Cutting Edge: Role of Macrophage Migration Inhibitory Factor in Inhibiting NK Cell Activity and Preserving Immune Privilege

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*J Immunol* 1998; 160:5693-5696; 
http://www.jimmunol.org/content/160/12/5693

**References**

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The absence of MHC class I Ags on the corneal endothelium, which lines the anterior chamber of the eye, makes this cell layer potentially vulnerable to lysis by NK cells. However, aqueous humor (AH), which bathes the corneal endothelium, contains a 12-kDa protein which inhibits the NK-mediated lysis of corneal endothelial cells. An amino acid sequence analysis of AH revealed that this factor shared >90% homology with macrophage migration inhibitory factor (MIF). The NK inhibitory effect of AH was neutralized with anti-human MIF Ab. Moreover, mouse rMIF produced a similar inhibition of NK cell activity. However, neither rMIF nor AH inhibited the CTL-mediated lysis of allogeneic cells. rMIF prevented the release of perforin granules by NK cells but not CTLs. Although MIF displays proinflammatory properties, these results indicate that it can also inhibit at least one immune effector element, NK cells, and thereby contribute to immune privilege in the eye.


Materials and Methods

**Aqueous humor**

AH was obtained from rabbit and mouse eyes by paracentesis as described previously (4).

**Mouse rMIF**

MIF belongs to a superfamily of small isomerases that are found in a wide variety of tissues, including the lens, cornea, brain, and pituitary gland (8–11). RT-PCR of mouse lens RNA was used to amplify the coding sequence for mouse MIF using primers 5'-TCCGCCCATATGCCTATGTCTACGTGAACACC and 3'-AGCGGTTGGATCCAAATGGGGCCACAGACTCAAAGC, which incorporated the NdeI (5') and BamHI (3') restriction sites. The cDNA was initially cloned into the pCRII vector (Invitrogen, San Diego, CA), sequenced, and then subcloned into the NdeI and BamHI sites of the pET-17b vector (Novagen, Madison, WI). Following the manufacturer's protocols, protein was expressed in pLyS3 cells and induced with 0.4 mM isopropyl-β-D-thiogalactoside for 3 h at 25°C. MIF protein was isolated by ion-exchange chromatography on High Performance Q-Sepharose (Pharmacia Biotech, Piscataway, NJ) and by gel filtration using a Superdex 75pg column (Pharmacia).
NK cell culture and cytotoxicity assays

Lymphokine-activated killer cells, which are highly enriched for NK cells, were prepared from C57BL/6 (H-2b) and C3H/HeJ (H-2k) mice as described elsewhere and used in a conventional 4-h 51Cr release assay (12). Neutralization of the NK inhibitory effect of AH was performed by incubating rabbit AH with polyclonal anti-human MIF IgG Ab (600 μg/ml; R&D Systems, Minneapolis, MN) for 1 h at 4°C. Controls consisted of rabbit AH treated in the same manner with normal goat IgG (600 μg/ml; Organon Teknika, West Chester, PA).

Corneal endothelial cell cultures and cytotoxicity assay

Mouse corneal endothelial cells are terminally differentiated and cannot undergo mitosis. Therefore, long term mouse corneal endothelial cell lines were established from C3H (H-2k) mice by transducing freshly isolated cells with the human papilloma virus genes E6 and E7 (13). These cells proliferate indefinitely while maintaining their original morphologic characteristics (13).

MIF assays

MIF activity was analyzed in a conventional capillary tube assay (14). The average area of macrophage migration (mm²) was calculated for each group by image analysis. Data are represented as the percentage of inhibition of macrophage migration after 24 h as compared with control medium. The percentage of inhibition of migration was calculated using the following formula: ([control migration – experimental migration] ÷ control migration) × 100.

MIF in rabbit and mouse AH was quantified using a direct competition ELISA as described previously (15). Mouse rMIF was used as the standard, and goat anti-human MIF Ab (IgG; R&D Systems) was used as the primary Ab. Rabbit anti-goat IgG conjugated with horseradish peroxidase (Accurate Chemical and Scientific, Westbury, NY) served as the secondary Ab.

Granule exocytosis assay

The release of sodium benzoyloxy-carbonyl-L-lysine-thiobenzylester-esterase from NK cells was determined using a modified method of Green and Shaw (16). One group of cells was frozen and thawed twice to obtain maximum granule release. Granule release was calculated according to the following formula: percentage of granule release = (experimental reading – background reading) ÷ (maximum reading – background reading) × 100.

Generation of allospecific CTLs

Allospecific CTLs were generated in vitro by incubating C57BL/6 (H-2b) spleen cells with γ-irradiated (3000 cGy) P815 mastocytoma (DBA/2; H-2b) stimulator cells for 5 days at 37°C. In vitro-primed C57BL/6 spleen cells were washed and used as effector cells in a 4-h 51Cr release assay using radiolabeled P815 cells as described previously (4). Cytotoxicity assays were performed in the presence of either MIF (10 μg/ml) or DMEM alone. Naive controls consisted of freshly isolated, unstimulated C57BL/6 spleen cells. The E:T ratio was 100:1.

Results

Partial aa sequence of NK cell-inhibitory factor

Rabbit AH was subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, stained with Coomassie blue, and subjected to tryptic digestion before HPLC microsequencing. The 14-aa internal sequence obtained was subjected to a BLAST net-work database search (National Center of Biotechnology Information, Bethesda, MD). Table I shows the strong sequence homology between the rabbit AH sequence and MIF (aa 95–108) sequences from various other species. This homology permitted us to confirm the presence of MIF in the AH from various species using anti-human MIF Ab. In multiple ELISAs, MIF was detected in mouse, rat, rabbit, cow, horse, and human AH (data not shown). Rabbit and mouse AH contained 8.6 ± 1.84 μg/dl and 5.4 ± 1.41 μg/dl of MIF, respectively. Moreover, we have also confirmed the presence of MIF in mouse and rat AH by Western blot analysis (data not shown). These findings are in agreement with the recent results of Matsuda et al. who detected MIF in cells lining the AC and in the AH of rats (9, 17).

Macrophage migration inhibitory activity of AH

MIF was originally defined by the capacity of supernatants from activated lymphocytes to prevent the random migration of macrophages (18). Additional experiments were performed to confirm that the 12-kDa protein in AH possessed functional MIF activity using a conventional macrophage migration inhibition assay (14). The results shown in Figure 1 demonstrate that AH and functional

Table I. Partial aa sequence of the 12.5-kDa NK cell-inhibitory factor in rabbit AH

<table>
<thead>
<tr>
<th>MIF Source</th>
<th>aa Sequence 95–108</th>
</tr>
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<tbody>
<tr>
<td>Mouse MIF</td>
<td>VYINYYDMNAANVG</td>
</tr>
<tr>
<td>Rat MIF</td>
<td>VYINYYDMNAANVG</td>
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</tr>
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</tr>
<tr>
<td>12.5-kDa rabbit AH protein</td>
<td>VYINYYDMNAANVG</td>
</tr>
</tbody>
</table>

* Underline represents the difference in aa sequence at that position upon comparison with mouse MIF.
rMIF significantly inhibited macrophage migration as compared with control medium. The MIF activity of AH was comparable with that shown by 10 μg/ml of wild-type murine rMIF.

Inhibition of NK-mediated cytosis by murine rMIF

If the NK inhibitory effect of AH was attributable to MIF, it should be possible to produce a similar inhibition of NK cell activity with murine rMIF. This possibility was tested using YAC-1 lymphoma target cells and lymphokine-activated killer cells as an enriched source of NK cells. rMIF showed a significant dose-dependent inhibition of the NK cell-mediated cytosis of YAC-1 cells (Fig. 2A). To test the physiologic relevance of these findings in the context of immune privilege, MIF was assayed for its ability to inhibit the NK cell-mediated lysis of syngeneic C3H corneal endothelial cells. The results show that rMIF produced a profound dose-dependent inhibition of the NK cell-mediated lysis of syngeneic corneal endothelial cells (Fig. 2B).

Neutralization of AH inhibitory activity with anti-MIF antiserum

To confirm that the inhibitory activity of AH was solely attributable to MIF, AH was treated with anti-MIF antiserum before use in NK cytolysis assays. Treatment with polyclonal goat anti-human MIF Ab almost completely eliminated the AH-mediated inhibition of NK cell cytotoxicity, while an isotype-matched control Ab had no effect on the AH-mediated inhibition of NK cytolyis (Fig. 4).

**Effect of AH on NK cell viability and cytotoxic granule release**

The capacity of MIF to produce an immediate and profound inhibition of NK cell-mediated cytosis suggests that MIF either kills NK cells or blocks their cytolytic function (i.e., the release of...
NK cells but not by CTLs (Fig. 5). This resulted in a marked inhibition of perforin granule exocytosis by treated and control medium groups. By contrast, exposure to MIF induced NK cell apoptosis (1% NK cell apoptosis in both AH-treated and control medium groups). Moreover, an assessment of apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling demonstrated that MIF did not induce NK cell apoptosis (<1% NK cell apoptosis in both AH-treated and control medium groups). By contrast, exposure to MIF resulted in a marked inhibition of perforin granule exocytosis by NK cells but not by CTLs (Fig. 5).

Discussion

The present results indicate that MIF is present in biologically significant concentrations in the AH and produces an immediate inhibition of the NK cell-mediated cytolyis of both neoplastic and normal target cells. MIF is not directly toxic to NK cells but appears to exert its inhibitory effects by impairing the release of cytolytic perforin granules from NK cells. As yet, we have no explanation as to why MIF does not inhibit the CTL-mediated cytolyis of allogenic target cells or affect perforin release by allspecific CTLs. It is noteworthy that AH contains two cytokines with immunosuppressive characteristics. However, unlike other AH-borne cytokines, such as TGF-β, the immunosuppressive effects of MIF are restricted to NK cells. The immediate suppression of NK cell-mediated lysis produced by MIF is crucial, because even minimal damage to the nonregenerative corneal endothelium can lead to blindness. The lens also fails to express MHC class I determinants; as such, it is theoretically vulnerable to NK cell-mediated injury. Immune damage to the lens would lead to cataract formation. However, the lens forms the posterior boundary of the AC and is bathed in AH. As a result, the lens is protected from NK cell-mediated damage.

The present study adds to a growing body of evidence indicating that MIF is ubiquitous and pleiotropic. The ability of MIF to produce an immediate and profound inhibition of NK cell-mediated lysis demonstrates for the first time that MIF can also function as an immunosuppressive cytokine.

References