

Human eosinophil granule major basic protein and its novel homolog

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For over 100 years, eosinophilia has been associated with allergic disease. Presently, the eosinophil is recognized as a proinflammatory leukocyte involved in protection against helminth infections and believed to play a major role in chronic allergic inflammation (1–6). The eosinophil is also an important source of cytotoxic proteins that can potentially induce pathologic changes in disease. Furthermore, in allergic inflammation, eosinophils can modulate immune responses by releasing growth factors, cytokines, and chemoattractants. Here, we summarize current information on human eosinophil granule major basic protein (MBP) and present new information on the existence of a novel MBP homolog.

Biochemical properties of eosinophil granule MBP

Initial studies of guinea pig eosinophils revealed a protein termed “major basic protein” (MBP), because

- 1) it accounts for approximately 55% of total granule protein
- 2) its isoelectric point (pI) is greater than pH 10
- 3) it is proteinaceous in nature (7–9).

Guinea pig MBP has a molecular mass of 11 kDa by SDS–PAGE, has a marked propensity to polymerize, and is arginine rich. Subsequently, a protein with comparable properties was isolated from human eosinophil granules; human MBP is a 13.8-kDa single polypeptide chain rich in arginine and with five unpaired cysteines (10), and

a calculated pI of approximately 11.4. The amino-acid sequence of human MBP has been determined by classical protein sequencing (11) and by deduction from its cDNA (12, 13). Homology searches show that MBP is unique and does not resemble the known sequence of other proteins, save for similarity to the lectin domain of the low-affinity IgE receptor, Fc ϵ R II (14, 15). The MBP cDNA indicates the existence of a prepromolecule with a 15-amino-acid leader sequence and a 90-amino-acid prosequence followed by the 117-amino-acid sequence of granule MBP (12, 13). The 90-amino-acid prosequence is markedly enriched in acidic amino acids, particularly glutamic acid; the pI of the prosequence is 3.9. The combination of the prosequence and MBP yields a molecule of 207 amino acids, with approximately equal numbers of strongly basic and strongly acidic amino acids and a resulting pI of 6.2. Although several possibilities can be entertained for the function of the prosequence of proMBP, one especially attractive possibility is that the prosequence of proMBP protects the cell during transport from the Golgi apparatus to the eosinophil granule (16). There, proMBP is processed to toxic MBP, and MBP crystallizes to form the granule core. Interestingly, the cDNA of another cationic toxin, barley toxin- α -hordothionin, codes for a nearly neutral precursor with a cationic toxin domain and an acidic prosequence (17). Thus, a precedent exists for a combined cationic effector molecule and an acidic prosequence.

Recently, the maturation of eosinophil granules was analyzed in cultures of interleukin (IL)-5

stimulated umbilical cord stem cells (18). By immunofluorescence analysis of these differentiating eosinophils, proMBP appeared at days 6–18, whereas MBP appeared at days 12–24. By day 6, Western blots detected heterogeneous glycosylated 33-kDa proMBP; its peak expression occurred on day 12. Western blots showed sequential processing of the 33-kDa proMBP to an 18-kDa intermediate form and, finally, to 14-kDa MBP. By dual label immunoelectron microscopy, proMBP was localized primarily to large, uncondensed eosinophil granules, whereas MBP was localized to granules containing a condensed central area. These results suggest that in differentiating eosinophils proMBP is expressed and processed as the granule condenses in a multistep process to 14-kDa MBP. Overall, these results seem consistent with the concept that the prosection functions to neutralize the toxic properties of MBP during the transport of proMBP to the eosinophil granule.

Two species of guinea pig MBP, gpMBP1 and gpMBP2, have been isolated and their cDNAs have been cloned (19, 20). Both species of guinea pig MBP cDNA showed a preproMBP with three domains: a signal peptide, an acidic prosection, and mature MBP. Guinea pig MBP1 and MBP2 were similar and their sequences resembled human MBP, particularly in the mature MBP domain, though not in the prosection. The calculated pIs of the guinea pig MBP1 and MBP2 were 11.7 and 11.3, respectively. In addition, the calculated pI for the prosection of MBP2 was 3.8; for proMBP2 itself, the pI was 5.6. Recently, the complete nucleotide sequence for the cDNA encoding rat eosinophil MBP was determined, and its structure is similar to that of the human and guinea pig MBP (21). Finally, murine MBP has been described, and the genes encoding proMBP have been cloned (22). By extraction of eosinophil granules, a murine MBP homolog was identified on the basis of strong (64%) N-terminal sequence homology with mature human MBP. The gene encoding MBP was isolated with a human MBP cDNA clone as a heterologous probe of mouse genomic and cDNA libraries under low stringency. The genomic nucleotide sequences of murine MBP exons are well conserved with the human gene, although homology alignments of the encoded protein show that extensive sequence conservation occurs only in the mature MBP. Expression data showed that this gene is transcriptionally active in tissues containing eosinophil progenitor cells, such as femoral bone marrow. Genomic Southern blots using the murine MBP gene at reduced stringency revealed the potential existence of a second, more divergent MBP-like sequence in the mouse, suggesting that, as with guinea pigs and man (see below), the

mouse genome may also encode MBP from more than one gene (22). Thus, the synthesis of MBP proceeds through a promolecule with a markedly acidic prosection in humans, guinea pigs, rats, and mice.

As noted above, a murine genomic MBP clone has been identified and is comparable in its organization to the human MBP genomic clone identified earlier (23). The human genomic MBP, 3.3 kb, is composed of six exons and five introns, one of which contains an Alu family repeat. The combined exon sequence is similar to the previously reported MBP cDNA sequence. The gene for MBP is preceded immediately by a putative promoter containing typical TATA and CCAATT boxes. Analyses of genomic DNA from four individuals of different racial groups showed identical restriction maps, suggesting that the MBP gene exhibits limited polymorphism (13, 23). Analyses of the promoter region of the MBP gene indicate the existence of a GATA-binding consensus site, and both GATA-1 and GATA-2 are bound to this site (24). GATA-1 cotransfection of Jurkat cells produced a 20-fold augmentation of MBP promoter activity. In contrast, cotransfection of GATA-1 and GATA-2 decreased the ability of GATA-1 to transactivate the MBP promoter by approximately 50%. These results indicate the existence of a GATA-1 target gene in eosinophils and point to a negative regulatory role for GATA-2.

MBP localization

Localization in eosinophil granules

MBP has been localized to the core of the guinea pig eosinophil granule by isolation of granule crystalloids and by immunoelectron microscopy (25). Gel electrophoresis of purified crystalloids showed a band in the expected position for MBP, which accounted for 79–91% of the dye staining. The core protein was immunochemically identical to MBP, and all of the core protein could be accounted for as MBP. Subsequently, immunoelectron microscopy studies of human eosinophils have shown that antibodies to MBP bind to granule cores (26, 27); MBP was not present within other eosinophil organelles, nor was it present in plasma cells, lymphocytes, or neutrophils (28). Recent immunoelectron studies found eosinophil MBP in coreless granules, where it was uniformly distributed throughout the granule, and in crystalloid-containing granules, where it was present only in the core (29). These findings are in keeping with those described above for the localization of proMBP and MBP, indicating a differentiation process that occurs in the granule.

Localization in basophils and mast cells

Isolation of basophils yielded a population of cells showing immunofluorescent staining for MBP in a granular pattern (30). Further, very small quantities of MBP were measurable in basophil extracts by radioimmunoassay (28). Although MBP may be synthesized by basophils, alternatively, basophils could internalize MBP in a similar manner to the internalization of eosinophil peroxidase by guinea pig basophils (31). Moreover, studies of mast cells have investigated MBP localization as a consequence of endocytosis (32). By immunofluorescence, mast cells from nasal polyps, ileal tissue, and cutaneous mastocytosis specimens contained MBP, whereas mast cells from normal skin did not stain for MBP. After MBP injection into normal skin, mast cells became MBP positive within 3 min, suggesting that MBP endocytosis by mast cells had occurred. Thus, the human mast cell may sequester toxic eosinophil granule proteins by endocytosis. Within the mast cell, MBP would presumably bind to the heparin-containing mast-cell granule, which, in turn, would probably neutralize the biologic activity of MBP. Thus, one function of the mast cell might be endocytosis and neutralization of cationic toxins by heparin (31, 33, 34).

Localization in placenta

Immunoreactive MBP, but not other eosinophil granule proteins, including eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), or Charcot-Leyden crystal protein, is elevated in the sera of all pregnant women and falls rapidly after parturition (35). Initial investigations showed that MBP levels in the blood of pregnant women rise strikingly before parturition and might form a useful predictor of the onset of term (36) and preterm (37) labor. However, a more recent study analyzing the relationship of MBP levels to the time of labor onset showed that 79% of the 112 pregnant women showed rises in MBP levels of $\geq 25\%$ above baseline during the third trimester (38). MBP levels were associated with placental weight, multiple gestation, and parity. However, these levels could not be used as a precise model to predict labor.

Using antibody to MBP and indirect immunofluorescence, MBP has been localized to placental X cells and placental-site giant cells (39); note that the placenta normally contains few eosinophils. Comparative analyses showed that the eosinophil granule MBP and the MBP derived from human placenta were biochemically and immunologically indistinguishable (40). Furthermore, the presence of MBP mRNA, identified by *in situ* hybridization,

indicates that MBP is being actively produced by the placental X cells (41). Recently, an association has been shown between the proform of MBP (proMBP) and pregnancy-associated plasma protein A (PAPP-A) (42). PAPP-A, isolated from pooled pregnancy serum, exists as a disulfide-bridged complex with proMBP in which the subunits of the constituents are present in a 1:1 molar ratio. Subsequently, the associations of proMBP with angiotensinogen in a 2:2 disulfide bond complex (molecular mass of approximately 200 kDa) and with angiotensinogen and C3dg in a 2:2:2 complex (with a molecular mass of approximately 300 kDa) were demonstrated (43). Thus, specific interactions between proMBP and other proteins occur in pregnancy, suggesting that these interactions are important, possibly in the pathophysiology of pregnancies associated with hypertension. Still, the function of MBP in pregnancy remains obscure.

MBP functions

Toxicity to parasites

Because MBP comprises the core of the eosinophil granule and accounts for over 50% of guinea pig eosinophil granule protein, it seems that elucidation of its functions should provide insights into the functions of the eosinophil itself. Nonetheless, early studies of MBP functions were not very revealing (8, 44). Subsequently, it was shown that MBP, along with other cationic eosinophil granule proteins, directly damages schistosomules of *Schistosoma mansoni* (45) and probably provides a strong, stable binding of eosinophils to the parasite target (46). MBP also damages other parasites, including newborn larvae of *Trichinella spiralis* (47, 48) and *Brugia pahangi* and *B. malayi* microfilaria (49). MBP also was found to kill a protozoan parasite, the bloodstream form (trypomastigote stage) of *Trypanosoma cruzi* (50, 51), the etiologic agent of Chagas' disease in man. In addition, amastigote forms of *T. cruzi*, the intracellular form of the organism, were ingested by eosinophils and damaged (52).

Effects on bacteria

Although initial studies of guinea pig MBP on bacterial growth failed to show striking effects (except for a 50% reduction in growth of *Escherichia coli*) (8), subsequent studies demonstrated marked activity against *Staphylococcus aureus* 502A and *E. coli* ML-35 (53). MBP was active against *E. coli* in medium containing 140 mM NaCl or 1 mM Ca^{2+} , but its activity was abolished

in the presence of 1 mM Mg^{2+} . In these studies, MBP caused outer and inner membrane permeabilization. Recent analyses of guinea pig MBP suggested that amino acids 54–67 might be involved in the MBP antibacterial activity (54). The 54–67 synthetic peptide exerted antibacterial activity; the activity was neutralized with a monoclonal antibody to human MBP, BMK-13; and the activity was lost after modification of arginine residues, suggesting that these arginine residues are important for the antibacterial activity.

Toxicity to mammalian cells

MBP is toxic to tumor cells (45) and other mammalian cells in a dose-related manner (55). Moreover, MBP concentrations in the toxic range are present in the body fluids of patients with diseases associated with eosinophilia (55). Thus, eosinophils probably release granule proteins *in vivo*, and the released MBP may damage tissues in hypersensitivity diseases. Investigation of this line of reasoning has shown that MBP damages respiratory epithelium, causing ciliostasis and exfoliation of respiratory epithelial cells (56–58), and mimicking the pathology of asthma (59).

MBP-mediated cell activation

MBP has been shown to activate peripheral blood basophils, causing a dose-dependent histamine release (60, 61). Because the histamine release stimulated by MBP was calcium-dependent, temperature-dependent, and energy-dependent, it did not appear to be a cytotoxic reaction (60). In contrast, EDN, ECP, and eosinophil peroxidase (EPO) did not stimulate histamine release from human basophils (61). MBP also caused histamine release from rat peritoneal mast cells, but here EPO and ECP (but not EDN) were also active (61). The mechanism of MBP-mediated basophil stimulation has been investigated; it is sensitive to extracellular calcium and is blocked by a calmodulin antagonist and by theophylline (62). More recent studies have shown that MBP stimulates an elevation in basophil cytosolic free Ca^{2+} concentrations, as judged by digital video microscopy (63). In some of these experiments, the average rise in cytosolic free Ca^{2+} concentrations was biphasic with an initial transient increase followed by a larger and more sustained elevation. Chelation of extracellular Ca^{2+} selectively blocked the second sustained rise in cytosolic free Ca^{2+} concentrations, as well as MBP-induced histamine release. Finally, the MBP-induced histamine release from basophils is potentiated by incubating basophils with IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor

(GM-CSF) (64). For example, cells preincubated with 10 ng/ml IL-3 or IL-5 required threefold to fourfold lower concentrations of MBP for histamine release and showed an enhanced rate of MBP-induced histamine release. MBP also stimulated low levels of leukotriene (LT) C_4 release from basophils; IL-3, IL-5, and GM-CSF potentiated the MBP-stimulated LTC₄ release up to threefold in parallel with histamine release. Thus, cytokines known to be present during allergic reactions may contribute to the pathophysiology of inflammation through potentiation of basophil-mediated histamine release stimulated by MBP.

MBP is also able to activate human neutrophils (65). For example, purified human peripheral blood neutrophils stimulated with MBP produced a concentration-dependent chemiluminescence response that peaked after 50–70 min. The MBP-induced chemiluminescence was abolished by the absence of calcium, and it was absent in neutrophils isolated from individuals with chronic granulomatous disease. More recently, it was found that incubation of MBP with neutrophils enhanced the expression of neutrophil CR3 (CD11b/CD18) and p150,95 (CD11c/CD18) (66). Analyses of the mechanisms by which MBP stimulates neutrophil superoxide anion production pointed to tyrosine kinase and calmodulin-dependent steps as being important in this nontoxic stimulation (67).

Finally, MBP and EPO are able to induce eosinophil activation as judged by release of EDN, whereas ECP was inactive (68). Pretreatment of eosinophils with dibutyryl cAMP or cytochalasin B completely abolished the EDN release induced by MBP and EPO, suggesting that the effects of these molecules are not due to cytotoxic eosinophil lysis. MBP also stimulated the production up to eightfold of IL-8 by eosinophils in a dose-dependent manner, and IL-8 mRNA could be demonstrated in eosinophils by reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, MBP and the calcium ionophore, ionomycin, synergistically induced production of LTC₄ from eosinophils. These results indicate that MBP and EPO may act as autocrine mediators in the pathophysiology of eosinophil-associated disease, especially bronchial asthma.

MBP and bronchial asthma

The ability of MBP to damage respiratory epithelium has already been noted (56–58). These studies showed that MBP acts directly on respiratory epithelium to cause ciliostasis and desquamation of respiratory epithelial cells. MBP also directly affects cilia (69) and most strikingly stops ciliary beating, possibly through inhibition of axonemal ATPase activity. MBP also activates canine res-

piratory epithelium, producing an increase in short-circuit current and an increase in net chloride secretion (70). In addition, MBP profoundly influences vascular permeability, as shown by experiments in the hamster cheek pouch. In these experiments, topical application of MBP at 0.1 and 0.5 nM caused a striking increase in vasopermeability, as well as muscle fasciculation and prominent arteriolar dilation (71). Instillation of all of the eosinophil granule proteins directly into monkey trachea implicated MBP as a molecule critical for induction of bronchial hyperreactivity (72). Finally, MBP is able to block the muscarinic M_2 receptor in guinea pigs sensitized and challenged with antigen, and MBP functions as an allosteric inhibitor of the M_2 receptor, inducing bronchial hyperreactivity in the guinea pig model of bronchial asthma. These studies are reviewed in detail elsewhere (73).

A novel MBP homolog

As indicated above, considerable evidence points to the eosinophil and in particular to MBP as a major effector in defense against helminths and as a critical mediator in allergic diseases. To identify novel eosinophil granule proteins, a cDNA library was constructed from eosinophil-like cells produced by IL-5 differentiation of umbilical cord mononuclear cells (UCMC). Subsequently, individual colonies were picked and their plasmid cDNA inserts sequenced. This resulted in the identification of a homolog of the eosinophil granule MBP. Interestingly, sequencing of 7995 plasmid clones from the UCMC showed that 8% coded for human prepro-MBP and 1.1% coded for the novel MBP homolog. The sequence of the homolog cDNA specified the existence of an N-terminal signal sequence, a highly acidic prosequence, and a basic MBP section. However, the pI of the MBP homolog at 8.7 is almost three orders of magnitude less than that of MBP itself, pI 11.4 (Table 1).

The tissue expression of proMBP and of the proMBP homolog has been investigated with PCR and specific primers for both molecules. ProMBP was expressed in bone marrow and placenta, whereas the proMBP homolog was expressed only in bone marrow.

The MBP homolog was subsequently isolated from lysates of eosinophil granules fractionated on columns of Sephadex G-50 and probed with a monoclonal antibody to a unique segment of the MBP homolog. The amino-acid sequence of the N-terminus of the MBP homolog corroborated its identity. Similarly, the molecular mass determined by mass spectrometry for the MBP homolog was in good agreement with the molecular mass calculated from its primary sequence.

Cellular toxicity and stimulation activities of MBP and the MBP homolog have also been compared. While both proteins were toxic to K562 cells in the micromolar range, about a twofold higher concentration of the homolog was necessary to achieve toxicity comparable to MBP itself. Neutrophil superoxide production was stimulated comparably by MBP and the MBP homolog while neutrophil IL-8 production stimulated by the MBP homolog was only 50% of that for MBP itself. Finally, MBP caused histamine release from basophils, whereas the MBP homolog was essentially inactive. Thus, the cytotoxicity and cyto stimulatory activities of the MBP homolog were qualitatively similar to MBP itself with the exception of histamine release.

Summary

Human MBP is associated with an array of *in vitro* cytotoxic and cyto stimulatory activities, mediated in part by its strong basicity. It is most prominently found in the eosinophil, but is also present in placental X cells and placental-site giant cells during pregnancy. Pathologically, its pattern of tissue deposition and its ability to induce characteristic pathophysiologic changes, such as bronchial

Table 1. Properties of human eosinophil granule MBP and MBP homolog and their encoding cDNA and genes

Protein	Site	M_r ($\times 10^{-3}$)	Isoelectric point*	Cell content ($\mu\text{g}/10^6$ eosinophils)	Molecular biology activities	cDNA	Gene
MBP	Core	13.8	11.4	9	Potent helminthotoxin and cytotoxin; causes histamine release from basophils and rat mast cells; neutralizes heparin; bactericidal; increases bronchial reactivity to methacholine in primates; unique strong platelet agonist; provokes bronchospasm in primates; activates neutrophils and eosinophils	~900 nt** (preproMBP)	3.3 kb** 5 introns 6 exons
MBP Homolog	??	13.4	8.7	??	Cytotoxin; activates neutrophils	~900 nt	??

*Calculated from amino-acid sequences deduced from cDNAs. **nt: nucleotides; kb: kilobases.

hyperreactivity and vasopermeability, strongly suggest a role for MBP in allergic and eosinophilic diseases. The role of MBP in protection from helminthic disease is also evident. Here, we present information on a novel homolog of eosinophil granule MBP with biologic activities similar to that of MBP. However, results from experiments on eosinophil granules suggest that the quantity of the homolog present in the eosinophil granule is significantly less than that of MBP itself. Further, preliminary experiments indicate that the two proteins are not synergistic in terms of their cytotoxic and cyto stimulatory biologic activities. Future experiments must determine whether the MBP homolog is deposited at sites of tissue damage and can be detected in biologic fluids at concentrations required for biologic activity.

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