Identification of macrophage migration inhibitory factor (MIF) in human skin and its immunohistochemical localization

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Received 14 December 1995; revised version received 26 January 1996

Abstract The presence and tissue localization of macrophage migration inhibitory factor (MIF) in human skin were examined. Reverse transcription-polymerase chain reaction analysis revealed that MIF mRNA was expressed in both surgically obtained normal human epidermis and primary cultured human keratinocytes. The expression of MIF was further confirmed by Western blot analysis, which demonstrated a single band at about 12.5 kDa using a polyclonal antibody against human recombinant MIF. Immunohistochemical studies showed that MIF existed in human epidermis, especially in the basal layer. The pathophysiological role of MIF in human skin remains undefined; however, the present results indicate that MIF may play an important role in immunity, inflammation and cellular differentiation of epidermal cells.

Key words: Human skin; Immunohistochemistry; Keratinocyte; Macrophage migration inhibitory factor; Reverse transcription-polymerase chain reaction

1. Introduction

The skin is an important barrier that protects the body against damage from the outside environment. It has been reported that various cytokines play important roles in homeostasis of normal skin and in its pathology [1]. Diverse stimuli trigger a cutaneous inflammatory response by activating specific pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1); however, the detailed interrelation of the cytokines to the pathophysiological mechanism has not been well elucidated.

Macrophage migration inhibitory factor (MIF) was the first lymphokine reported in the guinea pig to prevent the migration of macrophages out of capillary tubes [2]. Human MIF cDNA was first cloned from human T-lymphocytes, and it was revealed that MIF consists of 114 amino acid residues [3]. MIF has long been considered to be expressed exclusively in activated T-lymphocytes; however, a recent report indicated that macrophages are another major source of MIF [4]. We cloned rat MIF cDNA, and reported its physicochemical properties [5,6]. Moreover, we succeeded in the crystallization of both human and rat MIF [7,8]. During the course of our MIF study, we unexpectedly found the presence of MIF in human corneal epithelium (manuscript submitted for publication). This finding indicated that MIF might not be exclusively expressed in T-lymphocytes and monocytes/macrophages.

In the present study, we investigated the presence of MIF in human skin. Here we report the expression of MIF in human epidermal keratinocytes as revealed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses. Furthermore, we demonstrate the intracellular localization of MIF in human epidermis by immunohistochemistry. These novel findings suggest additional pathophysiological functions for MIF in cutaneous immunity, inflammation, and cellular differentiation.

2. Materials and methods

2.1. Materials

The following materials were obtained from commercial sources. Nitrocellulose membrane filters from Millipore (Bedford, MA, USA); Isogen RNA extraction kit from Nippon Gene (Tokyo, Japan); M-MLV reverse transcriptase from Gibco (Grand Island, NY, USA); Taq DNA polymerase from Perkin-Elmer (Norwalk, CO, USA); horseradish peroxidase-conjugated goat anti-rabbit antibody from Pierce (Rockford, IL, USA); Vector ABC Kit from Vector Laboratories (Burlingame, CA, USA); FITC from Pierce (Rockford, IL, USA); Konica immunostaining kit from Konica (Tokyo, Japan); Tissue-Tek O.C.T. Compound from Miles Scientific (Naperville, IL, USA) and Protein A Sepharose from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade.

2.2. Preparation of rabbit polyclonal antibodies against human MIF

Polyclonal anti-human MIF serum was generated by immunizing New Zealand White rabbits with purified recombinant human MIF. Human recombinant MIF was expressed in *E. coli* and purified to homogeneity as previously described [6]. In brief, the rabbits were inoculated intradermally with 100 μg of MIF diluted in complete Freund adjuvant at weeks 1 and 2, and with 50 μg of MIF diluted in incomplete Freund adjuvant at week 4. Immune serum was collected 1 week after the last inoculation. The IgG fraction was prepared using Protein A Sepharose according to the manufacturer's protocol.

2.3. Cell culture

The primary cultured normal human keratinocytes (second passage) were obtained from Kurabou Co. (Osaka, Japan). The cultures were fed on glass coverslips in a medium of consisting serum-free modified MCDB 153, supplemented with hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), epidermal growth factor (10 ng/ml), bovine pituitary extracts (150 μg/ml), and calcium (0.15 mM). The keratinocytes were grown in a humidified atmosphere under 5% CO_2 at 37°C. For the analyses of RT-PCR, Western blot and immunofluorescence microscopy, tertiary cultures of these cells were harvested before the colonies became confluent.

2.4. RT-PCR analysis

Human epidermis was surgically obtained from a normal subject, and was frozen immediately in liquid nitrogen until use. The epidermis was separated from the dermis by fine forceps. The total RNA was extracted from the epidermis or primary cultured keratinocytes with an Isogen RNA extraction kit. The reverse transcription of RNA was carried out by M-MLV reverse transcriptase using oligo-dT primer and subsequent amplification using Taq DNA polymerase. PCR was carried out for forty cycles of denaturation at 94°C for 1 min, annealing at 53°C for 2 min and extension at 72°C for 1 min using a thermal cycler (Perkin-Elmer, Model 2400). MIF primers used were

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SSDI S0014-5793(96)00120-2

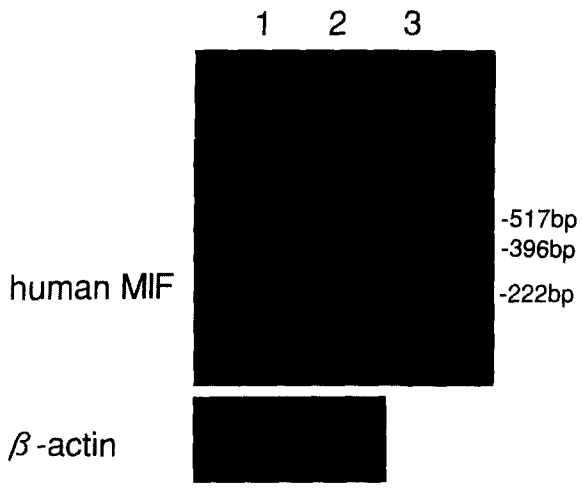

Fig. 1. RT-PCR analysis of MIF mRNA. RT-PCR was carried out as described in Materials and Methods. The PCR products were electrophoresed on 2% agarose gel. Lane 1, human epidermis; lane 2, primary cultured keratinocytes; lane 3, the molecular size marker (pBR322 DNA/AluI). The RT-PCR product of β-actin is shown at the bottom of each lane.

5′-CTCTCCGAGCTCACCCAGCAG-3′ (58–78) (forward) and 5′-CGCGTTCATGTCGTAATAGTT-3′ (292–312) (reverse). β-Actin primers used were 5′-CGTTCTGGCGGCACCACCAT-3′ (936–935) (forward) and 5′-GCAACTAAGTCATAGTCCGC-3′ (1170–1189) (reverse). After PCR, the amplified products were analyzed by agarose gel electrophoresis.

2.5. Western blot analysis

The tissue samples of homogenates of normal epidermis or the primary cultured keratinocytes were treated with 4 volumes of SDS-reducing buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.025% Bromophenol blue), and subjected to SDS-PAGE as described [9]. Proteins on the gel were transferred electrophoretically to a nitrocellulose membrane by the procedure of Towbin et al. In brief, the membrane was intensively washed with phosphate-buffered saline (PBS), and incubated with the polyclonal anti-human MIF antibody at room temperature for 1 h. After washing with PBS, the membrane was incubated with peroxidase-conjugated goat antibodies against rabbit IgG at room temperature for 1 h. After the reaction, proteins were visualized with a Konica immunostaining kit as recommended in the manufacturer's protocol.

2.6. Immunohistochemistry

Biopsy samples from normal human skin were immediately embedded in Tissue-Tek O.C.T. Compound, snap frozen in liquid nitrogen and stored at −80°C until further processing. The frozen embedded tissues were cut into 4-μm sections which were then fixed in acetone for 10 min at room temperature. They were stained with an avidin–biotin peroxidase complex procedure using a Vector ABC Kit according to the manufacturer's protocol. In brief, the sections were incubated overnight at 4°C with the anti-human MIF antibody. After three washes with PBS, the samples were reacted with biotinylated goat anti-rabbit IgG and avidin–biotin complex at room temperature for 30 min. The reaction was developed in 3,3′-diaminobenzidine tetrahydrochloride containing hydrogen peroxide (0.01%), and the tissue samples were mounted with alkylacrylates.

Immunohistochemical study of the primary cultured human keratinocytes was also carried out using a fluorescence-conjugated secondary antibody. The tertiary cultures of cells grown on cover slips were rinsed with PBS and dipped in −20°C acetone for 5 min. The fixed cells were incubated with the anti-human MIF antibody at 37°C for 1 h, followed by FITC-labeled goat anti-rabbit IgG (secondary antibody) at 37°C for 30 min. A fluorescence microscope (Nikon, Tokyo, Japan) was used for immunohistochemical observation.

3. Results

3.1. RT-PCR

MIF mRNA expression in the human epidermis and the primary cultured keratinocytes was examined by RT-PCR analysis. At the expected molecular size (255 bp), MIF RT-PCR products on the human epidermis and the cultured keratinocytes were observed in agarose gel (Fig. 1). They expressed MIF to similar extents assumed from the similar intensities of their PCR products normalized by the intensities of β-actin.

3.2. Western blot analysis

To further confirm the presence of MIF in the human epidermis, Western blot analysis was performed using the antibody against human MIF. The immunoblot analysis revealed that the normal human epidermis and the primary cultured keratinocytes could produce MIF that migrated to the corresponding molecular weight, about 12.5 kDa, visualized by a Konica immunostaining kit (Fig. 2). Pre-immune rabbit IgG did not react with human MIF in the immunoblot analysis (data not shown). This result indicated that MIF was biosynthesized in the human epidermis.

3.3. Immunohistochemisry

The epidermis obtained from a normal subject was positively stained by the antibody against human MIF in the cytoplasm, especially in the basal layer (Fig. 3a,b). The positive staining regions appearing in the middle and the left side of the spinous cell layers were considered to be keratinocytes of the epidermal basal cells. Their appearance was technically caused by the direction of the section. Some small positive staining spots were also observed in the dermis. These were considered to be endothelial cells of small blood vessels. Furthermore, eccrine sweat ductal cells and myoepithelial cells of eccrine sweat glands were also stained (data not shown). The positive staining was not observed when the tissue sample was stained with pre-immune rabbit IgG (Fig. 3c). To confirm that MIF localized in the cytoplasm of epidermal keratinocytes, we further examined the localization of MIF in cultured human keratinocytes using an FITC-labeled secondary antibody. It clearly demonstrated that the cytoplasm of cultured keratinocytes was positively stained (Fig. 3d). No specific positive staining was observed when the tissue was reacted with

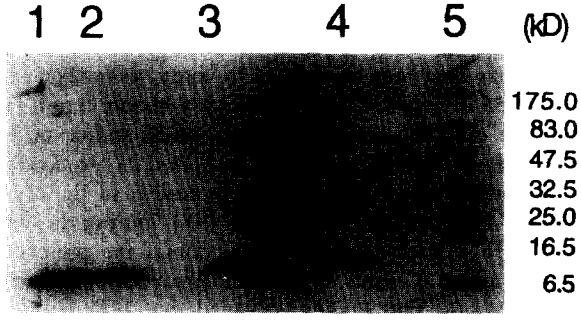

Fig. 2. Western blot analysis of the human epidermis for MIF. The tissue samples were collected, electrophoresed, transferred to a nitrocellulose membrane, and visualized by a Konica immunostaining kit as described in section 2. Lane 1, recombinant human MIF (200 μg); lane 2, recombinant human MIF (50 μg); lane 3, human epidermis (50 μg of protein); lane 4, primary cultured keratinocytes (5×10^5 cells); lane 5, prestained molecular marker (Biolabs).

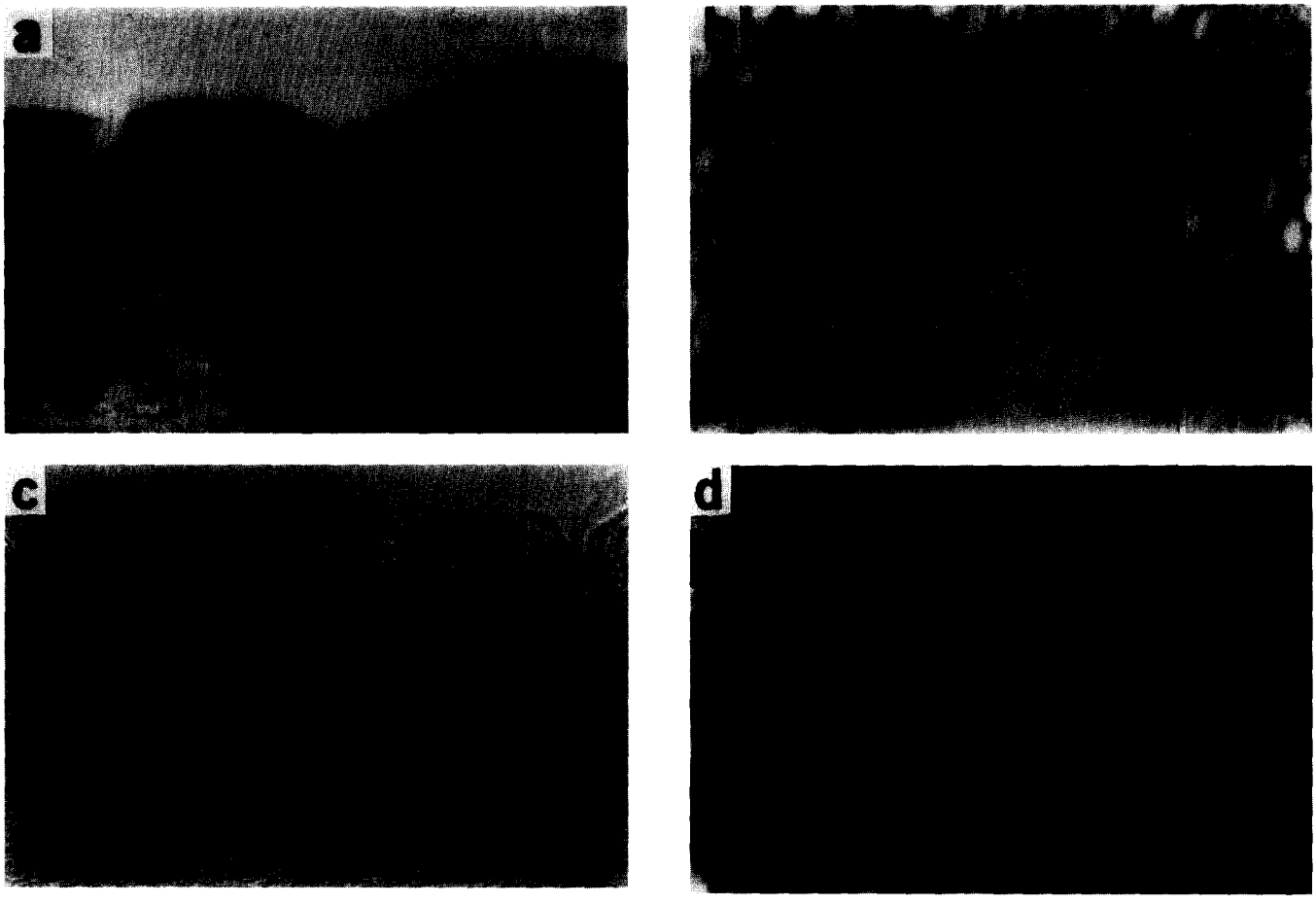

Fig. 3. Immunohistochemistry of MIF in normal human skin. a, a tissue specimen obtained from normal skin was stained using a Vector ABC immunostaining kit reacted with anti-human MIF antibody (×50). b, high-magnification of Fig. 3a (×200). c, a control tissue specimen reacted with pre-immune rabbit IgG (×50). d, primary cultured keratinocytes were reacted with anti-human MIF antibody followed by an FITC-labeled secondary antibody (×100).

pre-immune FITC-labeled rabbit IgG or with anti-human MIF IgG pretreated with an excess amount of recombinant MIF (data not shown).

4. Discussion

In the present study, we have demonstrated the expression of MIF in human epidermis by RT-PCR and Western blot analyses. Moreover, the presence of MIF was confirmed by immunohistochemistry which showed that MIF was distributed mainly in the cytoplasm in the keratinocytes. The unexpected tissue localization of the pro-inflammatory cytokine in tissue other than T-lymphocytes and monocytes/macrophages should help further understanding of the immunological reaction in human skin.

To date, several cytokines, including IL-1α, TNF-α, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factor-α (TGF-α), have been found in epidermal keratinocytes [1]; however, only IL-1α could be shown to be stored in normal skin in a biologically active form [10]. Cytokines released from keratinocytes can be induced by environmental stimuli such as trauma, bacterial toxins and ultraviolet light. For example, IL-1 and TNF-α release from keratinocytes are induced by ultraviolet radiation [11,12], and physical damage to keratinocytes can stimulate IL-1 release [13]. In addition, IL-1 in concert with other cytokines such as GM-CSF, IL-6, and TGF-α, stimulates keratinocyte proliferation [14,15]. That is, it is very likely that cytokines are a vital element of the pathological mechanism in inflammatory skin diseases.

The roles of MIF in regulating macrophages have been investigated in various tissues. MIF, originally identified as a lymphokine concentrating macrophages at inflammatory sites, is a potent activator of macrophages [16]. MIF has been considered to be exclusively expressed in activated T-lymphocytes and macrophages, and responses to stimuli such as wounds and infection [4,17]. In this study, we described the presence of MIF in human epidermal keratinocytes for the first time. However, the pathophysiological function of MIF in the human skin still remains undefined. Recently, Wistow et al. reported that MIF mRNA expression was correlated with cell differentiation of lens cells [18]. This finding suggests the possibility that MIF produced during the immune reaction and inflammatory processes may cause epidermal hyperplasia since skin epidermal hyperplasia and inflammation are considered to be linked to bioactions of localized cytokines and growth factors [14]. This possibility is supported by the fact that the basal cells of the epidermis are highly proliferative in nature.

In conclusion, the present results suggest that MIF is important in the cytokine network involved in human skin pathogenesis. Lately, MIF was found to be a glucocorticoid-

induced modulator of cytokine production [19]. This indicates that MIF is a critical component of the immune system and acts together with glucocorticoids to regulate immunity and inflammation. Considering this observation together with our present results, it is speculated that MIF may play an important role in cutaneous immunity and the inflammatory process in addition to its role in epidermal cell proliferation and differentiation. Experiments to test this possibility are currently underway.

Acknowledgements: This research was supported by a Grant-in-Aid for Research (no. 07670162) from the Ministry of Education, Science and Culture of Japan, The Akiyama Foundation, and The Ohyama Foundation. We are grateful to T. Kuriyama S. Tone for his technical assistance.

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