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Anatomical location of Macrophage Migration Inhibitory Factor in urogenital tissues, peripheral ganglia and lumbosacral spinal cord of the rat

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Abstract

Background: Previous work suggested that macrophage migration inhibitory factor (MIF) may be involved in bladder inflammation. Therefore, the location of MIF was determined immunohistochemically in the bladder, prostate, major pelvic ganglia, sympathetic chain, the L6-S1 dorsal root ganglia (DRG) and the lumbosacral spinal cord of the rat.

Results: In the pelvic organs, MIF immunostaining was prominent in the epithelia. MIF was widely present in neurons in the MPG and the sympathetic chain. Some of those neurons also co-localized tyrosine hydroxylase (TH). In the DRGs, some of the neurons that stained for MIF also stained for Substance P. In the lumbosacral spinal cord, MIF immunostaining was observed in the white mater, the dorsal horn, the intermediolateral region and in the area around the central canal. Many cells were intensely stained for MIF and glial fibrillary acidic protein (GFAP) suggesting they were glial cells. However, some cells in the lumbosacral dorsal horn were MIF positive, GFAP negative cells suggestive of neurons.

Conclusions: Therefore, MIF, a pro-inflammatory cytokine, is localized to pelvic organs and also in neurons of the peripheral and central nervous tissues that innervate those organs. Changes in MIF's expression at the end organ and at peripheral and central nervous system sites suggest that MIF is involved in pelvic viscera inflammation and may act at several levels to promote inflammatory changes.

Background

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that may play a role in several disease states, including arthritis, sepsis, Crohn's disease, allograft rejection, asthma and prostate cancer [1-3]. Although MIF was initially described as released from activated T-cells [4,5], subsequent investigations have localized MIF in a variety of other cells and tissues (for a recent

review [1]). For example, pre-formed MIF stores can be found in the epithelia of several organs and may be released in response to endotoxemia [6]. The role of MIF in the central nervous system remains to be determined, yet neurons in the brain synthesize and rapidly release MIF while glial cells appear to store MIF [7,8], and the anterior pituitary gland contains MIF that may be released during endotoxemia [9].

Recent evidence from our laboratory showed that MIF is located in the bladder of the rat and also in peripheral ganglia innervating the bladder and other pelvic viscera. The urothelium contains a rich store of pre-formed MIF [10,11]. Moreover, MIF protein and mRNA were also detected by Western blotting techniques and RT-PCR in the major pelvic ganglia (MPG) that provides efferent (parasympathetic and sympathetic) innervation to the bladder and other pelvic viscera [12,13]. In addition, the L6-S1 dorsal root ganglia (DRG) that provide afferent innervation to the bladder [13] and other pelvic viscera constitutively expressed MIF. Finally, the lumbosacral spinal cord of the rat where afferent and efferent information is integrated to result in effective pelvic viscera function [14], was also shown to contain MIF protein and mRNA.

Acute bladder inflammation induced by local (intravesical) administration of chemical irritants or lipopolysaccharide altered the levels of this cytokine in the bladder suggesting that MIF may be implicated in the early stages of bladder inflammation [10,11]. In addition, there were changes in the levels of MIF and cyclooxygenase-2 in the central and peripheral nervous tissues that innervate the bladder and other pelvic viscera. Therefore, in addition to the local inflammatory effect, MIF may also be involved in neurogenically mediated changes at both central and peripheral nervous system sites following visceral inflammation.

Our previous studies reported the anatomical localization for MIF only in the bladder, while the location of MIF in the peripheral ganglia (MPG; DRG) and in the lumbosacral spinal cord was not determined since the focus was on the change in MIF amounts in these tissues following bladder inflammation [10,11]. Therefore, as part of our ongoing investigations on the role of MIF in pelvic viscera inflammation, we investigated the location of MIF in two pelvic organs (bladder and prostate) and in peripheral ganglia that innervate these viscera using immunohistochemistry. We examined the major pelvic ganglia, where the parasympathetic and sympathetic neurons that innervate the bladder and the prostate are located. Also, we investigated the inferior mesenteric ganglia and the lumbar sympathetic chain that provide sympathetic innervation to the bladder and other pelvic viscera [13]. We also determined the location of MIF in the L6-S1 dorsal root ganglia that provide afferent innervation to these organs. Finally, we localized MIF within the lumbosacral spinal cord since the spinal cord integrates afferent and efferent information from the pelvic viscera [14].

Results

MIF in the bladder and the prostate

Intense MIF immunoreactivity in the urothelium was observed in the bladders of both male and female rats,

confirming our previous observations [11]. The staining was most intense in the basal and intermediate layers of the urothelium (Fig. 1A,1B). The superficial layer of the urothelium showed slight MIF immunostaining but also showed areas that appeared clear of MIF staining (Fig 1B). In addition, the fibroblasts in the lamina propria, immediately under the urothelium also showed MIF immunoreactivity (Fig 1B). Finally, MIF immunoreactivity was also observed in the smooth muscle in the bladder, confirming our previous findings. Some nuclei in smooth muscle fibers appeared to stain with MIF and weak, diffuse staining was also observed in the cytoplasm of smooth muscle fiber cells (Fig 1C).

In the prostate, MIF immunostaining was observed in the acini (Fig 1D,1E,1F) with the majority of the MIF staining localized to acinar epithelial cells. Occasionally, proximal acini, following the classification of Lee et al. [15], could be observed in some sections (Fig 1D,1E). Proximal acini could be distinguished from peripheral and central acini by the cuboidal (as opposed to columnar) epithelial cells and by the presence of surrounding smooth muscle layers. The MIF staining in the proximal acini was more intense than in the central acini where the MIF staining was limited to basal epithelial cells (Fig 1F). No immunofluorescence was observed in tissue sections exposed to preabsorbed antisera or where the primary antiserum had been omitted (data not shown).

The concentration of MIF in the bladder and the prostate was also determined using ELISA and expressed as ng of MIF protein/mg of total protein. In male rats, the bladder had a mean MIF protein concentration of 200.8 (\pm 5.75) ng/mg, whereas in the female bladder the MIF protein concentration was 67.3 (\pm 13) ng/mg. This difference was statistically significant ($p < 0.05$). The MIF protein concentration in the ventral lobe of the prostate was 144.1 (\pm 28.65) ng/mg.

MIF in the Major Pelvic Ganglia, Inferior Mesenteric Ganglia and Sympathetic Chain

MIF staining was observed in cells of the MPG of both males and female rats. The staining intensity varied with some cells showing intense staining (Fig 2A) and most cells showing moderate to weak staining. Some of the more intensely stained MIF containing cells also stained for TH (Fig. 2B). Thus, MIF staining was observed in sympathetic and parasympathetic cells of the MPG.

Neurons in the inferior mesenteric ganglia also displayed MIF immunofluorescence. Cells showing immunofluorescence for both MIF and TH were also observed (Fig 2C,2D). However, the MIF staining in the inferior mesenteric ganglion appeared less intense overall than in the MPG. Small intensely fluorescent (SIF) cells, intensely

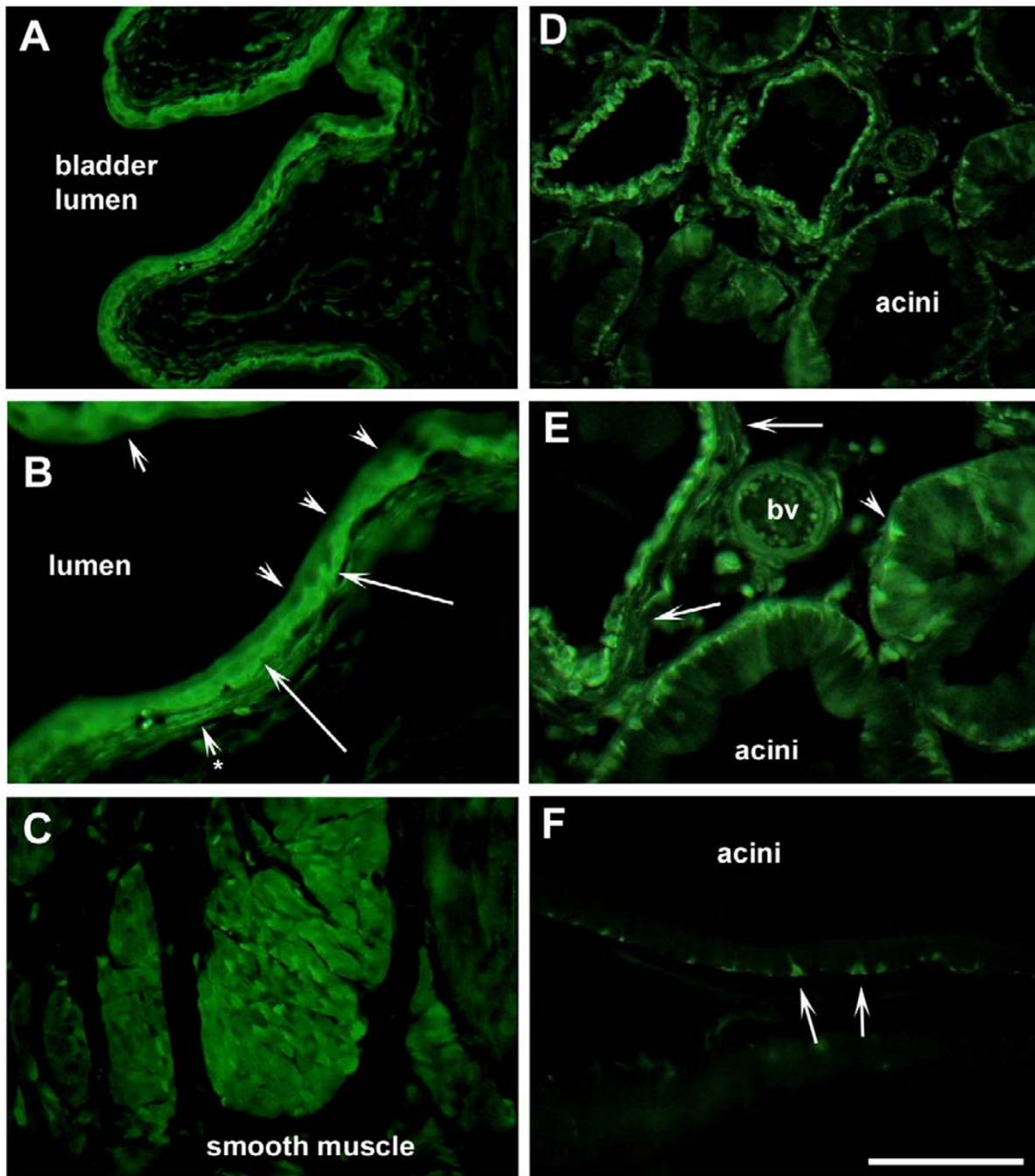


Figure 1

MIF immunohistochemistry in the bladder and prostate. The bladder showed strong MIF immunostaining in the urothelium (Fig 1A, 1B). The superficial layer of the urothelium had weak MIF immunostaining with some areas devoid of stain (Fig 1B; arrowheads), whereas the deeper layers of the urothelium showed intense MIF staining (long arrows). The fibroblasts immediately under the urothelium also exhibited MIF staining (arrow with asterisk). The smooth muscle of the bladder also showed MIF immunostaining (Fig. 1C) in the cytoplasm of muscle fibers and in nuclei. In the prostate, intense MIF immunostaining was observed in the acini (Fig 1D, 1E, 1F). Epithelial cells in the proximal acini showed strong MIF immunostaining (Fig 1D, 1E) and the smooth muscle surrounding those acini also showed MIF immunostaining (Fig 1E; long arrows). However, in central and peripheral acini, only basal epithelial cells showed MIF immunostaining (Fig 1E, arrowhead; F, long arrows). bv = blood vessel. Calibration bar: A, D = 200 μ m; B, C, E, F = 100 μ m.

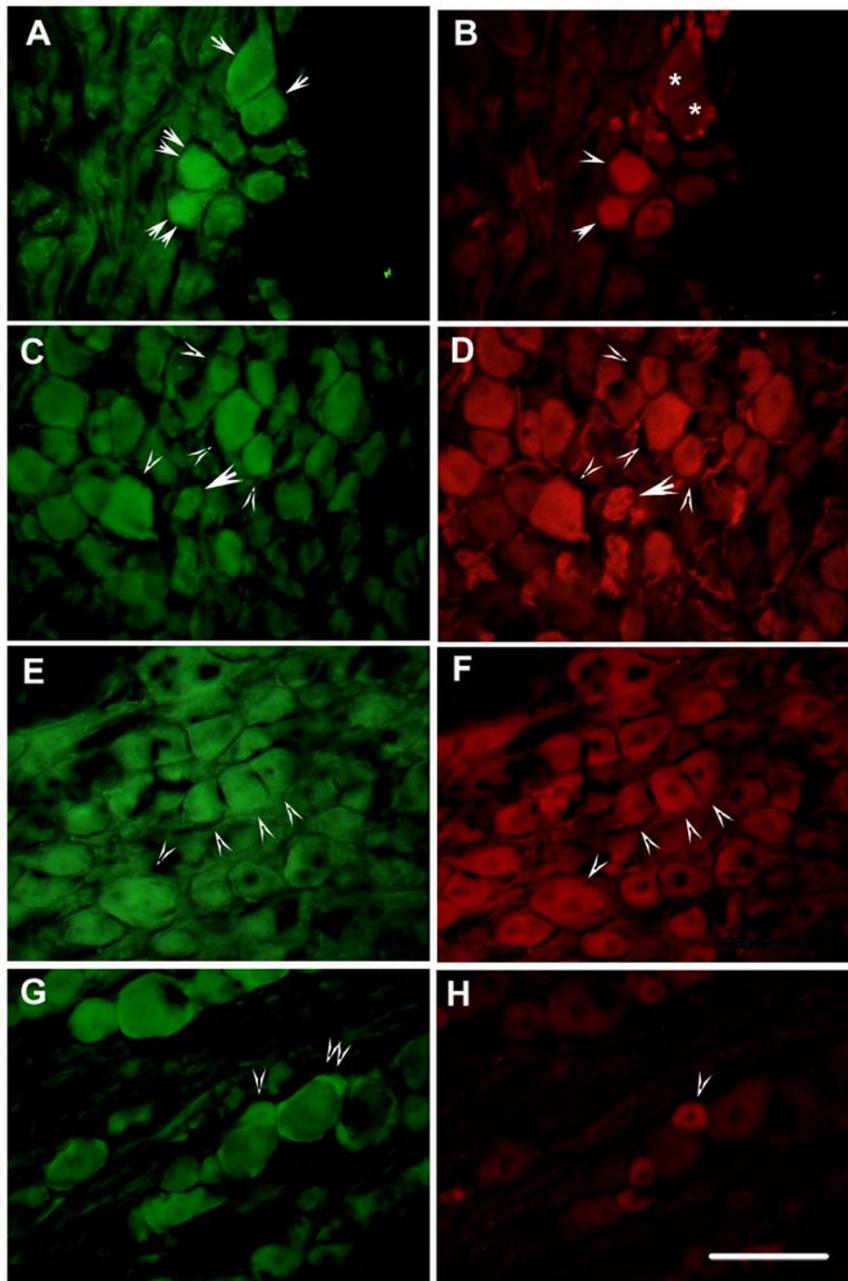


Figure 2

MIF immunohistochemistry in the peripheral ganglia of the rat. Neurons in the MPG showed weak to moderate MIF immunostaining (Fig 2A). Some of these neurons also co-contained TH (Fig. 2B). Double arrows illustrate neurons that contained MIF and also contained TH, marked in panel 2B with single arrows. However, some of the neurons that were stained with MIF (Single arrow) were not TH-positive (* in 2B). Therefore, both sympathetic and parasympathetic neurons in the MPG contained MIF. MIF was detected in neurons in the IMG (Fig 2C) and in neurons in the lumbar sympathetic chain (Fig 2E). Arrowheads indicate neurons that were identified as containing MIF and TH (Fig 2D,2F) in the IMG and the sympathetic chain. SIF cells in the IMG (large arrowhead) also contained MIF and TH (Fig. 2C,2D). Many of the neurons in the L6/S1 DRG stained for MIF. Weak to moderate immunostaining was observed in the cytoplasm of the neurons (Fig. 2G). Arrows illustrate some of the more intensely stained MIF containing neurons that also contained substance P (Figure 2H) In addition, the satellite cells surrounding the neurons were intensely stained for MIF. One such example is marked with double arrowheads in Fig. 2G, however, other examples are visible but not marked. Calibration bar = 100 μ m.

stained with TH, showed little to moderate MIF staining in the IMG (Fig. 2C) and MPG. Finally, nearly every cell in the sympathetic chain showed some degree of MIF immunoreactivity. More intensely staining cells could be located that also stained for TH (Fig. 2E,2F).

MIF in the dorsal root ganglia

MIF immunoreactivity was observed over most of the neurons in the L6/S1 DRG of rats of either sex. Some cells appeared intensely stained (Fig 2G) and some of these intensely stained MIF-containing neurons also stained for substance P (Fig. 2H). Many of the DRG neurons showed weak MIF immunostaining. The satellite cells surrounding the DRG neurons, however, were intensely stained for MIF.

Control sections that were exposed to both the MIF blocking peptide and the antiserum or those that did not receive primary antiserum to MIF, did not show any immunoreactivity (data not shown). Furthermore, in sections where one of the antisera (either MIF or TH/SP) had been omitted, but still processed for double immunofluorescence, only the appropriate immunoreactivity was observed, indicating that the secondary antibodies were not cross reacting to produce spurious labeling (data not shown).

MIF in the lumbosacral spinal cord

In the L6-S1 spinal cord, many cells were immunostained with MIF antisera. Intensely stained cells were observed throughout the white matter (Fig 3A). In addition, some intensely stained and some weakly stained cells were observed in the dorsal horn (Fig 3B). The dorsal horn also showed diffuse staining that could not be associated with particular cells but was limited to the dorsal horn and did not extend to the intermediate grey matter (Fig 3B).

In the intermediate gray matter, weakly staining MIF positive cells were located in the region around the central canal and in the IML region (Fig 3C,3D). The ventral horns and particularly the motor neurons were devoid of MIF immunostaining (data not shown).

In order to establish if the MIF stained cells were neurons or glia, we employed double-immunofluorescence with MIF and GFAP. All the MIF staining cells in the white matter also showed GFAP staining (Fig 4A), as would be expected. In the dorsal horn, although the great majority of the MIF cells also localized GFAP, indicating they were glial in origin, some cells also showed only MIF staining without GFAP suggesting that MIF could also be localized in neurons (Fig 4B,4C). In the IML region, only double-labeled cells were observed, suggesting that only glial cells containing MIF were detected in the IML region (Fig 4D).

Discussion

Results in the present study revealed the location of MIF protein in the bladder and prostate of the rat and in the central and peripheral nervous system tissues that innervate these organs.

MIF was localized mainly to epithelia of the bladder and the prostate. In the bladder, a relatively strong MIF staining was observed in the basal and intermediate layers of the urothelium of male and female rats confirming and extending our recent findings in male rats [10,11]. The superficial layer of the urothelium, on the other hand, contained relatively weak staining and areas devoid of MIF staining were often encountered. A similar pattern of MIF immunostaining was described for human epidermis with the basal layer displaying strong MIF staining and weaker staining in the more superficial layers [16]. MIF staining was also observed in the acini of the ventral lobe of the prostate. In the central acini, the labeling was limited to basal epithelial cells while columnar epithelial cells appeared devoid of MIF immunofluorescence. Proximal acini, however, showed intense staining in the cuboidal epithelia. These differences in epithelial MIF staining within the bladder and the prostate suggest a change in MIF synthesis as epithelia undergo differentiation as has been reported for the eye lens [17]. Basal cells in the urothelium undergo proliferation to give rise to the intermediate and superficial layers [18] and MIF expression was correlated with cell differentiation in the lens [17]. Therefore, it is possible that MIF is involved in cell differentiation in the urothelium and in other organs, as suggested by earlier investigations [16,17]. Alternatively, since the present study did not visualize MIF mRNA using *in situ* hybridization, it is possible that cells in the superficial layer of the urothelium synthesize MIF but release it more quickly than deeper epithelial cells.

In addition to the epithelial staining, we also observed significant MIF staining in the bladder smooth muscle cells with some fibers showing both cytoplasmic and nuclear staining. Weak MIF staining of the smooth muscle fibers around proximal acini was also observed. Other investigators have also reported MIF immunostaining in smooth muscle [6,19]. Fibroblasts in the lamina propria of the bladder were also stained for MIF. We observed a difference in the total bladder MIF protein content between male and female rats, with male rats having considerably more MIF protein. We were unable to locate reports of sex differences in MIF content in the literature. These preliminary findings are interesting but the physiological significance, if any, remains unclear at this point.

Previously, MIF was described in the epithelia of several organs of the rat [6]. The present findings provide anatomical evidence of MIF protein in the bladder and

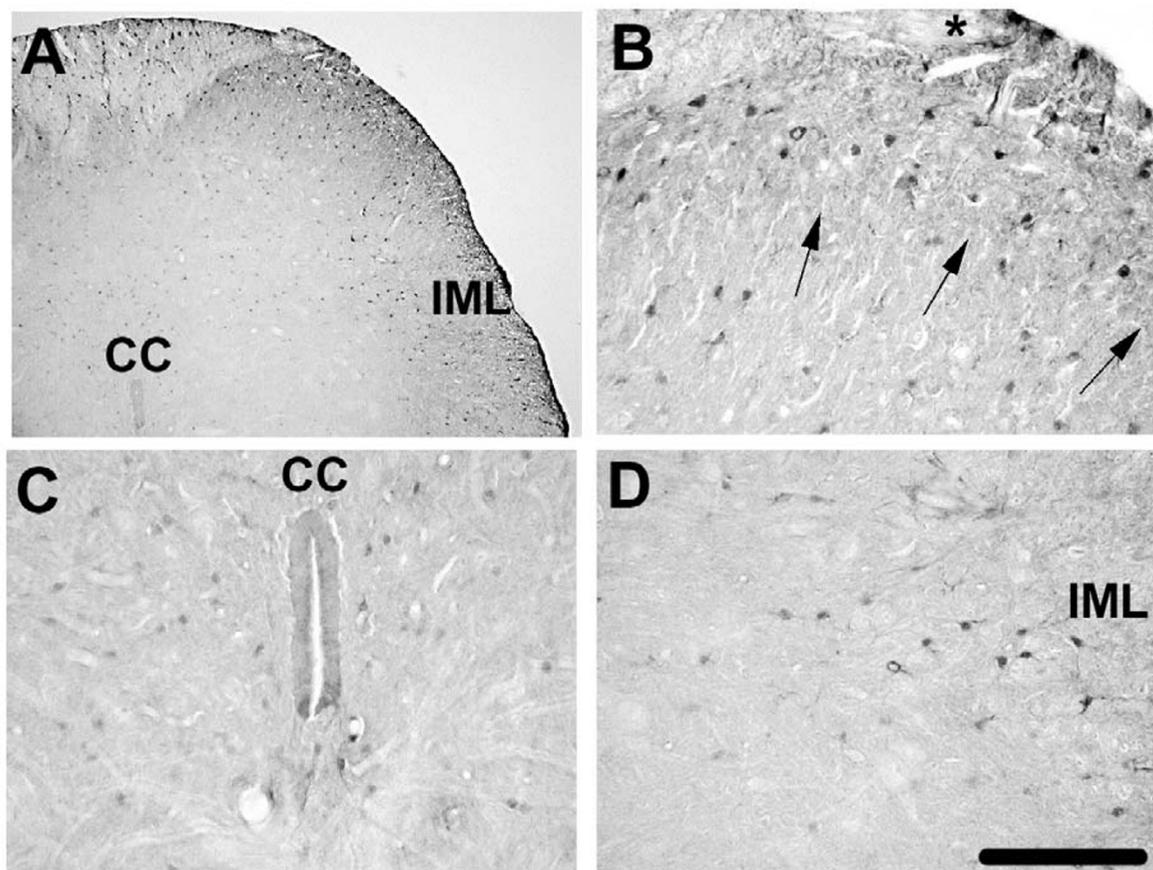


Figure 3

MIF immunohistochemistry in the lumbosacral spinal cord. A) Low power view of a section processed for MIF PAP. Many intensely labeled cells are observed in the white mater, the dorsal horn and in the IML region. Diffuse staining was observed in the dorsal horn. Moderately stained cells can be seen throughout the intermediate grey and in the area around the central canal. B) Higher magnification of the area of the dorsal horn showing labeled profiles. In addition, the dorsal root entry zone (marked with an asterisk) also appears labeled with MIF. Diffuse staining over the dorsal horn is marked by the arrows. C) Weak to moderately stained cells in the around the central canal. D) MIF stained cells were also observed in the intermediolateral cell region (IML), a region that contains sacral parasympathetic preganglionic neurons and interneurons. Calibration bar, A = 400 μ m; B-D = 100 μ m.

prostate epithelia of the rat and confirm our earlier findings demonstrating MIF expression in the bladder by immunohistochemistry, Western blotting analysis and RT-PCR [10, 11]. In other organs, the MIF stored in the epithelia was released upon endotoxemia [7]. As such, MIF may form part of the innate immune response of different organs to bacterial infection.

The exact role of MIF in the bladder and/or the prostate of the rat is under investigation. Previously, we had shown that a chemical cystitis model (intravesical hydrochloric

acid) decreased the overall MIF protein content in the bladder. This effect was likely due to epithelial denudation. However, histological evidence showed increased MIF staining over smooth muscle fiber bundles, perhaps indicating a role for MIF in the early stages of inflammation. Furthermore, intravesical LPS (another commonly used cystitis model in rodents) actually increased total bladder MIF protein and mRNA content [10]. Therefore, it is possible that MIF may be involved in the early responses of the bladder to inflammatory stimuli.

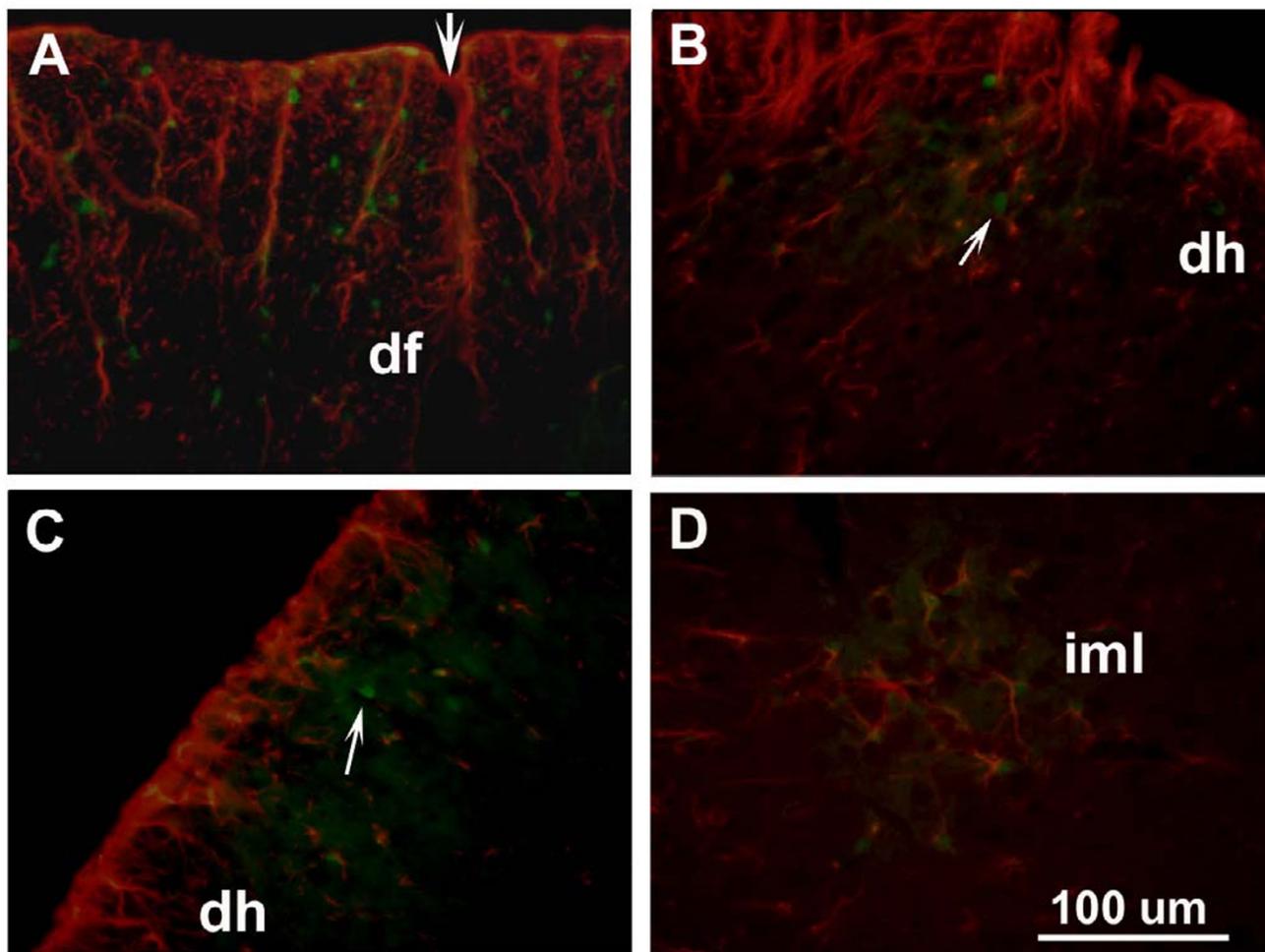


Figure 4

Double immunofluorescence for MIF and GFAP in the lumbosacral spinal cord. Each panel is a composite of two different photographs, arranged to overlap each other using Adobe Photoshop. A) In the dorsal funiculi, GFAP immunostaining (red) was very prominent. MIF immunostained cells (green) were seen associated with GFAP fibers. Arrow points to midline. In the dorsal horn (B & C), many of the MIF immunostained cells (green) were associated with GFAP immunofluorescence (red). However, some cells appeared to contain only MIF immunostaining (arrow). D. Several MIF immunostained cells are visible in the IML region. All of them also reveal GFAP staining. Calibration bar = 100 µm.

Interestingly, the MIF protein content in the prostate actually decreased after intravesical LPS (unpublished observations). These findings, although preliminary, suggest to us that MIF may be released from prostate epithelia because of inflammatory events in other pelvic viscera (in this case, the bladder) and this release may be mediated by neurogenic reflexes. If this is indeed the case, then MIF may play a role in neurogenic inflammation and in visceral referred pain. In humans, MIF protein has been localized in the prostate of cancer patients [2]. In fact, MIF may serve as a prognostic marker for prostate cancer since

serum levels correlate with disease progression [3]. Therefore, MIF may represent an important target for therapeutic intervention in bladder and prostate inflammation and cancer.

The anatomical location of MIF mRNA and protein has been described for the adult rat brain [7,8,20]. However, the spinal cord and peripheral nervous systems have received less attention [21,22]. In addition to the bladder and the prostate, MIF appeared to be ubiquitous in neurons of the peripheral ganglia of the rat.

Weak to moderate MIF immunostaining was observed in the neurons of the MPG. The major pelvic ganglia contain cholinergic and sympathetic neurons that innervate the bladder, prostate, colon and other pelvic viscera [12]. MIF immunostaining was localized in both parasympathetic (TH negative) and sympathetic (TH positive) cells in the MPG. In addition, the SIF (also TH positive) cells also contained MIF. These findings provide anatomical localization for our previous report of constitutive expression of MIF in the MPG previously [10,11]. Finally, sympathetic neurons (TH positive) in the inferior mesenteric ganglia and in the lumbar sympathetic chain also contained MIF. The lumbar sympathetic chain contains a large proportion of the sympathetic post-ganglionic neurons that innervate the bladder while the inferior mesenteric ganglion provides a much smaller contribution [12]. Although not directly tested in the present experiment, it is possible that some of the post-ganglionic neurons that also stained with MIF innervate the bladder and/or other pelvic viscera.

We also observed many L6/S1 DRG neurons that contained MIF, as well as intensely stained satellite cells. Some DRG neurons that were immunostained for MIF also co-contained the neurotransmitter substance P. Our findings of MIF in DRG neurons confirm earlier observations in the embryonic mouse [19]. However, our findings of MIF protein in afferent neurons in the DRG that also contain substance P represent a novel finding in the adult rat.

Finally, we observed substantial MIF immunostaining in the lumbosacral spinal cord of the rat. Glial cells (GFAP positive) in the white matter and in the gray matter were intensely immunostained for MIF. Earlier investigations also reported MIF immunostaining in the white matter of the spinal cord [21]. However, we also observed cells in the dorsal horn that stained for MIF but not for GFAP, suggesting they were neuronal rather than glial cells. In the brain, MIF immunohistochemistry is found strongly in glial cells with neurons displaying weak (or no) staining [7]. Therefore, neurons in the brain appear to synthesize MIF and quickly release it so that nearby glial cells accumulate MIF protein, without synthesizing it. It is possible that such a mechanism is at work at the level of the spinal cord.

In addition to cellular labeling with MIF, we also observed terminal-like labeling over the dorsal horn of the lumbosacral spinal cord. This labeling was seen as a band of MIF immunostaining that was darker in the superficial layers of the dorsal horn than in the deeper intermediate gray matter. This pattern of labeling is suggestive of the terminal fields of afferent neurotransmitters and corroborates previous reports of MIF terminal labeling in the

brain [7]. However, the staining was diffuse and individual nerve fibers (such as is the case with afferent neurotransmitter immunohistochemistry, for example, substance P) were not identified. Therefore, more work needs to be done to document whether the MIF staining over the dorsal horn represents afferent terminal labeling or diffuse, glial labeling.

MIF is a pro-inflammatory cytokine that may play a crucial role in initiating or maintaining several inflammatory conditions [1]. In experimental models, treatment with antibodies to MIF, reversed or prevented colitis and gastric ulcer formation [23,24]. MIF is upregulated in neurons in the brain and released into the CSF following intracisternal LPS or spinal cord injury [7,21]. In addition, MIF is upregulated in the brain after reperfusion subsequent to ischemia [25]. Therefore, MIF is likely involved in the brain's response infectious agents or trauma.

Conclusions

Results from the present study establish that MIF protein is located in epithelia and smooth muscle in two pelvic organs, the bladder and the prostate. Furthermore, post-ganglionic (parasympathetic and sympathetic) and afferent neurons also contain MIF protein. Finally, astrocytes and neurons in the lumbosacral spinal cord contain MIF. The role of MIF in the integration of pelvic viscera function is still under investigation. We have recently showed MIF upregulation in the bladder, in the L6/S1 DRG and in the lumbosacral cord after induction of acute bladder inflammation using two different models [10,11]. The upregulation of MIF in central and peripheral nervous tissues following localized (i.e. bladder) inflammation raises the interesting possibility that MIF may contribute to central and peripheral neurogenic events mediating inflammation. Although cytokines are traditionally associated with peripheral immune reactions as part of the innate response, evidence is accumulating that they may also be involved in normal brain function and may mediate pathological responses such as hyperalgesia [26,27]. In fact, the abundance of MIF in glia of the spinal cord is particularly interesting since glial cells have been suggested as involved in hyperalgesia through the release of cytokines and other substances [28]. Given the presence of MIF in the pelvic organs (bladder, prostate) and the central and peripheral nervous system tissues that innervate these organs, as well as the fact that bladder inflammation appears to upregulate MIF in these areas, it is possible that MIF represents a therapeutic target in reducing or preventing pelvic viscera inflammation.

Methods

All experiments were approved by the institutional Animal Studies Committee and conformed to the NIH guidelines for the conduct of experiments on animal subjects.

Perfusion and tissue collection

Six ($n = 6$) adult Sprague-Dawley (Harlan, IN) rats were used in this study. Four male (weight = 270–320 g) and two female (weight = 270–280 g) rats were deeply anaesthetized with sodium pentobarbital (80 mg/kg; i.p.) and perfused transcardially with cold (4°C) Krebs followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The following tissues were removed and placed in fixative at least overnight: bladder, ventral lobes of the prostate (in the males), both MPG, the inferior mesenteric ganglia (IMG), the sympathetic chain (L3-L5), the L6-S1 DRG and the L6-S1 spinal cord. In two of the males and the two female rats, the bladders and the ventral lobes of the prostate (in case of the males) were dissected prior to perfusion. The bladder was divided in half longitudinally, containing both dome and base. One half of the bladder and a piece of the ventral lobes of the prostate were then placed in fixative overnight. The MIF protein concentration of the remaining bladder and prostate was determined using an ELISA procedure (see below).

Immunohistochemical procedures

Cryostat sections (10 μ m) of the bladder (transverse sections through the dome and the base), prostate and all the ganglia were thaw-mounted onto gelatin coated slides. Free-floating cryostat sections (20 μ m) of the L6-S1 spinal cord were collected in phosphate buffer. MIF immunohistochemistry, either singly or in combination with other antigens was carried out in all the tissues according to the following protocols.

A) Single immunofluorescence

Sections were incubated with 3% normal serum (in 0.01 M phosphate buffer-saline, 0.1 % Triton X-100; 30 min) followed by incubation with MIF antibody (rabbit polyclonal, 1:200; Torrey Pines; Houston, TX) overnight. The antibody was visualized using a FITC-labeled secondary antibody (donkey anti-rabbit, 1:50; Jackson; West Grove, PA). The slides were cover slipped with fade-retardant media and examined using a Nikon microscope. Photographs were obtained using a microscope mounted digital camera (Pixera; Los Gatos, CA) attached to the microscope.

B) Double immunofluorescence

Sections from the MPG, the IMG and the sympathetic chain were processed for double-immunofluorescence in order to visualize both MIF and tyrosine hydroxylase, as follows: Sections were exposed to 3% normal serum (in 0.01 M phosphate buffer-saline, 0.1 % Tx-100; 30 min) followed by incubation with anti-MIF (1:200) and anti-tyrosine hydroxylase (sheep polyclonal antibody; 1:1000; Pel-Freez; Rogers, AR) overnight at 4°C. The antibodies were visualized using donkey anti rabbit and donkey anti-

sheep secondary antibodies (Jackson; 1:50), labeled with FITC and RITC, respectively.

Sections from the DRG were processed for the visualization of MIF and substance P as follows: sections were exposed to anti-MIF (1:200) and anti-substance P (guinea pig polyclonal; 1:1000; Chemicon; Tamecula, CA) overnight at 4°C. The antibodies were visualized using donkey anti rabbit and donkey anti-sheep secondary antibodies (Jackson; 1:50), labeled with FITC and RITC, respectively.

Some spinal cord sections were also processed for MIF and GFAP double-immunofluorescence as follows: Sections were incubated with anti-MIF (1:200) and anti-GFAP (mouse monoclonal; 1:1000; Chemicon). The antibodies were visualized using donkey anti rabbit and donkey anti-sheep secondary antibodies (Jackson), labeled with FITC and RITC, respectively.

C) Single PAP immunohistochemistry

Free-floating sections from the spinal cord were processed for MIF using a standard peroxidase-anti-peroxidase technique. Sections were exposed to 3% H₂O₂ to reduce endogenous peroxidase. The sections were then incubated with 3% normal serum (in 0.01 M phosphate buffer-saline, 0.1 % Tx-100; 30 min) followed by incubation with anti-MIF antibody (1:1600) overnight at 4°C and visualized as described previously [11]. Briefly, the sections were exposed to a goat anti-rabbit antiserum (Jackson; 1:50 in PBS) for 1 hour, followed by exposure to goat-PAP (Jackson; 1:100 in PBS). The sections were reacted with diaminobenzidine intensified with nickel ammonium sulfate. The slides were dehydrated, cleared in xylene and coverslipped with Permount.

Control experiments aimed to demonstrate the specificity of the staining with the MIF antibody and to rule out cross-reactivity of secondary antisera included: 1) preabsorption controls: incubation of the MIF antibody with the peptide used to produce the antibody; 2) omission of the primary antibody; 3) omission of either one of the primary antisera used for double-immunofluorescence.

ELISA procedures

Half of the bladders (containing both dome and base) of two male and two female rats, and the portions of the ventral lobes of the prostates of two male rats were assayed for MIF protein. Briefly, tissues were homogenized in CHAPS buffer 10 mM (3-[3-Cholamidopropyl] dimethylammonio]-1-propane-sulfonate, 2 mM EDTA, 4 mM iodoacetate in phosphate buffered saline (pH 7.2) with protease inhibitor cocktail (Invitrogen; Carlsbad, CA) and cleared tissue lysate prepared by centrifugation at 10,000 g for 15 minutes. MIF concentrations within cell lysates were determined using a rat MIF ELISA kit (Chemicon). Data

are presented as Mean (\pm S.E.M.) MIF concentration per mg of total protein of duplicate ELISA determinations for each tissue and from each experimental animal. The ELISA results were compared using a Student's t-test and a significant difference was defined as $p < 0.05$.

Authors' contributions

PLV performed the surgical procedures, tissue extraction and histology. KLMS performed the ELISA. Both authors participated in the design of the experiment, data analyses and interpretation. Both authors read and approved the final manuscript.

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