Normal Human Epidermal Keratinocyte-Derived Neutrophil Chemotactic Factor

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TAKEMATSU, H., ISONO, N., KATO, T. and TAGAMI, H. *Normal Human Epidermal Keratinocyte-Derived Neutrophil Chemotactic Factor*. Tohoku J. Exp. Med., 1990, **162** (1), 1-13 — Human epidermal keratinocytes constitutively produce a variety of cytokines, including neutrophil chemotactic peptide named epidermal cell-derived thymocyte-activating factor, which has been later confirmed to be interleukin 1 (IL-1). Because recombinant IL-1 lacks chemotactic activity, in the present study, we examined the exact nature of the neutrophil chemotactic peptide in the culture supernatant of normal human epidermal keratinocytes. Normal human epidermal keratinocytes produced a neutrophil chemotactic factor, which was also chemotactic for T lymphocytes. Molecular sieve chromatography revealed an approximate molecular size of 11,000 daltons. The activity was retained after heating at 100°C for 10 min, and at a pH between 4 and 11, but was partially inactivated at pH 3, or by trypsin treatment. The chemotactic activity was not inhibited by the treatment with anti-IL-1 antibody. Its production by keratinocytes was stimulated by IL-1 and lipopolysaccharide but not by UV irradiation, tumor necrosis factor- α or by interferon- γ . The neutrophil chemotactic activity in vivo was confirmed by the intradermal injection of the factor into guinea pigs. Blocking study with monoclonal antibodies against NAP-1/IL-8 confirmed that the neutrophil chemotactic factor is IL-8. ——— keratinocyte; chemotactic factor; interleukin 8

Inflammatory reactions in skin diseases such as psoriasis are mediated by cytokines produced by epidermal keratinocytes (Takematsu et al. 1986a, b; Elder et al. 1989; Grossman et al. 1989; Kupper 1989; Takematsu and Tagami 1990); human epidermal keratinocytes constitutively produce a variety of cytokines, including interleukin 1 (IL-1) (Luger et al. 1983b; Sauder et al. 1983; Hauser et al. 1986; Kupper et al. 1986), IL-3 (Danner and Luger 1987), IL-6 (Grossman et al. 1989), transforming growth factor α (Coffey et al. 1987), β (Fisher et al. 1988), and granulocyte-macrophage colony-stimulating factor (Chodakewitz et al. 1988).

One of the salient histologic features of psoriatic lesions is an accumulation of neutrophils in intra- and subcorneal or spongiform pustules (Ackerman

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et al. 1982). Previously, we suggested the presence of 12,000 Da chemotactic peptides other than anaphylatoxin C5a in psoriatic lesional skin (Takematsu et al. 1986a). Production of a neutrophil chemokinetic peptide was also supposed from the presence of chemoattractant activity in the stratum corneum of psoriatic skin lesions (Fincham et al. 1988; Schröder et al. 1989). Neutrophil chemotactic peptides have been found to be produced by a variety of cells including macrophages (Kownatzki et al. 1986; Schröder et al. 1987; Yoshimura et al. 1987; Matsushima et al. 1988), lymphocytes (Schröder et al. 1988), endothelial cells (Strieter et al. 1989a), and fibroblasts (Strieter et al. 1989b). On the other hand, there has been scant direct evidence to show that a neutrophil chemotactic factor is produced by normal human epidermal keratinocytes except for epidermal cell-derived thymocyte-activating factor (ETAF) that shows the activity of IL-1 (Luger et al. 1983b).

The purpose of this study was to examine neutrophil chemotactic properties of the culture supernatant of normal human epidermal keratinocytes, particularly in relation to IL-1. We present here direct evidence to support the concept that normal human keratinocytes produce a neutrophil chemotactic factor IL-8.

MATERIALS AND METHODS

Chemicals. The following materials were purchased from Sigma Chemical Company (St. Louis, MO, USA): trypsin-Agarose beads; mouse IgG; Agarose beads coated with rabbit IgG; phorbol 12-myristate 13-acetate (PMA); protein A-Sepharose CL-4B. EpiPack's Normal Human Epidermal Keratinocytes and Keratinocyte Growth Medium were from Clonetic Corporation, San Diego, CA, USA. MCDB153 from Kyokuto Pharmaceutical Company, Tokyo, Japan. Uni-Pore polycarbonate membrane was from Bio-Rad Laboratories, Richmond, CA, USA, and nitrocellulose membrane was from Nihon Millipore Kogyo, Yonezawa, Japan. Human T-Cell Recovery Column was from Scican Diagnostics, Alberta, Canada. Human recombinant (r) IL-1 α and β , human recombinant tumor necrosis factor- α , human recombinant interferon- γ , human r-granulocyte-macrophage colony-stimulating factor (GM-CSF) were from Genzyme Corporation, Boston, MA, USA. Lipopolysaccharide (LPS; *E. coli* 055: B5) was from Difco Laboratories, Detroit, MI, USA.

Antibodies. Rabbit anti-human IL-1 antibody was from Genzyme Corporation, Boston, MA, USA. Monoclonal antibody against NAP-1 was a gift from Dr. T. Yoshimura (Immunopathology Section, Laboratory of Immunobiology, National Cancer Institute, Frederick, MD, USA), and monoclonal antibody against NAP-1/IL-8, 46ES was from Dr. Schröder (Department of Dermatology, University of Kiel, Kiel, Germany).

Cell culture. Normal human epidermal keratinocytes were grown in Keratinocyte Growth Medium. After appropriate culture time, the conditioned medium was collected.

Molecular sieve chromatography. Culture supernatant of normal epidermal keratinocytes was loaded onto a TSK-G2000SW exclusion high performance liquid chromatography column (Tosoh, Tokyo; 7.5 $\times$ 30 cm). Phosphate-buffered saline was used as the eluent. Flow rate was 0.5 ml/min, and fractions of 0.5 ml were collected. The protein content was estimated by the measurement of absorbance at 280 nm. The fractions were assayed for neutrophil chemotactic activity.

Temperature stability. The column fraction was subjected to heating for 30 min at 60°C, for 10 min at 100°C, or repeated freeze/thawing three times.

pH treatment. pH of the column fraction was altered for 1 hr then adjusted to pH 7.4, and tested for neutrophil chemotactic activity.

Trypsin sensitivity. The column fraction (400 μ l) was incubated for 5 min at 34°C with 100 μ l of trypsin-Agarose beads (5 units) or 100 μ l of Agarose beads coated with rabbit IgG. The beads were removed by centrifugation, and the supernatants were assayed for chemotactic activity (Sheridan et al. 1987).

Blocking studies. To assess the relationship between the neutrophil chemotactic factor and IL-1, blocking study was performed. Rabbit anti-human IL-1 antibody or an IgG fraction of normal rabbit serum was incubated with the neutrophil chemotactic fraction for 2 hr at room temperature followed by 48 hr at 4°C. Samples were then centrifuged at $2,000 \times g$ for 30 min at 4°C, and the supernatant was then assayed for chemotactic activity.

To assess the relationship between the neutrophil chemotactic factor and IL-8, blocking study was performed. Neutrophil chemotactic fraction was incubated with monoclonal antibody against NAP-1, monoclonal antibody against NAP-1/IL-8, or mouse IgG adsorbed to protein A-Sepharose CL-4B at for 1 hr at room temperature. Samples were then centrifuged at $2,000 \times g$ for 10 min at 4°C, and the supernatant was then assayed for chemotactic activity.

Effect of cytokines, PMA and LPA. Normal human epidermal keratinocytes were stimulated with 100 U/ml human rIL-1 α , 100 U/ml human rIL-1 β , 100 U/ml rTNF- α , 100 U/ml human rIFN- γ , 60 U/ml rhGM-CSF, 1 ng/ml PMA, and 10 μ g/ml LPS in MCDB153 medium. After stimulation for 24 hr (Matsushima et al. 1988), the chemotactic activities in the culture supernatants were assayed.

UV irradiation. Normal human keratinocytes were irradiated with 0, 25, 50, and 200 J/m² of ultraviolet (UV) light in the 280- to 320-nm range by a FL 20S fluorescent sun lamp (Matsushita Electric Company, Tokyo). Neutrophil chemotactic activity in the culture supernatant was assayed 24 hr after UV irradiation (Gahring et al. 1984).

Synergism with C5a/C5a des Arg. In order to assess the synergism with chemotactic complement fragment C5a/C5a des Arg, the column fraction was tested for neutrophil chemotactic activity in the presence or absence of equal amount of a fraction of zymosan-activated serum containing C5a/C5a des Arg (Takematsu et al. 1986b). After incubating for 30 min, neutrophil chemotactic activities in the samples were assayed.

Neutrophil chemotactic assay. Neutrophil chemotactic activity was measured using a Blind Well Chamber with a 3- μ m Uni-Pore polycarbonate membrane. Human neutrophils collected after centrifugation of heparinized blood on Ficoll-Isopaque solution were suspended in Hanks' solution containing 0.5% bovine serum albumin and 100 μ g/ml streptomycin. A suspension containing 2×10^5 cells in 30 μ l was placed in the upper compartment of the chamber, while 25 μ l of a chemoattractant was placed in the bottom compartment. The chamber was incubated for 45 min at 37°C in a 5% CO₂ moist atmosphere. The chemotactic activity was expressed as an average number of the cells reaching the bottom surface of the filter in five fields for each filter at a magnification of $\times 400$.

Lymphocyte chemotactic assay. In lymphocyte chemotactic assay, a stimulus was placed in the lower compartment of a chemotactic chamber, and human T-lymphocyte suspension (2×10^6 /ml) obtained through Human T-Cell Recovery Column was poured into the upper compartment, separated by a 5 μ m-pore size nitrocellulose membrane. After incubation of the chambers for 3 hr at 37°C, the membranes were detached, stained with Giemsa, and cleared with xylene. Cell migration was assessed by the leading front technique: measurement of distances that the leading 2 cells moved through the membrane; a migration index was defined as the difference between movements in response to the medium alone and in response to a stimulus (Okamoto et al. 1985).

Chemiluminescence assay. The chemiluminescence assay was performed by using a Biolumat (LB9500, Berthold, FRG) as described previously (Kato et al. 1983).

Thymocyte assay of IL-1. IL-1 activity was assessed by potentiation of thymocyte reactions to a mitogen (comitogenic assay) (Luger et al. 1983b). Mouse thymocytes were

suspended in RPMI 1640 containing 10% fetal calf serum and cultured for 72 hr at 1.5×10^6 cells/0.2 ml/well in flat-bottomed microtiter plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA). Cultures were pulsed with $0.5 \mu\text{Ci}$ /well of [^3H]thymidine (specific activity, 5.0 Ci/mmol) for the final 16 hr of incubation. Cells were collected on glass filter paper by an automatic cell microharvester and [^3H] activity was counted by a liquid scintillation counter.

Intradermal injection. A suspension of the column fractions in $100 \mu\text{l}$ collected through molecular sieve chromatography was injected intradermally into the skin of back of guinea pigs. A 3 mm punch biopsy was performed 0, 1, 2, 3, and 6 hr after the injection. All biopsies were processed for light microscopy, and hematoxylin and eosin-stained sections were examined.

RESULTS

Kinetics

The conditioned medium of the normal human keratinocyte showed a neutrophil chemotactic activity (Fig. 1). The chemotactic activity was mainly produced at the logarithmic growth phase of the cells.

Molecular sieve chromatography

Characterization by molecular sieve chromatography revealed that the culture supernatant of the normal human keratinocyte had neutrophil chemotactic activity in the fraction with an approximate mol. wt. of 11,000 (Fig. 2). The 11,000 dalton fraction thus obtained was pooled and used in the following experiments.

Fig. 1. Kinetics of production of the neutrophil chemotactic factor. Normal human epidermal keratinocytes were grown. After the indicated time, the numbers of cells were counted, and neutrophil chemotactic activity in the culture supernatant was assayed. Data are means $\pm$ s.e. from six replicate determinations.

Fig. 2. Molecular sieve chromatography of the keratinocyte-derived neutrophil chemotactic factor. Culture supernatant of normal epidermal keratinocytes was loaded onto a TSK-G2000SW gel filtration column (7.5×30 cm). Phosphate-buffered saline was used as the eluent. The fraction size was 0.5 ml, and the flow rate was 0.5 ml/min. The protein content was estimated by the measurement of absorbance at 280 nm. The fractions were assayed for neutrophil chemotactic activity.

Chemotactic and chemokinetic activity

Checkerboard analysis revealed that the peak fraction of the factor collected through the molecular sieve chromatography was predominantly chemotactic but mildly chemokinetic for neutrophils (Table 1).

T lymphocyte chemotactic activity

The keratinocyte-derived factor was also mildly chemotactic but less chemokinetic for T lymphocytes (Table 2).

Temperature stability

Studies of the effect of temperature on the stability of the neutrophil chemotactic factor revealed that heating at 60°C , 100°C , or repeated freeze/thawing had no effect on the neutrophil chemotactic factor activity (data not shown).

pH stability

When the neutrophil chemotactic factor was tested for pH stability, the activity was stable from pH 4 to 11. Acid pH of 3 partially inactivated the neutrophil chemotactic activity (from 155 ± 9 to 80 ± 6).

TABLE 1. *Chemokinetic and chemotactic activity of the keratinocyte-derived factor for neutrophils^a*

	KDNCf to cell compartment					
	Buffer	1 : 100	1 : 50	1 : 10	1 : 5	1 : 2
Buffer	1±0.5					
1 : 100	1±0.5	4±1				
1 : 50	2±0.7		6±1			
1 : 10	9±2			9±2		
1 : 5	53±5				12±2	
1 : 2	116±5					36±3

^aSerially diluted peak fraction from the molecular sieve chromatography, tentatively termed keratinocyte-derived neutrophil chemotactic factor (KDNCf), was put into upper or lower compartment of the chemotaxis chamber. For the “buffer” condition, no KDNCf was present. Increasing chemotaxis is shown on the vertical and increasing chemokinesis along the diagonal. Data are means±s.e. from four replicate determinations.

TABLE 2. *Chemokinetic and chemotactic activity of the keratinocyte-derived factor T-lymphocytes^a*

	KDNCf to cell compartment			
	Buffer	1 : 10	1 : 2	1 : 1
Buffer	1.0			
1 : 10	1.5±0.04	1.4±0.2		
1 : 2	1.7±0.1		1.5±1.1	
1 : 1	2.1±0.1			1.6±0.1

^aSerially diluted KDNCf was put into upper or lower compartment of the chemotaxis chamber. Data are means±s.e. from four replicate determinations.

Trypsin sensitivity

Treatment with trypsin significantly reduced the activity of the neutrophil chemotactic factor (from 125±11 to 30±5).

Effect of antibody to human IL-1

Rabbit anti-human IL-1 antibody was found to inhibit the action of recombinant IL-1 α and in the thymocyte assay. The chemotactic activity was not inhibited by the treatment with the anti-IL-1 antibody, indicating that the neutrophil chemotactic factor is antigenically distinct from IL-1 α and β (data not shown).

TABLE 3. *Effect of monoclonal antibodies against NAP-1/IL-8 on the activity of the neutrophil chemotactic activity^a*

Treatment	Chemotactic activity
Mouse IgG	102 ± 9
Anti-NAP-1	25 ± 3
46ES	20 ± 2

^aThe neutrophil chemotactic factor preparation was incubated with monoclonal antibodies against NAP-1/IL-8 or mouse IgG adsorbed to protein A-Sepharose CL-4B at for 1 hr at room temperature, and the supernatant was then assayed for neutrophil chemotactic activity. Data are means ± s.e. from six replicate determinations.

Effect of antibody against NAP-1/IL-8

Monoclonal antibodies against NAP-1/IL-8 inhibited the activity of the neutrophil chemotac activity (Table 3).

Effect of cytokines, PMA and LPS

Treatment with the cytokines did not induce specific proliferation of the keratinocytes. IL-1 α and β stimulated the production of the neutrophilic

Fig. 3. Stimulation of production by cytokines. Normal human keratinocytes were incubated with MCDB153 medium, human (h) rIL-1 α , β , hrTNF- α , hrIFN- γ , hrGM-CSF, PMA, and LPS in MCDB153. After stimulation for 24 hr, the neutrophilic chemotactic activities of the stimulators (open columns) and those of the culture supernatants (shaded columns) were measured. Data are means ± s.e. from six replicate determinations.

chemotactic factor, and LPS augmented chemotactic factor production, while $\text{TNF-}\alpha$ $\text{IFN-}\gamma$, GM-CSF, or PMA did not at the concentrations tested (Fig. 3).

Effect of UV irradiation

When the normal human keratinocytes were irradiated with 25, 50, and 200 J/m^2 of UV light in the 280- to 320-nm range, there was no significant increase in the neutrophil chemotactic activity in the culture supernatant (data not shown).

Synergism with C5a/C5a des Arg

A mixture of the neutrophil chemotactic factor and C5a/C5a des Arg showed a much higher neutrophil chemotactic activity than the sum of the activities of the chemotactic factor and that of C5a/C5a des Arg, suggesting a synergism between the neutrophil chemotactic factor and C5a/C5a des Arg (Fig. 4).

Chemiluminescence assay

The neutrophil chemotactic factor had no effect on the oxidative metabolism of neutrophils as measured by chemiluminescence (data not shown).

Intradermal injection

Intradermal injection of the neutrophil chemotactic factor into guinea pigs induced an accumulation of neutrophils and mononuclear cells in the dermis and within the blood vessels 3 to 6 hr after injection (Fig. 5).

Fig. 4. Synergism of the neutrophil chemotactic factor and C5a/C5a des Arg. The neutrophil chemotactic factor were tested for neutrophil chemotactic activity in the presence or absence of equal amount of a fraction of zymosan-activated serum containing C5a/C5a des Arg. Data are means $\pm$ s.e. from six replicate determinations.

Fig. 5. Intradermal injection. Intradermal injection of the neutrophil chemotactic factor into guinea pigs induced an accumulation of neutrophils and mononuclear cells in the dermis.

DISCUSSION

Neutrophil chemotactic peptides have been found to be produced by epidermal cells as well as by inflammatory cells (Schröder et al. 1987 ; Yoshimura et al. 1987 ; Strieter et al. 1989a, b ; Watanabe et al. 1989). In the present study we showed that normal human epidermal keratinocytes produced in vitro a neutrophil chemotactic factor, which was also chemotactic for T lymphocytes. The activity was produced predominantly at the logarithmic growth phase. Molecular sieve chromatography revealed an approximate molecular size of 11,000 daltons. The activity was retained after heating at 100°C for 10 min, and at a pH between 4 and 11, but was partially inactivated at pH 3, or by trypsin treatment. The chemotactic activity was not inhibited by the treatment with anti-IL-1 antibody. Its production by keratinocytes was stimulated by IL-1 but not by UV irradiation. Its in vivo neutrophil chemotactic activity was confirmed by the intradermal injection of the factor into guinea pigs. These results suggest that human keratinocytes are capable of producing a neutrophil chemotactic peptide distinct from IL-1.

Previously, ETAF with an approximate mol wt of 12,000 Da that was indistinguishable from IL-1, was shown to be chemotactic for neutrophils (Luger et al. 1983a ; Sauder et al. 1984). Although later, rIL-1 itself was shown to lack

chemotactic activity (Yoshimura et al. 1987), no studies have been done on the nature of the chemotactic peptides reported as ETAF. Our present study showing the distinction of the keratinocyte-derived neutrophil chemotactic factor from IL-1 clearly indicates that the concurrent expression of both IL-1-like and neutrophil chemotactic activities in ETAF preparation seems to be due to the incomplete dissociation of IL-1 in the past from the neutrophil chemotactic factor in the column fractions as once suggested by Yoshimura et al. (1987). Gahring et al. (1984) showed that UV irradiation enhanced the release of ETAF/IL-1. In the present study, however, we found no effect of UV light on the production of the neutrophil chemotactic activity. Again, this discrepancy seems to be due to the difference in the release mode of IL-1 and the chemotactic peptide by keratinocytes. Sauder et al. (1988) showed that ETAF preparation stimulated the autocrine growth of human keratinocytes. Whether this stimulatory effect was due to IL-1 or to the neutrophil chemotactic factor is yet to be clarified.

A neutrophil chemotactic peptide with a mol wt of 9,000 Da produced by macrophages (Kownatzki et al. 1986; Schröder et al. 1987; Yoshimura et al. 1987; Matsushima et al. 1988), lymphocytes (Schröder et al. 1988), endothelial cells (Strieter et al. 1989a), and fibroblasts (Strieter et al. 1989b) has been termed IL-8/neutrophil-activating peptide (NAP)-1, and its genomic structure has been determined (Matsushima et al. 1988; Mukaida et al. 1989; van Damme et al. 1989). It is chemotactic for both neutrophils and T lymphocytes (Larsen et al. 1989a). The physicochemical and biochemical characteristics of the keratinocyte-derived neutrophil chemotactic factor demonstrated in the present study suggest that IL-8 seems to be the most likely candidate for it. Our subsequent blocking study with monoclonal antibodies against NAP-1/IL-8 confirmed that the neutrophil chemotactic factor is IL-8. Our present data are consistent with the recent report by Larsen et al (1989b) on an induction of expression of mRNA for IL-8 in normal human keratinocytes.

While Matsushima et al. (1988) showed the induction of IL-8 mRNA by TNF- α as well as IL-1 in monocytes, we failed to stimulate the production of the neutrophil chemotactic factor by TNF- α . Whether the discrepancy was due to the difference in utilized cell source, i.e., monocytes vs keratinocytes, or in method, i.e., mRNA vs peptide, awaits further study.

Inflammatory reactions in the epidermis seem to be intricately regulated, because, although normal keratinocytes secrete neutrophil chemotactic factor in vitro, we see scant infiltration of neutrophils and mononuclear cells into the normal epidermis in vivo. In addition to IL-1, normal human epidermal keratinocytes were found to produce an inhibitor of IL-1 (Takematsu et al. submitted). It is, therefore, intriguing to speculate whether, in the same fashion, normal human epidermal keratinocytes produce inhibitor(s) of the neutrophil chemotactic factor in vivo, thereby suppressing the inflammatory reactions in the epidermis.

Based on the results of our present study and those of others (Hauser et al.

1986), we can conclude that normal human epidermal keratinocytes constitutively produce IL-1 and IL-8. The levels of IL-1 in the psoriatic scales are significantly lower than that in the normal stratum corneum (Takematsu et al. 1986b; Cooper et al. 1990). Thus, it is intriguing to speculate how the production of proinflammatory IL-8 is regulated in the psoriatic lesional skin.

Complement-derived chemotactic factors, C5a/C5a des Arg, is another chemotactic peptide in psoriatic lesional skin (Takematsu et al. 1986a). The synergism of the keratinocyte-derived chemotactic factor with C5a/C5a des Arg observed in the present study suggests that the keratinocyte-derived chemotactic factor and the complement derived factors are capable of augmenting the chemotactic activity each other in the lesional skin of psoriasis or sterile pustular dermatoses.

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