Identification and characterization of macrophage inflammatory protein 2

(polymorphonuclear leukocyte/endotoxin/chemotaxis/platelet factor 4/heparin)

STEPHEN D. WOLPE^{*†}, BARBARA SHERRY^{*}, DIANE JUERS[‡], GEORGE DAVATELIS^{*}, ROGER W. YURT[‡],
AND ANTHONY CERAMI^{*}

^{*}Laboratory of Medical Biochemistry, Rockefeller University, 1230 York Avenue, New York, NY 10021-6399; and [‡]Department of Surgery, Cornell University Medical Center, 1300 York Avenue, New York, NY 10021

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ABSTRACT In response to endotoxin, macrophages secrete a protein with a molecular mass of ≈ 6000 Da and with an affinity for heparin. This protein, which we term "macrophage inflammatory protein 2," is a potent chemotactic agent for human polymorphonuclear leukocytes. In addition, subcutaneous administration of the monokine causes a localized inflammatory reaction. Partial N-terminal sequence data reveal similarity to a family of proteins, the archetype of which is platelet factor 4. Although macrophage inflammatory protein 2 is a distinct member of the platelet factor 4 family, its sequence is most closely related to that of the *gro*/KC gene product, which is expressed in transformed or platelet-derived growth factor-treated cells.

One hallmark of the acute inflammatory state is the recruitment and activation of polymorphonuclear leukocytes (PMNs). Numerous mediators have been shown to be involved in this process, including leukotrienes (1, 2); complement components (3, 4); cachectin/tumor necrosis factor (TNF) (5–7); bacterial products (4, 8); neutrophil-activating protein 1 (NAP-1), a recently described monokine with sequence relatedness to the platelet factor 4 (PF4) family (9–15); and another monokine, macrophage inflammatory protein 1 (MIP-1) (16).

We have previously shown that endotoxin-stimulated macrophages secrete two proteins that bind to heparin-Sepharose and elute only with high-salt buffer (16). One of these proteins, MIP-1, is a doublet of approximate molecular mass of 8000 Da on SDS/PAGE, and it activates PMNs to undergo an oxidative burst and can cause a local inflammatory reaction when administered subcutaneously (16). We show here that the other major heparin-binding protein from endotoxin-stimulated macrophages is also an inflammatory protein with a sequence that is related to those of a recently recognized family of mediators, the archetype of which is PF4. This protein, which we call "macrophage inflammatory protein 2" (MIP-2), has a molecular mass of ≈ 6000 Da and can induce a localized inflammatory reaction. Furthermore, this protein is chemotactic for human PMNs but does not induce chemokinesis or an oxidative burst.

MATERIALS AND METHODS

Materials. Supernatants from COS cells transfected with a plasmid containing the hamster or human *gro* gene or a control plasmid alone were provided by A. Anisowicz and R. Sager (Dana-Farber Cancer Institute, Boston, MA). Partially purified human NAP-1 protein was a gift from J. Van Damme (Rega Institute for Medical Research, Leuven, Belgium), and

purified human NAP-1 protein was given by T. Yoshimura and E. Leonard (National Cancer Institute, Bethesda, MD). All other reagents were obtained from Sigma.

Cell Culture. The mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection. The cells were maintained in culture and stimulated with endotoxin to produce conditioned medium as previously described (16).

Purification of MIP-2. MIP-2 was purified by using methodology previously described for MIP-1 (16). The degree of purification was followed by SDS/PAGE with silver staining. In brief, 2 liters of conditioned supernatant from endotoxin-stimulated RAW 264.7 cells were concentrated and diafiltered against 20 mM Tris-HCl (pH 8.0) and applied to a Mono Q 10/10 (anion-exchange) column (Pharmacia LKB Biotechnology, Rahway, NJ). Greater than 90% of the MIP-2 was observed not to bind to the column and was recovered in the effluent.

The peak MIP-2-containing fractions were applied to a heparin-conjugated Sepharose (Pharmacia LKB Biotechnology) column equilibrated with 20 mM Tris-HCl (pH 8.0) and eluted with a 0–2 M NaCl linear gradient in the same buffer. MIP-2 eluted at ≈ 0.75 M NaCl. Peak fractions were concentrated in a Centricon ultrafiltration device with a molecular weight cutoff of 3000 (Amicon, Danvers, MA) and applied to a Superose 12 (gel-filtration; Pharmacia LKB Biotechnology) column equilibrated with 100 mM ammonium acetate. From 2 liters of RAW 264.7 conditioned medium (which equaled ≈ 100 mg total protein), we generally isolated 0.5 mg of MIP-2 as assessed by the Bradford protein assay (Bio-Rad, Rockville Center, NY) with bovine gamma globulin as standard. By comparison, ≈ 2 mg of MIP-1 and 1 mg of cachectin/TNF could be purified from the same batch of conditioned medium.

Immunoblot Analysis. Antisera to MIP-2 were produced in rabbits injected once subcutaneously with 10 μ g of purified protein emulsified in complete Freund's adjuvant and once 1 month later with 10 μ g of purified protein in incomplete Freund's adjuvant. Antisera were collected 1 week after the second immunization. Approximately 50 ng of pure MIP-2 or NAP-1 protein or roughly equivalent amounts of human or hamster *gro* protein from the serum-free supernatants of COS cells transfected with the appropriate vector were subjected to SDS/PAGE in 10–18% linear gradient gels and transferred to nitrocellulose by using a transblot apparatus (Bio-Rad). Blots were blocked in 5% dry milk (Alba) for 1–2 hr and incubated in antiserum diluted 1:100 for 1 hr at room

Abbreviations: PMN, polymorphonuclear leukocyte; MIP, macrophage inflammatory protein; PF4, platelet factor 4; NAP-1, neutrophil-activating protein 1; TNF, tumor necrosis factor.

[†]To whom reprint requests should be addressed at the following address: Genetics Institute, 87 Cambridge Park Drive, Cambridge, MA 02140.

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FIG. 1. Purification of MIP-2. The final positions of the molecular mass markers (in kDa) are shown on the left as are those of cachectin/TNF and MIP-1 for this 10–18% SDS/PAGE system. The four lanes show successive stages of purification of MIP-2. Lanes: 1, concentrated and diafiltered crude RAW 264.7 supernatant; 2, effluent from Mono Q (anion-exchange) column; 3, pooled peak fractions after purification on heparin-Sepharose; 4, pure MIP-2 after Superose 12 (gel filtration).

temperature. The blots were washed three times in phosphate-buffered saline containing 0.05% Tween 20 and 0.05% thimerosol, and bound antibody was detected with an alkaline phosphatase-conjugated second antibody (Promega Biotech, Madison, WI).

PMN Chemotaxis and Activation. The assay for chemotaxis was conducted as described (16). In brief, chemotaxis was assayed by placing 25 μ l of chemoattractant [fMet-Leu-Phe (10 nM)], MIP-2, or buffer (Gey's balanced salt solution, pH 7.4/2% bovine serum albumin) in the lower wells, and the top wells were filled with 45 μ l of buffer containing 1.1×10^4 PMNs (isolated by Ficoll/Hypaque density gradient centrifugation and dextran sedimentation). The two wells were separated by a cellulose nitrate membrane with a 3- μ m pore size (SM 11302; Sartorius Balances, Westbury, NY). Chambers were incubated at 37°C in a humidified 5% CO₂/95% room air chamber for 45 min. Membranes were removed and stained, and the number of PMNs migrating into the membrane was counted every 10 μ M up to 130 μ M by using an

automated Optomax Imaging system (Optomax, Hollis, NH). Random migration was also determined under conditions where the gradient of chemotactic agent was abolished by including equal concentrations in the upper and lower chambers.

The ability of MIP-2 to elicit the release of hydrogen peroxide from adherent PMNs was assayed as described (16).

RESULTS

Purification of MIP-2. As judged from silver-stained SDS/PAGE gels, >90% of MIP-2 remained in the effluent of the Mono Q column (Fig. 1). This one-step purification was sufficient to remove contaminating MIP-1 and cachectin/TNF; as shown (16), these two proteins bind to the Mono Q column under these loading conditions and elute at ≈ 0.37 M NaCl. Successive steps of heparin affinity chromatography and gel filtration were sufficient to purify MIP-2 protein to homogeneity (Fig. 1). MIP-2 eluted from heparin-Sepharose at ≈ 0.75 M NaCl and migrated with an apparent molecular mass of 10,000 Da on gel filtration (data not shown).

Analysis of partial N-terminal amino acid sequence data of purified MIP-2 (Fig. 2) indicated a unique sequence. Comparison with other sequences present in the Dayhoff bank by using the FASTP program (24) revealed similarity to a family of proteins with sequence relatedness to PF4. Fig. 2 shows the sequences of the various members of this family aligned by a conserved cysteine residue. Table 1 compares the percent sequence identities over the region corresponding to the partial amino acid sequence obtained for MIP-2. The closest relationship to MIP-2 was with the predicted amino acid sequence for the *gro* gene product (referred to here as *gro*). The MIP-2 sequence is 62.5% identical with human *gro* and 68.7% identical with hamster *gro*; this increases to 88% in both cases when amino acid changes that could result from a single base change are taken into consideration.

The similarity in sequence between MIP-2 and *gro* suggested that MIP-2 could be the murine equivalent of *gro*. The predicted murine KC gene product (KC), however, showed a closer relationship (65.6% identity to human *gro* and 81.2% identity to hamster *gro* for KC versus 62.5% and 68.7%, respectively, for MIP-2) when compared over the same region as the partial sequence for MIP-2. Similar results were obtained when the comparisons were conducted over the entire sequence; KC was 68% identical to human *gro* and 85% identical to hamster *gro*. Human and hamster *gro* were 68%

FIG. 2. Sequences of MIP-2 and the other members of the PF4 family. Sequences were obtained from the literature and were aligned by means of a conserved cysteine residue. Platelet basic protein (BPB) is the precursor for β -thromboglobulin and connective tissue activating protein III. Amino acids enclosed in boxes are conserved among the various members of the PF4 family. Lowercase prefixes refer to species: m, murine; h, human; b, bovine; r, rat; c, chicken; ham, hamster. Sequence references are as follows: this work, MIP-2; ref. 17, hPF4; ref. 18, bPF4; ref. 19, rPF4; ref. 20, BPB; ref. 9, NAP-1; ref. 21, IP10; ref. 22, c9E3; ref. 23, hGro and hamGro; and C. Stiles and coworkers (36), mKC.

Table 1. Percent sequence identity

	bPF4	rPF4	hPBP	c9E3	hNAP-1	hIP10	hGro	hamGro	mKC	mMIP-2
hPF4	68.7	65.6	53.5	26.6	39.2	37	33.3	37	33.3	39.2
bPF4	—	60	66.6	30	39.2	33.3	44.4	39.2	42.8	40.7
rPF4		—	55.1	33.3	46.4	32.1	39.2	42.8	46.4	41.3
hPBP			—	53.5	57.1	32.1	60.7	55.5	62.9	53.5
c9E3				—	50	27.2	48.3	48.3	54.8	41.9
hNAP-1					—	26.6	46.6	46.6	50	46.6
hIP10						—	Ins	Ins	Ins	Ins
hGro							—	65.6	65.6	62.5
hamGro								—	81.2	68.7
mKC									—	59.3

Sequences were compared using the FASTP program. Comparisons are only for the portion of the each sequence corresponding to that available for MIP-2 as aligned in Fig. 2. Ins, insignificant; PBP, platelet basic protein. Lowercase prefixes refer to species: m, murine; h, human; b, bovine; c, chicken; ham, hamster.

identical when compared to each other over their entire sequence.

Immunoblot Analysis. To further characterize the relationship between MIP-2 and the other members of the PF4 family, a rabbit polyclonal antiserum was raised against MIP-2. The antiserum reacted monospecifically against serum-free supernatants from endotoxin-stimulated RAW 264.7 cells or thioglycolate-elicited mouse macrophages but did not recognize any proteins in supernatants from unstimulated cells (an example of such a blot with antibody against MIP-2 is shown in Fig. 3). Preimmune serum also showed no reactivity (data not shown). In particular, antiserum to MIP-2 did not cross-react with MIP-1 or cachectin/TNF (see, for example, lane 2 of Fig. 3). Rabbit anti-MIP-2 cross-reacted weakly with human and hamster gro but not with purified human NAP-1 protein (Fig. 3). Similarly, no cross-reaction was seen with partially purified human NAP-1 from another laboratory [supplied by J. Van Damme (data not shown)]. In addition, the human myelomonocytic cell line HL60 secreted a cross-reacting protein after stimulation with 0.1 μ M phorbol 12-myristate 13-acetate (data not shown), further suggesting that the lack of reactivity of anti-MIP-2 antibody with the human NAP-1 protein is due to lack of immunological relatedness rather than to species specificity. The nature of the cross-reacting material from HL60 cells is under investigation.

PMN Chemotaxis and Activation. Because other members of the PF4 family have been shown to be chemotactic for and to activate PMNs (9–15, 25–27), we studied the effect of MIP-2 on these cells. MIP-2 was significantly chemotactic for human PMNs at 10 ng/ml and was more chemotactic for PMNs than fMet-Leu-Phe at concentrations greater than 100 ng/ml (Fig. 4). When equal concentrations of MIP-2 were added to both sides of the membrane, no increase in migration was observed. Thus the activity of MIP-2 at the concentrations tested appears to be due to stimulation of directed migration rather than random migration.

PMNs did not undergo an oxidative burst (as measured by production of hydrogen peroxide) when treated with concentrations of MIP-2 ranging from 10 ng/ml to 1 μ g/ml (data not shown). The relative abilities of MIP-1 and MIP-2 to trigger various PMN functions will be detailed in another report.

In addition to these *in vitro* assays, MIP-2 was tested *in vivo* by injection of 100 ng into the footpads of endotoxin-resistant C3H/HeJ mice. This injection induced a leukocyte infiltrate similar in magnitude to that previously shown for MIP-1 (data not shown; ref. 16).

DISCUSSION

We have identified two heparin-binding proteins that are secreted by endotoxin-stimulated macrophages. One of these

FIG. 3. (A) Silver stain of a duplicate of the gel used for immunoblot of MIP-2. (B) Immunoblot of MIP-2. Lanes: 1, purified MIP-2; 2, supernatant from endotoxin-stimulated RAW 264.7 cells; 3, supernatant from unstimulated thioglycolate-elicited mouse peritoneal macrophages; 4, supernatant from endotoxin-stimulated, thioglycolate-elicited mouse peritoneal macrophages; 5, purified NAP-1 protein; 6, supernatant from COS cells transfected with plasmid alone; 7, supernatant from COS cells transfected with plasmid containing the human *gro* gene; 8, supernatant from COS cells transfected with plasmid containing the hamster *gro* gene. Lanes 7 and 8 are reversed in the immunoblot (B). Molecular mass markers (in kDa) are shown on the left.

FIG. 4. Human PMN chemotaxis and chemokinesis in response to MIP-2. Chemotaxis (■) was measured by including MIP-2 in the lower well of the chamber only, and chemokinesis (▨) was measured by including the same concentration of MIP-2 in the upper and lower chambers. fMLP, fMet-Leu-Phe (10 nM).

proteins (MIP-1) has inflammatory properties (16). We show here that the other protein, which we term macrophage inflammatory protein 2 (MIP-2), is also an inflammatory monokine.

MIP-2 has a molecular mass of ≈ 6000 Da on SDS/PAGE and fractionates from a gel filtration column with an apparent molecular mass of 10,000 Da. In contrast to MIP-1 or cachectin/TNF, MIP-2 is cationic and does not bind to an anion-exchange column equilibrated at pH 8.0. This made separation of these activities and subsequent purification of MIP-2 relatively straightforward. After removal of the majority of contaminating proteins by anion-exchange chromatography, MIP-2 was purified to homogeneity by sequential heparin affinity chromatography and gel filtration.

MIP-2 is an extremely active chemotactic agent but induces little or no chemokinetic activity at the doses tested. At 10 ng/ml (1.7 nM) MIP-2 is significantly chemotactic for human PMNs, and at concentrations >100 ng/ml (17 nM) MIP-2 exhibits a higher leukotactic index than fMet-Leu-Phe at the latter's optimum concentration of 10 nM. Studies indicate that MIP-2 can also induce degranulation of PMNs with release of lysozyme but not β -glucuronidase (S.D.W., A.C., and H. Cohen, unpublished observations). Murine MIP-2 did not, however, induce a respiratory burst in human PMNs; it is not yet clear whether this is due to species specificity or is an inherent property of the molecule.

These effects on PMNs are similar to observations made recently with a protein isolated from human mononuclear cells by a number of investigators and variously referred to as "310C," "MDNCF," "MONAP," "NAF," or "GCP" (9–15); this protein is now known as neutrophil activating protein 1 (NAP-1). Because of the striking similarity in properties of MIP-2 and NAP-1, the possibility was considered that MIP-2 might be the murine equivalent of NAP-1. This appears not to be the case based on both sequence and immunological analyses. Comparison of the N-terminal sequence of MIP-2 with the PF4 family by using the FASTP program shows a 47% identity with NAP-1 but a 63% and 69% identity with human and hamster gro, respectively. This agrees well with the immunoblotting results, which demonstrated cross-reactivity with hamster and human gro but not NAP-1 from two different laboratories.

It also appears unlikely that MIP-2 is the murine equivalent of gro as the predicted murine KC gene product shows an

even higher sequence identity to gro, particularly in the case of hamster gro. We therefore conclude that MIP-2 is closely related to, but separate from, gro or KC.

It is of interest that the gro and KC genes were originally found in studies on the control of cell growth. The gro gene was isolated by differential hybridization of DNA from transformed cells (23); the KC gene was isolated by differential hybridization of DNA from cells treated with platelet-derived growth factor (28). Transfection of cells with the gro gene does not lead to transformation, however (23). Similarly, treatment of density-arrested 3T3 fibroblasts with MIP-2 or MIP-2 plus limiting amounts of serum or plasma does not increase uptake of [3 H]thymidine (S.D.W. and A.C., unpublished observations). In light of these data, as well as the sequence similarity and immunological cross-reactivity between MIP-2 and gro/KC, it will be of interest to see if the latter have inflammatory properties as well.

In comparison to MIP-2, MIP-1 is weakly chemotactic (16). It is not clear at this point if this relatively small response of human PMNs to MIP-1 is intrinsic or due to species specificity or to a reduction in specific activity due to the tendency of this protein to form large aggregates during purification. MIP-1 does induce a respiratory burst in PMNs; data reported here indicate that MIP-2 does not do so under the same conditions. These two proteins may therefore act in a complementary fashion to trigger full activation of PMNs. The possibility of synergy between these two monokines is being examined.

MIP-1 is a doublet of ≈ 8000 Da on SDS/PAGE. The cDNAs for each band of the doublet (i.e., MIP-1a and MIP-1b) have been cloned and shown to code for proteins with $\approx 60\%$ sequence identity (refs. 16, 29, and 37). Recently, the genes for a number of putative cytokines with sequence similarity to MIP-1 have been identified in activated T cells and fibroblasts, suggesting that this is a member of a larger family (30–34). At least one member of this family (TCA3) shows significant sequence relatedness to the PF4 family (S.D.W. and A.C., unpublished results). Although there is little apparent sequence similarity between MIP-1 and MIP-2, it is possible that these two proteins are representative of two families that have diverged from a common precursor (S.D.W. and A.C., unpublished results).

MIP-1 and MIP-2 are major secreted products from stimulated macrophages, comprising $\approx 2\%$ and 0.5% , respectively, of the proteins secreted by endotoxin-stimulated RAW 264.7 cells. Other bioactivities of these monokines remain to be discovered. MIP-2 is not pyrogenic in a rabbit model (G.D., S.D.W., and A.C., unpublished observations). Moreover, neither MIP-1 nor MIP-2 are mitogenic for bovine endothelial, rat smooth muscle, mouse 3T3 fibroblasts, or human T cells (S.D.W. and A.C., unpublished observations). As a number of inflammatory monokines such as cachectin/TNF and interleukin 1 have overlapping activities (35), it will be of interest to see which are shared with MIP-1 and MIP-2.

Finally, it is intriguing that an inflammatory mediator is closely related to gene products thought to be involved in tumorigenicity and control of cell growth. Just as gro and KC gene products now must be examined for possible inflammatory properties, the possible role of MIP-2 in oncogenesis and cell proliferation should also be considered.

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