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REVIEW ARTICLE

Milk fat globule epithelial growth factor VIII (MFG-E8) sustains survival of cancer cells by prompting tumor angiogenesis and suppressing host immunities*

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Abstract

Milk fat globule epithelial growth factor VIII (MFG-E8) is a novel adhesion protein mainly produced by macrophages and dendritic cells; it is expressed in most of the human tissues and functions to prompt cancer progression and survival. MFG-E8 contains a signal sequence for secretion, two epidermal growth factor (EGF)-like domains at the NH2 terminus and two discoidin domains with blood-clotting factor V/factor VIII (C1 and C2) at the COOH terminus. The second EGF domain contains an arginine-glycine-aspartic (RGD) integrin-binding motif that engages $\alpha_v\beta_5$ integrins to facilitate cell adhesion and induce integrin-mediated signal transduction. Integrin $\alpha_v\beta_3$ associates with VEGF receptor 2, engagement of integrins can promote angiogenesis, which plays key roles in growth, proliferation, and survival of cancer cells. VEGF stimulates the expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on angiogenic vasculature, thereby potentiating effects of VEGF receptor engagement. Mice expressing a mutant form of $\alpha_v\beta_3$ integrin are unable to undergo tyrosine phosphorylation, confirming the important role that this integrin plays in pathological angiogenesis and providing important mechanistic insights. The C-terminus discoidin-like domains promote binding to membrane phospholipids, functioning close to VEGF like angiogenesis. MFG-E8 is an opsonin for apoptotic cells, and it acts as a bridging protein between apoptotic cells and phagocytes. It also influences cell immunities by altering $CD4^+$ and/or $CD8^+$ cells. Antibody or small peptide works with MFG-E8 at different functional sites or interacts with EGF-like domains and/or discoidin-like domains may play an important role in anti-angiogenesis or immune restoration. Altering the structures and/or functions of MFG-E8 and/or its domains is promising for development of novel anti-cancer strategies.

Key words: milk fat globule epithelial growth factor VIII (MFG-E8); carcinoma; target therapy; angiogenesis; apoptosis

List of abbreviations: milk fat globule epithelial growth factor VIII (MFG-E8); vascular endothelial growth factors (VEGFs); fibroblast growth factor (FGF); tripeptide Arg-Gly-Asp (RGD); granulocyte/monocyte colony-stimulating factor (GM-CSF); cyclin-dependent kinase inhibitor 1 (P21WAF1/CIP1); B-cell lymphoma 2/Bcl-2 associated X protein (Bcl-2/Bax); platelet-derived growth factor receptor β (PDGFRβ); tumor cells proliferation rate index (Ki-67); toll-like receptor (TLR)

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Research on cancer pathways targeting therapeutic approaches is imperative and a promising new hope in cancer management. Cancer angiogenesis, which is regulated by angiogenic and anti-angiogenic factors, is a prerequisite for solid tumor growth, proliferation, and metastasis; its biological process has been developed in cancer treatment [1–2]. The angiogenic factors include vascular endothelial growth factors (VEGFs), and

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fibroblast growth factors (FGF1, FGF2). These angiogenic factors promote signal transduction and endothelial cell proliferation by engaging trans-membrane receptor tyrosine kinases on endothelial cells, prompting new blood vessel formation, and cancer cell proliferation and survival.

Milk fat globule epithelial growth factor VIII (MFG-E8), is a newly discovered glycoprotein with VEGF-like structures and functions [3], which is mainly produced by macrophages and dendritic cells. It is an opsonin for apoptotic cells and it acts as a bridging protein between apoptotic cells and phagocytes. It is highly expressed in breast cancer and melanoma, and contains two N-terminus EGF-like domains and two C-terminus discoidin-like domains. The second EGF repeat includes the integrin-binding motif Arg-Gly-Asp (RGD) which can interact with integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, where the discoidin-like domains promote binding to membrane phospholipids, making MFG-E8 a "bridging factor" in cell apoptosis as its main function [4]. MFG-E8 binding to integrins regulates a variety of signaling pathways, prompting VEGF-dependent angiogenesis by activating Akt in mice with pancreatic cancer [5], enhancing PDGF-PDGFR beta signaling mediated by integrin-growth factor receptor crosstalk [6], and inducing the expression of the transcription factors Twist and Snail, promoting motile mesenchymal phenotype in melanoma cells [7]. It also prompts proliferation of colon epithelial cells through integrin-mediated cellular signaling, and treatment with an siRNA targeting α_v-integrin reduced the proliferation of Colon-26 cells that was stimulated with recombinant MFG-E8 [8]. MFG-E8 is highly expressed in patients with cholangiocarcinoma (CCA), which was significantly characterized with a poor differentiation, pathological advanced stage, and metastasis of CCA [9].

The two discoidin-like domains promote binding to membrane phospholipids and removal of apoptotic cells by macrophages, clearing epithelial cells in involution, maintaining intestinal epithelium and facilitating vascularization [10–11]. MFG-E8 promotes tumor progression in oral squamous cell carcinoma and it might be involved in the clearance of apoptotic SCC cells by living SCC cells [6, 12]. It plays a key role in T reg cell homeostasis, by influencing $CD4^+$ and $CD8^+$ effector T cell activation and function, and regulating tumor immunity balance and tolerance [13]. Interaction on RGD or C1 and/or C2 functions to regulate cancer angiogenesis, immunities, proliferation, and survival or help to develop new cancer treatment modalities or agents.

MFG-E8 structure and expression

Milk fat globule epithelial growth factor VIII (MFG-E8), a 66-kDa glycoprotein, was initially identified in mice, and then isolated from the mammary gland of several other mammalian species, such as bovines and humans [3]. It is abundantly expressed in the mammary glands, spleen, lymph nodes, brain, and lungs; however, its expression is low in the liver and small intestine [10, 14]. MFG-E8 contains a signal sequence for secretion, two epidermal growth factor (EGF)-like domains at the NH2 terminus and two discoidin-like domains with blood-clotting factor V/factor VIII (C1 and C2) at the COOH terminus [3, 15]. The second EGF domain contains an RGD integrin-binding motif that engages $\alpha_v\beta_5$ integrins to facilitate cell adhesion and to induce integrin-mediated signal transduction. Integrin $\alpha_v\beta_3$ associates with VEGF receptor 2 [16], and engagement of integrins can enhance endothelial cell survival and migration [17]. VEGF also stimulates the expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on angiogenic vasculature, thereby potentiating the effects of VEGF receptor engagement. Recent studies in mice, expressing a mutant form of $\alpha_v\beta_3$ integrin that is unable to undergo tyrosine phosphorylation, confirm the important role that this integrin plays in pathological angiogenesis for providing important mechanistic insights [18]. Pharmacological inhibitors of VEGF and FGF receptor kinases and antibodies that bind to these growth factors or their receptors have been extensively studied clinically and in animal models of cancer.

Two mRNA MFG-E8 variants were found in mice by Oshima [19] – 66 kDa long form of MFG-E8 (MFG-E8L) containing a 37 amino acid proline/threonine rich (P/T rich) domain (between the second EGF domain and the first dicoidin domain), and 53 kDa short form of MFG-E8 (MFG-E8S) that lacks the P/T-rich domain [19–20]. The expressions of the two variants show spatial and temporal specificity but similar biological activity. MFG-E8S is distributed widely, whereas MFG-E8L has been found in activated mice macrophages, immature dendritic cells, skin Langerhans cells, and epidermal keratinocytes [21–23].

MFG-E8 contributes to phagocytes and removal of apoptotic cells

Apoptosis is an ordinary process of cell suicide that, unlike necrosis, does not elicit inflammation. Recently, it has been shown that if the removal process of apoptotic cells fails, apoptotic cells undergo post apoptotic secondary necrosis and release inflammatory cytokines.

The function of MFG-E8 is associated with promoting removal of apoptotic cells by macrophages, clear epithelial cells in involution, maintenance of intestinal epithelium, and facilitating vascularization [10–11]. The MFG-E8 production from macrophages is increased by granulocyte/monocyte colony-stimulating factor (GM-CSF) and fractalkine (CX3CL1) [24–25]. Its expression is downregulated in autoimmune disease [10], Alzheimer

disease, atherosclerosis, and sepsis [26–28], but it is upregulated in some disease conditions and cancer [7, 29].

Apoptotic cells were quickly removed by phagocytic cells such as monocytes, macrophages, and dendritic cells. The apoptotic cells express "eat me" signals, i.e., express phosphatidylserine (PS) on the external plasma membrane surface; MFG-E8 and the C-terminus discoidin domains mediate attachment to PS on apoptotic cells and the RGD motif of N-terminal domains engages $\alpha_v\beta_3/\alpha_v\beta_5$ integrins expressed on the advancing phagocytes [30–32]. This process downregulates cyclin-dependent kinase inhibitor 1 (P21WAF1/CIP1) expression and increases the B-cell lymphoma 2/Bcl-2 associated X protein (Bcl-2/Bax) ratio, which prompts endothelial cell survival by decrease in apoptosis [33]. MFG-E8 acts as a "bridge molecule" in the process of phagocytosis, recognizing and binding the apoptotic cells to the phagocyte. Decreased MFG-E8 level by sepsis or inflammation could be detrimental to apoptotic cell clearance [22]; however, high levels of it can inhibit cell engulfment [34]. This phenomenon complicated MFG-E8 in the involvement of disease pathogenesis and progression. MFG-E8-deficient mice develop an autoimmune phenotype that is characterized by autoantibody production, splenomegaly, and histological evidence of glomerulonephritis [10].

MFG-E8 prompts cancer angiogenesis

MFG-E8 contains two epidermal growth factor (EGF)-like domains, which contain an RGD sequence at the NH2 terminus. This EGF-like structure confers MFG-E8 with angiogesis-like function, and this angiogenesis promoting activity was attributed to enhancement of VEGF-induced Akt phophorylation and endothelial cell survival in a $\alpha_v\beta_3/\alpha_v\beta_5$ integrin-dependent manner [35–36]. At least 8 integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$) of its family, especially $\alpha_v\beta_3$, contributed to endothelial cell migration, proliferation, and apoptosis. It plays an important role in new blood vessel formation via junctional adhesion molecule 1/A (JAM-1/A) [37], increasing frequencies of blood vessels and myofibroblasts in wound area of wild type mice and reducing frequencies in knockout mice [38–39]. It was also found to enhance MFG-E8 expression in the dermis and granulation tissue with localization near blood vessels during wound healing. MFG-E8 accumulated around $CD31^+$ endothelial cells and co-localized with α-smooth muscle antibody$^+$ (αSMA^+) or platelet-derived growth factor receptor β^+ ($PDGFR\beta^+$) in human and murine dermis affects angiogenesis via actions on pericyte precursors (PCs) as well as endothelial cells (ECs) [36, 38]. In both xenograft tumors and clinical specimens of melanoma, MFG-E8 expression was increased near blood vessels where bone marrow-derived mesenchymal stromal cells (MSC) could be found, and MFG-E8 increased tumor angiogenesis by increasing VEGF and endothelin-1 expression in MSC and by enhancing M2 macrophage polarization [40].

Neutzner *et al* [5] found that MFG-E8 promoted tumorigenesis in Rip1-Tag2 transgenic mice. MFG-E8 mRNA was easily detected in pancreatic tumors and in cell lines derived from these tumors, but were not readily identified in normal pancreas. MFG-E8 deficient mice exhibited aggregate tumor burdens that were 2-fold lower and angiogenic islets that were ¼ lower than those in corresponding control wild type mice.

MFG-E8 derived from either tumor or host myeloid cells, promoting B16 melanoma growth *in vivo* through coordinated Akt and Twist signaling in the tumor microenvironment in C57Bl/6 mice. The vascularity of B16 melanoma growing and tumor cells proliferation rate index (Ki-67) in the MFG-E8 knockout mice were both reduced by 30%–50% compared to that of tumors in wild-type (WT) mice [36]. $\alpha_v\beta_3$ integrin overexpression in the vertical growth phase melanoma promotes cancer progression through coordinated $\alpha_v\beta_3$ integrin signaling in tumor cells, vascular elements, and infiltrating myeloid cells [41–43]. This promotes signal transduction and endothelial cell proliferation by engaging trans-membrane receptor tyrosine kinases on endothelial cells, eventually leading to new blood vessel formation and cancer cell proliferation and survival [35].

MFG-E8 suppresses host cellular and humoral immunities

The two discoidin domains are closely related to immune responses, which, like blood-clotting factor V/factor VIII (C1 and C2) at the COOH terminus, are the binding sites for apoptotic cell. Anti-tumor immune response was observed by using the purified exosomes. The immunosuppressive effects include T-cell killing due to enrichment of Fas ligand on tumor exosomes, NK cell inhibition or inhibition of monocyte differentiation in dendritic cells, possibly mediated by TGF [44]. The C1C2 domain in lactadherin can target other amino acid sequences to exosomes, fused to interleukin 2 or GM-CSF. The fusion protein can be secreted by live cells associated with exosomes [45]. Fusing C1C2 domain to a chicken egg ovalbumin (OVA) antigen in OVA-secreting tumor cell lines could induce more efficient anti-tumor immune responses and cause tumor cells to grow slower than tumor cells secreting the same antigen without fusion [46]. MFG-E8 may also affect the quality and magnitudes of antigen-specific responses by modulating apoptotic cell engulfment and processing [24]. It mediates and interacts with sentinels of apoptotic death signals, such as FADD and pro-apoptotic Bcl-2 family members. It interferes with the TNF and IL-6 mediated inflammatory signal

cascade and Rip kinase activities in dying tumor cells [13, 47]. It plays a key role in T reg cell homeostasis; combined use of anti-MFG-E8 antibodies with chemotherapy enhances cross-presentation of tumor antigens on dendritic cells, and markedly increases $CD4^+$ and $CD8^+$ effector T cell activation and function, resulting in high levels of surface CD44, INF-γ production, and tumor-specific cytotoxicity [24, 48]. MFG-E8-secreting tumor showed impaired IFN-γ production and CD107a immobilization by influencing $CD8^+$ cells with dense infiltrate of $CD4^+$ $FoxP3^+$ T regs [48–49]. It elicits STAT-3 activation, which promotes tumor cell survival and immune suppression; knockdown of MFG-E8 with shRNAs decreased phosphorylated Fak, phosphorylated Src, and phosporylated Akt levels, and increased apoptosis [43]. MFG-E8 altered IgG2a and IgG2b antibodies which might contribute to tumor destruction through Fc-dependent cytotoxicity [24, 48], it also helped dendritic cells to regulate the delicate balance between immunity and tolerance by fine-tuning recognition of dying cells [13]. MFG-E8 suppresses T cell activation/proliferation and inhibits Th1, Th2, and Th17 subpopulations while increasing regulatory T cell subsets. Neutralizing MFG-E8 substantially abrogates these effects, whereas addition of recombinant MFG-E8 to differentiated embryonic stem cells restores immunosuppression. Furthermore, MFG-E8 suppresses T cell activation and regulates T cell polarization by inhibiting protein kinase Cθ phosphorylation through the $\alpha_v\beta_{3/5}$ integrin receptor [50].

Discussion

MFG-E8 is found in almost every organ in humans. It is highly expressed in advanced stages of breast cancer, oral squamous cell carcinoma, colorectal cancer, and metastatic melanoma associated with a poor prognosis [12, 43, 51]. It is also highly expressed in triple negative phenotype or lower ErbB2 amplification breast cancer, but ER and/or PR levels reversed MFG-E8 expression. MFG-E8 expression decreases during tumor progression in ER^+ and $erbB2^+$ human breast cancers, but is highly increased in progressed triple negative breast cancers [52]. MFG-E8 was present in high levels in triple negative (ER^-, PgR^-, $erbB2^-$) breast cancer cell lines and in serum of patients. Transcription of MFG-E8 was controlled by p63 or TP63, and is a target gene of P63/P73 [53–54]. Abnormalities in p63/p73 regulation are important features of triple negative (basal) breast cancers. P63 gene regulates MFG-E8 expression, and MFG-E8 knockdown sensitizes triple negative breast cancers to cisplatin treatment [54]. MFG-E8 is expressed in triple negative breast cancers as a target gene of the p63 pathway, but may serve a suppressive function in ER^+ and $erbB2^+$ breast cancers.

Dendritic cell and tangible and tumor-associated macrophages (TAMs) produce large amount of MFG-E8 [43, 55]. Through a study on cancer stem cells and TAMs, Jinushi *et al* [56] found that MFG-E8 modulates oncogenic signals and triggers chemotherapy drug resistance in cancer stem/initiating cells by inducing Stat3 phosphorylation and smoothened expression, and downregulates sonic hedgehog pathways (Shh). MFG-E8 in association with IL-6 plays a critical role in mediating human cancer stem cell tumorigenicity. Its processes include several pathways, like PI3K/Akt, STAT3/SOCS3, β-catenin, mTOR, and TGF-β/FOXO, that regulate anti-cancer drug responsiveness and cancer cell efferocytosis [10, 57–58].

Yamazaki *et al* [12] reported that MFG-E8 promotes tumor progression in oral squamous cell carcinoma (SCC) and that it might be involved in the clearance of apoptotic SCC cells by living SCC cells by studying surgical samples of oral SCC and carcinoma *in situ*. MFG-E8 expression was correlated with clinicopathological features such as tumor size, pathological stage, locoregional recurrence, and scattering invasion pattern. By IHC staining, MFG-E8 was enhanced in apoptotic SCC cells, and some of which were apparently engulfed by the neighboring SCC cells. Transient MFG-E8 knockdown by siRNA in ZK-1 cells decreased cell proliferation and invasiveness and increased cell death.

Administration of anti-MFG-E8 antibodies alone achieved modest tumor destruction in colon adenocarcinoma in mice [48]. Combined use of cytotoxic agents (gemcitabine, 5-Fu, or CPT-11) with anti-MFG-E8 antibodies in mice colon adenocarcinoma model showed enhanced caspase-3 activation and anti-tumor effects compared with that after chemotherapy or antibody alone, and it also resulted in a loss of mitochondrial membrane potential [48]. MFG-E8 also plays an important role in T cell and dendritic cell function by influencing the cytokine productions. Anti-MFG-E8 antibodies attenuate tumor cell resistance to cytotoxic treatments, likely because of the inhibition of Akt activation. Some degree of intrinsic tumor cell sensitivity to the cytotoxic agent appears necessary for this enhancement. Efficient cross presentation of immunogenic antigens serves a key role in achieving a multiple array of anti-tumor immune responses by DC-targeted vaccines; and the activation of innate immune signals mediated by TLR, NLR, and/or CD40 may sense DC to facilitate cross presentation of immunogenic tumor antigens and triggering specific T cell responses [13, 59].

An additional mechanism by which anti-MFG-E8 antibodies might increase tumor cell killing, particularly in conjunction with anti-VEGFR-2 antibodies, may involve a more robust inhibition of the tumor blood supply, as MFG-E8 is required for VEGF-induced

angiogenesis. Moreover, knockdown of MFG-E8 in MC38 carcinoma cells exposed to chemotherapy also reduces VEGF production. Fens *et al* [60] found that RGD-modified erythrocytes show specific binding and internalization by tumor endothelial cells *in vivo* and *in vitro*, and widespread necrosis in tumors with a typical viable rim and central core necrosis in mice with B16.F10 melanoma. This phenomenon possibly by endothelial cell damage induced by degradation of erythrocytes and released large amounts of iron, causing intoxication of the endothelial phagocytes.

MFG-E8 not only prompts cancer vascular angiogenesis, but also regulates multi cancer pathways (p63/p73, PI3K/Akt, STAT3/SOCS3, β-catenin, mTOR, TGF-β/FOXO, etc.), which makes cancer cell resistant to chemotherapy and host immunity suppression. The multi-domain structure of MFG-E8 allows production of truncated proteins that are stable and that could be used as therapeutic agents. Truncated forms of MFG-E8 (such as the entire NH2 terminus) may bind to $\alpha_v\beta_3$ with higher affinity than RGD containing peptides that have little secondary structure and thus are more effective inhibitors. Additionally, MFG-E8 gene knockout influences tumor regeneration, development, and progression; the influences of MFG-E8 gene blockade in immunity also needs further concurrent study. It is likely that MFG-E8 fragments will also inhibit Del1: $\alpha_v\beta_3$ interactions, blocking at least one of the compensatory pathways that are apparently upregulated in MFG-E8 gene knockout tumors.

Conclusion

MFG-E8 is present widely in human tissues and can easily be targeted by its special multi-domain structures. It can be used as a marker of tumor progression in melanoma, cancer burden in metastatic breast cancer and its role in other types of cancer will be revealed in the future. Treatment modalities may focus on developing small molecules as inhibitors targeting $\alpha_v\beta_3/\alpha_v\beta_5$ integrin to influence cancer angiogenesis, targets on C1 and/or C2 domains to restore host cellular or humoral immunities, and/or to elicit new anti-cancer agents by coupling MFG-E8 antibodies. Development of MFG-E8-based therapeutics on cancer will be focused in the future studies.

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Conflicts of interest

The authors indicated no potential conflicts of interest.

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