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Macrophages and the hypoxic tumour microenvironment

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. TAM Accumulation Within Solid Tumours
3.1. TAM Infiltration Predicts Poor Outcome
3.2. Mechanisms of TAM Accumulation
3.2.1. Extravasation of Circulating Monocytes
3.2.2. TAM Accumulation in Hypoxic Tumour Tissue
3.2.2.1. Effect of Hypoxia-Regulated Chemoattractants
3.2.2.2. Hypoxia-Induced Arrest of Macrophage Migration
4. TAM, Hypoxia and Angiogenesis
4.1. Hypoxia-Inducible Factor (HIF)
4.2. TAM and Angiogenesis
4.2.1. Mechanisms of Angiogenesis
4.2.2. Lymphangiogenesis
4.3. Migration and Metastasis
4.4. Immunosuppression
5. Anti-Tumour Effects of TAMs
6. Macrophages and Anticancer Therapy
7. Conclusions
8. References

1. ABSTRACT

One characteristic of solid tumour tissue is the presence of large numbers of tumour-associated macrophages. These migrate down gradients of chemo-attractive agents to accumulate within hypoxic and / or necrotic areas where they are generally related to poor clinical prognosis. In this review we will discuss the molecular mechanisms that underlie recruitment of macrophages into tumours and their pro-tumourigenic activities with respect to stimulation of angiogenesis, lymphangiogenesis, tumour cell migration, metastasis and immuno-suppression. The potential of macrophage-related anticancer therapies will be discussed in the light of this phenotype.

2. INTRODUCTION

Solid tumours manifest as a mass comprising neoplastic cells, stroma, vasculature and inflammatory cells within a complex microenvironment. Multiple interactions between these various components combine to determine clinical outcome. Increasing interest has focussed on the macrophage component of the leukocytic infiltrate, highlighting the generally pro-tumourigenic phenotype of these cells and the striking contribution of tumour hypoxia to this phenotype.

Tumour-associated macrophages (TAMs) are derived predominantly from circulating peripheral blood

Table 1. Clinical correlations of outcome with TAM infiltration in different cancer types

Cancer Type	Correlation with TAM number	No. of cases	Ref.
Breast	Poor prognosis	40	6
	Reduced disease-free survival	249	7
	Reduced relapse-free and overall survival	101	8
	High tumour stage, increased recurrence	80	9
	Increased tumour grade	207	10
Bladder	Higher in invasive vs superficial cancers. Increased metastases and vascular invasion, reduced 5 year survival	63	11
	Reduced rate of recurrence	53	12
Esophagus	Increased vascular invasion and metastases	56	13
	Enhanced survival	377	14
Cervix	No correlation with stage, grade or survival	56	15
	Reduced tumour stage. No correlation with tumour grade or lymph node status	24	16
Pulmonary adenocarcinoma	Reduced survival	113	17
NSCLC	No correlation with tumour stage	63	18
	Increased 5 year and median survival	175	19
Colorectal	Reduced tumour stage, improved prognosis	30	20
	More superficial invasion, fewer lymph node metastases, increased 5 year survival	60	21
	More superficial invasion, reduced vascular invasion, fewer metastases	97	22
Prostate	Poor outcome, reduced survival	85	23
Follicular lymphoma	Reduced overall, disease-free and progression-free survival	99	24
	No association with early transformation	25	25
Head and neck	Increased lymph node metastases	102	26
Gastric	Increased 5 year survival	84	27

monocytes and can comprise up to 50% of the cellular mass of a tumour (1, 2). It was originally assumed that TAMs functioned as a major part of the host defence against developing tumours based on their ability to kill tumour cells in vitro (3) and that this host response was eventually overwhelmed, enabling progression to an invasive and ultimately metastatic phenotype.

Conversely the majority of reports now describe an array of pro-tumourigenic macrophage functions, stemming from the observation that high levels of macrophage infiltration are associated with poor prognosis in different tumour types (4). This is largely due to the fact that TAM generally represent an alternatively activated M2 population of macrophages with functions including promotion of angiogenesis, matrix remodelling and suppression of adaptive immunity, rather than classically activated M1 macrophages with good antigen-presenting and cytotoxic abilities (5). TAMs produce a multitude of tumour-promoting factors including mitogenic and immuno-suppressive cytokines, pro-angiogenic growth factors and proteolytic enzymes. It has additionally been shown that tumour microenvironmental factors, such as hypoxia, actively contribute to this phenotype by both stimulating macrophage infiltration and inducing TAMs to express various growth factors and enzymes that stimulate angiogenesis (4).

In this review we will outline the mechanisms by which circulating monocytic cells are induced to accumulate within tumour tissue. We will discuss the therapeutic implications of TAM infiltration with respect to clinical prognosis and examine in detail the various means by which the hypoxic component of the tumour microenvironment contributes to the regulation of macrophage gene expression and function. Finally, we will examine the implications of these findings in the light of development of novel macrophage-based anti-tumour therapies.

3. TAM ACCUMULATION WITHIN SOLID TUMOURS

3.1. TAM infiltration predicts poor clinical outcome

The importance of the macrophage component of solid tumour tissue is most strikingly represented in breast cancer, where the vast majority of clinical data ascribes a poor prognosis to tumours with high numbers of infiltrating macrophages. Initially observed by Steele *et al* in 1984 (6), numerous studies have now reported a strong positive association between high macrophage index and unfavourable clinical parameters as regards survival (7, 8), regression (8, 9) and tumour stage (9, 10). Less data is available in other cancer types and the association between macrophage infiltration and prognosis in these situations is unclear (Table 1).

3.2. Mechanisms of TAM accumulation

The initial migration of circulating monocytes across the vascular wall is regulated by a range of adhesion molecules and chemotactic factors, many of which are expressed by both tumour endothelium and tumour cells themselves. This is followed by fine-tuning of TAM localisation within the tumour mass by migration along intra-tumoural gradients of chemotactic factors and is regulated to a large degree by tumour hypoxia (Figure 1).

3.2.1. Extravasation of circulating monocytes

Transendothelial migration involves sequential steps of monocyte tethering to the endothelium, loose rolling along the vascular surface, firm adhesion to the endothelial layer and diapedesis between adjacent endothelial cells. Monocyte rolling is mediated by interactions between selectins and their ligands and by monocytic beta(1) and beta(2) integrins interacting with endothelial VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular cell adhesion molecule 1) respectively. Hypoxic conditions within tumor tissue up-regulate expression of all three endothelial cell adhesion molecules (29-30), most likely via tumour and stromal cell

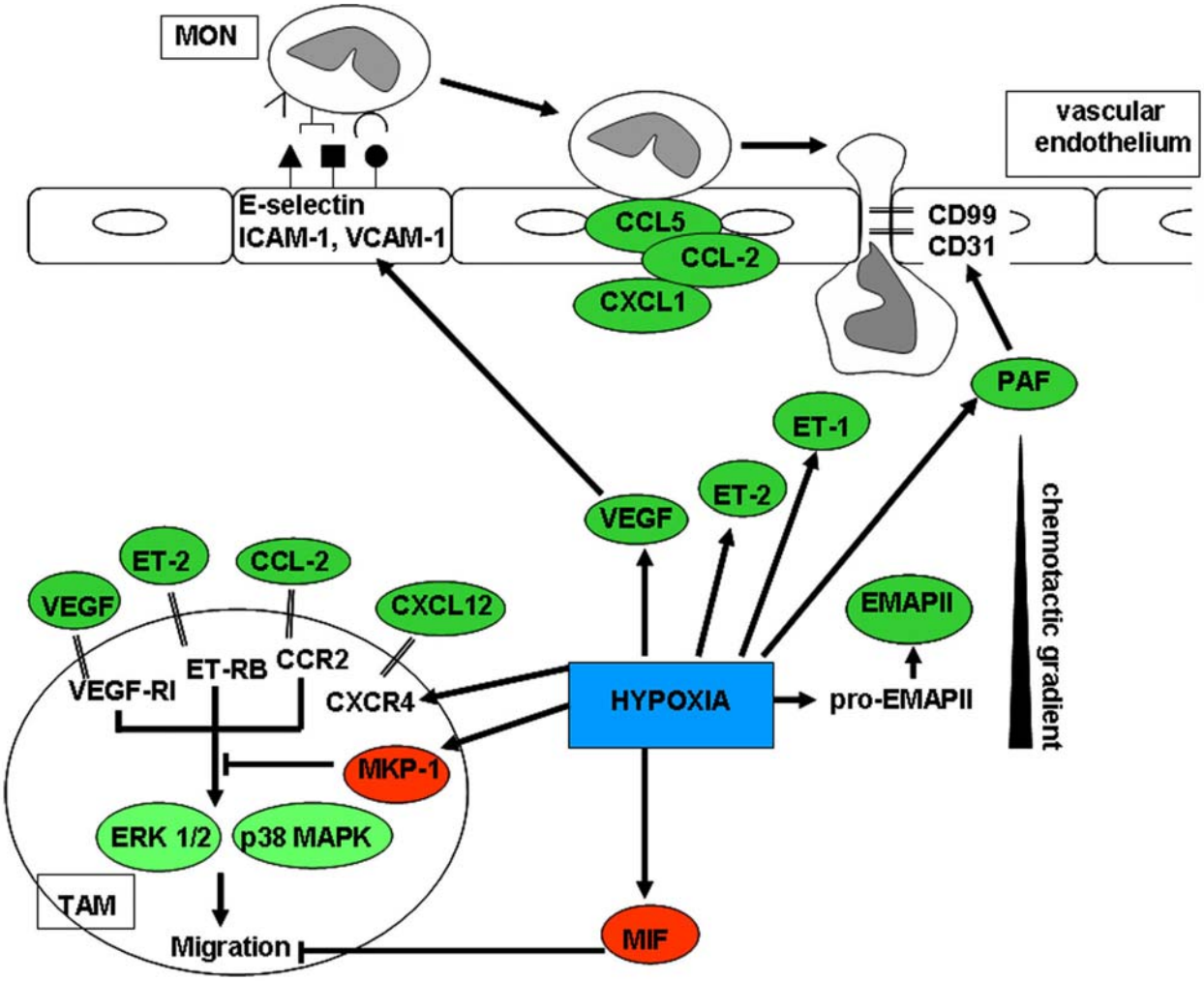

Figure 1. Transendothelial migration of circulating monocytes across the vascular wall is governed by adhesion molecules and chemotactic factors which regulate the sequential steps of monocyte tethering, loose rolling and firm adhesion to the endothelial layer and subsequent diapedesis between adjacent endothelial cells. Fine-tuning of TAM localisation within the tumour mass is largely regulated by tumour hypoxia via specific induction of monotactic cytokines and inhibition of migration within areas of hypoxia.

secretion of hypoxia-induced genes such as VEGF (vascular endothelial growth factor) (31).

Firm adhesion of monocytes to the endothelial layer is initiated by immobilised chemokines present on the endothelial surface. CCL2 / MCP-1 (monocyte chemoattractant protein-1), CCL5 / RANTES (regulated on activation normal T cells expressed and secreted) and CXCL1 / KC (GRO alpha/keratinocyte-derived chemokine) are currently the only chemokines shown to function as arrest molecules under physiological conditions (32). RANTES and CXCL1 are expressed in a variety of tumours including breast (33, 34), cervical (34) and colorectal (35, 36) cancers and melanoma (37, 38). MCP-1 is also expressed in breast (39), ovarian (40) and lung cancers (NSCLC)(41), in all of which it shows a positive correlation with TAM accumulation (39-41). Murine studies *in vivo* have revealed a biphasic effect of MCP-1 over-expression. Gene transfer to xenografts or syngeneic tumours promoted macrophage infiltration to a level correlating with the amount of MCP-1 produced (42). Low-level MCP-1 secretion resulted in tumor formation (42), whereas high level secretion was associated with massive monocyte/macrophage infiltration and marked tumour necrosis (42, 43).

As well as being chemoattractant molecules, MCP-1 and RANTES stimulate monocytes to express other proteins that enhance monocyte migration and tumour progression. RANTES stimulates human monocytes to express MCP-1, CCL3 / MIP-1 alpha (macrophage inflammatory protein 1 alpha), CCL4 / MIP-1 beta (macrophage inflammatory protein 1 beta) and CXCL8 / IL-8 (interleukin-8) as well as the RANTES receptor CCR1 (44). It is likely that these and additional chemokines also function to enhance extravasation by mechanisms whereby

chemokine activation of monocytes amplifies the recruitment signal.

The final stage of extravasation involves transendothelial diapedesis, with initial interaction between CD31 / PECAM-1 (platelet endothelial cell adhesion molecule 1) expressed on both monocytes and endothelial cells followed by similar homophilic adhesion via CD99 / MIC-2 (45). Diapedesis is enhanced under hypoxic conditions by induction of PAF (platelet-activating factor) which acts in an autocrine fashion to activate protein kinase C and phosphorylate PECAM-1 (46). Additionally, CD99 is over-expressed in both cervical (47) and prostate (48) carcinomas and is also regulated by hypoxia (49).

3.2.2. TAM accumulation in hypoxic tumour tissue

Hypoxia (an oxygen tension of 0.1-0.5% O_2) generally occurs >100μm from functional blood vessels (50) and, as human malignancies have an inadequate vasculature, is widespread in both primary tumours and their metastases (51). Many macrophage chemoattractants are regulated by hypoxia, resulting in a gradient of chemotactic proteins down which macrophages migrate to accumulate in the most severely hypoxic regions of tumour tissue (52). A brief over-view is provided here, although the subject has been reviewed in detail by Balkwill (53).

3.2.2.1. Effect of hypoxia-regulated chemoattractants

The most widely studied of these cytokines is VEGF. VEGF stimulates migration via activation of its receptor VEGF-R1 / flt-1 on monocytes / macrophages (54), resulting in the positive correlation observed in breast tumours between elevated VEGF expression and the number of infiltrating macrophages (55). VEGF is induced by hypoxia in a broad range of tumour cells via stabilization of the transcription factor Hypoxia-Inducible Factor-1 alpha (HIF-1 alpha, see section 4.1) (56). Several studies have described elevated levels of VEGF in tumour cells and macrophages within avascular / peri-necrotic (and therefore presumably hypoxic) areas of human tumors (57, 58). This has led to the suggestion that positive feedback mechanisms might serve to amplify the monotactic signal via pathways whereby hypoxia-induced VEGF in both TAMs and tumour cells exerts a chemotactic action on other macrophages, aiding their migration to avascular tumour sites. It is also evident that macrophages up-regulate chemokine receptors under hypoxic conditions. The CXCR4 receptor for the monotactic CXC chemokine SDF-1 (stromal cell-derived factor-1, CXCL12) is induced by hypoxia via a HIF-dependent pathway (59), another mechanism whereby the hypoxic micro-enviroment amplifies chemotactic signals.

Endothelins are small vasoactive and mitogenic peptides that are expressed in a wide range of human tumours (60, 61). ET-1 (endothelin-1) and ET-2 (endothelin-2) are both chemotactic and bind the ET-RA receptor on monocytes (62) and the ET-RB receptor on macrophages (63) respectively, implying that ET-1 might recruit circulating monocytes into tumour tissue with ET-2 functioning to localize macrophages within the tumour mass. Both genes are additionally induced by hypoxia (49, 63), suggesting that this localization might target areas of reduced pO_2.

Another possible component of the hypoxic gradient of chemoattractants is EMAP II (endothelial-monocyte activating peptide II). EMAP II is synthesized as the precursor protein pro-EMAP II which is post-translationally cleaved by proteases released from apoptotic and necrotic cells to form the active cytokine. Cleavage of pro-EMAP II appears to be enhanced by hypoxia. Despite no effect on the mRNA, increased levels of mature EMAP II were detected in the supernatant of hypoxic tumor cells *in vitro* (64) and in kidney tissue in a murine model of ischaemia-reperfusion (65). It has been suggested that hypoxia might enhance production of mature EMAP II as part of a mechanism to attract macrophages to areas of hypoxia / necrosis, supported by the observation that macrophages accumulate in areas of high EMAP II expression in uveal melanoma (66).

Other factors involved in macrophage migration are down-regulated by hypoxia. Both constitutive and cytokine-induced expression of the CCR5 chemokine receptor was antagonised by hypoxia in murine macrophages (67). As CCR5 mediates MIP-1 alpha, MIP-1 beta and RANTES-induced chemotaxis, this may represent a way to retain macrophages at hypoxic sites. Interestingly, MCP-1 expression is inhibited by hypoxia at both the transcriptional and post-transcriptional level in ovarian cancer cells (68) and murine and human monocytic cell lines (69). This raises questions as to whether this is also the case *in vivo*. Although a positive correlation has been observed between pan-tumour MCP-1 expression and TAM accumulation (39-41), macrophages also accumulate in areas of necrosis where there is little MCP-1 (70). MCP-1 may therefore be primarily involved in general macrophage recruitment, with specific localisation within areas of tumour hypoxia being achieved by a different group of hypoxia-induced cytokines.

3.2.2.2. Hypoxia-induced arrest of macrophage migration

A complementary and / or alternative mechanism whereby macrophages are induced to accumulate in regions of hypoxia involves the hypoxia-regulated inhibition of macrophage chemotaxis. This is partly mediated by induction of cytokines that inhibit macrophage migration. MIF (macrophage migration inhibitory factor) is hypoxia-inducible in cancer cell lines (71, 72) and macrophages (73) and is expressed in tumour cells around necrotic areas in glioblastoma multiforme (71). Initially characterised as inhibiting the random migration of macrophages (74, 75) it also promotes malignant cell transformation, stimulates angiogenesis and inhibits tumour cell cytolysis via poorly defined mechanisms (76). Recent transcriptional profiling of primary human monocytes under hypoxia has revealed the GRO-family chemokines CXCL2 (GRO beta) and CXCL3 (GRO gamma) as hypoxia-inducible (77). These have been identified as specialized monocyte arrest chemokines in an *in vitro* flow chamber model of monocyte recruitment (78). Data is limited as regards expression of

these proteins *in vivo*, although CXCL3 has been described in tumour biopsies of metastatic renal cell carcinoma (79).

More importantly however, migration induced by many chemoattractants seems to be directly inhibited by hypoxia itself. MCP-1-induced migration requires phosphorylation and activation of p38 MAPK (p38 mitogen-activated protein kinase) and ERK1/2 (extracellular signal-regulated kinase 1/2). Grimshaw *et al* found that hypoxia inhibited migration induced by MCP-1 *in vitro* and that hypoxic macrophages rapidly up-regulated MKP-1 (MAPK phosphatase 1) by a pathway also induced by chemical activators of HIF-1 (80). MKP-1 dephosphorylates ERK 1/2 and p38 MAPK, suggesting that hypoxic up-regulation of MKP-1 might be responsible for the observed inhibition of MCP-1-induced chemotaxis (80). This additionally has implications for other chemoattractants. ET-2-mediated macrophage migration is also inhibited by hypoxia (61) and both VEGF signaling via VEGF-R1 (81) and ET-2 signalling via ET-RB (61) require signal transduction via the MAPK pathway.

It therefore appears that hypoxic induction of macrophage chemoattractants followed by specific inhibition of migration within the hypoxic region acts to direct macrophages to areas of hypoxia / necrosis where they accumulate and function as tumour-associated macrophages.

4. TAM, HYPOXIA AND ANGIOGENESIS

Due to the functional phenotype of M2 macrophages as regards matrix remodelling functions and secretion of pro-angiogenic cytokines (5), the predominant clinical effect of macrophage activation manifests as a striking pro-angiogenic phenotype in tumours with high focal TAM infiltration (82). Angiogenesis is the main mechanism whereby a solid tumour mass can develop a nutritive blood supply, enabling it to overcome micro-environmental limitations on proliferation and to expand. This requires a switch from the constitutive anti-angiogenic phenotype, involving a change in the balance of pro- and anti-angiogenic molecules that are secreted from the tumour and from infiltrating immune cells including macrophages (83). This switch is elicited by a combination of genetic and microenvironmental stimuli including hypoxia.

4.1. Hypoxia-Inducible Factor (HIF)

Hypoxia regulates the expression of many genes by stabilisation of the transcription factors HIF-1 and HIF-2. HIF initiates a complex programme of gene expression including genes involved in processes such as angiogenesis, apoptosis, glycolysis and cell cycle control which are central to survival and expansion of the malignant cell population in an oxygen-deficient environment (84). HIF is a heterodimeric transcription factor composed of a hypoxia-inducible alpha subunit and a constitutively expressed beta subunit. Under normoxia HIF alpha is post-translationally hydroxylated by the prolyl hydroxylase domain enzymes targeting it for proteasomal degradation (85, 86). However these enzymes have an absolute requirement for O_2 and are therefore inactive under hypoxic conditions, allowing HIF alpha protein to accumulate and activate transcription of downstream target genes. Comprehensive *in vivo* studies of the HIF-mediated molecular response to hypoxia have generally demonstrated the importance of angiogenesis and tumour vascularisation to the growth and expansion of solid malignant tumours. Abolition of the angiogenic response to hypoxia in HIF-1 alpha $^{-/-}$ embryonic stem cells and HIF-1 beta-deficient fibroblasts produced poorly vascularised xenografts with a reduced rate of growth compared with their wild-type counterparts (87, 88).

Hypoxic up-regulation of HIF-1 alpha, HIF-2 alpha and downstream target genes has been demonstrated in primary human macrophages (89) and TAMs *in vivo* (90, 91). A strong correlation has been observed between increased numbers of HIF-2 positive macrophages and high levels of angiogenesis in cancers of the breast (92) and bladder (93). Additionally, adenoviral over-expression of HIF alpha in primary human macrophages revealed HIF-2 to be the primary mediator of the hypoxic response in these cells (73).

Conversely HIF-1 alpha has recently been shown to be essential for normal macrophage function. There is now substantial evidence that HIF is also induced by non-hypoxic stimuli including oncogenic mutation (ras, src, PTEN), growth factor stimulation (insulin, insulin-like growth factor-1, and angiotensin) (84) and macrophage-associated stimuli such as oxidized low-density lipoprotein (94), lipopolysaccharide (95), the macrophage-derived peptide PR39 (96) and differentiation itself (91, 97). Conditional ablation of HIF-1 alpha in murine myeloid cells greatly inhibited homotypic adhesion, motility and invasion of *ex vivo* peritoneal macrophages with associated reductions of *in vivo* macrophage infiltration, edema formation, and tissue destruction in murine models of inflammation (98). The authors additionally demonstrated an essential role for HIF-1 alpha in the maintenance of intracellular energy homeostasis in macrophages. Even under normoxic conditions HIF-1 alpha null macrophages exhibited a greater than 80% reduction in intracellular ATP compared with control cells (98). We have recently demonstrated that macrophage expression of HIF-1 alpha is also intrinsically linked to the regulation of intracellular iron homeostasis by iron transport proteins such as Nramp1 (Natural resistance-associated macrophage protein 1) (97). These results suggest a substantial role for HIF in macrophage physiology over a wide range of microenvironmental conditions and raise questions as to the mechanism of activation and functional importance of the response.

4.2. TAM and angiogenesis

Angiogenesis, the development of new blood vessels from an existing vascular bed, is necessary in order to supply a nutritive blood flow to an expanding malignant tumour. The relationship between tumour angiogenesis and prognosis was unclear until a study by Folkman and Weidner in early stage breast carcinoma revealed that the

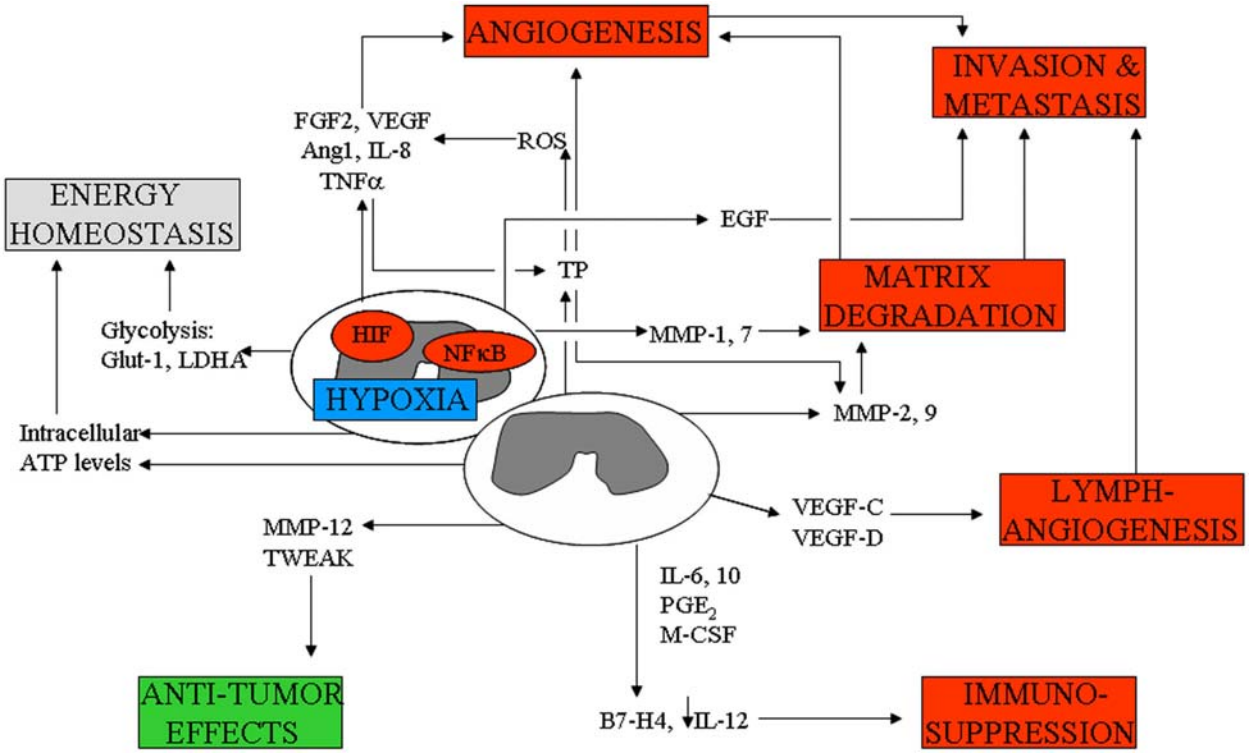

Figure 2. Micro-environmental hypoxia plays a central role in regulating tumour-associated macrophages. Both HIF-dependent and HIF-independent hypoxia-inducible genes interact to form a complex network of effects that combine to determine the TAM phenotype. Shown in red boxes are key pathways involved in the tumorigenic phenotype, to which macrophages contribute.

presence of 'hotspots' of high angiogenic activity, rather than an overall high level of angiogenesis, was the critical factor predicting an unfavorable prognosis (99). This was due to the biological importance of the 'hotspots' as points of entry into the vasculature for metastatic tumour cells.

Early studies in a series of invasive breast carcinomas revealed a strong positive correlation between TAM infiltration and high vascular grade (8) which has since been observed in a number of other cancer types (100-102). Interestingly, the most angiogenic tumours are also the most necrotic and have the highest macrophage indices, implying an important role for hypoxia in angiogenic activation of macrophages (103).

4.2.1. Mechanisms of angiogenesis

The molecular basis of the pro-angiogenic activity of TAMs is similar to that of tumour cells themselves in that hypoxia induces the expression of a variety of cytokines and growth factors (Figure 2). Indeed, cDNA array analysis of primary human macrophages demonstrated that hypoxia can up-regulate the mRNA of over 30 proangiogenic genes including VEGF, IL-8 (interleukin 8), FGF2 (fibroblast growth factor 2) and angiopoietin (73).

Probably the most extensively studied of these is VEGF which as well as being a macrophage chemoattractant is also a major pro-angiogenic molecule, being both an endothelial cell mitogen and acting to increase the vascular permeability of tumour blood vessels (83). VEGF is significantly up-regulated in response to hypoxia in tumour cells *in vitro* (56) and in invasive breast carcinoma was specifically expressed in TAMs in avascular areas where tumour cells also expressed VEGF (58). This is the only report to date to specifically describe expression of VEGF by hypoxic TAMs *in vivo*, although many others have reported a positive association between numbers of infiltrating TAM and immunoreactivity for VEGF (55, 57, 104). A recent study in nude mice demonstrated a key role for macrophage VEGF in the initiation of tumour angiogenesis. Bingle *et al* implanted T47D human breast tumour spheroids pre-infiltrated with human macrophages

under the dorsal skin. After 3 days, VEGF expression and surrounding blood vessel counts were much greater in spheroids containing infiltrating macrophages than those containing tumour cells alone (105). EGF (epidermal growth factor) and the HIF-regulated TGF-alpha (transforming growth factor alpha) (106) are also macrophage-produced mitogenic cytokines. The receptor for both, EGF-R, is up-regulated in breast cancers of poor prognosis where it is associated with macrophage infiltration, suggesting that TGF-alpha and EGF-producing TAMs might select for aggressive tumour phenotypes(55).

TAMs also secrete proteolytic enzymes including a range of matrix metalloproteinases, MMP-1 (107) and MMP-7 (108) of which are hypoxia-inducible with MMP-1 being regulated by HIF-2 alpha (107). MMPs digest the

tumour extracellular matrix, releasing heparin-bound growth factors and facilitating endothelial cell migration to enhance angiogenesis. MMP-9 also promotes the migration and invasion of cancer cells and macrophages by mediating type IV collagen degradation in the vascular basement membrane. The importance of MMP-9 for angiogenesis has been demonstrated in MMP-9 knock-out (MMP-$9^{-/-}$) mice, which produce $MMP9^{-/-}$ macrophages (109). Human tumour xenografts in $MMP9^{-/-}$ mice were smaller and contained fewer infiltrating macrophages than those in wild-type animals, associated with reduced tumour angiogenesis (109). Similarly, suppression of MMP-9 expression by TAMs in an HPV16-induced murine model of cervical carcinogenesis severely impaired the angiogenic response (110).

As well as being intrinsically angiogenic, secretion of some macrophage-derived cytokines initiates a cascade of other pro-angiogenic factors. TNF-alpha (tumour necrosis factor alpha) is another hypoxia-inducible macrophage-derived pro-angiogenic cytokine expressed predominantly in the TAM population of breast cancer tissue (111). TP / PD-ECGF (thymidine phosphorylase / platelet-derived endothelial cell growth factor) is expressed by both TAMs and tumour cells (112), is pro-angiogenic (2) and protective versus hypoxia- and DNA damage-induced apoptosis (113). Tissue culture assays have demonstrated that TNF alpha can enhance expression of TP and in breast cancer TNF-alpha expression in TAMs has been correlated with TP protein production (114), which in turn correlates with tumour angiogenesis and prognosis in invasive breast cancer (112) and astrocytic tumours (115). Macrophage production of TNF-alpha was also enhanced by co-culture with cancer cell lines, which in turn enhanced cancer cell invasiveness due to TNF-alpha-dependent induction of MMP-2 and MMP-9 in the macrophages (116).

CSF-1 (colony-stimulating factor 1), a pro-angiogenic factor and potent monocytic chemoattractant (117), also regulates MMP expression. Antisense or RNAi-mediated blockade of CSF-1 expression in MCF-7 mammary carcinoma xenografts reduced expression of MMP-2, MMP-12 and VEGF, inhibited macrophage infiltration and suppressed angiogenesis and tumour growth (118). The importance of the macrophage phenotype has been demonstrated in a cross of CSF-1 knock-out mice with transgenic mice susceptible to mammary cancer. This produced null mice with tumours containing a significantly reduced macrophage infiltrate correlating with delayed tumour progression (119). Re-introduction of CSF-1 increased TAM infiltration, accelerated tumour progression and increased the number of metastases (119). The central role played by this system has been extensively reviewed by Condeelis and Pollard (120).

This suggests the possibility of two arms to the TAM-mediated angiogenic response. A primary potent response mediated by hypoxic up-regulation of pro-angiogenic cytokines and growth factors via the induction of HIF (and potentially other transcription factors such as NF-kappaB (121), Erg-1 (122) and Ets-1 (123)). Secondly, in the presence of oxygen, cancer cells themselves might induce macrophages to express pro-angiogenic cytokines. Addtionally under normoxic conditions TP may induce tumour cell oxidative stress by the generation of reactive oxygen species which upregulate the angiogenic factors VEGF, MMP-1 and IL-8 (124).

4.2.2. Lymphangiogenesis

The importance of lymphangiogenesis as a mechanism to aid the spread of malignant tumour cells to the lymph nodes has been controversial. However, intratumoral lymphatics and lymphangiogenesis have recently been detected in head and neck cancer (125), thyroid carcinoma (126), melanoma (127) and breast cancer (128). Additionally, peri-tumoural lymphangiogenesis has been observed in cervical cancers associated with expression of VEGF-C and VEGF-D, the main lymphatic growth factors, and their receptor VEGFR-3 in TAMs (129). The importance of TAMs as a source of VEGF-C and VEGF-D has been demonstrated using a corneal model of injury lymphangiogenesis. Specific neutralisation of VEGF-A by VEGF TRAP depleted recruitment of macrophages to the site of injury and completely inhibited formation of new lymphatic vessels, suggesting that macrophages play a crucial role in signalling and / or amplifying the signals essential for stimulating division and migration of lymphatic endothelial cells (130). An additional mechanism whereby macrophages might support lymphangiogenesis was recently elucidated in a mouse corneal transplant system. Following transplant, CD11b+ macrophages infiltrated the corneal stroma and trans-differentiated into lymphatic endothelial cells that joined existing lymphatic vessels (131). Macrophage trans-differentiation might therefore provide an alternative or complementary mechanism whereby TAMs can promote tumour lymphangiogenesis.

4.3. Migration and metastasis

Many of the growth factors and cytokines mentioned with reference to their pro-angiogenic capacity also directly stimulate the growth of tumour cells themselves and/or promote tumour cell migration and metastasis, as evidenced by the additional effects observed in the angiogenic models cited. Using a chemotaxis-based invasion assay and multiphoton-based intravital imaging in mammary tumours of MMTV-PyMT mice it has been shown that tumour cells and macrophages co-migrate towards EGF and CSF-1 (132). The migratory response of carcinoma cells to these chemotactic agents was dependent on the presence of macrophages, as in mice that were also CSF-1-deficient, and therefore macrophage-depleted, there was a large reduction in the number of migrating tumour cells (132). The basis for this co-dependence for migration and invasion is thought to be a positive feedback loop whereby macrophage-derived EGF promotes tumour cell migration and CSF-1 expression and tumour cell-derived CSF-1 promotes the expression of EGF by macrophages (133). This elegantly demonstrates that, even in the presence if the relevant mitogenic cytokines, TAMs are necessary for optimal migration (and metastasis) of tumour cells.

4.4. Immunosuppression

An additional characteristic of alternatively activated M2 macrophages is that of suppression of

adaptive immunity through mechanisms including suppression of T cell proliferation and activity, lack of tumour cell lytic ability and poor antigen-presenting capabilities (5).

A number of studies have shown that a major contributory factor to the M2 phenotype is tumour cell secretion of molecules such as PGE_2 (prostaglandin E_2), M-CSF, TGF-beta1 and interleukins IL-4, IL-6 and IL-10 (134-136), implying that tumour-derived molecules direct the potential of activated macrophages to promote tumour development. In ovarian cancer patients, activated monocytes / macrophages from the peripheral blood and ascites were recently shown to demonstrate defective antibody-dependent cell-mediated cytotoxicity and phagocyte functions in comparison with normal controls (137). This may in part be due to IL-6 and IL-10-mediated induction of macrophage B7-H4 expression, a molecule implicated in the suppression of tumour-associated antigen-specific T cell immunity (134). Tumour-derived cytokines such as PGE2, M-CSF and IL-10 are also implicated in the down-regulation of macrophage release of immunostimulatory cytokines such as IL-12 (134, 138). In an orthotopic murine model of prostate cancer intratumoural injection of IL-12-expressing macrophages, which showed markedly increased surface MHC expression, induced significant suppression of tumour growth associated with increased infiltration of CD4+ and CD8+ T cells (139). At least some of these immunosuppressive activities might be regulated by over-activation of transcription factors such as Stat3. Stat3 inhibition has been shown to enhance the cytotoxicity and antigen-presenting function of activated macrophages associated with retardation of tumour growth and accompanied immune activation (140).

Hypoxia is also likely to contribute to suppressing the anti-tumour activity of TAMs, as it amplifies secretion of potent immunosuppressive agents such as IL-10 and PGE_2. Additionally, hypoxic up-regulation of 'pro-angiogenic' molecules such as MMP-7 by macrophages has a potentially broader role in determining the M2 phenotype. MMP-7 is known to mediate cleavage of the pro-apoptotic Fas and so to protect tumour cells from cytolysis by cellular components of the immune system (141).

5 ANTI-TUMOUR EFFECTS

It is evident from the wealth of data already discussed that the majority of TAMs function to promote tumour progression and suppress the host anti-tumour immune response. However there is also evidence for anti-tumour effects of TAMs, some of which may be cancer-type specific. Contrary to the generally positive association between TAM infiltration and tumour progression discussed in section 3.1, high numbers of TAMs have been associated with enhanced tumour cell apoptosis and improved disease-free survival in gastric (142), oesophageal (14) and other cancers (Table 1). Similarly, the presence of infiltrating macrophages has been shown to be essential for the spontaneous tumour regression observed in a murine model of intra-ocular tumour formation (143).

The mechanisms behind the anti-tumour effects of TAMs in these tumours are under-reported, although could potentially be ascribed to the presence of significant numbers of classically activated M1 macrophages. Alternatively TAMs may represent a unique population of macrophages with key properties of M2 cells which additionally express selected M1-associated genes. This was recently described by Biswas *et al* (144) who characterised the gene expression profile of murine fibrosarcoma TAMs by cDNA microarray analysis. These TAMs displayed the gene expression profile of a classical M2 population (immunosuppressive cytokines, scavenger molecules, inflammatory chemokines etc) and additionally expressed high levels of the IFN-inducible M1 chemokines CXCL9, CXCL10 and CXCL16 (144). IFN-inducible CXC chemokines are generally potent inhibitors of angiogenesis and chemo-attractant for T cells (145, 146). Overexpression of CXCL10 in murine mammary adenocarcinoma cells had no effect in culture but elicited a strong T cell-dependent anti-tumour effect *in vivo* (147). Similarly, intratumoural injection of adenoviral vectors co-expressing either CXCL9 or CXCL10 with IL-12 resulted in marked tumour regression and enhanced survival time compared with any agent alone (148). This was accompanied by both enhanced cytotoxic T cell activity and reduced angiogenesis. Analysis of CXCL9 and CXCL10 expression in human lung cancer has identified no significant association of either agent with recurrence or disease-free survival (149). Less data is available for CXCL16, although low level expression has been observed in TAMs in human colorectal adenocarcinoma (150). Despite the limited data available regarding expression and clinical function of these genes, it is evident that coincident expression of a small range of IFN-inducible M1 chemokines might produce significant anti-tumour activity.

Other genes are also implicated in macrophage-mediated mechanisms of anti-tumour activity, including macrophage-derived MMP-12. In a murine model of Lewis lung carcinoma, deletion of macrophage MMP-12 resulted in enhanced tumour-associated microvessel density and a generally angiogenic phenotype in comparison with wild-type controls (151). This suggested a role for macrophage MMP-12 in retarding tumour growth that is in accordance with observations in human squamous cell carcinoma, where macrophage expression of MMP-12 was predictive of improved outcome (152). Another factor implicated in mediating macrophage anti-tumour mechanisms is TWEAK (TNF-like weak inducer of apoptosis) (153). Administration of a neutralizing anti-TWEAK monoclonal antibody significantly promoted tumour growth and reduced tumour rejection and survival of mice inoculated with TWEAK-sensitive tumour cells. These effects were abolished by the inhibition of macrophage infiltration, suggesting that TWEAK might mediate anti-tumour effects of macrophages *in vivo* (153). Despite such reports however, it is obvious that the balance of the reported effects of tumour-infiltrating macrophages is biased towards a generally pro-tumourigenic phenotype.

6. MACROPHAGES AND ANTI-CANCER THERAPY

From the above review it is clear that macrophages are major contributors to tumour growth,

invasion and metastasis (figure 2) and it is therefore appropriate to consider them as a potential target for therapy. Given the propensity of macrophages to accumulate within solid tumour tissue, it is an attractive proposition to utilise this as a method of delivering therapies to sites of tumourigenesis. At the most basic level, autologous monocyte-derived macrophages stimulated with IFN-gamma *ex vivo* have demonstrated an ability to kill tumour cells and inhibit their proliferation *ex vivo* by mechanisms involving mediators such as TNF-alpha (154). Other strategies have included *ex vivo* transfection of monocyte-derived macrophages with immuno-stimulatory cytokines such as M-CSF and IFN-gamma (155) Few side-effects have been reported clinically, although significant anti-tumour activity has yet to be demonstrated (156, 157). This may be because successful delivery of macrophage immunotherapies only occurs in a proportion of cases, as demonstrated in metastatic ovarian carcinoma patients where injected radio-labelled autologous macrophages were tracked to the tumour site in only 12 / 22 patients (158). This implies that incorporation of tracking studies in the early stages of immunotherapy might enable selection of patients suitable for this type of strategy.

As macrophages are specifically attracted to areas of hypoxia, it is also an appealing proposition to use macrophages to deliver gene therapy that is specifically activated under hypoxic conditions and so lend a greater element of tumour-targeting. A step in this direction has been achieved using hypoxia response elements (HREs), that are activated by the transcription factors HIF-1 and HIF-2, to regulate expression of the enzyme that activates the pro-drug cyclophosphamide (89). Human cytochrome P450 linked to an HRE was transduced into macrophages which were subsequently able to migrate into the hypoxic centre of tumour spheroids. Expression of cytochrome P450 was induced specifically in hypoxic regions, conferring sensitivity to cycloheximide only in those spheroids containing the transgene (89). Strategies involving HRE-driven expression of IFN-gamma have been demonstrated in murine ANA-1 macrophages in culture (159). Similar tumour regions can also be targeted using the glucose-starvation sensitive grp94-promoter (160). In transgenic mice, LacZ transgene expression driven by the grp94 promoter was strongly activated in a variety of spontaneous and chemically induced tumours in both tumour cells and TAMs. In contrast, LacZ was not detected in normal tissues or in macrophages associated with normal organs (160). Another method of encouraging accumulation of macrophages within murine tumour tissue has recently been described by Guiducci *et al* (161). Intra-tumoural injection of a CCL16 adenoviral construct induced expression of the monotactic chemokine within the tumour site associated with accumulation of tumour macrophages. Administered in combination with CpG and an anti-IL10-receptor antibody, infiltrating macrophages were switched from an M2 to an M1 phenotype triggering an innate immune response and marked tumour necrosis (161).

An alternative strategy for overcoming the macrophage delivery problem would be to use gene delivery techniques to specifically target gene therapy to macrophages in tumour tissue. Systemic delivery of plasmid-based ribozymes targeting NF-kappaB in a murine model of melanoma suppressed NF-kappaB expression in tumour and stromal cells, including TAMs, and significantly reduced metastatic spread (162). For such as approach to be clinically viable it would be necessary to develop a more targeted gene delivery vehicle to minimise effects on other cell types or normal tissues. The NF-kappaB system does highlight the efficacy of targeting transcription factors however. Activation of PPAR gamma (peroxisome proliferator-activated receptor gamma) in TAM for example, can almost completely ameliorate macrophage-mediated suppression of the cytotoxic T cell response (163).

Finally, it is also possible to use the cytotoxic potential of tumour-resident macrophages to mediate and / or enhance the effects of radiation-, chemotherapy- and cytokine-based therapies. *In vitro* studies have shown that in the presence of the clinical immuno-modulator lipid A, RAW 264.7 macrophages secrete nitric oxide, TNF-alpha and other cytokines which greatly enhance the hypoxic cell radio-sensitivity of EMT-6 cells (164). Macrophage activation has also been implicated as a central mediator of the anti-tumour effects of CD40 ligation in murine neuroblastomas (165) and of IL-2 and IL-12 in murine syngeneic lymphoma (166). Indeed NO-mediated autocrine induction of IL-12 secretion has been suggested as a mechanism whereby paclitaxel (67), prolactin (68) and nitroaspirin (69) may correct tumour-induced immune dysfunction, so generating tumouricidal macrophages and inducing a host anti-tumour response. As a note of caution however, it is not yet clear whether such results would translate into the clinical situation. A recent prospective study in patients with metastatic melanoma suggested that high numbers of infiltrating TAMs might correlate negatively with response to IL-2-based immunotherapy (170).

7. CONCLUSIONS

It seems clear that, at least in the majority of situations, TAM function to promote tumour growth and metastasis. Combined with the fact that they are naturally attracted to the hypoxic microenvironment within tumour tissue, this suggests them as strong candidates for development of tumour-specific targeted therapies. Some caution should be addressed as regards the clinical situation however as [A] not all tumours display a convincing association between prognosis and TAM accumulation and [B] no macrophage-based therapies have yet been successfully translated into the clinic. This implies that, as for other anti-cancer therapies in development, it might be necessary to tailor macrophage-based strategies to the individual situation.

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Abbreviations: CSF, colony stimulating factor; EGF, epidermal cell growth factor; EMAP, endothelial-monocyte activating peptide; ERK, extracellular signal-regulated; ET, endothelin; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; ICAM, intercellular adhesion molecule; IL, interleukin; KC, keratinocyte-derived chemokine; MAPK, mitogen-activated protein kinase; MCP, monocyte chemotactic protein; MIF, macrophage migration inhibitory factor; MIP, macrophage inflammatory protein; MKP, MAPK phosphatase; MMP, matrix metalloproteinase; Nramp1, natural resistance-associated macrophage protein 1; PAF, platelet activating factor; PD-ECGF, platelet-derived endothelial cell growth factor; PECAM, platelet-derived endothelial cell adhesion molecule; PGE, prostaglandin E; PPAR, peroxisome proliferators-activated receptor; RANTES, regulated on activation normal T cells expressed and secreted; TAM, tumour-associated macrophage; TGF, transforming growth factor; TNF, tumour necrosis factor; TP, thymidine phosphorylase; TWEAK, TNF-like weak inducer of apoptosis; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial cell growth factor.

Key Words: Tumour-associated macrophage, TAM, Angiogenesis, Hypoxia, Immunosuppression, Migration, Metastasis, therapy, Review

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