

Chapter 2

Role of Tumour-Associated Macrophages in the Regulation of Angiogenesis

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Introduction

As mentioned in previous chapters, tumours consist not only of malignant cells but also of various stromal cell types including tumour-associated macrophages (TAMs) (Sica et al. 2008). One early sign that TAMs might influence tumour angiogenesis was the finding that TAM numbers positively correlate with tumour angiogenesis in breast carcinomas (Leek et al. 1996). Several subsequent studies have confirmed such a link in a wide array of tumour types (Aharinejad et al. 2004; Bailey et al. 2007; Koide et al. 2004; Ohta et al. 2003; Saji et al. 2001) and showed that high-TAM numbers are also often linked to poor prognosis (Bingle et al. 2002; Lewis and Pollard 2006). However, definitive evidence for the pro-angiogenic effect of TAMs in tumours was provided using various murine tumour models. First, we demonstrated that macrophage infiltration into small breast tumour nodules (tumour spheroids) markedly enhanced their ability to induce neovascularisation following implantation into window chambers on the flanks of mice (Bingle et al. 2006). Then Lin and colleagues showed that, when MMTV-PyMT mice (which develop mammary tumours) were crossed with transgenic mice carrying a colony stimulating factor-1 (*Csf1*) null mutation (*Csf1^{lop/op}*), the absence of CSF-1 markedly decreased macrophage infiltration in pre-malignant tumours, which, in turn, resulted in the inhibition of tumour angiogenesis and delayed tumour progression (Lin et al. 2006; Lin et al. 2001). Indeed, they showed that TAMs control the ‘angiogenic switch’ associated with the malignant transition of the spontaneous mammary tumours that form in MMTV-PyMT mice (Lin et al. 2006). Other strategies employing an antibody to CSF-1 (Paulus et al. 2006) or clodronate liposomes (Zeisberger et al. 2006) to deplete TAMs have also resulted in a marked reduction in tumour angiogenesis.

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Elevated expression of such macrophage chemoattractants as the CC-chemokine CCL2 (MCP-1) in solid tumours correlates both with increased TAM recruitment (Hoshino et al. 1995; Leung et al. 1997) and with increased tumour neovascularisation (Barleon et al. 1996; Zhu et al. 2004). Additionally, the accumulation of TAMs in solid tumours is associated with the increased expression of such pro-angiogenic molecules as VEGFA, and bFGF, and correlates with increased formation of angiogenic blood vessels (Bolat et al. 2006; Tsutsui et al. 2005).

Functional plasticity is a hallmark feature of macrophages and they can respond to diverse environmental signals with a wide array of functional phenotypes. Consistent with this, a variety of overlapping macrophage activation states have been reported with classically activated, 'M1' and alternatively activated, 'M2' macrophages representing opposing extremes of this continuum. Exposure to pro-inflammatory cytokines and microbial products such as interferon gamma (IFN γ) and lipopolysaccharide (LPS) polarizes macrophages into the pro-inflammatory, M1 phenotype, which is characterized by the increased expression of their own pro-inflammatory mediators including inducible nitric oxide synthase (iNOS), reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI) and their increased microbicidal/tumoricidal activity. Anti-inflammatory molecules such as the Th2 cytokines, IL-4, IL-13 and IL-10, as well as apoptotic cells and immune complexes in combination with Toll-like receptor (TLR) stimulation induce macrophages to express an M2 activation phenotype. Such 'M2' macrophages are thought to be involved in promoting angiogenesis and tissue remodelling/repair, dampening inflammation by producing high levels of anti-inflammatory cytokines (e.g. IL-10 and transforming growth factor- β , TGF β) and up-regulating products of the arginase pathway (arginase II, ornithine and polyamines). They also display a high level of phagocytic activity and express high levels of both mannose and scavenger receptors (Gordon 2003; Mantovani et al. 2004; Mantovani et al. 2002; Mills et al. 2000; Siciliano et al. 2006). However, it should be noted that variations within the M2 phenotype of macrophages have recently been described leading to the subdivisions, M2a, -b and -c. Interleukins 4 and -13 stimulate the onset of the M2a phenotype in macrophages (Mantovani et al. 2002; Martinez et al. 2009), whereas exposure to immune complexes in combination with TLR activation induces M2b-type macrophages. Finally, exposure to glucocorticoids or IL-10 results in the production of M2c-type macrophages, with greater immunosuppressive, pro-angiogenic and tissue remodelling functions (Goerdts and Orfanos 1999).

Murine TAMs show many characteristics of an M2-polarized state (Biswas et al. 2008; Mantovani et al. 2002). For example, TAMs often up-regulate pro-angiogenic mediators, such as VEGFA (Balkwill and Mantovani 2001), cyclo-oxygenase (COX)-2 (Nakao et al. 2005) and MMP9 (Coussens et al. 2000). They also have impaired expression of such pro-inflammatory mediators as (e.g. IL-12 and TNF α), RNI and major histocompatibility complex (MHC) II, which functionally correlate to reduced cytotoxicity and antigen presenting capacity. Conversely, TAMs up-regulate anti-inflammatory molecules such as IL-10 and TGF β . Other hallmark markers of M2 activation, such as up-regulated levels of the M2-specific genes, arginase,

macrophage galactose-type C-type lectin-2 (Mgl2), found in inflammatory zone 1 (Fizz1) and Ym1 (Mantovani et al. 2003; Van Ginderachter et al. 2006) are also expressed by murine TAMs. However, it should be noted that some TAMs in mouse tumour models, as well as human tumours (e.g. breast, ovarian and renal cell carcinoma), exhibit typical M1 cytokines such as TNF α , IL-1 β , IL-6, IL-23p19 and CXCL8, depending on tumour type and stage (Biswas et al. 2006; Dinapoli et al. 1996; Langowski et al. 2006). This has led to the suggestion that the phenotype of TAMs may vary between different stages of tumour development and possibly different regions of the same tumours (Murdoch et al. 2008; Ohno et al. 2004).

Pro-angiogenic Functions of Macrophages in Tumours

TAMs express a broad number of cytokines that are capable of directly inducing the formation of blood vessels within tumours including VEGFA and bFGF (Bolat et al. 2006; Tsutsui et al. 2005). However, TAMs also produce a wide range of pleiotrophic factors including IL1 β (Voronov et al. 2003), TGF- β 1, and TNF α (Luo et al. 2006; Yoshida et al. 1997), which promote new blood vessel formation by promoting the release of such factors as VEGFA from other cell types commonly found within the tumour micro-environment (Pertovaara et al. 1994). TAMs also indirectly promote the formation of blood vessels in tumours using a variety of TAM-derived enzymes such as the matrix metalloproteinases (MMPs)- 2, -7, -9 and -12 (Giraudo et al. 2004; Hildenbrand et al. 1995; Houghton et al. 2006; Huang et al. 2002; Nozawa et al. 2006). These are extracellular matrix (ECM) remodelling enzymes that alter the structure of the ECM to support the attachment, migration and invasion of activated ECs. In addition, the interaction of MMPs with the ECM can also increase the bioavailability of such pro-angiogenic factors as VEGFA and bFGF. Accordingly, the recruitment of myeloid cells expressing MMP9 is important for the liberation of VEGFA from the ECM. It was, therefore, hardly surprising to find that MMP9-expressing myeloid cells were essential for tumour angiogenesis in a murine model of glioblastoma (Du et al. 2008). In addition, mice lacking MMPs 2 and 7 show a reduced rate of tumour progression when implanted with B16 melanoma and Lewis Lung carcinoma cells or in a murine colorectal carcinoma model (Itoh et al. 1998; Wilson et al. 1997). TAMs also express the important pro-angiogenic enzyme thymidine phosphorylase in tumours (TP) and this correlates with tumour neovascularisation and poor prognostic outcome in patients with gastric cancer (Kawahara et al. 2010). This enzyme is capable of modifying DNA released by dead or dying cells by converting the nucleoside, thymidine, into thymine and the potent pro-angiogenic molecule, deoxyribose 1-phosphate. Taken together, these findings highlight the importance of TAM-derived enzymes in the regulation of tumour neovascularisation.

TAMs might also promote the formation of a tumour vasculature in a number of additional ways. These can include the recruitment of CD34+ AC133+ endothelial

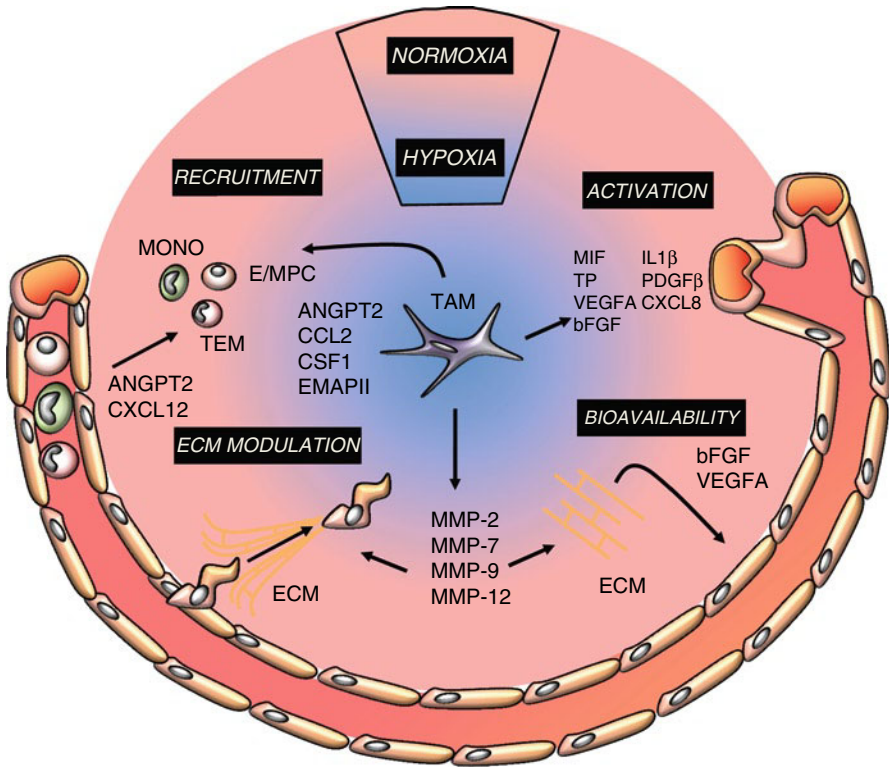


Fig. 2.1 Mechanisms by which TAM promote the formation of the tumour vasculature. TAMs promote the formation of the tumour vasculature by a variety of mechanisms including the recruit of such pro-angiogenic cell types as monocytes (MONO) and TIE2-expressing monocytes (TEM). TAMs also actively recruit endothelial and myeloid progenitors (E/MPCs), capable of directly incorporating into the tumour vasculature. Furthermore, TAMs release a variety of ECM-modifying enzymes. These enhance the ability of activated EC to migrate and invade the tumour micro-environment by restructuring the ECM. Additionally, these same matrix-remodelling enzymes also increase the bioavailability of such pro-angiogenic cytokines and growth factors as VEGFA and bFGF via their liberation from the ECM. Finally, TAMs also directly promote angiogenesis through the release of such pro-angiogenic cytokines and growth factors that act directly on EC, this induces their activation, migration, proliferation and differentiation into tumour neo-vessels

progenitors or CD34[−] CD14⁺ human peripheral blood monocytes (Gill et al. 2001; Peichev et al. 2000). Indeed, CD14⁺ CD34[−] peripheral blood mononuclear cells have been found expressing the classic EC markers VEGFR2⁺ and vWF⁺ (Von Willebrand Factor) (Schmeisser et al. 2001). The presence of VE-cadherin⁺ VEGFR2⁺ GR⁺ CD11b⁺ MMP9⁺ cells has been found in solid tumours where they contributed to tumour neovascularisation by becoming incorporated into the tumour endothelium (Yang et al. 2004) (Fig. 2.1).

Re-education of TAMs by Tumour-Derived Signals

The tumour micro-environment is now thought to stimulate many of the pro-angiogenic functions of TAMs. For example, TNF α secreted by ovarian tumour cells, enhances TAM expression of VEGFA, MMP-9 and other important pro-angiogenic factors (Hagemann et al. 2006). Antibodies released from tumour-infiltrating B cells can also stimulate macrophages to promote tumour angiogenesis (Andreu et al. 2010).

As mentioned in the chapter by Cramer and colleagues in this book, tumour hypoxia (very low oxygen levels) also plays an important part in re-educating macrophages in tumours. The oxygen tension in normal tissues ranges from 20 to 80 mmHg. Hypoxia, or low oxygen tension, defined as an oxygen tension less than 10 mmHg, arises when an imbalance occurs between the supply of O₂ and its consumption by local cells. This usually results from the abnormally high proliferation of tumour cells or the presence of a defective vasculature (Shannon et al. 2003; Thews et al. 1998; Vaupel et al. 1989). This leads to cellular dysfunction and ultimately cell death. Most tumours with a diameter greater than 1 mm contain areas of hypoxia (Brown, 1979; Hill et al. 1996; Shannon et al. 2003). The oxygen tensions in head and neck tumour metastases were found to be less than 30 mmHg with the median being 10 mmHg indicating the presence of severe hypoxia (Brown 1999). Additional studies have reported similar findings in a variety of other tumour types including prostate (Movsas et al. 1999), cervical (Hockel et al. 1991), breast, brain, head/neck and soft tissue sarcoma (Vaupel et al. 1989; Vaupel et al. 1991).

Interestingly, TAMs have been shown to accumulate in such hypoxic and/or necrotic areas of breast, endometrial, ovarian, colorectal and buccal tumours (Lewis and Pollard 2006; Murdoch et al. 2008). Indeed, a positive correlation between the accumulation of TAMs and the presence of hypoxia has also been reported in the liver metastases of breast and colorectal tumours (Stessels et al. 2004). It is thought that these areas attract TAMs by releasing such hypoxia-induced chemoattractants as VEGFA, endothelins, EMAPII (also known as SCYE1) and CXCL12 (SDF-1) (Kioi et al. 2010; Murdoch et al. 2004; Murdoch et al. 2008). It might also involve the release of factors such as high-mobility group box 1 (HMGB1) by necrotic cells in sites of chronic tumour hypoxia as this has been shown to alter the phenotype of macrophages (Andersson et al. 2000; Stros 2010). However, it remains to be seen whether necrotic debris can act as a chemoattractant to recruit monocytes into tumours.

It has been suggested that once drawn into such hypoxic sites TAMs become trapped and unable to leave due to their reduced expression of chemokine receptors and the reduced expression of chemokines by tumour cells (Negus et al. 1998; Sica et al. 2000). Exposure to hypoxia can stimulate the expression of macrophage migration inhibitory factor (MIF) by both macrophages and tumour cells (Koong et al. 2000; White et al. 2004). This may inhibit the ability of TAMs to migrate out of hypoxic sites into other areas of the tumour. Recent studies have also shown the up-regulated expression of CXCR4, the receptor for CXCL12 (SDF1) by hypoxic

macrophages (Fang et al. 2009; Schioppa et al. 2003) and that hypoxia-inducible transcription factor-1 (HIF-1) influences the positioning and function of TAMs by regulating their expression of CXCR4 (Schioppa et al. 2003). Moreover, HIF-1 activation also up-regulates CXCL12 in hypoxic endothelial cells (Ceradini et al. 2004).

Hypoxic Signalling and Regulation of Transcription in TAMs

A number of recent studies have suggested that, in hypoxic areas, TAMs express a highly pro-angiogenic phenotype mainly due to their up-regulation of HIFs-1 and -2 (Burke et al. 2003; Fang et al. 2009). This is known to trigger increased transcription of many HIF target genes that promote angiogenesis such as VEGFA, MIF, ANGPT2 and CXCL8 (Fang et al. 2009). Moreover, we showed that TAMs up-regulate the pro-angiogenic chemokine 'VEGFA' in avascular, peri-necrotic tumours (Lewis et al. 2000).

However, there is considerable debate regarding the relative contributions of HIFs-1 and -2 to hypoxia-induced pro-angiogenic gene expression in macrophages. Some studies have suggested that HIF-2 is the dominant form of HIF up-regulated by hypoxia in such cells (Griffiths et al. 2000; Talks et al. 2000). Additionally, White and colleagues used adenovirally transfected human macrophages to over-express HIFs-1 and -2 and showed that HIF-2 rather than HIF-1 appeared to mediate the hypoxic induction of various pro-angiogenic genes such as VEGFA, IL8, COX2 and bFGF (White et al. 2004). However, their data is difficult to interpret as transfected macrophages expressed super-physiological levels of these two transcription factors, and normoxic rather than hypoxic cultures were used throughout.

Other studies have shown that human macrophages accumulate high levels of both HIF-1 than HIF-2 when exposed to tumour-specific levels of hypoxia in vitro (Burke et al. 2002). When siRNA was used to knock down the alpha subunit of each HIF, the majority of hypoxia-induced pro-angiogenic genes were seen to have equal dependence on both HIFs, suggesting that each HIF was capable of compensating for the loss of the other (Fang et al. 2009). An intriguing recent study by Johnson and colleagues has shown that IL-4 up-regulates HIF-2 but not HIF-1 levels in macrophages and in doing so, skews them towards a more M2-like phenotype. This suggests that HIF-2 may play a part in mediating the induction by hypoxia and/or IL-4 of the M2 phenotype expressed by some TAMs (Takeda et al. 2010) and accords well with the finding that high numbers of HIF-2 expressing TAMs correlate with increased tumour vascularity in breast carcinomas (Leek et al. 2002). Furthermore, the macrophage expression of HIF-2 has recently been found to impact on the progression of murine hepatocellular carcinomas (HCC) – as evidenced by their reduced growth in myeloid-specific HIF-2 α conditional knock-out mice. Moreover, tumour cells exhibited a lower mitotic index in these tumours (Imtiyaz et al. 2010). Finally, it should be noted that Elbarghati and colleagues showed that HIFs-1 and -2 may be differentially regulated during re-oxygenation of macrophages after the cessation of hypoxia (for example, in hypoxic tumour areas undergoing revascularization).

HIF-1 up-regulation was rapidly reversed during re-oxygenation, whereas HIF-2 levels took longer to drop to normoxic levels (Elbarghati et al. 2008).

Recently, the activation of another transcription factor, NF- κ B, has also been implicated in the transcriptional regulation of hypoxic macrophages. Short-term exposure (2–4 h) of murine macrophages to hypoxia activates NF- κ B, which in turn up-regulates HIF-1 α levels (Rius et al. 2008). This study demonstrated the impaired ability of IKK β –/– macrophages to up-regulate HIF-1 α in the presence of hypoxia. Recent data from our group has now shown that although macrophages exposed to more prolonged periods of hypoxia (e.g. 18 h) up-regulate the expression of such NF- κ B members as p65, p50 and IKK β , inhibition of canonical NF- κ B signalling had no effect on their up-regulation of various genes in hypoxia (Fang et al. 2009). Together these findings suggest that NF- κ B may be more important in the initial, early responses of macrophages to tumour hypoxia than when they experience it for longer periods in tumours.

TIE2-Expressing Monocyte/Macrophages

A subset of human and murine monocyte/macrophages have been identified that display highly pro-angiogenic functions – those that express the tyrosine kinase with Ig and EGF homology domain-2 (TIE2) receptor, which is typically expressed on endothelial cells, (De Palma et al. 2007; De Palma et al. 2005; De Palma et al. 2003; Murdoch et al. 2007; Nowak et al. 2004) and are known as TIE2-expressing monocyte/macrophages (TEMs). TEMs have been found in a variety of human tumours including kidney, colon, pancreas, lung and in soft tissue sarcomas (Venneri et al. 2007).

The recruitment of TEMs into solid tumours is thought to be via mechanisms that are distinct from those used by TIE2–TAMs. As mentioned previously, MCP-1 (CCL2) plays a prominent role in the recruitment of monocytes from the peripheral blood into solid tumours, a process mediated by the expression of CCR2, the receptor for CCL2, on the majority of circulating monocytes. TEMs do not express CCR2 (Venneri et al. 2007) and therefore CCL2 appears not to play a significant role in their recruitment. Interestingly, we and others have shown that TEMs could be recruited into tumours by the TIE2 ligand, angiopoietin 2 (ANGPT2) (Gu et al. 2006; Murdoch et al. 2007; Stratmann et al. 1998; Venneri et al. 2007), a cytokine up-regulated by endothelial cells in tumour blood vessels. ANGPT2 is typically stored in Weibel-Palade bodies in these cells, secretory granules that rapidly fuse with the cell membrane upon endothelial cell activation, and release stored cytokines and growth factors, including ANGPT2, into the extracellular space (Fiedler et al. 2004). TEMs appear to be inherently better at promoting the formation of tumour blood vessels than TAMs. Accordingly, circulating TEMs were found to express higher levels of important pro-angiogenic mediators such as VEGFA, MMP9, COX2, TP and Cathepsin B. Consistent with the elevated expression of pro-angiogenic effector molecules, TEMs promoted angiogenesis to a greater extent in both in vitro endothelial cell and murine in vivo tumour assays (Coffelt et al. 2010).

When human U87 glioma cells were co-injected subcutaneously into nude mice with TEMs, a significantly higher level of tumour vascularisation was seen compared with mice receiving non-TEMs (De Palma et al. 2005). Moreover, De Palma and colleagues used a conditional genetic TEM depletion model to show the essential pro-angiogenic effect of these cells in murine tumours. To do this, sub-lethally irradiated mice were implanted with orthotopic human glioma cells and their bone marrow reconstituted from a murine transgenic strain in which the expression of the thymidine kinase gene was under the control of the *Tie2* promoter. When the pro-drug, ganciclovir, was then administered to tumour-bearing mice to specifically ablate the TEM population, it resulted in a marked reduction in both tumour angiogenesis and growth (De Palma et al. 2005). Taken together, these data clearly demonstrate the role that TEMs play in regulating tumour angiogenesis.

Concluding Remarks

The findings discussed in this chapter highlight the multifaceted way in which TAMs stimulate tumour angiogenesis. These highly versatile cells up-regulate a plethora of cytokines and growth factors in response to such micro-environmental signals as cytokines secreted by tumour cells and hypoxia. These factors either activate endothelial cells directly in the tumour vasculature or indirectly via a variety of ECM-remodelling enzymes, such as various MMPs that re-shape the ECM to facilitate EC invasion and migration and increase the bioavailability of various pro-angiogenic mediators. Taken together, these findings indicate that the development of therapeutic strategies aimed at re-directing the phenotype of macrophages back towards more M1-like, anti-tumour functions in vivo might prove efficacious in the treatment of cancer.

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