Angiogenesis and Multiple Myeloma

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Abstract The bone marrow microenvironment in multiple myeloma is characterized by an increased microvessel density. The production of pro-angiogenic molecules is increased and the production of angiogenic inhibitors is suppressed, leading to an “angiogenic switch”. Here we present an overview of the role of angiogenesis in multiple myeloma, the pro-angiogenic factors produced by myeloma cells and the microenvironment, and the mechanisms involved in the myeloma-induced angiogenic switch. Current data suggest that the increased bone marrow angiogenesis in multiple myeloma is due to the aberrant expression of angiogenic factors by myeloma cells, the subsequent increase in pro-angiogenic activity of normal plasma cells as a result of myeloma cell angiogenic activity, and the increased number of plasma cells overall. Hypoxia also contributes to the angiogenic properties of the myeloma marrow microenvironment. The transcription factor hypoxia-inducible factor-1 α is overexpressed by myeloma cells and affects their transcriptional and angiogenic profiles. In addition, potential roles of the tumor suppressor gene inhibitor of growth family member 4 and homeobox B7 have also been recently highlighted as repressors of angiogenesis and pro-angiogenic related genes, respectively. This complex pathogenetic model of myeloma-induced angiogenesis suggests that several pro-angiogenic molecules and related genes in myeloma cells and the microenvironment are potential therapeutic targets.

Keywords Multiple myeloma · Angiogenesis · Angiogenic switch · Microenvironment · Hypoxia

Introduction

Angiogenesis is a multistep process characterized by the formation of new blood vessels from a pre-existing vasculature that occurs in physiologic conditions, such as normal growth and tissue healing, and pathologic states, such as cancer [1, 2]. The process begins with vasodilatation and increased permeability of existing vessels. The surrounding matrix degrades and allows activated and proliferating endothelial cells to migrate and form lumens. The endothelial cells subsequently differentiate and mature into a complex network of vessels supported by peri-endothelial cells and the matrix [1, 2]. Tumoral angiogenesis differs significantly from physiologic angiogenesis due to the chaotic and aberrant production of pro-angiogenic factors resulting in aberrant vascular structure, altered endothelial cell-pericyte interactions, abnormal blood flow, and increased permeability [4, 5]. Cancer cells induce an “angiogenic switch” by either a direct overproduction of pro-angiogenic molecules or by the induction of pro-angiogenic molecules in the microenvironment. Several years ago it was hypothesized that angiogenesis is involved in tumor growth and metastasis [3]. It is now well established that tumor cells stimulate vessel formation through the over-expression or induction of pro-angiogenic molecules in the tumor microenvironment, a process termed “angiogenic switch” [2–4]. Under normal conditions the angiogenic switch is “off,” and there is a balance between pro-angiogenic and anti-angiogenic molecules. When the switch is “on” the balance is tipped in favor of pro-angiogenic molecules

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leading to increased angiogenesis [2–4]. The angiogenic switch can occur at any stage of tumor progression, depending on the type of tumor and its microenvironment [2–4]. Many tumors initially have an avascular growth phase before reaching a steady state of proliferating cells. The angiogenic switch occurs at a later stage, resulting in perivascular detachment and vessel dilation followed by angiogenic sprouting, new vessel formation, and the recruitment of perivascular cells [4, 5], thus allowing for exponential tumor growth.

In the last years, increased angiogenesis has been demonstrated in the bone marrow (BM) microenvironment in hematologic malignancies, including multiple myeloma (MM), suggesting a potential pathophysiologic role for angiogenesis in MM. MM is a plasma cell malignancy characterized by a tight relationship between tumor cells and the BM microenvironment that supports myeloma cell growth and survival [6]. In MM, as in solid tumors, disease progression is characterized by a pre-angiogenic stage of slow tumor progression followed by an angiogenic switch and a subsequent angiogenic stage associated with progressive tumor growth [7]. Here we present an overview of the role of BM angiogenesis in MM, the production of pro-angiogenic factors by myeloma cells and the BM microenvironment, and the mechanisms involved in the MM-induced angiogenic switch.

Bone Marrow Angiogenesis in Multiple Myeloma Patients

Increased BM angiogenesis in patients MM was first demonstrated by Vacca et al. [8], who described increased *in vitro* pro-angiogenic activity of isolated plasma cells from patients with active MM as compared with inactive MM and monoclonal gammopathy of undetermined significance (MGUS). Thereafter, others confirmed this observation, showing that MM patients with active disease have increased BM angiogenesis compared to patients with smoldering MM or early stage MM [9, 10]. In a large cohort of patients with monoclonal gammopathies it has been shown that BM microvessel density (MVD) as assessed by immunohistochemical staining for CD 34 was significantly higher in patients with symptomatic MM as compared to MGUS and healthy controls. An increased incidence of high-grade angiogenesis was also demonstrated in patients with relapsed MM as compared to newly diagnosed MM [10]. In this study a relationship between BM angiogenesis and the number of BM plasma cells and plasma cell labeling index was demonstrated [10]. Others showed a correlation between MVD, the proliferation index Ki-67 and plasma cells burden [11]. Overall these results suggest that increased BM angiogenesis correlates with the

progression of monoclonal gammopathy to overt MM and the extent of plasma cell infiltration.

Several groups have demonstrated a significant relationship between increased BM angiogenesis and prognosis in MM patients [10, 12–21]. In multivariate analysis of separate cohorts of MM patients, MVD emerged as an independent prognostic factor for overall survival together with beta2-microglobulin and C-reactive protein [10, 13, 14, 18, 20, 21]. A relationship between the increased BM microcirculation and the presence of deletion 13 [22], and the gain of 1q21 has also been demonstrated, but no relationship has been identified with the deletion of 17p13 [23]. The prognostic impact of angiogenesis in solitary bone plasmacytoma has been reported, showing that patients with high MVD were more likely to progress to MM with a shorter event-free survival [15]. Moreover, a significant relationship between increased angiogenesis, as assessed by immunohistochemistry, and a diffuse magnetic resonance imaging (MRI) pattern of infiltration was recently reported in newly diagnosed MM patients treated with novel agents and correlates with poor prognosis [17]. MVD has been also identified as a good predictor of complete response to therapy in MM.[13, 16]. Treatment related changes in BM angiogenesis have been reported in MM patients undergoing high dose therapy in relation to their response to therapy [24], although not all authors have shown a significant reduction of MVD in MM patients responsive to treatment [25, 26]. Interestingly, recent data also shows a significant decrease of MVD in patients responding to treatment with thalidomide [27].

Vasculogenesis in MM

Together with angiogenesis, new blood vasculature in the BM of MM patients is due to a vasculogenic process [28, 29]. In contrast to angiogenesis, vasculogenesis is a *de novo* formation of blood vessels from endothelial progenitor cells (EPCs) recruited from the BM. EPCs are incorporated into nascent vessels at the angiogenic sites, and proliferate and differentiate into endothelial cells [30, 31]. The presence of EPCs has been demonstrated in the peripheral blood together with the circulating endothelial cells (CEC) in patients with malignancy. However, the real contribution of EPCs to the tumor vasculature and their role in the vasculogenic process in cancer is still unknown [30, 31]. Circulating EPCs and CEC have also been demonstrated in MM patients and their levels correlate with disease activity [32].

Peripheral mononuclear cells also contribute to vasculogenesis [29]. Both monocytes and macrophages may give rise to endothelial cells in presence of pro-angiogenic cytokines. Interestingly, it has been reported that the

contribution of monocytes to the increased vasculature that occurs in the BM of MM patients is higher in MM patients with active disease as compared to those with MGUS or healthy subjects [33]. Recent data show that pleiothrophin production by myeloma cells induced transdifferentiation of monocytes into vascular endothelial cells in combination with macrophage colony stimulating factor (M-CSF), suggesting a critical role of this protein in vasculogenesis in MM [34].

Production of Pro-angiogenic Factors by Myeloma Cells and the Microenvironment

The increased BM angiogenesis in MM is sustained by an imbalance between the production of pro-angiogenic and anti-angiogenic factors by both myeloma cells and the microenvironment. Myeloma cells interact with several BM microenvironment cells including stromal cells, fibroblasts, osteoblasts, osteoclasts, T lymphocytes, monocytes/macrophages and mast cells that produce growth and survival factors that sustain myeloma cell survival and trigger endothelial cell proliferation and angiogenesis.

In the last years several factors produced by myeloma cells and the microenvironment have been identified as critical in MM-induced angiogenesis as summarized in Fig. 1.

Vascular Endothelial Growth Factor (VEGF)

The pro-angiogenic molecule vascular endothelial growth factor (VEGF) is the primary growth and survival factor for endothelial cells and is essential for vascular

development [1, 35]. Different isoforms of VEGF that share common receptors have been identified: VEGFR-1 or Flt-1, VEGFR-2 or KDR/Flk-1 and VEGFR-3 or Flt-4. VEGFR-1 and VEGFR-2 are expressed on endothelial cells and mediate the stimulatory effect of VEGF on endothelial cell proliferation, survival and migration [1, 35]. Placental growth factor (PlGF) also belongs to the VEGF family, binds VEGFR-1 and enhances the angiogenic effect of VEGF [1, 36]. In contrast, the soluble form of the VEGFR-1, (sVEGFR-1 or sFLT-1), generated by alternative splicing, acts as an inhibitor of VEGF.[1, 35].

The pro-angiogenic activity of myeloma cells is sustained by VEGF production. It has been demonstrated that myeloma cells directly produce VEGF [37–39]. Moreover, VEGF produced by myeloma cells stimulates interleukin-6 (IL-6) and VEGF secretion by BM stromal cells that in turn induce VEGF production by myeloma cells in a paracrine fashion [40, 41]. An increase in the angiogenic activity of mesenchymal stromal cells has also been demonstrated in MM with VEGF overexpression compared with healthy donors [42]. A VEGF autocrine loop has also been postulated based on the presence of VEGFR-1 on myeloma cells, by which VEGF stimulates myeloma cell proliferation through the MEK-1/ERK pathway [43]. MEK-1 specific inhibitors inhibit VEGF secretion by myeloma cells and by the BM microenvironment, suggesting that this pathway is involved in VEGF secretion by myeloma cells [44].

A potential correlation between VEGF secretion and CD45 expression by myeloma cells has been hypothesized on the basis of experimental data from the 5T2MM mouse model [7]. In this model the angiogenic switch is correlated with an increased number of CD45⁺ myeloma cells, with a higher secretion of VEGF as compared to control [7].

Fig. 1 Pro-angiogenic factors produced by myeloma cells and the microenvironment. Myeloma cells directly produce several pro-angiogenic molecules and induce their expression in bone marrow stromal cells. This in turn stimulates myeloma cell growth in a

paracrine fashion. *VEGF* Vascular endothelial growth factor, *bFGF* basic fibroblast growth factor, *HGF* hepatocyte growth factor, *OPN* osteopontin, *Ang-1* Angiopoietin-1, *IL-6* interleukin-6, *IL-8* interleukin-8. *MMP-1,-2,-9* metalloproteinase-1,-2,-9

Freshly purified CD138⁺ myeloma cells express VEGF mRNA in almost all MM patients (80–90%) and high VEGF levels are detected in BM samples of MM patients [37, 38, 45, 46]. However, only a few MM patients produce the VEGF soluble antagonist sVEGFR-1 and have a higher VEGF/sVEGFR-1 ratio as compared to healthy subjects [45]. This further supports the importance of VEGF in the increased BM angiogenesis in MM patients.

Basic-Fibroblast Growth Factor-2 (bFGF)

Basic fibroblast growth factor (bFGF) is another pro-angiogenic molecule that stimulates endothelial cell proliferation, survival, migration and mobilization in vascular development [1, 2, 4]. bFGF's role in MM-induced angiogenesis has been demonstrated through the capacity of an anti-bFGF blocking antibody to inhibit the in vitro vessel formation induced by BM plasma cell samples [8]. Myeloma cells may produce bFGF, however not all MM patients' plasma cells produce bFGF [41, 47, 48]. Bisping et al. [47] have reported that human myeloma cell lines and sorted CD138⁺ cells obtained from 12 of 15 MM patients produced bFGF, concluding that myeloma cells are the predominant source of bFGF. Others have shown that neither human myeloma cells nor EBV-positive B cell lines secrete bFGF [41]. In our cohort of newly diagnosed MM patients we have demonstrated that 11 out of 35 patients expressed bFGF mRNA and a lower number of patients produced bFGF protein [48]. In contrast, it has been clearly shown that BM stromal cells express b-FGF that contributes to the pro-angiogenic activity of the microenvironment cells in MM patients [48].

Angiopoietins

The maturation and stabilization of the vascular wall is critical and is regulated by angiopoietin-1 (Ang-1), a factor that binds primarily to Tie2 receptor expressed on the endothelium [1, 2, 49]. Ang-1 does not induce endothelial cell proliferation directly [50], but acts as a survival factor for endothelial cells [51], induces vessel stabilization [52], tubule formation [53] and plays a key role in mediating interactions between endothelial and matrix cells [54]. Ang-1 is also produced by mast cells in the microenvironment that promotes plasma cell growth and stimulates angiogenesis together with tumor-derived VEGF [59].

Angiopoietin-2 (Ang-2) is the natural antagonist of Ang-1, and blocks Ang-1 mediated Tie2 activation on endothelial cells and induces vessel destabilization [1, 2, 49, 55]. This process may lead either to vessel regression or promote angiogenesis [55]. Because Ang-1 has a critical role in the angiogenic switch its potential role in myeloma-induced angiogenesis has been investigated [56]. First, it

has been shown that myeloma cells express and secrete Ang-1 but not its antagonist Ang-2 [56]. Ang-1 is expressed in about 47% of patients of newly diagnosed MM patients. However, Ang-2 is not present in any patients tested [56]. In addition, the potential role of Ang-1 in MM-induced angiogenesis has been confirmed in an experimental model of angiogenesis. In this system, the conditioned medium of myeloma cells increased vessel formation in comparison with either control or VEGF treatment [56]. The presence of an anti-Tie-2 blocking antibody completely blunted tubule formation induced by myeloma cells [56]. In line with this evidence, other authors demonstrated inhibition of tumoral angiogenesis using a Tie2 blocking soluble receptor in solid cancer murine models [57].

In addition to the direct production of Ang-1, myeloma cells alter the expression of angiopoietins and the Tie2 receptor in the BM microenvironment. In an in vitro co-culture system it has been demonstrated that several human myeloma cell lines up-regulated expression of Tie-2 by endothelial cells at both the mRNA and protein level [56]. This finding has been confirmed in vivo, with the demonstration that over-expression of Tie-2 in isolated BM endothelial cells of MM patients as compared to normal endothelial cells [58], supporting the hypothesis that the response of MM endothelial cells are activated by Ang-1 and are more sensitive to the effect of Ang-1 produced by myeloma cells themselves. Higher levels of Ang-2 have been detected in MM patients as compared to controls, an observation with a potentially prognostic impact [60]. However, the source of circulating Ang-2 is not clear. It has been reported that myeloma cells lack expression of Ang-2 mRNA and protein [56], and consistent with this observation, the *ANGPT2* gene expression levels in a large dataset of MM patients were very low [61]. In contrast, other authors have reported Ang-2 expression by myeloma cells [60]. Since endothelial cells and EPCs production of Ang-2 is well-established, [49, 62] these results suggest that Ang-2 serum levels may be primarily derived from endothelial vascular cells.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of enzymes that proteolytically degrade components of the extracellular matrix (ECM) promoting tumor invasion, metastasis and angiogenesis [63]. MMPs enhance angiogenesis by detaching pericytes from vessels undergoing angiogenesis, releasing and activating ECM-bound angiogenic factors, exposing cryptic pro-angiogenic integrin binding sites in the ECM, and by cleaving endothelial cell-cell adhesions [64]. MMPs also negatively regulate angiogenesis through the generation of endogenous angiogenesis inhibitors by proteolytic cleavage [64].

Myeloma cells produce both MMP-2 and MMP-9. MMP-2 expression by plasma cells from MM patients with active disease is increased as compared to patients with MGUS [8]. However MMP-9 expression is similar between MM and MGUS patients [8]. MMP-9 secretion by myeloma cells is enhanced by their interaction with endothelial cells in the microenvironment [65, 66]. Myeloma cells also upregulate MMP-1 secretion by BM stromal cells [67] and MMP-7 secreted by MM cells induces activation of the pro-MMP-2 [68].

Osteopontin

Osteopontin (OPN) is a pro-angiogenic factor that promotes endothelial cell migration and survival [60–70], adhesion of both endothelial and smooth muscle cells [71], and has been associated with tumoral angiogenesis in mouse models [72, 73]. OPN is produced directly by myeloma cells and is critical for the stimulation of endothelial cells. In an *in vitro* model of angiogenesis, OPN-immuno-depleted CM from myeloma cells failed to induce a pro-angiogenic effect and an anti-OPN Ab blocked myeloma-induced angiogenesis [74]. These observations support the potential role of OPN as a direct pro-angiogenic factor in MM. However, it is possible that OPN contributes to the increased angiogenesis in MM indirectly through the modulation of matrix metalloproteinase-2 (MMP-2). OPN increases MMP-2 expression and activates pro-MMP-2 in tumor cells [75]. OPN is secreted by osteoclasts in the myeloma microenvironment [76], suggesting that these cells may contribute to the angiogenic activity of this factor in MM. Interestingly, recent data suggest that osteoclasts act as angiogenic cells and regulate angiogenesis in the BM [77].

Hepatocyte Growth Factor (HGF), Syndecan-1 and Heparanase

Hepatocyte growth factor (HGF), a heparin-binding cytokine, is primarily expressed by mesenchymal cells and has pleiotropic effects mediated via its binding to the proto-oncogenic c-met receptor [78]. HGF has been shown to stimulate endothelial cell proliferation and survival, and acts as a pro-angiogenic molecule [79]. HGF is involved in tumoral angiogenesis and metastasis [80, 81]. In addition, HGF is synthesized by myeloma cells [82] and enhances MMP-9 secretion by myeloma cells [83]. HGF activity and signaling in myeloma cells is enhanced by syndecan-1, a cell surface heparan sulfate-bearing proteoglycan highly expressed by myeloma cells [84, 85]. Syndecan-1 can be cleaved and shed into the microenvironment and plays a role in myeloma cell growth, survival and angiogenesis [86, 87]. A relationship between HGF and syndecan-1 levels has

also been observed with BM angiogenesis [12]. Heparanase is involved in syndecan-1 shedding through the upregulation of MMP-9 and in HGF expression and activity [88, 89], enhancing the pro-angiogenic properties of myeloma cells. Moreover, recent data suggest that heparanase expressed by myeloma cells upregulates VEGF production. Together these factors result in shedding of syndecan-1, which promotes MM-induced angiogenesis [90, 91].

Interleukin-6 and Interleukin-8

IL-6 is the major growth and survival factor for myeloma cells and is over-produced in a paracrine fashion by BM stromal cells in contact with myeloma cells [6]. IL-6 stimulates VEGF and OPN production by myeloma cells [6, 40, 54] and may exert a direct, pro-angiogenic effect [92], suggesting that this cytokine is involved both directly and indirectly in MM-induced angiogenesis.

IL-8, also called CXCL8, is a chemokine that exerts potent angiogenic activity through binding to the CXCR1 and CXCR2 receptors present on endothelial cells [93]. Studies indicate that myeloma cells and BM stromal cells [94, 95] directly produce IL-8, and elevated BM levels of IL-8 have been demonstrated in MM patients [95]. Tumor cell expression of IL-8 has been linked to the metastatic potential of many solid tumors [96, 97]. In myeloma cells, IL-8 expression has also been correlated with aberrant CD28 expression and consequently with MM progression and extra-medullary localization [98].

Relationship Between Angiogenic Factors and BM Angiogenesis in Multiple Myeloma

The over-production of pro-angiogenic factors by myeloma cells and the microenvironment is reflected by the overall increase in their levels in the BM plasma (i.e. VEGF, bFGF, OPN and HGF) and peripheral serum of MM patients [9, 12, 46, 99, 100]. Cytokine levels are generally higher in the BM than in the peripheral blood [46]. However, there is not a strict relationship between BM angiogenesis and the levels of pro-angiogenic factors, suggesting that elevated cytokine levels may reflect myeloma cell burden rather than BM angiogenesis.

Different studies have investigated the potential relationship between the production of pro-angiogenic factors by myeloma cells and the increased BM angiogenesis observed in MM patients. Although VEGF and bFGF are considered the primary angiogenic growth factors produced by myeloma cells, recent evidence indicates that there is not a significant difference in the plasma cell expression levels of bFGF and VEGF and their receptor amongst MGUS, smoldering MM and

active MM patients [101]. This suggests that the increased BM angiogenesis occurring in MM patients as compared to MGUS could be due to the higher numbers of plasma cells rather than the overexpression of pro-angiogenic molecules by myeloma cells.

Other authors have evaluated the expression of pro-angiogenic genes in normal BM plasma cells and in malignant myeloma cells by microarray analysis [102]. Expression of other angiogenic genes such as *HGF*, *ANG*, *TGFA*, was higher in malignant myeloma cells as compared to normal plasma cells [102]. Overall these data suggest that the increased BM angiogenesis in MM patients as compared to healthy subjects is due either to the increased number of BM plasma cells in MM patients or to the aberrant expression of angiogenic genes by myeloma cells that further increase the pro-angiogenic activity of normal plasma cells. In line with this hypothesis, a relationship between the expression of pro-angiogenic factors other than bFGF and VEGF and BM angiogenesis in MM patients has been reported. A significantly higher MVD and number of microvessels per field has been demonstrated in MM patients positive for Ang-1 expression as compared to those negative for Ang-1 [56]. Similar results have been obtained in comparisons of Ang-1 expression in myeloma cells versus healthy twin plasma cells. A 5.8 fold increase of Ang-1 transcripts was detected in myeloma cells, supporting an association with increased angiogenesis [103]. OPN expression by myeloma cells was also correlated with increased BM angiogenesis in MM patients. In particular, it has been demonstrated that OPN positive MM patients have a significantly higher MVD and number of microvessels per field as compared to OPN negative patients [74].

Finally, other investigators have noted that BM samples obtained from MGUS subjects have a decreased capacity to inhibit vessel formation in an in vitro model of angiogenesis as compared to BM samples from MM patients, and postulated that the increased angiogenesis that occurs in MM patients is a result of decreased production of normal angiogenesis inhibitors by myeloma cells, tilting the balance between pro-angiogenic and anti-angiogenic factors.[101].

Hypoxia and Hypoxia-Inducible Factor-1 in Myeloma-Induced Angiogenesis

Hypoxia is a common feature of solid tumors that is associated with angiogenesis and the malignant phenotype and is critical in the regulation of the angiogenic switch [104, 105]. Tumor adaptation to hypoxia is mainly due to the hypoxia-inducible factor (HIF)-1, a key transcription factor that regulates angiogenesis and tumor progression in

solid cancers [106–111]. HIF-1 is a heterodimeric DNA binding complex composed of two basic helix-loop-helix proteins, including the constitutively expressed HIF-1 β and the hypoxia-inducible α -subunit HIF-1 α [106–109]. HIF-1 α is over-expressed in many tumors [110, 111]. Under normoxic conditions, HIF-1 α has a very short life and undergoes proteasomal degradation by oxygen-dependent hydroxylation. In contrast under hypoxic conditions, hydroxylation is suppressed and HIF-1 α protein escapes proteasomal destruction and accumulates and translocates to the nucleus [106–109].

The role of hypoxia and HIF-1 α has also been investigated in MM [112]. Myeloma cells grow in a hypoxic microenvironment. Myeloma BM has relatively low pO₂ and sO₂ as compared with healthy subjects [113]. It has also been reported that there is no significant difference in the reduced BM pO₂ and sO₂ in MM patients as compared with MGUS patients [112]. However, in a MM mouse model it has been reported that both normal and MM-infiltrated BM are hypoxic, although the level of oxygen was lower in MM BM [104]. This discrepancy could be due either to the different method used in the detection of hypoxia or to the lower physiological oxygen tension reported in mice as compared to human [112–114].

It is assumed that myeloma cells in the BM microenvironment are chronically exposed to low oxygen levels. This is supported by the finding that HIF-1 α protein is highly expressed in myeloma cells [112]. Interestingly, the presence of HIF-1 α protein was also observed in CD138⁺ purified cells of about 28% of MM patients analyzed under normoxic conditions [112], suggesting that hypoxia-independent stabilization of HIF-1 α may occur in myeloma cells together with HIF-1 α over-expression induced by the hypoxic microenvironment. Others also reported that myeloma cells express HIF-1 α in normoxia, showing that constitutive expression of HIF-1 α by myeloma cells is associated with oncogenic c-Myc, delineating a common signaling pathway in myeloma cells [115].

The role of hypoxia and HIF-1 α in the production of pro-angiogenic molecules by myeloma cells has also been highlighted. By microarray analysis it has been reported that hypoxia affects both the transcriptional and angiogenic profiles of myeloma cells, as summarized in Fig. 2. Among the pro-angiogenic genes, *VEGFA* and *IL8* were found to be significantly induced in CD138⁺ MM cases under hypoxic conditions [112]. Finally, it was reported that VEGF, IL-8, OPN and PGF are regulated by HIF-1 α at both the mRNA and protein levels in myeloma cells [112], and that HIF-1 α silencing consistently and significantly suppresses the pro-angiogenic properties of myeloma cells in vitro [112]. Overall these observations support the critical role of hypoxia and its transcription factor HIF-1 α in the angiogenic switch induced by myeloma cells.

Fig. 2 Effect of hypoxia on the transcriptional and pro-angiogenic profile of myeloma cells. Important gene and related pathways significantly up-regulated by hypoxia treatment in CD138⁺ purified cells. Among the pro-angiogenic genes *VEGFA* and *IL8* were induced by hypoxia (Ref. ¹⁰²)

Genes Involved in the Regulation of the Pro-angiogenic Profile of Myeloma Cells

Although HIF-1 α seems to be a major regulator of the pro-angiogenic profile of myeloma cells and MM-induced angiogenesis, more recently the potential involvement of other genes in the production of pro-angiogenic factors by myeloma cells has been elucidated [95, 116].

The new candidate tumor suppressor gene inhibitor of growth family member 4 (ING4) has been recently implicated in tumors as a repressor of tumor growth and angiogenesis through its association with NF- κ B [117, 118]. Interestingly, it has been shown that tumors lacking ING4 showed increased vascularization compared with ING4 expressing tumors [118]. Moreover, ING4 down-regulated angiogenesis related molecules including IL-8 and HIF-1 α [117, 119]. In line with these observations it has been shown that ING4 nuclear level is reduced in myeloma cells as compared to normal plasma cells, and that ING4 regulates IL-8 and OPN production by myeloma cells both in normoxia and hypoxia [95]. In addition, ING4 has a suppressive effect on HIF-1 α activity in myeloma cells because blocking ING4 in myeloma cells increases both HIF-1 α activity and the expression of its target gene NIP-3 [95]. The involvement of ING4 as an angiogenic repressor in MM was further confirmed in an established in vitro angiogenesis model showing that the supernatant of myeloma cells with suppressed ING4 levels significantly stimulates vessel formation in normoxic and hypoxic conditions [95]. Finally, these results were expanded in vivo by the finding in MM patients that ING4 expression levels are negatively correlated with the expression of pro-angiogenic molecules and the extent of BM angiogenesis [95]. These observations support the hypothesis that ING4 may act as angiogenic repressor in MM, in a manner similar to that previously demonstrated in glioblastoma [117].

The deregulation of the homeobox (HOX) B genes has been previously correlated to the angiogenic process as well as to tumoral-induced neoangiogenesis and tumor progres-

sion in solid cancer [120–123]. Particularly, the overexpression of HOXB7 has been associated with the tumor-related angiogenic switch, cell proliferation, and the production of bFGF by breast cancer and melanoma cells [122, 123]. In addition, HOXB7 overexpression may also regulate VEGF, IL-8 and Ang-2 by tumor cells [121]. In MM it has recently been shown that HOXB7 overexpression in myeloma cells regulated VEGFA, FGF2, MMP2 and PDGFA gene expression and the corresponding proteins VEGF, bFGF, MMP-2 and PDGF-AA. The capacity of HOXB7 to regulate bFGF and VEGF was confirmed by silencing HOXB7 in myeloma cells that constitutively overexpressed HOXB7. The role of HOXB7 as a regulator of pro-angiogenic factors in myeloma cells was confirmed in a large independent dataset of primary MM tumors showing a significant relationship between HOXB7 expression and other angiogenic factors including bFGF and VEGF. These data suggest that HOXB7 could be an important gene in the regulation of the angiogenic profile of myeloma cells.

Anti Angiogenic Effect of the Novel Anti-MM Agents and Future Perspectives

New agents showing a significant anti-MM clinical activity such as thalidomide, its derivatives, the immunomodulatory drugs (IMiDs), and the proteasome inhibitor Bortezomib, have anti-angiogenic activity both in vitro and in vivo. Firstly it was demonstrated that thalidomide inhibits bFGF by a rabbit corneal assay [124]. Others showed that thalidomide inhibits VEGF, HGF, bFGF, IGF-1 and Ang-2 expression by BM endothelial cells isolated from MM patients [125, 126]. All IMiDs, including lenalidomide and pomalidomide, have anti-angiogenic activity independent of their immunomodulatory effects [125, 127, 128], although thalidomide is probably a more prominent inhibitor of angiogenesis than its derivatives. IMiDs inhibit VEGF and bFGF secretion from both myeloma and BM stromal cells and block endothelial cell migration rather

than endothelial cell proliferation through the interference of PI3K/AKT signaling [128, 129]. Finally, in pre-clinical in vivo mouse MM models both thalidomide and lenalidomide induced a significant reduction of tumor-associated MVD [130, 131]. Although this evidence suggests that thalidomide and IMiDs have an anti-angiogenic activity, a reduction of MVD as well as VEGF levels is not always seen with thalidomide response in MM patients [100, 125, 127, 132], suggesting that both the in vivo anti angiogenic effect of these drugs and the contribution of their anti-angiogenic activity to the overall anti-tumor effect is uncertain.

In addition to a direct anti-MM effect, proteasome inhibitors have been shown to have an anti-angiogenic effect as well [125]. These drugs inhibit vascular formation on matrigel and induce apoptosis of endothelial cells in a dose-dependent manner [133]. Similarly, it has been shown that Bortezomib at clinically achievable concentrations inhibits endothelial cell proliferation and targets VEGF-induced caveolin-1 phosphorylation in endothelial cells [134]. Bortezomib also blocks MM interaction with BM stromal cells and indirectly blocks the production of pro-angiogenic factors [125]. Pre-clinical in vivo studies confirm the potential anti-angiogenic effect of the proteasome inhibitors [135] and it has been consistently reported that MM patients treated with Bortezomib show a significant reduction in MVD although serum VEGF are not consistently reduced [136]. However, a significant reduction in Ang-2 levels has been reported in MM patients treated with Bortezomib. Further studies are necessary to clarify whether the anti-angiogenic effect of Bortezomib is clinically relevant in vivo in MM patients [137].

Other new drugs with demonstrated anti-angiogenic activity in ongoing clinical phase I/II trials in myeloma include PI3K/AKT/mTOR inhibitors (i.e. Perifosine, Rapamycin), MAPK signal pathway inhibitors, and heat shock protein 90 (HSP90) inhibitors, either as single agents or in combination with bortezomib or IMiDs [138].

VEGF is a well known target of anti-angiogenic therapy in solid tumors. Drugs targeting VEGF and its receptor have been developed and include the anti-VEGF monoclonal antibody (Bevacizumab), a soluble fusion protein of the extracellular domain of VEGFR-1 and VEGFR-2 (Aflibercept), and several receptor tyrosine kinase (RTK) inhibitors targeting VEGFR [138]. Some of these have shown anti-MM activity in preclinical models (Sunitinib, Sorafenib, Semaxinib, Vandetanib, and the pan-VEGFR inhibitor Pazopanib) [138–140], however preliminary data as single agents has failed to show a relevant clinical effect despite their inhibitory effect on VEGF [141–143]. The lack of effect of VEGF inhibitors could be due either to the capacity of myeloma cells to produce other pro-angiogenic factors, allowing an escape from the block of VEGF and its

signaling, or independence from the angiogenic switch of myeloma cells in end-stage disease. Future clinical studies should be done to explore the potential role of anti-VEGF drugs in combination with Bortezomib and IMiDs to improve their cytotoxic effects or in the early stage of disease.

Conclusions

MM is characterized by increased angiogenesis in the BM microenvironment that is related to myeloma cell infiltration and may support myeloma cell growth and survival. BM angiogenesis correlates with disease progression and prognosis in MM patients. The presence of an imbalance in the production of pro- and anti-angiogenic molecules in favor of pro-angiogenic factors leads to the angiogenic switch in MM. Myeloma cells directly produce or induce several pro-angiogenic molecules in the microenvironment, including VEGF, bFGF, Ang-1, OPN, HGF and IL-8. Current data suggests that the increased BM angiogenesis in MM patients is due either to the increased number of BM plasma cells or aberrant expression of angiogenic factors by myeloma cells that may further increase the pro-angiogenic activity of normal plasma cells. The lack of production by of angiogenic inhibitors by myeloma cells may contribute to the high grade of BM angiogenesis in MM patients. Molecular mechanisms underlying the angiogenic switch in MM have recently been identified. The BM microenvironment is hypoxic and the hypoxia induced transcription factor HIF-1 α is critically involved in the production of angiogenic factors by myeloma cells. HIF-1 α is overexpressed by myeloma cells and affects their transcriptional and angiogenic profiles. In addition, a potential role of ING4 as a repressor of angiogenesis and of HIF-1 α activity, as well as the role of the pro-angiogenic gene HOXB7 has also been recently highlighted.

The complex pathogenetic model of myeloma-induced angiogenesis suggests that VEGF and other pro-angiogenic factors produced by myeloma cells and the microenvironment are potential targets for anti-angiogenic therapy in MM patients. Recent data suggests that the novel anti-MM agents such as thalidomide, IMiDs and Bortezomib have anti-angiogenic in vitro, either through the blockade of the production of pro-angiogenic factors or a direct effect on endothelial cells, although the clinical relevance of this effect as well as the role of anti-angiogenic therapy in MM patients are yet to be determined

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