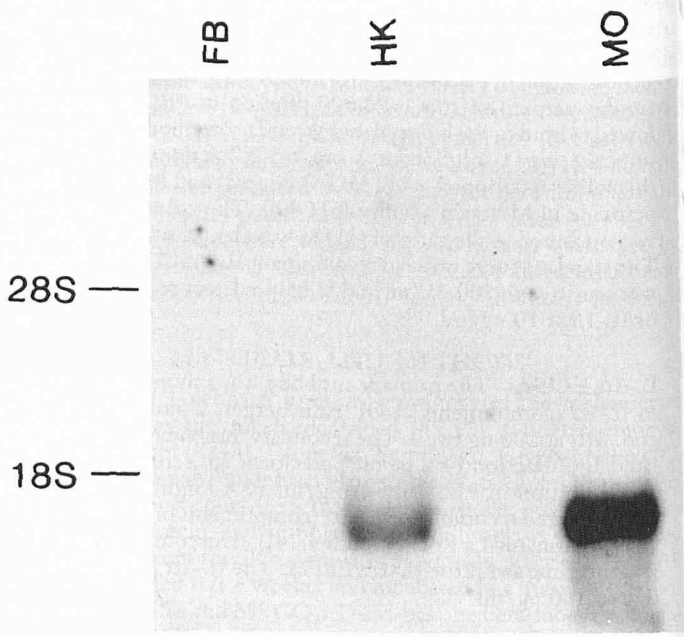

Figure 2. Northern blot analysis of 15 μg human fibroblast (FB) RNA, 10 μg human keratinocyte (HK) RNA, and 500 ng IgG-stimulated human monocyte (MO) RNA. A full-length cDNA probe for IL-1ra binds specifically at approximately 1.8 kb to RNA from keratinocytes and stimulated monocytes.

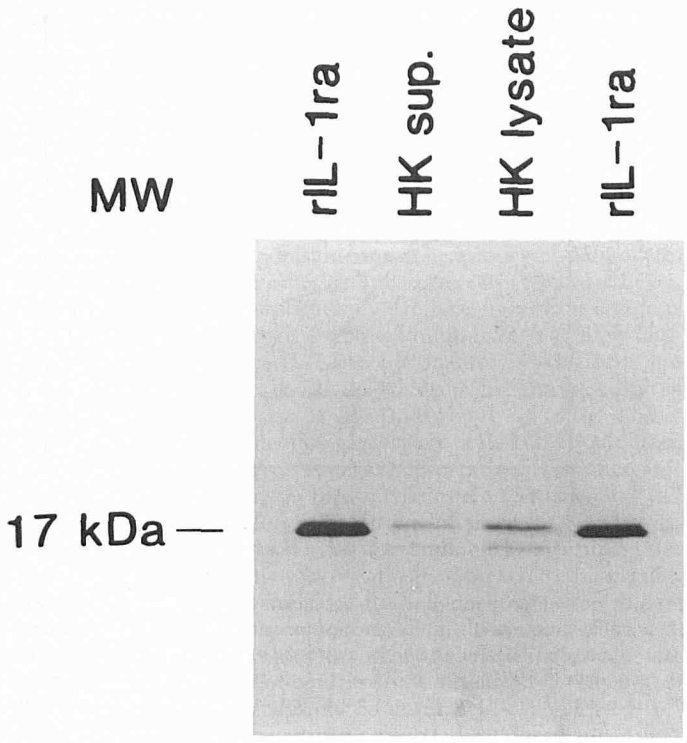

Figure 3. Western blot analysis of IL-1ra protein in lysates and supernatants of keratinocytes. Human keratinocyte supernatant (HK sup) protein was precipitated from 2 ml of 0.03 mM calcium-conditioned medium (IL-1ra 6 ng by ELISA). Keratinocyte lysate (HK lysate) protein from cells cultured in the same medium was precipitated from 0.5 ml lysate (20 ng IL-1ra). For comparison, 17.1-kD recombinant monocyte IL-1ra (30 ng) is shown in *lanes 1* and *4*.

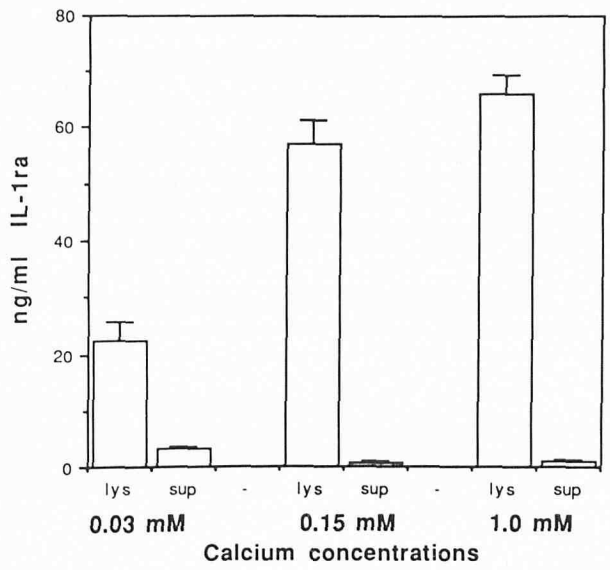

Figure 4. Human keratinocyte (HK) IL-1ra production increases with increasing extracellular calcium concentrations. Human keratinocytes were cultured for 72 h in KGM with 0.03 mM, 0.15 mM, or 1.0 mM calcium. The data represent the mean ± SD of four experiments. Statistical comparison using t test of lysates is as follows: $p < 0.001$ for 0.03–0.15 mM; $p < 0.001$ for 0.03–0.1 mM; $p = 0.02$ for 0.15–1.0 mM.

concentrations. However, when corrected for total cellular protein this increase was no longer seen (Table I). In supernatants, IL-1α was only detectable in small quantities (0.08–0.12 ng/ml). The ratio of IL-1ra to IL-1α in keratinocyte lysates increased from ≈12:1 to 25:1 with increasing extracellular calcium concentrations. We have verified by quantitating cellular levels of the keratin k5 and involucrin that keratinocytes grown in those calcium concentrations show progressively greater differentiation as extracellular calcium increases.*

IL-1β also was present in small quantities in keratinocyte lysates and decreased during keratinocyte differentiation from 0.141 ± 0.015 to 0.106 ± 0.008 to 0.097 ± 0.006 ng/ml with increasing extracellular calcium concentrations. Supernatant IL-1β was near or below the ELISA detection limit of 10 pg/ml. When expressed per mg total protein, IL-1β in keratinocyte lysates decreased by over 50% during cell differentiation (Table I).

Biologic Activity of Keratinocyte IL-1ra Keratinocyte and monocyte IL-1ra were compared for inhibitory activity towards IL-1 augmentation of PHA-induced murine thymocyte proliferation. In order to determine relative biologic activities, human keratinocyte IL-1ra was affinity purified to separate IL-1ra from other keratinocyte cytokines including IL-1. Increasing concentrations of affinity-purified keratinocyte IL-1ra or recombinant monocyte IL-1ra were added to 100 pg/ml IL-1β in the murine thymocyte assay and biologic activity was expressed as percent inhibition of control (Fig 5). The inhibition curves were similar with 50% inhibition of IL-1β effects occurring at a ratio of IL-1ra to IL-1β of 25 to 1 for either keratinocyte or monocyte IL-1ra. Adding later elution fractions from the affinity column as a control had no effect on IL-1β stimulation in the murine thymocyte assay (data not shown).

DISCUSSION

A specific receptor antagonist of IL-1 (IL-1ra) is synthesized and secreted by human monocytes after stimulation with adherent IgG [1]. A cDNA has recently been cloned for a variant form of IL-1ra that is found in the lysates of keratinocytes and other epithelial cells [4]. The predicted amino acid sequence of epithelial IL-1ra, termed icIL-1ra (intracellular), is identical to that of the mature extracellular form of monocyte IL-1ra, termed sIL-1ra (secretory), except for the presence of an additional seven amino acids on the NH_2-terminus of the icIL-1ra molecule [4]. This structural variation is probably due to the presence of a different first exon for keratinocyte icIL-1ra and alternative DNA splicing. Synthesized monocyte sIL-1ra additionally possesses a 25-residue leader sequence; keratinocyte icIL-1ra lacks such a signal peptide and, thus, is predicted to remain inside the cell. Initial attempts to sequence the purified keratinocyte IL-1ra in our studies were unsuccessful due to the presence of a blocked NH_2-terminus.

Our studies have extended the initial observation on the presence of a variant form of IL-1ra in keratinocytes [4] by quantifying its production, examining the effects of cell differentiation, and comparing the relative production of IL-1ra, IL-1α, and IL-1β. The results establish that cultured human keratinocytes constitutively produce icIL-1ra in an amount per mg of total cell protein that is equivalent to the amount of sIL-1ra produced by stimulated monocytes over 24 h. The major portion of extracellular monocyte sIL-1ra is glycosylated to varying degrees and is 22–25 kD in size, whereas very little sIL-1ra inside monocytes is in the larger glycosylated form [4]. In contrast, icIL-1ra is found predominantly in keratinocyte lysates; both inside and outside the cell icIL-1ra is almost

* Kashihara-Sawami M, Norris DA: The level of differentiation of cultured keratinocytes determines the level of intracellular adhesion molecule 1 (ICAM-1) induction by γ-interferon and can be modulated by retinoids and steroids (submitted).

Table I. Effect of Differentiation on the Production of IL-1α, IL-1β, or IL-1ra by Human Keratinocytes[a]

	ng Cytokine in Lysates per mg Total Cellular Protein[b]		
Cytokine	0.03 mM Ca^{++}	0.15 mM Ca^{++}	1.0 mM Ca^{++}
IL-1α	14.9 ± 0.5	13.5 ± 1.0	13.9 ± 1.0
IL-1β	1.19 ± 0.04	0.58 ± 0.04	0.52 ± 0.02
IL-1ra	186 ± 29	320 ± 40	359 ± 19

[a] Human keratinocytes were cultured for 72 h in growth medium containing 0.03, 0.15, or 1.0 mM calcium (Ca^{++}). The cells were lysed by serial freeze-thaw in fresh growth medium.

[b] The concentrations of IL-1α, IL-1β, or IL-1ra in cell lysates were determined using specific ELISA (mean of three wells) and the total protein was determined by a standard colorimetric assay. The data were expressed as ng cytokine per mg total protein ± SD, based upon three lysates. For IL-1ra, $p = 0.002$ for 0.03 mM – 0.15 mM; $p < 0.001$ for 0.03 mM – 1.0 mM as determined by t test.

entirely in the 17-kD nonglycosylated form. Thus, unlike monocytes, keratinocytes appear not to secrete IL-1ra and do not glycosylate the basic polypeptide. However, both forms of IL-1ra possess identical specific biologic activities when assayed against IL-1 in the murine thymocyte assay. Thus, the extra seven NH_2-terminal amino acids in icIL-1ra appear not to affect the receptor-binding properties of this molecule. Lastly, an IL-1ra mRNA of ≈ 1.8 kb was detected by Northern blot analysis in both keratinocyte and monocyte extracts.

We observed that icIL-1ra production is enhanced when keratinocytes are differentiated by culture in medium containing a high concentration of calcium. Human keratinocytes also constitutively produce both IL-1α and IL-1β; most IL-1 remains inside the cell and IL-1α is the predominant form. During keratinocyte differentiation, IL-1α production was unchanged and IL-1β decreased; the ratio of IL-1ra to IL-1α increased from ≈ 12 : 1 to over 25 : 1. Recent studies have established that IL-1ra must be present in 10 – 100-times excess over IL-1 in order to block cell stimulation [27], although both IL-1ra and IL-1 bind to the same receptors with an identical affinity [28,29]. Target cells possess 200 – 5000 or more IL-1 receptors per cell but exhibit a full biologic response when only 1 – 2% of these receptors are occupied by IL-1. Thus, because of this exquisite sensitivity to IL-1, excess IL-1ra must be present to block IL-1 – induced biologic responses. Human keratinocytes synthesize sufficient IL-1ra so that inhibition of IL-1 effects may result when this molecule is released along with IL-1 from dying keratinocytes in the stratum corneum of the skin.

It cannot be assumed that the results of our studies with cultured keratinocytes will be paralleled by studies on intact epithelium. Keratinocyte icIL-1ra could be induced by the growth medium and not represent a constitutive product of the normal epidermis. KGM medium does not contain added serum, but the bovine pituitary extract or hydrocortisone present could stimulate keratinocytes.

Table II. Effect of Differentiation on IL-1ra Production by Human Keratinocytes[a]

	ng IL-1ra in Cell Lysates[b]	
Culture Conditions	Per μg DNA	Per Million Cells
0.03 mM Ca^{++}	14.1	108
0.15 mM Ca^{++}	45.8	276
1.0 mM Ca^{++}	45.7	273

[a] Human keratinocytes were cultured for 72 h in growth medium containing 0.03, 0.15, or 1.0 mM calcium (Ca^{++}). The cells were lysed by serial freeze-thaw in fresh medium.

[b] The IL-1ra concentrations in cell lysates were determined by specific ELISA (performed in triplicate for each sample) and the data represent the mean of four lysates. Cell number and DNA quantification were determined in two parallel cultures.

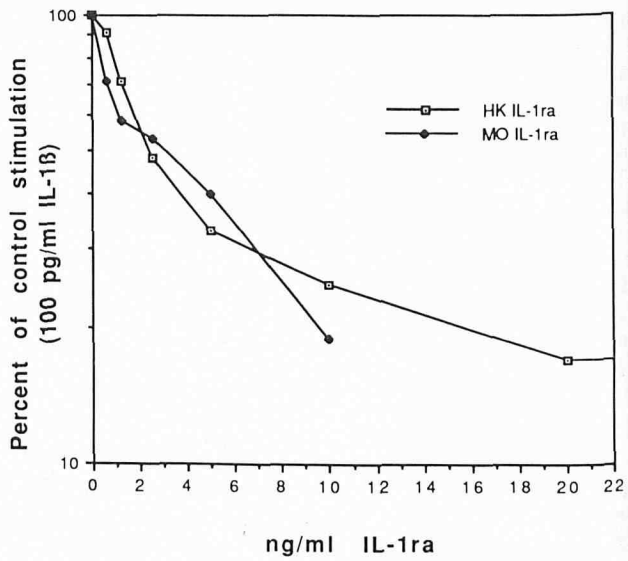

Figure 5. Biologic activities of keratinocyte (HK) and monocyte (MO) IL-1ra. The amounts of affinity-purified keratinocyte IL-1ra and recombinant monocyte IL-1ra in ng/ml are expressed on the ordinate. Activity was assayed against 100 pg/ml IL-1β in the murine thymocyte assay (mean of three wells) with the abscissa expressed as percent of control stimulation.

However, keratinocytes cultured over 72 h produced identical amounts of IL-1ra in the absence or presence of these substances. Furthermore, preliminary results of immunofluorescence studies indicate that IL-1ra is present in the normal epidermis in a band immediately beneath the stratum corneum (Bigler CF, Arend WP, unpublished observations).

It is possible that an apparent increase in IL-1ra production by differentiated cultured keratinocytes could be secondary to enhanced cell viability or to a nonspecific increase in total protein production. However, these possibilities are rendered unlikely by the observation that IL-1ra production was equally increased when expressed per cell number, per μg DNA, per total protein, or per ng IL-1α protein. Alternatively, lysis by freeze-thaw could have preferentially lysed more differentiated cells. However, identical results were obtained when keratinocytes were lysed by the detergent NP-40. Enhanced production of IL-1ra by more differentiated keratinocytes is paralleled in human monocytes where in vitro maturation into macrophages leads to a large increase in constitutive IL-1ra production [30].

IL-1 production by cultured keratinocytes is usually measured as activity in a bioassay. IL-1 activity in human keratinocytes is predominantly cell associated and increases directly with the calcium concentration of the culture medium [31]. IL-1α and IL-1β proteins have been measured by ELISA in epidermal keratomes of skin [11] and recently in cultured keratinocyte lysates [32]. A decrease or absence in expected IL-1 activity in keratinocyte lysates could be secondary to the concomitant presence of IL-1ra. An IL-1 inhibitor of 40 kD was partially purified by HPLC from UVB-stimulated epidermis of mice [33,34] and may represent a different molecule than the 18-kD keratinocyte IL-1ra that is described in this paper.

We suggest that both IL-1ra and IL-1α may serve primarily intracellular roles. IL-1α has been hypothesized to contribute to cell senescence; transfection of an antisense deoxynucleotide to IL-1α into human diploid endothelial cells led to prolonged in vitro survival of these cells [35]. IL-1ra could antagonize a possible intracellular agonist effect of IL-1α on limiting cell growth. Our studies have not established whether icIL-1ra in keratinocytes is primarily membrane bound or free in the cytoplasm. An additional possible function for intracellular IL-1ra could be in regulation of expression

of cell-surface receptors for IL-1 by binding these receptors inside the cell. Whereas virtually all of the keratinocyte IL-1ra remains cell bound, up to half of the IL-1ra in fresh monocytes and 50–60% of the IL-1ra in alveolar macrophages is found in cell lysates.† Thus, in both keratinocytes and monocytes the major physiological function for IL-1ra could be served inside the cells.

There are significant differences in the forms and function of IL-1 and IL-1ra in monocytes and keratinocytes. IL-1β is the predominant IL-1 receptor agonist in human monocytes; IL-1α is the predominant IL-1 receptor agonist in human keratinocytes. Monocytes require stimulation to synthesize and secrete IL-1β and sIL-1ra, whereas keratinocytes constitutively produce significant amounts of IL-1α and icIL-1ra, which is secreted minimally, if at all. Monocytes process 31-kD pro-IL-1β to the mature 17-kD form and glycosylate 17-kD sIL-1ra to 22–25-kd forms. In contrast, cultured keratinocytes do not process 31-kD IL-1α or IL-1β to the 17-kD mature forms [32] and do not glycosylate icIL-1ra. In addition, keratinocytes and monocytes function differently within their microenvironments. Monocytes must be recruited to tissue sites and present antigen in addition to secreting inflammatory mediators. The epidermis in its function as an immunologic barrier must react quickly and nonspecifically to any damage or breach to this barrier. Preformed IL-1α could be released from damaged or dying epidermal cells, initiating and augmenting an inflammatory response. Keratinocyte icIL-1ra may play an important role in suppressing the pro-inflammatory effects of constitutively produced IL-1α in normal, non-inflamed epidermis. In addition, icIL-1ra released following epidermal damage may modify IL-1–induced inflammation and healing. Differential expression of IL-1α and icIL-1ra by keratinocytes may be important in inflammatory skin diseases, wound healing, or immune suppression following exposure to UVB light.

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