Identification and immunohistochemical localization of macrophage migration inhibitory factor in human cornea

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Abstract We identified macrophage migration inhibitory factor (MIF) mRNA expression in human cornea, and demonstrated its immunohistological localization. Reverse transcription-polymerase chain reaction analysis revealed that MIF mRNA was expressed in both the corneal epithelial and endothelial cells. Immunohistochemical study using the polyclonal antibody prepared from immunizing a rabbit with human recombinant MIF showed that MIF was present in the basal cells of corneal epithelium and endothelial cells. The fact that MIF exists in those cells of the cornea indicates that MIF may play an important role in corneal cell immunity and cellular differentiation.

Key words: Macrophage migration inhibitory factor; Corneal epithelium; Corneal endothelium; Immunohistochemistry; Reverse transcription-polymerase chain reaction

1. Introduction

Corneal epithelial cells and endothelial cells have different embryogenic origins, and distinct functions. Corneal epithelial cells are nonkeratinized, stratified squamous cells derived from surface ectoderm [1]. The basal epithelial cells proliferate throughout life, and the progeny cells migrate from the basal layer to the surface [2]. Epithelial cells form a layer protecting the surface of the cornea, and perform a barrier function in the regulation of corneal hydration. In response to wounding, surface defects are healed by proliferation and migration of the epithelial cells. On the other hand, corneal endothelial cells are derived from neural crest cells [3]. Endothelial cells have a limited capacity to proliferate following wounding, and endothelial defects are closed by a combination of cell enlargement and migration.

Several studies have suggested that epidermal growth factor (EGF), basic fibroblast growth factor (basic FGF), and interleukin-1α (IL-1α) can regulate specific functions in corneal epithelial cells [4-6]. EGF and basic FGF have been shown to stimulate proliferation of corneal epithelial cells in culture and to increase the rate of healing of epithelial wounds. On the other hand, there is limited information available concerning factors that might regulate human corneal endothelial cells. Recently it has been reported that basic FGF stimulates corneal endothelial cell growth and wound healing [7].

Macrophage migration inhibitory factor (MIF) was the first lymphokine reported in the guinea pig [8]. It has the ability to prevent the migration of macrophages out of capillary tubes. Human MIF cDNA has been cloned, and it was shown that MIF consists of 114 amino acid residues [9]. 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 [10]. Recently, we cloned rat MIF cDNA, reported its physicochemical properties [11,12], and succeeded in the crystallization of both human and rat MIF [13,14]. Moreover, we presented the tertiary structure of rat MIF at 2.2 Å resolution [15].

During the course of our MIF study, we unexpectedly found that MIF mRNA was expressed in the human cornea. In the present study, we report for the first time that MIF is highly expressed in corneal epithelial and endothelial cells as demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical analysis. The novel localization of MIF may indicate a new biological aspect of MIF.

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); 4-chloro-1-naphthol substrate for horseradish peroxidase from Promega Corp. (Madison, WI, USA); Tween 20 from Kanto (Tokyo, Japan); complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and 3,3'-diaminobenzidine tetrahydrochloride from Wako (Osaka, Japan); alkylacrylates from Merck (Darmstadt, Germany); poly(L-lysine) from Sigma (St. Lous, MO, USA); Vector ABC Kit from Vector Laboratories (Burlingame, CA, USA); Konica immunostaining HRP-1000 from Konica (Tokyo, Japan), and Protein A-Sepharose from Pharmacia (Uppsala, Sweden). All other chemicals used were of analytical grade.

2.2. Preparation of antisera

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 [12]. In brief, the rabbits were inoculated intradermally with 100 μg of MIF emulsified in CFA at weeks 1 and 2, and with 50 μg of MIF in IFA 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. RT-PCR analysis

Two normal corneas and lens specimens were obtained from two patients with malignant orbital tumors after receiving their written informed consent. No apparent inflammation or tumor invasion was observed in the ocular tissues examined in the present study. Quarters of the corneal button and lens were used for RT-PCR analysis without fixation. Total RNAs were extracted from the corneal epithelium, endothelium and lens with an Isogen RNA extraction kit. The corneal epithelium and endothelium were separated from the corneal stroma by fine forceps. The reverse transcription of the RNAs was carried out by M-MLV reverse transcriptase using oligo(dT) primer and subse-

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quent amplification using Taq DNA polymerase. PCR was carried out for 40 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 5′-CTCTCCGAGCT-CACCCAGCAG-3′ (58–78) (forward) and 5′-CGCGTTCATGTCG-TAATAGTT-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.

2.4. Western blot analysis

Half of the remaining corneal button and lens was used for Western blot analysis as described [16]. In brief, the samples were dissolved in 20 μl of Tris-HCl (50 mM, pH 6.8) containing 2-mercaptoethanol (1%), sodium dodecyl sulfate (SDS) (2%), glycerol (20%) and bromophenol blue (BPB) (0.04%), and heated at 100°C for 5 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described [17]. The electrophoresed proteins were transferred onto a nitrocellulose sheet at 50 mA for 1 h using a semi-dry blot transfer apparatus (Bio-Rad). Then the sheet was intensively washed with phosphate-buffered saline (PBS), and incubated with an anti-human MIF polyclonal antibody (1:1000 dilution) for 1 h at room temperature, and reacted with peroxidase-conjugated anti-rabbit IgG serum (1:1000 dilution) for 1 h at room temperature. After the reaction, proteins were visualized with a Konica immunostaining HRP-1000 as recommended in the manufacturer's protocol. Protein concentration was determined with a Micro BCA protein assay reagent kit (Pierce).

2.5. Immunohistochemistry

The remaining quarters of the corneal button and lens were fixed overnight in paraformaldehyde (4%) in (PBS). Then, 8-μm thick frozen sections were cut with a Leica 1720 cryostat (Nussloch, Germany) and mounted on poly(L-lysine)-coated slides. The tissue samples were immersed in 100% methanol containing hydrogen peroxide (0.3%) for 30 min to quench internal peroxidase reactivities. Subsequently, they were stained with an avidin-biotin-peroxidase complex procedure using a Vector ABC Kit according to the manufacturer's protocol. Non-specific staining was blocked by incubation for 30 min with normal goat serum (10%). The sections were further incubated overnight at 4°C with the anti-human MIF polyclonal antibody. After three washes with PBS, the samples were reacted with biotinated 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.

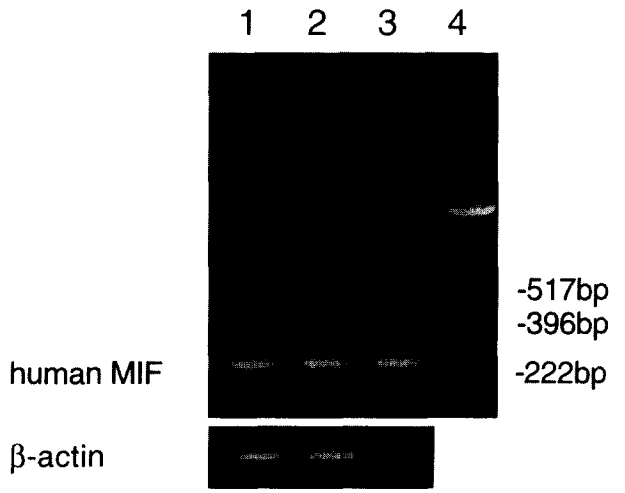

Fig. 1. RT-PCR analysis of MIF mRNA. RT-PCR was carried out as described in section 2. The PCR products were electrophoresed on 2% agarose gel. Lanes: 1, corneal epithelium; 2, corneal endothelium; 3, lens; 4, molecular size marker (pBR322 DNA/*Alu*I). The RT-PCR product of β-actin is shown at the bottom of each lane.

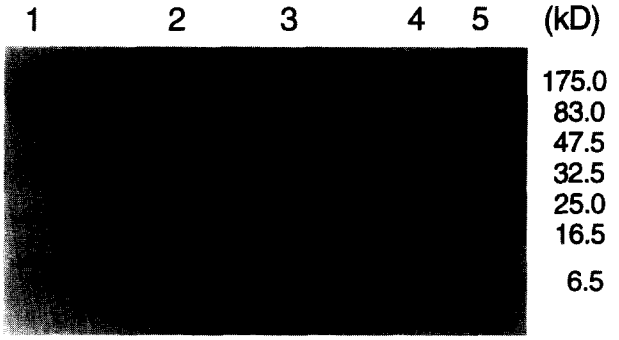

Fig. 2. Western blotting analysis of the corneal epithelium, endothelium, and lens for MIF. Each tissue sample was collected, electrophoresed, transferred to a nitrocellulose membrane, and visualized by Konica immunostaining HRP-1000. Lanes: 1, recombinant human MIF (50 ng); 2, corneal epithelium (40 μg protein); 3, corneal endothelium (50 μg protein); 4, lens (20 μg protein); 5, prestained molecular marker (Biolabs).

3. Results

3.1. RT-PCR

MIF mRNA expression in the human corneal epithelium, endothelium and lens was examined by RT-PCR analysis. At the expected molecular size (255 bp) of MIF RT-PCR products were observed on the agarose gel on the epithelium and endothelium of the cornea and lens (Fig. 1). Even though RT-PCR is not necessarily quantitative, the relative MIF mRNA expression appeared to be greatest in the lens, and lesser in the epithelium and endothelium as assumed from the fluorescence intensities of their PCR products normalized by the intensities of β-actin.

3.2. Western blot analysis

To further confirm the presence of MIF, Western blot analysis was carried out. The tissue samples from the corneal epithelium, endothelium, and lens were electrophoresed on a polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. The transferred protein bands visualized by the immunostaining kit migrated to the corresponding molecular weight of MIF, about 12.5 kDa (Fig. 2). Preimmune rabbit IgG did not react with human MIF in the immunoblot analysis (data not shown). This result indicated that MIF was biosynthesized in each of the ocular tissues.

3.3. Immunohistochemistry

We carried out immunohistochemical analysis for the specific detection of MIF using an anti-human MIF antibody. Positive MIF staining was observed on the basal cell layers of both corneal epithelium (Fig. 3a) and endothelial cells (Fig. 3c). In detail, the cytosols of these cells were strongly and exclusively stained; however, stromal cells did not react well with the antibody. Lens epithelial cells of the equator also showed positive staining (Fig. 3d). In the absence of the anti-human MIF primary antibody or with the addition of an excess amount of recombinant MIF, this staining procedure did not result in any specific staining (Fig. 3b). These facts revealed that MIF was present in the cytosols of human epithelial and endothelial cells as well as that of lens cells.

4. Discussion

In the present study, we demonstrated for the first time that MIF was expressed in human corneal epithelial and endothelial cells by RT-PCR, and the presence of MIF was further confirmed by Western blot and immunohistochemical analyses. MIF was considered to be largely expressed in T-lymphocytes and macrophages. A major problem for research in this area has been the limited availability of human tissue and lack of an appropriate animal model. To solve this problem, PCR was effectively used to demonstrate the presence of MIF in human corneal epithelial and endothelial cells. This procedure revealed that MIF mRNA was expressed in human corneal epithelial and endothelial cells. This fact may provide novel information on the physiological functions of MIF.

Recently, the essential roles of growth factors in regulating cells have been shown in various tissues. They contribute to normal homeostasis and responses to stimuli such as wounding or infection. As for the physiology of the human eye, the endogenous production of growth factors by human corneal epithelial and endothelial cells has been reported [6]. It has been demonstrated that EGF, and basic EGF can modulate corneal epithelial cell proliferation and migration [4,5]. Wistow et al. recently described MIF mRNA expression in differentiating chicken lens cells, and reported that this correlated with cell differentiation [18]. Here, we demonstrated the positive immunostaining of MIF for differentiating human lens cells, in particular around the equatorial epithelium (Fig. 3d). The immunohistological results obtained on the human lens are consistent with their findings.

On the other hand, MIF in the human cornea has not been investigated. The present study demonstrated that MIF mRNA was expressed in normal corneal epithelium and endothelium (Fig. 1). This constant expression of MIF mRNA suggests the possibility that the protein plays an important role in the maintenance of cellular functions. Moreover, MIF may possibly contribute to differentiation of the epithelial cells, because the basal cells of the corneal epithelium are considered to have a similar potential for differentiation, as in lens equatorial epithelium. Taken together, these results suggest that MIF is important in the cytokine network for the differentiation of the human cornea.

MIF, originally identified as a lymphokine concentrating macrophages at inflammatory sites, is a potent activator of macrophages [19]. Langerhans cells are known to be present as unique inflammation-related cells in cornea. It is often observed that Langerhans cells, antigen-presenting cells of macrophage origin in the cornea, migrate toward the central region during inflammation [20]. Considering these facts, MIF may play a critical role in enhancement of Langerhans cell migration in the case of corneal inflammation. Furthermore, MIF may contribute to the formation of the keratic precipitate in granulomatous uveitis, where macrophages tend to adhere to each other and to the corneal endothelium to form large precipitates [21]. It is widely accepted that leakage of cellular proinflammatory proteins from the injured cornea enhances ocular inflammation.

Although we discovered the presence of MIF in human corneal epithelial and endothelial cells, the essential function of MIF in ocular regions remains ambiguous. Recently, MIF was found to be a glucocorticoid induced modulator of cytokine production [22], indicating that MIF is a critical compo-

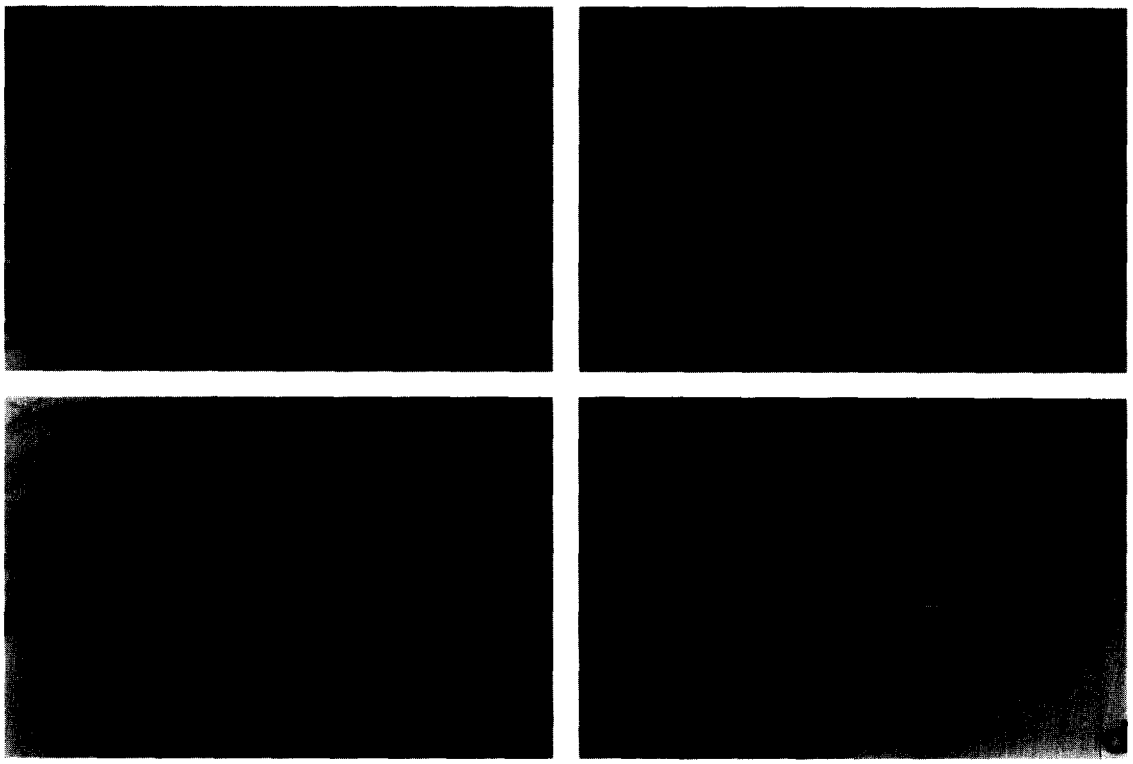

Fig. 3. Immunohistochemical study of MIF in the human corneal epithelium, endothelium, and lens. Tissue preparation and immunohistochemical staining were carried out as described in section 2. (a) Corneal epithelium (×60); (b) control tissue specimen reacted with pre-immune rabbit IgG (×60); (c) endothelium (×60); (d) lens epithelium (×60). En, Ep, and C denote the corneal endothelium, lens epithelium, and lens capsule, respectively.

nent of the immune system and acts together with glucocorticoids to regulate immunity and inflammation. Considering this fact, it is speculated that MIF may play an important role in corneal cell immunity, its inflammatory process, and possibly cell proliferation. Further in vivo and in vitro investigations are underway to elucidate the pathophysiological roles of MIF.

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References

[1] Snell, R.S. and Lemp, M.A. (1989) in: Clinical Anatomy of the Eye, pp. 128–134, Blackwell, Boston.
[2] Pepose, J.S. and Ubels, J.L. (1992) in: Adler's Physiology of the Eye, vol. 9, (Hart, W.M., Jr. ed.) pp. 29–70, Mosby Year Book, St. Louis.
[3] Maurice D.M. (1984) in: The Eye, vol. 3 (Davson, H. ed.) pp 1–129, Academic Press, Orlando, FL.
[4] Gospodarowicz, D., Mescher A.L., Brown, K.D. and Birdwell, C.R. (1977) Exp. Eye Res. 25, 631–649.
[5] Boisjoly, H.M., Laplante, C., Bernatchetz, S.F., Salesse, C., Giasson, M. and Joly, M.C. (1993) Exp. Eye Res. 57, 293–300.
[6] Li, D.Q. and Tseng, S.C.G. (1995) J. Cell. Physiol. 163, 61–79.
[7] Hoppenreijs, V.P.T., Pels, E., Vrensen, G.F.J.M. and Treffers, W.F. (1994) Invest. Ophthalmol. Vis. Sci. 35, 931–944.
[8] Bloom, B.R. and Bennett, B. (1966) Science 153, 80–82.
[9] Weiser, W.Y., Temple, P.A., Witek-Giannotti, J.S., Remold, H.G., Clark, S.C. and David, J.R. (1989) Proc. Natl. Acad. Sci. USA 86, 7522–7526.
[10] Calandra, T., Bernhagen, J., Mitchell, R.A. and Bucala, R. (1994) J. Exp. Med. 179, 1895–1902.
[11] Nishihira, J., Kuriyama, T., Sakai, M., Nishi, S., Ohki, S. and Hikichi, K. (1995) Biochim. Biophys. Acta 1247, 159–162.
[12] Nishihira, J., Kuriyama, T., Nishino, H., Ishibashi, T., Sakai, M. and Nishi, S. (1993) Biochem. Mol. Biol. Int. 31, 841–850.
[13] Suzuki, M., Murata, E., Tanaka, I., Nishihira, J. and Sakai, M. (1994) J. Mol. Biol. 235, 1141–1143.
[14] Sugimoto, H., Suzuki, M., Nakagawa, A., Tanaka, I., Fujinaga, M. and Nishihira, J. (1995) J. Struct. Biol. 115, 331–334.
[15] Suzuki, M., Sugimoto, H., Nakagawa, A., Tanaka, I., Nishihira, J. and Sakai, M. (1996) Nature (Struct. Biol.), in press.
[16] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[17] Schagger, H. and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[18] Wistow, G.J., Shaughenessy, M.P., Lee, D.C., Hodin, D. and Zelenka, P.S. (1993) Proc. Natl. Acad. Sci. USA 90, 1272–1275.
[19] Weiser, W.Y., Pozzi, L.M. and David, J.R. (1991) J. Immunol. 147, 2006–2011.
[20] Tagawa, Y., Takeuchi, T., Saga, T., Matsuda, H. and Silverstein, A.M. (1985) in: Advances in Immunology and Immunopathology of the Eye (O'Connor, R.G. and Chandler, J.W. eds.) pp. 203–207, Masson, New York.
[21] Hogan, M.J., Kimura, S.J. and Thygeson, P. (1959) Am. J. Ophthalmol. 47, 155–170.
[22] Calandra, T., Bernhagen, J., Mets, C.N., Spiegel, L. A, Bacher, M., Donnelly, T., Cerami, A. and Bucala, R. (1995) Nature 377, 68–71.