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REVIEW

Role of milk fat globule-epidermal growth factor 8 in osteoimmunology

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Milk fat globule-epidermal growth factor 8 (MFG-E8) is a glycoprotein that is abundantly expressed in various tissues and has a pivotal role in the phagocytic clearance of apoptotic cells. However, MFG-E8 has also gained significant attention because of its wide range of functions in autoimmunity, inflammation and tissue homeostasis. More recently, MFG-E8 has been identified as a critical regulator of bone homeostasis, being expressed in both, osteoblasts and osteoclasts. In addition, it was shown that MFG-E8 fulfils an active role in modulating inflammatory processes, suggesting an anti-inflammatory role of MFG-E8 and proposing it as a novel therapeutic target for inflammatory diseases. This concise review focusses on the expression and regulation of MFG-E8 in the context of inflammatory bone diseases, highlights its role in the pathophysiology of osteoimmune diseases and discusses the therapeutic potential of MFG-E8.

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Introduction

Milk fat globule-epidermal growth factor 8 (MFG-E8, also known as lactadherin) was identified in the early 1990s as one of the most abundant proteins in the membranes of milk fat globules.[1] However, it took another 10 years to identify one of the most important functions of this protein, namely the bridging of apoptotic cells and phagocytes leading to the subsequent engulfment and clearance of apoptotic cells.[2] Considering the massive involution the mammary glands undergo when milking ceases and the large number of epithelial cells that enter apoptosis and must be cleared, it is not surprising that MFG-E8 is abundantly expressed in the lactating mammary gland and has a crucial role as an opsonin to ensure proper remodeling of the gland. The critical role of the clearance of apoptotic cells is further underlined by studies in mice. Loss of MFG-E8 in mice leads to the development of a lupus-like disease. This is likely caused by an increased production of autoantibodies due to insufficient clearing of apoptotic cells, as indicated by the presence of numerous apoptotic lymphocytes in the germinal center of the spleen and lymph nodes that were not properly engulfed by macrophages.[3]

The clearance of apoptotic cells, however, is not only important for developmental processes but also to purge a variety of tissues from pathogen-loaded phagocytes or cell debris. As such, research of the past years has unveiled a broad expression pattern of MFG-E8 throughout various tissues and cell types, indicating that its role as opsonin reaches far beyond the regulation of the mammary gland. In fact, current studies highlighted an important function of MFG-E8 in the maintenance of proper bone remodeling, as well as in the homeostasis of reproductive organs, epidermal tissue and blood.[4–8]

In addition to the regulation of tissue homeostasis, MFG-E8 has been shown to modulate immune reactions. Especially in models of sepsis and ischemia–reperfusion (I/R) injury but also in chronic inflammatory disease models, the expression of MFG-E8 is decreased in affected tissues, leading to a stronger inflammatory response. Various studies have shown that by treating such conditions with recombinant MFG-E8, the disease burden can be reduced. Even though the proper clearance of apoptotic cells likely also contributes to the pathogeneses of inflammatory disease,[9,10] numerous studies have unequivocally attributed direct anti-inflammatory actions to MFG-E8, for example by suppressing inflammatory pathways such as the NF-κB pathway.[11]

Thus, this concise review summarizes the most recent findings concerning the role of MFG-E8 in osteoimmune diseases. We will focus on the regulation of MFG-E8 during inflammation, its putative role as an immunomodulator and its impact on bone homeostasis in health and disease. Finally, we will discuss the potential of MFG-E8 as a therapeutic target for chronic inflammatory bone diseases.

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Structure and Expression of MFG-E8

MFG-E8 is a glycoprotein that consists of a N-terminal cysteine-rich region containing a signal sequence for secretion into the extracellular region and two epidermal growth factor (EGF)-like domains. The second EGF repeat contains an arginine-glycine-aspartate motif, which can be recognized by $\alpha v\beta_3$ and $\alpha v\beta_5$ integrins located on phagocytes. The C-terminal site has two discoidin-like domains (C1 and C2), showing a high sequence similarity to the blood coagulation factors V/VIII, which have a high binding affinity to phosphatidylserine (PS) of apoptotic cells.[1,12,13] In mice as well as humans, different isoforms of MFG-E8 have been identified. In humans, the full-length isoform contains 387 amino acids giving rise to a protein of about 43 kDa, whereas a shorter isoform lacks the aa 291–342 (37 kDa protein). Because of the post-translational modifications (glycosylation, sialyation) the protein sizes in western blots vary among the different reports. In mice, the full-length isoform contains 463 amino acids (51 kDa protein) and the shorter isoform lacks aa 110–147, giving rise to a 47 kDa protein. Even though the shorter isoform in the mouse lacks exon 4 of the original transcript, both isoforms retain the conserved binding domains and thus exhibit similar functions. Apart from different expression patterns, the function of these different isoforms is so far unknown.

Initially, MFG-E8 was identified in the lactating mammary gland as part of the milk fat globule membrane surrounding the lipid droplets,[1] where it is important for mammary gland remodeling and clearance of apoptotic cells during involution.[14,15] However, MFG-E8 has been identified in a variety of other cell types including antigen-presenting cells, fibroblasts, splenocytes, vascular smooth muscle cells, adipocytes, oligodendrocytes, glial cells, astrocytes, epidermal cells, osteoblasts and osteoclasts.[4,7,16–19] Especially in immune cells, high expression levels of MFG-E8 could be observed, such as CD68-positive macrophages located in germinal centers of the spleen and lymph nodes, which are necessary for the engulfment of apoptotic B cells.[3] Also in immature dendritic cells, differentiated from murine bone marrow cells, gene expression of MFG-E8 is highly induced.[7] The presence of MFG-E8 in these cell types has been shown to suppress effector T-cell functions and support the differentiation into regulatory T cells, thereby highlighting the importance of this protein for the immune system and subsequently for the pathogenesis of inflammatory diseases.[17,20] However, the functionality of MFG-E8 goes beyond immune regulations. For instance, MFG-E8 expressed in and around blood vessels has been shown to act as a downstream effector of pro-angiogenic vascular endothelial growth factor signaling, thereby controlling neovascularization after ischemic injury.[21]

Functions of MFG-E8

Role as an opsonin

The main function of MFG-E8 is the engulfment of apoptotic cells by acting as a bridging molecule between apoptotic cells and phagocytes.[2] The clearance of apoptotic cells is critical for developmental processes, immune tolerance, the maintenance of tissue integrity and the prevention of pro-inflammatory conditions.[22–24] Senescent cells, virally infected cells and cells dying during development and tissue turnover provoke the degradation process by exposing PS from the inner to the outer membrane, which can be recognized by opsonins such as MFG-E8. MFG-E8 then links the apoptotic cells to phagocytes by binding to the adhesion receptors integrin receptor $\alpha v\beta_3$ on macrophages or $\alpha v\beta_5$ on dendritic cells. This results in conformational changes of the integrin receptor, leading to cytoskeletal reorganization of phagocytes, which promotes the engulfment of cells/cell debris.[25,26] An impaired clearance of apoptotic cells is associated with autoimmune and inflammatory diseases including acute lung injury, cystic fibrosis and systemic lupus erythematosus.[27–29] The importance of MFG-E8 in apoptotic cell engulfment becomes particularly clear in MFG-E8 knock-out mice. These mice suffer from splenomegaly and spontaneously develop lupus-like symptoms as a result of an accumulation of apoptotic B lymphocytes in the spleens.[3]

Role in bone metabolism

Already in 2002, MFG-E8-expressing human osteoblasts have been identified, and, thereupon, also human monocyte-derived osteoclasts were shown to express MFG-E8.[18,30] Both cell types also express integrins, osteoclasts having a particular high expression of $\alpha_v\beta_3$, making them amenable to bind MFG-E8.[31] However, the functional role of MFG-E8 in the bone microenvironment was discovered just recently. Abe *et al.*[32] demonstrated that MFG-E8 expressed by osteoclasts negatively regulates osteoclastogenesis and that MFG-E8 deficiency contributes to alveolar bone loss induced by inflammatory conditions during periodontitis. In more detail, they could show that MFG-E8-deficient mice exhibit an increased RANKL-induced osteoclastogenesis *ex vivo*, especially after ligature-induced periodontitis, in which these mice underwent more periodontal bone loss compared with control animals (**Figure 1**). Interestingly, the increased osteoclast activity could be prevented in both cases after administering recombinant MFG-E8.[32] These findings were extended by a recent study showing that bone loss associated with MFG-E8 deficiency is not restricted to inflammatory conditions but is also relevant in the steady-state bone metabolism in healthy, as well as in ovariectomized mice as a model for postmenopausal osteoporosis. Herein, it was shown that MFG-E8 is not only relevant for osteoclastogenesis but is also important for osteoblast function. MFG-E8-deficient mice exhibited an impaired bone formation as well as poor mineralization compared with wild-type animals, whereas the osteoclast number and resorption activity increased at the same time.[4] Increased osteoclastogenesis in MFG-E8 mice was further exacerbated after ovariectomy. As MFG-E8 binds to $\alpha_v\beta_3$ integrin, it is perceivable that it might use this integrin as the main signaling mediator. However, even though integrin β_3-knock-out mice also display an increased number of osteoclasts, the osteoclasts are non-functional as they do not form a podosome belt that is necessary for bone resorption.[33] Thus, β_3-knock-out mice become osteosclerotic over time, which suggests that, even though MFG-E8 might associate with this integrin subtype, likely additional factors are involved in the regulation of osteoclastogenesis by MFG-E8. One possible function of MFG-E8 within the bone was proposed by Harre *et al.*[30] who suggested that osteoclasts, which derive from monocyte precursors just as macrophages and dendritic cells, might be necessary for apoptotic cell removal. The deletion of MFG-E8 could cause an accumulation of apoptotic cells in the

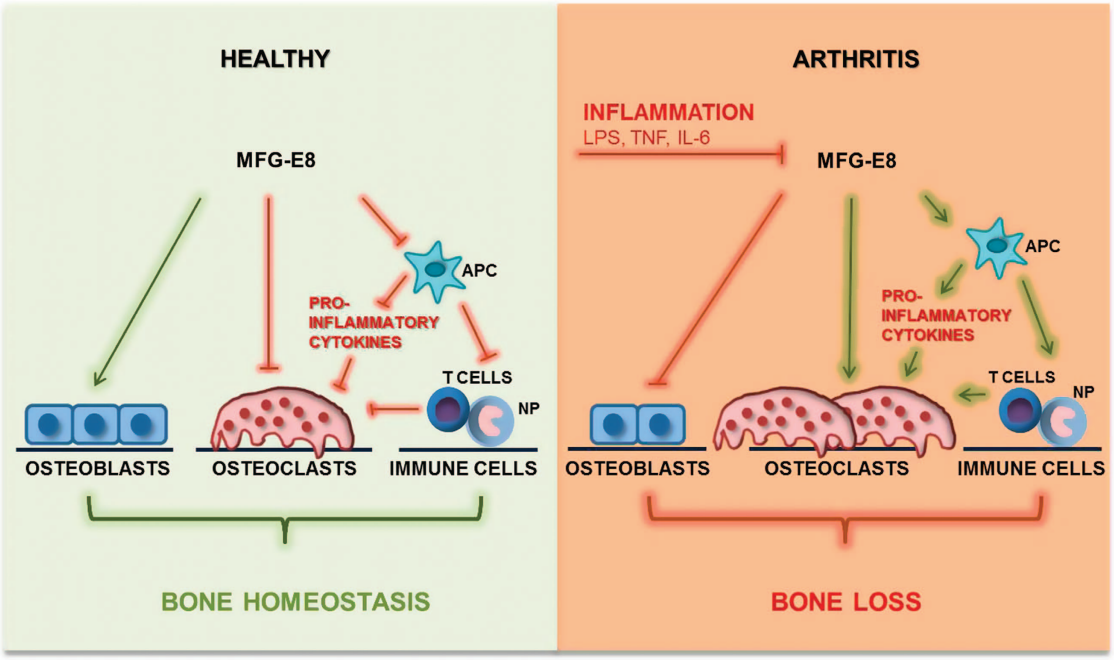

Figure 1 Role of MFG-E8 in health and disease. (Left) Healthy state. MFG-E8 promotes osteoblast differentiation and suppresses osteoclastogenesis. In addition, antigen-presenting cells (APCs) are not activated, thus, preventing the activation of T cells and the recruitment of neurophils (NPs). (Right) Inflammatory arthritis. Inflammatory stimuli suppress MFG-E8 expression, which leads to suppression of osteoblastogenesis and support of osteoclastogenesis. The activation of APCs by the decreased MFG-E8 expression leads to an enhanced production of pro-inflammatory cytokines, an activation of T cells and a stimulation of NP recruitment, thereby, further stimulating osteoclasts, leading to bone loss.

bone because it is not accessible for classical phagocytes, thereby resulting in poor bone quality.

As MFG-E8 seems to be critical for a balanced bone metabolism, especially under pathological conditions, it might be a promising target to therapeutically influence bone metabolism.

Role in inflammatory diseases

Besides the described function of MFG-E8 in the process of apoptotic cell engulfment, MFG-E8 gained special attention with regard to inflammatory diseases. In pathophysiological conditions such as sepsis,[34] I/R injury,[35,36] ulcerative colitis[37,38] and atherosclerosis,[39] a significant downregulation of MFG-E8 was reported (**Figure 1**). For instance, tissue concentrations of MFG-E8 in septic rats have been described to decrease while the number of apoptotic cells increased simultaneously. Interestingly, when dendritic cell-derived exosomes containing MFG-E8 are transferred into these rats, the inflammatory response in sepsis is reduced.[34] According to Komura *et al.*,[40] the downregulation of MFG-E8 seems to be dependent on LPS signaling via the CD14-TLR4 receptor complex, which is one of the most important signaling pathways after Gram-negative infections. In CD14-deficient mice and TLR4-mutated mice, respectively, splenic MFG-E8 production remained unchanged after induction of sepsis, thereby maintaining the phagocytic activity of peritoneal macrophages.[40] Similar results were shown for hepatic I/R injury wherein MFG-E8 levels in murine liver tissue were decreased. By administration of recombinant MFG-E8 liver injury could be diminished, which was accomplished via an upregulation of PPARγ resulting in a downregulation of the NF-κB pathway and consequently decreased TNFα and IL-6 levels.[41] Also in a colitis model, MFG-E8 reduced NF-κB signaling and modulated $\alpha_v\beta_3$ integrin expression.[37] The study of Albus *et al.*,[5] which investigated the role of MFG-E8 in rheumatoid arthritis, further underlines the observation that an inflammatory milieu reduces MFG-E8 levels. Patients suffering from rheumatoid arthritis had lower MFG-E8 serum concentrations compared with healthy controls, which rose again after complete or partial remission. Similarly, arthritic mice presented with decreased MFG-E8 expression levels in the paws and MFG-E8-deficient mice exhibited a stronger disease burden accompanied with more extensive bone loss compared with wild-type animals. They could further show that the MFG-E8 expression in osteogenic stromal cells, which greatly contribute to the inflammatory bone loss,[42] is downregulated by LPS and TNFα.[5] This means that MFG-E8 not only regulates the inflammatory response but is also regulated by inflammatory stimuli itself[17] (**Figure 1**). With respect to arthritis, in which regulatory T cells have a crucial role, it is particularly interesting to notice that MFG-E8 has been implicated to control the release of TGF-β and CC chemokine ligand 22, which are both known to attract and maintain the regulatory T-cell phenotype. In addition, MFG-E8 has been shown to limit neutrophil invasion by reducing the expression of CXCR2 in an α_v integrin-dependent manner.[43] Thus, besides studying the impact of MFG-E8 on inflammation-induced bone loss, more research is needed to unravel the role of MFG-E8 in the inflammatory response itself, as it is likely that MFG-E8 also modulates the differentiation and function of other T-helper cell subsets and cells of the innate immune system.

Finally, MFG-E8 has also been studied in periodontitis. In this inflammatory disease, which is caused by microorganisms of the oral cavity, leading to alveolar bone loss, MFG-E8 expression decreased as well. However, after 24 h, MFG-E8

gradually increased again until day 8 after ligature placement, which correlated with the number of osteoclasts causing periodontitis-dependent bone loss. Abe *et al.*[32] proposed that MFG-E8 might derive from osteoclasts during the course of periodontitis. Microinjection of recombinant MFG-E8 into the gingiva inhibited periodontitis-induced bone loss accompanied by a decrease in inflammatory mediators such as IL-1β, IL-6 and IL-17. Hence, this study also points towards the therapeutic potential of MFG-E8 to treat inflammatory bone diseases.

Therapeutic Implications

In the osteoimmunological context, recombinant MFG-E8 has so far only been used to locally treat periodontitis-induced bone loss by microinjections into the gingiva. However, there are several additional examples in other tissues that testify to the efficacy of MFG-E8 treatment to ameliorate disease burden in inflammatory and ischemic conditions. For example, treatment with recombinant MFG-E8 accelerated mucosal healing in various models of I/R injury models as well as in septic mice and mice with radiation-induced intestinal mucosal damage.[36,44–48] In most cases, the increased clearance of apoptotic cells, the reduced inflammation and an increase in epithelial cell proliferation have been described as underlying mechanisms. Importantly, treatment with recombinant MFG-E8 has been shown to both, prevent colitis when given before disease onset and ameliorate disease burden when given once the full-blown disease was established.[49] This suggests that, also in a more relevant clinical setting, in which patients have already developed symptoms of intestinal failure, recombinant MFG-E8 may be a viable treatment choice.

In addition to gut inflammation, MFG-E8 treatment has also been tested in ischemic and inflammatory brain injury models. After the induction of stroke, treatment with recombinant MFG-E8 decreased acute ischemic brain injury by mitigating inflammation and apoptosis and by promoting neurogenesis. Thus, MFG-E8 may not only be useful to limit ischemia but also to restore neuronal function.

In another set of studies, MFG-E8 has been identified to promote angiogenesis. Using a comprehensive set of *in vitro* and *in vivo* models, Silvestre *et al.*[21] showed that MFG-E8 is required for vascular endothelial growth factor-induced Akt phosphorylation and subsequent vessel growth. The intramuscular overexpression of MFG-E8 in an ischemic hindlimb model resulted in pro-angiogenic activities indicated by an increase in the capillary density and angiographic score. A subsequent study investigating skin wounds in mice confirmed the pro-angiogenic effect, showing that local treatment with MFG-E8 increased the number of vessels, reduced inflammation and accelerated wound healing.[50]

Taken together, numerous studies have underlined the potential of MFG-E8 as a therapeutic option, even though the efficacy of MFG-E8 treatment still remains to be tested for osteoimmune diseases. Further, the use of recombinant MFG-E8 appears to be safe, as none of the described studies have reported adverse effects on the liver or kidney. Zhang *et al.*[45] have injected mice with $>2\,mg\,kg^{-1}$ MFG-E8 intravenously and have observed no alterations in liver transaminases. Nevertheless, more detailed pharmacological studies are required to carefully examine the pharmacodynamics and toxicity of recombinant MFG-E8.

Finally, it should be noted that, in some diseases, treatment with MFG-E8 may be contraindicated. Patients with chronic pancreatitis, for example, have increased levels of MFG-E8 in the pancreatic stellate cells, and the increased expression levels are correlated with the extent of fibrosis and pain.[51] Also, MFG-E8 has been suggested to promote obesity and tumorigenesis of epithelial cells in the mammary and colon. Thus, treating with recombinant MFG-E8 may be walking on a thin line: although it may be useful to limit tissue damage and promote regeneration, excessive MFG-E8-induced proliferation may result in tumor development. Clearly, more studies are required to address the long-term efficacy and safety of MFG-E8 treatment to resolve this important issue. Also, larger prospective studies are needed to investigate the regulation of MFG-E8 in various human diseases and validate its potential therapeutic benefit in humans.

Conclusion

Research of past years has generated an incredible wealth of knowledge on the role and regulation of MFG-E8 in a broad variety of diseases. Most recently, the emerging role of MFG-E8 in osteoimmunology has been defined and shows a similar pattern to gut diseases, in which the therapeutic potential of MFG-E8 has been most extensively studied. Even though these studies have been very positive, more research is required to understand the molecular function of MFG-E8 in the physiology, immunology and pathophysiology of metabolic and chronic inflammatory skeletal diseases and evaluate its therapeutic potential, especially considering the chronicity of musculoskeletal diseases.

Conflict of Interest

The authors declare no conflict of interest.

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