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IL1/ETAF ACTIVITY AND UNDETECTABLE IL1 β mRNA AND MINIMAL IL1 α mRNA LEVELS IN NORMAL ADULT HEAT-SEPARATED EPIDERMIS

Stephen Demczuk

Division of Immunology and Allergy, Department of Medicine, University of Geneva, Hospital Cantonal, CH-1211 Geneva Switzerland

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SUMMARY: Interleukin 1 (IL 1), a cytokine expressed from a number of cell types, plays a vital part in immune and inflammatory processes. Epidermal IL1-like activity, also known as epidermal cell thymocyte-activating factor (ETAF), is associated with whole skin, epidermis, Langerhans cells and cultured keratinocytes and keratinocyte cell lines. We show that preparations of heat-separated epidermis possess abundant IL1/ETAF activity as indicated by PGE/collagenase induction in cultured synovial cells and proliferation of mouse thymocytes. However, using cDNA probes to interleukin-1 α and β we show that freshly isolated heat-separated epidermis from healthy adult donors contains only a slight detectable level of IL1 α mRNA by dot blot analysis and no IL1 β and IL1 α mRNA by Northern blot analysis. The implications of these observations will be discussed.

In addition to lymphoid tissues and resident lymphoid cells, immune systems consist also of cells and tissues of nonlymphoid origin. Only recently, has it become evident that the epidermis plays a vital role in many complex immune reactions. The predominant cells of the epidermis include the keratinocyte, melanocyte and Langerhans cells. Ontogenetically and functionally, bone-marrow-derived Langerhans cells appear as descendants from the monocyte-macrophage-histiocyte linage (1,2). The keratinocyte, which comprises the vast majority of the epidermal cell population, has recently received considerable attention as a participant in the immune response (1,3,4).

IL1 is an important regulator hormone of immune and inflammatory responses (5,6). The predominant source of IL1 is the peripheral monocyte-macrophage. Several names have been given to IL1 or IL1-like activity that reflect the cell-type from which it originates or the physiological response attributed to it. The sources of IL1-like/ETAF activity in the epidermis have been linked to the keratinocyte and the Langerhans cells (7,8,9,10). Although these studies focused on IL1/ETAF release from the epidermis, enriched populations of cultured keratinocytes and Langerhans cells, and keratinocyte cell lines under various culture conditions and stimuli, no study has addressed whether IL1/ETAF is expressed from freshly isolated heat-separated adult epidermis.

Abbreviations: poly(A)$^+$ RNA, polyadenylated RNA; cDNA, complementary DNA; IL1, interleukin-1; ETAF, epidermal thymocyte activating factor; PMA, phorbol myristic acetate; MCF, mononuclear cell factor; LAF, lymphocyte-activating factor; DMEM, Dulbecco's modified Eagle's medium; PHA, phytohemagglutinin; IFN, interferon.

Current Address: Department of Microbiology and Immunology, Bowman Gray School of Medicine, Winston-Salem, NC 27103.

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In this report, we demonstrate that even though abundant IL1/ETAF activity was present in freshly isolated heat-separated epidermis, only a low level of IL1 α mRNA and no IL1 β mRNA were detected by hybridization analysis of total or poly(A)$^+$ epidermal RNA.

MATERIALS AND METHODS

Human Peripheral Mononuclear Cells (PBMC): Human donor blood (Blood Transfusion Unit, University Hospital, Geneva) was prepared from buffy coats, centrifuged through Ficoll-Hypaque gradients and stimulated or not for 12 to 16 h with 3 ug/ml phytohemagglutinin (PHA) and 12 ug/ml concanavalin A (ConA) as previously described (11). Blood mononuclear cells were also obtained from a female acute monocytic leukemia patient (M5 of the French, American, British classification). Following centrifugation through Ficoll-Hypaque gradients patient mononuclear cells were stimulated for 16 h with 50 ng/ml phorbol myristic acetate (PMA).

Human Monocytic Lymphoma Cell Line U937: U937 cells were first differentiated for 3 days in RPMI-1640, 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin and 250 U/ml human recombinant γ-interferon (rγ-IFN) (Biogen S.A., Geneva). The cells were then washed 2 times in sterile PBS, resuspended in media containing 0.1 ug/ml Con A and 0.05 ug/ml PMA at a cell density of 2 x 10^6 cells/ml and cultured for an additional 24 h. These cells were later determined to be contaminated with mycoplasmas (see ethidium bromide stained gel (Fig 1d), 16S and 23S bands and reference 10).

Heat-Separated Epidermis: Normal epidermis from the upper arms and back of patients with plastic surgery was isolated as described (12). Briefly, skin was received on ice minutes after surgery, placed in 0.9% NaCl (containing 2 mM ZnCl in two cases) at 60°C for 60 sec. The epidermal layer was scraped off using a scalpel and quenched in liquid N_2 for RNA extraction and intracellular IL1 activity.

Human Squamous Carcinoma Cell Line A431: Squamous epidermal carcinoma A431 cells were cultured in Dulbecco's modified Eagles medium (DMEM; Gibco), 10% FCS, 2 mM glutamine and penicillin/streptomycin. Cells were washed two times in cold phosphate buffered saline and quenched in liquid N_2 for RNA extraction (10).

IL1/ETAF Bioassays: Freshly isolated synovia from rheumatoid arthritis patients were the source of target cells in the IL1/ETAF-MCF (PGE_2 and collagenase induction) bioassay (13). Lymphocyte activating factor activity (IL1/ETAF-LAF) was performed on cultured (66 h) thymocytes from 6 to 8 week old C3H/HeJ mice as described (14).

RNA Extraction: Frozen (-80°C) pellets of PBMC, heat-separated epidermis and A431 cells were extracted in 4 M guanidine thiocyanate/0.2 M β-mercaptoethanol (15). Total RNA and poly(A)$^+$RNA were isolated as previously described (16).

Cell-associated IL1 and IL1/ETAF Activities: Cell-associated material was prepared by washing isolated cells and epidermal tissue 3 times with phosphate buffered saline followed by freezing (liquid N_2) and thawing two times in 3 ml DMEM-10% FCS. The resuspended cells were homogenized in a Polytron homogenizer three times on ice, cleared by centrifugation at 4°C, filter-sterilized and assayed for IL1/ETAF activities.

Northern Blot Analysis: Human IL1 α and β cDNA clones were isolated from cDNA gene banks prepared from PMA-induced monocytic cell line U937 as previously described (10,17,18). The β_2-microglobulin cDNA clone (19) was a gift from S. Suggs. RNA gel electrophoresis and Northern blot analysis were performed with 3.0 μg of poly(A)$^+$RNA and 10 μg or 20 μg of total RNA as described (11). After 18-20 h of hybridization at 65°C membranes were washed reaching a stringency of 0.1 x sodium chloride sodium citrate (SSC), 0.1% SDS at 68°C.

RESULTS

IL1 and IL1/ETAF activities in PBMC and heat-separated epidermis: Freshly isolated (non adhered) PBMC possessed little if any detectable cell-associated IL1 activity (Table I). However, after stimulation with PHA/ConA, significant extracellular IL1/MCF and IL1/LAF activities were observed. On the contrary, cell-associated IL1-MCF/ETAF and IL1-LAF/ETAF activities were present in freshly isolated heat-separated human epidermis.

IL1 α and β mRNA steady-state levels in PBMC and heat-separated epidermis: The significant level of cell-associated IL1/ETAF activity in nonstimulated heat-separated epidermis suggested that IL1 α and β mRNA would be abundant provided that either contributed to this activity. Northern blot analyses using cDNA probes to IL1 α and β mRNA were performed to verify this hypothesis. However, as seen in Fig 1a (lanes 7,8) no detectable hybridization was observed by the IL1 β probe to total or poly(A)$^+$RNA from heat-separated epidermis. This is in contrast to the significant hybridization to RNA

Table 1

IL1/MCF and IL1/LAF Activities in Mononuclear Cells and Heat-Separated Epidermis

	Mononuclear Cells		H-S Epidermis
	NS[a]	S[b]	NS
PGE_2 (ng/ml)	3.0	278 ± 17	105 ± 5
Collagenase (U/ml)	< 0.1	1.9 ± 0.2	1.20 ± 0.1
LAF (cpm x 10^{-4})	1.0	3.4 ± 0.2	1.9 ± 0.3

[a]Nonstimulated freshly isolated mononuclear cells were centrifuged through Ficoll' Hypaque and assayed for intracellular IL 1 activity.

[b]Stimulation with PHA/ConA; Data are Mean ± SEM.

from stimulated PBMC (lane 2), an enriched population of stimulated monocytes (lane 4) from a monocytic leukemia patient and stimulated U937 cells (lane 6). Even after extensive film exposure to membrane (Fig. 1b), there was no indication of IL1 β probe hybridization to heat-separated epidermal RNA, suggesting that adult epidermis is void of any detectable level of IL1 β messenger. Figure 1c shows that in each lane intact RNA was transferred to the membrane prior to hybridization with the β_2-microglobulin cDNA probe. As seen in lane 7, β_2-microglobulin mRNA levels in total applied RNA from heat-separated epidermis is lower than that found in enriched mRNA of total poly(A)$^+$RNA (lane 8). Furthermore, nearly equivalent levels of intact total RNA were added to each lane as indicated by the ethidium bromide stained gel in Fig. 1d, an example of the RNA transferred to each membrane.

The cell-associated IL1/ETAF activity from heat-separated epidermis may have resulted from IL1 α encoded by its cognate mRNA. However, as seen in Fig. 2 (lanes 1,2), no detectable mRNA was evident following hybridization to a nick-translated cDNA

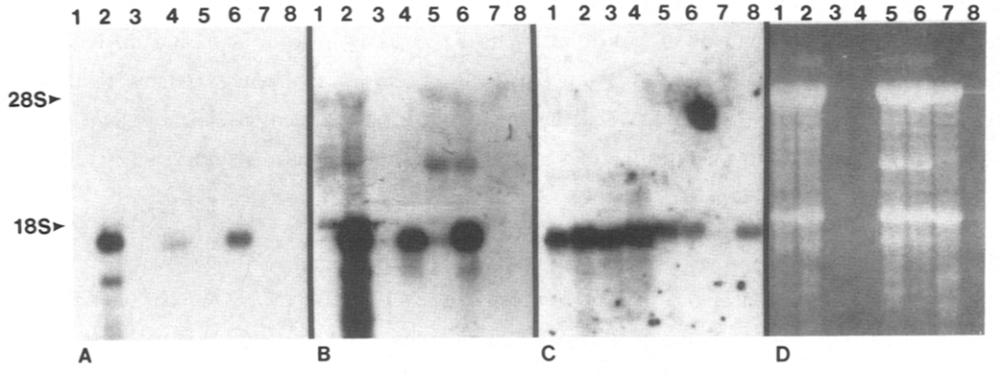

Fig. 1. Northern blot analysis of IL1 β mRNA. Total RNA (20 μg) from PBMC (lanes 1 and 2), polyA$^+$ RNA (3.0 μg) from mononuclear cells of a monocytic leukemia patient (lanes 3,4) and 20 μg total U937 RNA (lanes 5 and 6), and 20 μg total RNA and 3.0 μg polyA$^+$ RNA (lanes 7 and 8) from heat-separated adult epidermis were electrophoresed, transferred to membrane and hybridized to nick translated cDNA probes to IL1 β (a,b) and β_2-microglobulin (c). RNA in lanes 2 and 4 were from cells stimulated with PHA/ConA and PMA, and RNA from lanes 5 and 6 were extracted from cells exposed to γIFN; and γIFN followed by ConA and PMA. The photo in (b) is an overexposure of the same membrane in (a). (d) shows an example of an ethidium bromide stained gel prior to RNA transfer.

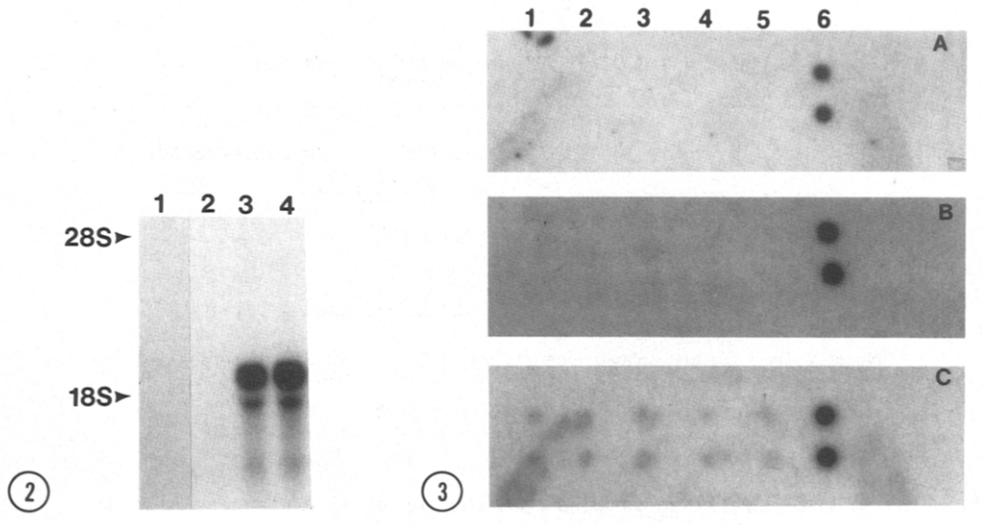

Fig. 2. Northern blot analysis of IL1 α mRNA. Total RNA (10 μg) from heat-separated adult epidermis preparations 5 and 6 (lanes 1 and 2), and control and PMA-stimulated A431 cells (lanes 3 and 4) were hybridized to a nick translated probe to IL1 α.

Fig. 3. Dot blot hybridization of RNA from heat-separated epidermis employing a nick-translated cDNA probe to IL1 β (a) and to IL 1 α (b). Total RNA was spotted in duplicate from epidermal preparation 4 (1) 1.0 μg (2) 2.0 μg (3) 4.0 μg and from preparation 1 (4) 2.5 μg and (5) 5.0 μg and (6) 1.0 μg poly(A)$^+$ RNA from PHA/ConA stimulated PBMC. Panel (c) is the same dot blot as (b) but the membrane was washed at a lower stringency to show that RNA was added as indicated.

IL1 α probe, even after 25 days of membrane-film exposure (long exposure not shown). Parallel lanes contain IL1 α mRNA from the constitutive IL1 producing squamous carcinoma cell line A431 (lanes 3,4). Again, an ethidium bromide staining showed that the RNA was intact prior to blotting and that transfer was complete (data not shown).

Even though RNA dot blots give less information concerning RNA quality and mRNA size distribution and produce a higher signal to noise ratio than that of Northern gel blot analysis, the level of sensitivity is greater. As seen in Fig. 3a, a probe to IL1 β mRNA did not hybridize to RNA from freshly isolated heat-separated epidermis. In Fig. 3b, there occurred a faint amount of hybridization to RNA using a probe to IL1 α mRNA. Figure 3c is the same membrane as 3b, but washed at a lower stringency to show that RNA was added to each point. In all, five tissue preparations were tested in Northern blot and dot blot analysis and only in dot blot analysis was IL1 α mRNA slightly detected.

DISCUSSION

This communication is the first to compare the level of IL1 mRNA to IL1/ETAF activity in freshly isolated adult heat-separated epidermis. In this study skin was not taken from areas that were likely exposed to UV light from the sun. *In vivo* exposed murine epidermis and cultured human and murine keratinocytes exposed to UV light contain elevated levels of IL1/ETAF activity and IL1 mRNA (4,20,21) and UV-irradiated mice and rabbits have increased serum IL1 levels (22,23). Although the epidermis is composed mainly of three major cell types, the keratinocyte is the predominant cell comprising

over 90% of the epidermal population. Keratinocytes that undergo differentiation while surfacing from the lower to the upper epidermal layer in forming the stratum corneum also exhibit considerable changes in gene expression (24). Several reports have demonstrated that cultured keratinocytes (4,25,26), keratinocyte cell lines (4,10,26) and keratinocytes isolated from newborn mice (4) express detectable and at times abundant messenger to IL1 α or β or both, whereas terminally differentiated keratinocytes had no detectable steady-state IL1 messenger RNA (4).

However, in this report we show that adult human heat-separated epidermis in which considerable IL1/ETAF activity is present possesses a slight detectable level of IL1 α mRNA. Even after five epidermal samples were analyzed no IL1 β mRNA was detected. Although, Ansel *et al.* (4) have detected abundant IL1 α levels in normal epidermal keratinocytes of newborn mice, this may be due to the undifferentiated state of the keratinocytes in this tissue. In addition, their results may have resulted from the method of prolonged isolation and trypsin digestion of keratinocytes (27). Others who have shown IL1 α and β expression from cultured keratinocytes have utilized longterm cultures of infant foreskin keratinocytes (26). Under these conditions keratinocytes do not differentiate as *in vivo*, requiring a feeder layer of 3T3 cells, (24,28) and were cultured in the absence of hydrocortisone. The absence of hydrocortisone may increase IL1 mRNA steady-state levels. Glucocorticoids suppress IL1 mRNA expression through decreased IL1 transcription and mRNA stability (29,30,31).

We have utilized skin isolated from mid-winter to early spring months from the upper arm and back which would minimize epidermal exposure to UV radiation. RNA degradation was further minimized by employing heat-separation as a rapid method of isolating the epidermal layer of skin without the contamination from cells of the dermal layer. As evident by the intact RNA bands in the stained gel Fig. 1d, the intact hybridized band of β_2-microglobulin mRNA in Fig. 1c, lane 6, and the high molecular weight (~100 kD) rabbit reticulocyte translation products of heat-separated keratinocytes RNA (data not shown), the absence of IL1/ETAF mRNA in these cells was not due to RNA degradation.

The substantial amount of cell-associated IL1/ETAF activity in these preparations may indicate that 1) another IL1/ETAF factor exists, 2) IL1/ETAF is expressed from perhaps the dermis but accumulates in the epidermis and 3) although the level of IL1 β mRNA is below detection by the current methodologies employed, the efficient translation of IL1 α or β mRNA coupled with a low IL1 turnover could generate conditions which facilitate the detection of IL1 activities. This may possibly be the case since Hsu and Zhao (8) have detected by immunohistological staining a "paucity" of IL1 in Langerhans cells in epidermal skin samples but did not report any evidence of IL1 in the surrounding keratinocytes. Oxholm et al. (32) have detected by immunohistological staining IL1 α and β on human epidermal cell membranes. However, they could not block the staining by preincubating the antisera with excess purified recombinant or native IL1. They suggested that ETAF/IL1 and peripheral blood monocyte derived IL1 are the products of different genes.

Protein turnover is dramatically affected by the age and differentiation of keratinocytes and other cell types (33). For example, the reduction with age of ubiquitin in lens epithelium is in concert with enhanced levels of modified proteins (34). Ubiquitin is a required cofactor for initiation of cytoplasmic degradation of damaged and/or rapidly turned over proteins (35,36). Through cellular aging and development, there occurs an increased inactivation of exopeptidase and endopeptidase activity (34,37). Such conditions could explain the IL1/ETAF activity where the level of IL1 β mRNA is below detection and that IL1 α is minimal but their rate of translation and reduced IL1 turnover allow for IL1/ETAF assayable activity.

In conclusion, we show that heat-separated adult epidermis from normal donors does not possess detectable IL1 β mRNA and only a slight detectable level of IL1 α mRNA by dot blot and not by Northern blot analysis, but does contain readily detectable IL1/ETAF activity. These data suggest the possibility that another IL1/ETAF molecule(s) other than an IL1 α or β product elicits the IL1/ETAF activity in adult epidermal tissue. Recently, IL6, formerly called B-cell stimulatory factor 2, which is expressed in many cell types has been shown to possess LAF activity (38) and other biological activities associated with IL1. It will be critical to determine if the high IL1-ETAF and IL1-MCF activities are due to this multibiological faceted cytokine.

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