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Modulation of tumor associated macrophages in solid tumors

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TABLE OF CONTENTS

1. ABSTRACT

It is becoming increasingly clear that interactions between cancer cells, stroma cells and the extracellular matrix (ECM) are pivotal to the processes of neovascularization and tumorigenesis. As tumor stromal components known as tumor associated macrophages (TAMs), mononuclear phagocytes can play a key role in tumor type specific neoangiogenesis by promoting remodeling of the ECM through the production of matrix metalloproteases (MMPs), secreting pro-angiogenic growth factors and stabilizing the tumor vasculature. The growth factor colony-stimulating factor-1 is produced by a wide variety of cancer cells and tumor stroma cells and influences the migration, survival and phenotype of TAMs. Thus, understanding the relationships of the cancer cell with the host environment is key for specifically exploiting tumor growth promoting tumor host interactions for new therapeutic strategies. This review outlines the strategies for targeting CSF-1 in malignancies to influence TAMs in tumor development.

2. INTRODUCTION

Solid tumors develop at primary and secondary sites when malignant, and genetically unstable cancer cells interact with stromal cells comprising primarily macrophages, inflammatory cells, fibroblasts and endothelial cells to proliferate within a supporting extracellular matrix (1, 2). During primary and secondary metastatic tumor development, an adequate blood supply enabled by the processes of angiogenesis and vasculogenesis is essential to allow oxygen supply and removal of waste products. Interactions between cancer cells, macrophages and the extracellular matrix (ECM) are pivotal to tumor-specific neovascularization and tumorigenesis (1, 2). Accompanying tumor growth and invasion is the release of angiogenic growth factors and matrix metalloproteinases (MMPs) by cancer cells and cells derived from the stromal compartment (3-6). These processes allow the growth of tumors to macroscopic levels (7-10). Anti-cancer therapies are increasingly being

developed that target the tumor stromal compartment (11, 12).

3.1 The origin and immunophenotype of tumor associated macrophages (TAMs)

The reticuloendothelial system is a diverse group of mononuclear phagocytes which includes liver Kupffer cells, brain microglea and lung alveolar macrophages that perform specialized, tissue-specific functions. Macrophages are derived from $CD34^+$ progenitors, mobilized from the bone marrow as promonocytes which then develop into monocytes and extravasate into tissues where differentiation into a specific type of resident, tissue macrophage occurs (13). Together with other inflammatory cells such as T cells and granulocytes, macrophages form an integral component of the stromal compartment in solid tumors (14). A subset of blood $CD14^+/VEGFR2^+$ monocytes have been shown to exert direct pro-angiogenic activity (15). Evidence has also been provided that monocytes can trans-differentiate into endothelial cells (16) and mimic endothelial progenitor cells in culture (17). It has recently been shown that TAMs represent a minor subset of myeloid $CD11b^+$ tumor-infiltrating cells that are characterized by expression of the Tie-2/TEK angiopoietin (Ang) receptor, a receptor tyrosine kinase previously thought to be restricted to endothelial cells (18). TAMs are derived from circulating Tie-2 expressing monocytes (TEM). TEM lack expression of inflammatory monocyte surface molecules such as CD62 or CCR2 (monocyte chemoattractant protein-1/CCL2), but express a myeloid profile which includes the receptor for colony-stimulating factor-1 (CSF-1) CD115/c-fms (19). Interaction between both Ang-2 and Tie-2 and CSF-1 with c-fms are known to promote TAM migration to solid tumors (19, 20).

3.2 The role of TAMs in tumorigenesis

Within tissues, macrophages phagocytize dead cells and debris, promote remodeling of the extracellular matrix (ECM) and secrete cytokines and pro-angiogenic growth factors. The prognosis associated with TAMs is dependent on tumor type but in breast cancer (21) and prostate cancer (22), TAM accumulation has been linked to decreased survival. Tumors recruit macrophages which may be classified as inflammatory type 1 macrophages which have anti-tumor activity or more commonly to type 2 (M2) macrophages which are pro-angiogenic and stimulate tumor growth (19, 23, 24). These latter macrophages within the tumor stroma can modify the ECM and influence capillary growth by different mechanisms (25, 26). Tumors may therefore recruit macrophages and create a microenvironment that causes macrophages to suppress immune function and adopt trophic roles during development and repair (27). By producing growth factors, cytokines and proteolytic enzymes that act directly to stimulate vascularization, macrophages can stimulate endothelial cell (EC) proliferation, migration and differentiation *in vitro* and angiogenesis *in vivo* (10, 25). Macrophages can also modify the ECM either through the direct production of ECM components or the production of proteases that alter ECM structure and composition (28, 29). Remodeling of the ECM is crucial to both angiogenesis and tumorigenesis and primarily involves the MMP family of proteolytic enzymes. Macrophages produce a number of MMPs including MMP-2. MMP-2 has been identified as a key enzyme mainly produced by tumor stroma cells and is capable of degrading type-IV collagen, the major component of basement membranes (30). MMPs degrade the ECM including the basement membrane and in conjunction with soluble growth factors, foster the migration and proliferation of ECs. This process promotes both angiogenesis and metastasis (31). Strict regulation of MMP expression is critical for maintenance of proper ECM homeostasis, however, in malignancies high levels of MMPs are often synthesized by cancer cells and, more importantly, also by adjacent and intervening stromal cells (32, 33).

3.3 The role of TAMs in tumor neo-vascularization

TAMs can stimulate angiogenesis by secreting angiogenic factors, or indirectly by producing ECM-degrading proteases, which in turn release sequestered angiogenic factors. TAMs migrate to regions of hypoxia in tumors and metastatic lesions (34) where production of pro-angiogenic molecules such as VEGF, Ang-1 and MMP-12 are induced. These effects are mediated in part by binding of the hypoxia-inducible factor transcription factor to hypoxia-response elements in target promoters such as the macrophage-specific metalloelastase MMP-12 (35, 36) which is also detected adjacent to developing capillaries in wound healing (37). However, while macrophages affect neovascularization by secreting angiogenic and ECM-degrading factors, TAMs may directly contribute to vessel formation in the tumor. TAM accumulation correlates with microvessel density and negatively with outcome in lung cancer (38). In angiogenesis, new blood vessels originate from the pre-existing vasculature through the proliferation of ECs and the recruitment of vascular mural cells (39). Besides the origin of tumor ECs from pre-existing vessels, the process of vasculogenesis in which endothelial progenitor cells (40) are recruited to tumors and incorporated into new blood vessels also plays a crucial role in neovasularization and tumor progression (41, 42). The interaction of monocytes/macrophages and ECs originates in embryogenesis. Embryonic vasculogenesis is initiated by the hemangioblast, a precursor of both hematopoietic and endothelial systems (43) and $CD45^+$ progenitor cells precede the advancement of ECs into avascular zones in developing mouse embryos (44). The ability to acquire endothelial properties was observed in monocytes/macrophages placed in angiogenic conditions *in vitro* and *in vivo* (45-47). Moreover, cells with a hybrid phenotype, positive for both the myeloid marker CD11c and endothelial VE-cadherin have been isolated from tumors (48). These findings favor the ability of monocytes to contribute to neovascularization by vascular mimicry. Many examples of vascular mimicry have been recently described including from tumor (49) and epithelial (50) cells, all of which can function in an endothelial capacity in various physiological or pathological settings. Studies of monocyte/macrophage migration and assembly in a subcutaneous Matrigel containing basic fibroblast growth factor (bFGF) indicates that infiltrating mononuclear cells are able to assist other precursor cells present in the

infiltrate to develop a lumen and thus initiate capillary-like structures (51).

3.4 CSF-1 and TAM regulation

Monocyte and macrophage generation and chemotaxis is primarily regulated by the growth factor CSF-1 also known as macrophage (M-) CSF (14, 52) through interaction with the receptor tyrosine kinase c-fms proto-oncogene (53) which is expressed by both TEMs and TAMs (19, 33). CSF-1 is produced by a variety of cell types such as fibroblasts, monocytes, activated macrophages and tumor cells (54-56). CSF-1 is overexpressed in human cervical cancer (57), gliomas (58), embryonic cancer (59), breast cancer, ovarian and endometrial cancers (60, 61). Circulating CSF-1 levels are a marker of breast cancer progression to metastasis (62) and serum levels of CSF-1 correlate to poor prognosis and lymph node metastasis in colon carcinoma (63). CSF-1 augments the production of a variety of cytokines by macrophages such as TNF-α (64) and also stimulates monocytes to secrete biologically active VEGF (65) which leads to accelerated angiogenesis *in vivo* (66). VEGF is a key factor in tumor angiogenesis and is upregulated in numerous malignant tumors. Genetic deletion of the CSF-1 gene ($Csf1^{op/op}$) significantly attenuated the development of invasive carcinomas in tumor-prone transgenic mice, a phenotype associated with the failure to recruit macrophages into neoplastic tissues in the absence of CSF-1 (20). CSF-1 modulated TAM populations regulate not only the formation of high density vascular networks in breast cancer models, but also work to maintain and remodel the existing tumor vasculature (67).

3.5 Targeting tumoral stromal cell-derived CSF-1

Due to the role of CSF-1 in TAM regulation and the elevated serum levels of CSF-1 observed frequently in cancer patients, it is tempting to speculate that blocking stromal cell-derived CSF-1 expression would retard tumor growth. Targeting CSF-1 production from genetically stable stromal cells offers the advantage of bypassing genetically unstable cancer cells avoiding the development of therapy resistance. To evaluate the role of stromal cell-derived CSF-1, we xenografted human embryonic cancer cells (CRL-2073) and SW620 colon cancer cells, which have no detectable mRNA or protein for human CSF-1 or c-fms, into immune deficient mice. A key observation in this cancer model was that both human embryonic cancer cells and colon cancer cells stimulated increased host tissue expression of CSF-1. Associated with increasing CSF-1 tissue expression was an enhanced infiltration of macrophages within and surrounding the tumor (59).

To block CSF-1, we initially designed CSF-1 phosphorothioate (PT)-modified antisense oligodeoxynucleotides (ODNs). Antisense ODNs are chemically modified stretches of single-stranded DNA complementary to pre-mRNA and mRNA regions of a target gene that are capable of inhibiting gene expression. To examine the efficacy of the CSF-1 antisense ODNs, they were first tested on cells expressing CSF-1 and then in immune-deficient mice xenografted with human embryonic and colon cancer cells.

SCID mice bearing established human embryonic tumors were treated systemically with CSF-1 antisense ODNs. Systemic treatment with CSF-1 antisense ODN significantly down-regulated tissue CSF-1 mRNA and protein levels and suppressed the growth of embryonic tumors to dormant levels. In addition, the density of vascular sprouts was reduced in mouse testis. Similarly, VEGF-A and KDR/flk-1 mRNA levels as well as MMP-2 protein expression were significantly reduced in CSF-1 antisense ODN-treated mice (59).

Using an established flank model of SW620 colon cancer cells in nude mice, we showed that host CSF-1 tissue mRNA and protein levels increase with tumor progression, similar to the embryonic cancer model. After 2 weeks of CSF-1 antisense ODN treatment, CSF-1 mRNA and protein expression was significantly downregulated compared to controls. Tumor growth was markedly retarded in mice following CSF-1 blockade and tumor weights were significantly decreased compared to controls. MMP-2 protein expression in tumor lysates markedly increased with tumor progression and declined significantly following CSF-1 inhibition. Long-term (6 month) survival was observed in 8 of 14 mice following CSF-1 blockade, whereas all mice were dead after 65 days in the control groups. At sacrifice 6 months after therapy, no metastases were detected. At 65 days (at which time the last animal in the control groups died), 85.7% of CSF-1 antisense-treated mice were still alive (59).

Macrophage recruitment in embryonic tumors and colon cancer is associated with tumor growth. Likewise, macrophages are also recruited into mammary gland carcinomas (68, 69). Since metastatic progression of mammary gland tumors is profoundly reduced in the absence of such tumor-associated macrophages (20) and CSF-1 blockade suppresses tumor growth, MMP production and macrophage recruitment in embryonic and colon cancer tumors support the paradigm that CSF-1 enhances progression of malignancies through effects on the recruitment and control of macrophages that regulate tumor cell growth, angiogenesis and the ECM. The recent discovery of highly specific, small interfering (si)RNA molecules as promising candidate therapeutics to specifically and potently modify gene expression led us to hypothesize that blocking CSF-1 using this approach would efficiently suppress breast cancer development. For this purpose, human MCF-7 mammary carcinoma cells were used in a xenograft model in nude mice. MCF-7 cells upregulate host (mouse) CSF-1 production but lose their ability to express human CSF-1 after xenografting to mice. Furthermore, macrophage invasion in the tumor xenografts was observed. In association with this, host (mouse) MMP-2 and MMP-12 were strongly expressed during tumor progression in control animals (33).

Before *in vivo* administration, CSF-1 and CSF-1R siRNAs were tested for sequence and dose dependent suppression of target gene expression *in vitro*. Mice bearing human MCF-7 mammary carcinoma xenografts were

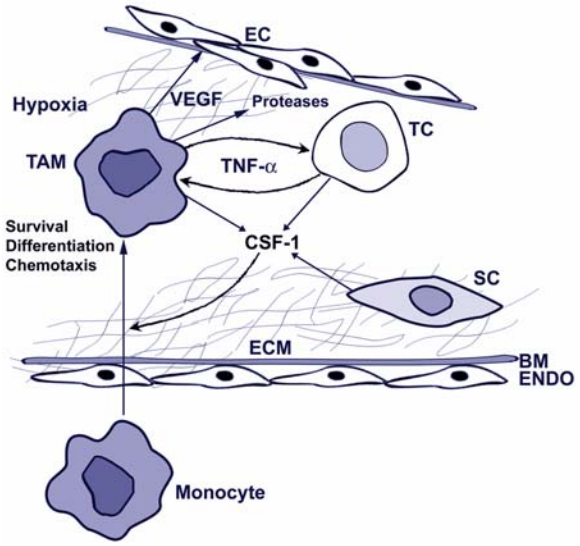

Figure 1. Proposed model for CSF-1 and TNF-α regulated tumor development. A subset of circulating monocytes transverse the basement membrane (BM) and endothelium (ENDO) and migrate to areas of tumor hypoxia as tumor associated macrophages (TAM). TAM survival, differentiation and chemotaxis is influenced by CSF-1 derived from TAMs, stromal cells (SC) and tumor cells (TC) and TNF-α derived from tumor cells. TNF-α induces the expression of CSF-1 in macrophages, which leads to secretion of a variety of proteases to breakdown the extracellular matrix (ECM) and the BM, thereby promoting invasion into the surrounding stroma. The cooperation between TAMs and tumor cells in areas of tumor hypoxia also induces upregulation of angiogenic growth factors, like VEGF, thereby stimulating endothelial cell (EC) migration, proliferation, and differentiation into new vessels. TAMs modulated by cancer cells can also secrete TNF-α or other factors to stimulate tumor cell proliferation.

treated with five intratumoral injections of CSF-1 siRNA and c-fms siRNA. siRNA treatment was well tolerated and no significant changes in the cellular blood count of treated mice was observed. siRNA treatment against CSF-1 and c-fms significantly suppressed mammary tumor growth and selectively downregulated target protein expression in tumor lysates. In addition, treatment with CSF-1 siRNA or c-fms siRNA reduced macrophage recruitment to the tumor and intratumoral levels of both MMP-2 and MMP-12 (33).

Histomorphometrical analysis of mammary tumors showed an increased density of proliferating ECs with tumor progression that was decreased after CSF-1 and CSF-1R siRNA blockade. In addition, VEGF-A mRNA levels increased with tumor progression and were reduced in CSF-1 and CSF-1R siRNA-treated mice. CSF-1 and CSF-1R blockade, however, did not significantly affect tissue mRNA expression of the VEGF-A receptors Flt-1 and KDR. Importantly, CSF-1 blockade increased survival in mice with mammary tumor xenografts. The median survival of animals in the control group was 62 days, which was significantly increased in mice after treatment with CSF-1 siRNA (103 days) and slightly increased after treatment with CSF-1R siRNA (76 days) (33).

3.6 Targeting TAMs by interrupting TNF-α-mediated tumor-host interaction

It is clear that cancer is not a single-cell disease and that the existence and behavior of cancer cells are constantly modulated by the host stroma compartment. However, tumor cells secrete many factors, cytokines and chemokines that directly or indirectly affect the tumor microenvironment. Tumor necrosis factor (TNF)-α is a key cytokine produced by both malignant cells and macrophages (70). In macrophages, TNF-α can induce translation of the type-IV collagenase MMP-9 by macrophages which degrades ECM collagen (71). There is also evidence that TNF-α can induce tumor cell apoptosis in neoplastic tissues (72). However, colon carcinoma cells frequently lose sensitivity to the induction of apoptosis during tumor progression (73). Moreover, TNF-α may even promote tumor growth at lower levels (5). TNF-α has been identified as an inducer of CSF-1 production (74).

In this context, siRNA may be utilized to investigate the mechanisms by which colon cancer cells up-regulate host factors to promote tumorigenesis. Exploring the interaction between colon cancer cells and macrophages by performing cytokine profiling of human SW620 colon carcinoma cells grown in nude mice (75), we found that in addition to CSF-1, levels of host and cancer cell-derived TNF-α mRNA expression increase dramatically during tumor development. In support of these findings, high CSF-1 serum levels correlate to poor prognosis in colon carcinoma patients (63) and soluble TNF-R1 is increased in colorectal cancer patients (76). By using siRNA *in vitro*, an autocrine regulation of TNF-α in colon cancer cells was identified which leads to increased tumor cell proliferation (75). In line with this, TNF-α can provide a survival signal for the cancer cell, and hence, it has been referred to as a tumor-promoting factor (77).

Since TNF-α regulates the expression of CSF-1 in macrophages, it was then shown by co-culturing colon cancer cells with macrophages, that TNF-α expression by SW620 cells leads to the induction of macrophage TNF-α and CSF-1, and that CSF-1 in turn increases macrophage VEGF-A and MMP-2 expression. In accordance with this findings, cocultures of breast cancer cell lines with macrophages led to TNF-α–dependent MMP induction in the macrophages associated with enhanced invasiveness of the malignant cells (78). In addition, tumor cell-derived TNF-α can influence the migration of macrophages *in vitro* (75). Moreover, intratumoral treatment of colon cancer xenografts with TNF-α siRNA, mouse CSF-1 siRNA or combined TNF-α siRNA/mouse CSF-1 siRNA suppressed tumor growth by 34%, 47% and 50%, respectively. In addition, cell proliferation and the number of tumor-infiltrating macrophages were reduced following treatment. Blocking human TNF-α reduced mRNA levels of human and host (mouse) TNF-α, host CSF-1, MMP-2 and VEGF-A mRNA levels in tumor lysates. In contrast, neither TNF-α nor CSF-1 blockade affected human VEGF-A expression

by cancer cells. Moreover, the significant reduction of total VEGF-A and MMP-2 protein expression in the tumor tissue following CSF-1 and combined TNF-α and CSF-1 blockade further supports the importance of host cell gene expression for tumor growth (75).

Another potential tumor-promoting effect of TNF-α has been shown in TNF-α –deficient mice, which were resistant to skin carcinogenesis (79, 80). Overexpression of TNF-α increased metastatic activity of tumor lines (80), and pretreatment of animals from an experimental fibrosarcoma model with TNF-α increased lung metastases (81). Thus, several studies indicate that increases in TNF-α concentrations within the tumor microenvironment promote cancer spread. In agreement with these reports, other studies have confirmed tumor and stromal expression of TNF-α in breast, ovarian, colorectal, and prostate cancers (82).

4. PERSPECTIVE

TAMs represent the major inflammatory component of the stroma of many tumors and accelerate tumor growth in neoplastic tissue by promoting angiogenesis, remodeling of the ECM and suppressing adaptive immunity. In many human tumors, a high frequency of TAMs is associated to poor prognosis (83). Thus, increasing knowledge of the mechanisms that allow cancer cells to "educate" macrophages should allow development of novel therapeutics to target cancer cells and the tumor microenvironment in the treatment of solid tumors.

In this context, inhibition of upregulated host CSF-1 in human embryonic, colon and breast cancer xenografts in mice suppresses tumor growth and increases mouse survival. Associated with this suppression, decreased tumor vascularity, reduced expression of angiogenic factors and MMPs and decreased macrophage recruitment to the tumors was observed (33, 59). These results combined with the recently recognized role of macrophages as VEGF-secreting cells (65), suggest that certain cancer cells upregulate host CSF-1 leading to macrophage modification of the ECM and facilitating angiogenesis and tumor development. Moreover, some cancer cells produce CSF-1 and directly influence macrophages (84). Thus, interaction between cancer cells and the surrounding tumor microenvironment leads to upregulation of CSF-1 which in turn leads to macrophage recruitment. The tumor microenvironment educates these tumor-associated macrophages to perform supportive roles that promote tumor progression and metastasis (42). In colon cancer, TNF-α derived from colon cancer cells is an autocrine growth factor for cancer cells, increases macrophage migration and stimulates CSF-1 production by stromal macrophages and possibly other cell types. CSF-1, in turn, induces VEGF-A and MMP-2 overexpression in macrophages in an autocrine manner, thereby modulating angiogenesis and colon cancer growth (75). Thus, studies demonstrating the key role of CSF-1 and TNF-α signaling for tumor growth promoting activities of TAMs could lead to the development of therapeutic targets in cancer treatment.

Many macrophage chemoattractants are regulated by hypoxia, resulting in migration and accumulation of macrophages in hypoxic tumor regions (85). A key regulator of hypoxia induced genes is the transcription factor hypoxia-inducible factor (HIF), which regulates several factors in tumor cells and TAMs (35, 86, 87), including VEGF. Thus, hypoxia- and CSF-1-induced VEGF in TAMs probably exerts a chemotactic action on other macrophages, aiding their migration to hypoxic tumor sites. It is also evident that macrophages upregulate chemokine receptors under hypoxic conditions, such as the CXCR4 receptor for the monotactic CXC chemokine SDF-1 (stromal cell-derived factor-1, CXCL12), as an example, which is induced by hypoxia via a HIF-dependent pathway (34, 88)

In addition to factors regulated by CSF-1 and TNF-α signaling events between TAMs and tumor cells, on which the current review focuses (Figure 1), TAMs can respond to the presence of stimuli in tumors with the release of a variety of growth factors, cytokines, chemokines and enzymes that regulate tumor growth, angiogenesis, invasion and metastasis (89), which all constitute potential therapeutic targets for inhibition of tumor promoting TAM activity.

Increasingly siRNAs are finding application *in vivo* to specifically modulate target gene expression (90). Since intratumoral injections of siRNA can block the function of target genes in macrophages as described above and a report showing that pretreatment of tumor cells with siRNA against macrophage migration inhibitory factor (MIF) normally induced during hypoxia effectively inhibits metastasis to the liver (91), favors the use of these nucleic acid-based constructs for large-scale human studies (92) whereby a more sustained therapeutic modality may be required to increase therapeutic efficacy. siRNAs induce sequence-specific gene silencing superior to the inhibition of gene expression mediated by single-stranded antisense oligonucleotides (93). RNA interference is therefore a powerful tool to block gene function directly in TAMs or cancer cells or surrounding stromal cells producing factors, which regulate TAMs and thereby influence solid tumor development.

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Abbreviations: CBC: complete blood count; CSF-1: colony-Stimulating Factor-1; CSF-1R: CSF-1 receptor; EC: endothelial cell; ECM: extracellular matrix; HIF: hypoxia-inducible factor; MMP: matrix metalloprotease; SDF-1: stromal cell-derived factor-1; TAM: tumor associated macrophage; TNF-alpha: tumor necrosis factor alpha; TNFR: TNF-α receptor; VEGF: vascular endothelial growth factor;

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