

Chapter 2

Angiogenesis in Multiple Myeloma

2.1 General Features of Multiple Myeloma

Multiple myeloma (MM) is the second most common hematological malignancy and is responsible for approximately 2% of cancer death. Clonal expansion of malignant terminally differentiated, B-lymphocyte-derived plasma cells is characteristic of MM and results in excessive production of monoclonal immunoglobulins, thereby contributing to renal failure as well as other complications, such as hyperviscosity (Table 2.1). Diagnosis is characterized by the presence of monoclonal immunoglobulin, the presence of bone marrow plasmacytosis and typical skeletal lesions. Survival for patients with MM range from <6 months to more than 10 years based on disease stage and prognostic factors (Table 2.2). For several decades, the treatment of MM included combinations of chemotherapeutic agents with steroids for elderly patients and high-dose melphalan followed by autologous stem-cell transplantation after an induction with the combinations vincristine/doxorubicin/demethasone for patients <65–70 years.

In MM the neoplastic population consists of 3 clonally related compartments (a) a small stem-cell compartment, composed of totipotent cells that are self-maintaining and supply cells to the downstream compartments, (b) an expansion compartment or “growth fraction” (GF), whose proliferative activity expands the small population entering from the stem-cell compartment, (c) a differentiation compartment—the largest where proliferation stops and differentiation into mature plasma cells takes place (Billadeau et al. 1993). Cell loss by apoptosis and necrosis occurs in this compartment.

The kinetics of the stem-cell is unknown, but should be quite negligible, given the very small size of its compartment. Instead, we have plenty of information about the kinetics of the GF, the basic proliferative nucleus that contains cells in the G1, S, G2 and M phases, as well as resting G0 cells, i.e., cells outside the proliferative cycle, but capable of joining it again following different stimuli (Drewinko et al. 1981). The balance between GF cell production and cell loss from the differentiation compartment determines growth, steady state or reduction of the myeloma cell mass.

Table 2.1 Multiple Myeloma staging system

<i>Stage I</i>	
1	Low M-component levels
2	Absent or solitary bone lesions
3	Normal hemoglobin, serum calcium, Ig levels (non-M component)
<i>Stage III (any one or more of the following)</i>	
1	High M-component
2	Advanced, multiple lytic bone lesions
3	Hemoglobin <8.5 g/dL, serum calcium >12 mg/dL
<i>Stage II (overall values between I and III)</i>	
Subclassification based on renal function: A, serum creatinine <2mg/dL; B, serum creatinine >2 mg/dL)	

Table 2.2 Diagnostic criteria for multiple myeloma

A	The diagnosis of myeloma requires a minimum of one major and one minor criteria or three minor criteria which must include (1) and (2). These criteria must manifest in a symptomatic patient with progressive disease
B	<i>Major criteria</i>
1	Plasmacytoma on biopsy
2	Marrow plasmacytosis (>30%)
3	M-component
C	<i>Minor criteria</i>
1	Marrow plasmacytosis (10–30%)
2	M-component: present but less than above
3	Lytic bone lesions
4	Reduced normal immunoglobulins

GF proliferative activity is measured by means of the labeling index (LI%), which identifies the S-phase (DNA synthesis) cell fraction (Durie and Salmon 1975). At diagnosis, the LI% is generally low (median = 1 % range 0–5) (Salmon 1975) and is calculated on the basis of the overall neoplastic population. If other methods are used and the LI% is solely related to the GF, which is generally small (mean <1 %, range <1–3.7) (Drewinko et al. 1981), it is much higher (median = 30 %, range 15–100), indicative of a very lively proliferation. This is confirmed by the fact that the myeloma cell mass doubling time is relatively long (5–15 months), with a huge cell loss (>90 %) given the remarkably large size of the differentiation compartment (Drewinko et al. 1981; Durie and Salmon 1975). It is then inferred that cell production by the GF is quite high. In short, the bone marrow myelomatous population consists of a small nucleus of rapidly proliferating cells, and a huge amount of non proliferating differentiated cells that are eventually lost.

MM is clinically diagnosed when the cellular mass is $>10^{12}$ cells -a stage reached following Gompertzian growth (Salmon and Seligman 1974). The initial growth is extremely fast (exponential), and then progressively reduced (LI% and GF decrease) according to a log factor, just as cell density continues to increase, until a steady state is reached (about 5–25 months after neoplastic changeover)

(Salmon and Seligman 1974). The length of this factor differs from one patient to another and determines whether the steady state is reached at high or low cell mass levels. When diagnosed, the plasma cell proliferative activity is extremely varied, depending on the steady state level (a) if the steady state has been reached, further growth is moderate (low LI% and GF), cell loss is high ($>90\%$), and the disease is poorly aggressive (non-active) (b) if the steady state is still a long way ahead, even though the cell mass is large enough ($>10^{12}$ cells) to elicit clinical symptoms, growth is exponential (high LI% and GF), cell loss is reduced ($50\text{--}60\%$), the tumor mass expands rapidly, and the disease is aggressive (active). There is no connection between LI% and the clinical stage (Durie et al. 1980).

In relapse which is usually detected when the cell mass is $<10^{12}$ cells, growth is exponential because the steady state is distant, and the disease is active. In the post-treatment stable remission, or plateau phase ($>50\%$ reduction of the M component for at least 6 months), the cell mass and the growing steady state have been reduced to a level allowing no further reduction, most cells are in G0 and growth is therefore limited (LI $<1\%$, GF 10.5%), and the disease is quiescent (non-active). While on treatment, after an initial and transient recruitment of G0 cells into the proliferative cycle and an increase in LI% (Salmon 1975) responsive patients exhibit growth conditions close to the plateau phase and the disease is non-active.

According to the Gompertzian growth pattern, the neoplastic population in MGUS reaches the steady state at very low cell mass levels ($10^{10}\text{--}10^{11}$ cells). LI is very low ($<1\%$). The Gompertzian model relates active MM (exponential growth phase) to experimental solid tumors in the vascular phase, and non-active MM and MGUS (slow growth phase) to their avascular (“dormant”) phase.

Mean survival is shorter in patients with LI 21% (15 months) than in those with LI $<1\%$ (40 months), irrespective of the cell mass. In the Gompertzian pattern growth, MM with elevated LI% is still far from the steady state, since its mass is in the exponential portion of the growth curve. Patients rapidly respond to induction cytostatic treatment (“early responders”), but their response is short lived and followed by an equally rapid relapse due to massive recruitment of G0 plasma cells, and hence by shorter overall survival. Since LI% refers to the whole myelomatous population, not to GF alone, very small variations (e.g., by 1%) really denote substantial changes in GF proliferative activity. This must be taken into account when forming a prognosis.

2.2 Angiogenesis in Multiple Myeloma

In 1994, Vacca and colleagues demonstrated for the first time that bone marrow MVD was significantly increased in MM compared to MGUS and moreover in active versus non-active forms.

The close association between angiogenesis and active MM indicates that it is the vascular phase of plasma cell tumors, and thus the counterpart to the vascular tumor implants of the rabbit iris and of locally invasive and metastatic solid tumors.

Conversely, MGUS and non-active MM represent the avascular phase, and correspond to tumor spheroids in agar, to tumor implants of the rabbit eye anterior chamber or to *in situ* tumors. Since most patients with active MM have a microvessel area 22 % and LI 21 %, a 2 % “vascular bone marrow threshold” seems to be required for this phase and rapid tumor growth.

The microvessel area and the LI% are closely associated with the MM activity phase and are mutually correlated. A disease in a given steady state (MGUS, non-active MM) can thus be supposed to at risk of progression towards the subsequent larger-mass steady state (active MM) if the bone marrow shows angiogenesis, and that this risk is greater the larger the microvessel area, namely 3.9 times higher for each 1 % increment. The risk is the same with 0.6 % increments of the LI. Microvessel area variations are therefore less restricted than those of LI% as a risk assessment parameter and could be a useful guide to prognosis just as LI% is utilized in plasma cell proliferative diseases. Angiogenesis correlates with LI%, but not with plasmacytosis, which mostly measures the compartment.

These findings as a whole suggest that, like solid tumor cells, highly proliferating-but not slowly proliferating nor differentiated myeloma cells, possess an angiogenic capability that could be expressed by the release of a variety of factors as follows: (a) Recruitment and activation of the bone marrow microenvironment to release interleukin-6, -8 (IL-6, IL-8), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage CSF (GM-CSF), tumor necrosis factor alpha (TNF- α), (b) Extracellular matrix proteolysis with fibroblast growth factor-2 (FGF-2) release, (c) Secretion of transforming growth factor beta (TGF- β) and IL-1 β stimulating platelets to secrete platelet derived growth factor (PDGF). It should be noted that IL-6, IL-8, GM-CSF and IL-1 β are mostly secreted in the MM active phase where angiogenesis is found. Because in turn angiogenesis favours plasma cell growth, it could thus complete a positive loop.

The progression of MGUS and non-active MM to active MM could take place when plasma cells become able to induce angiogenesis as described, resulting in formation of new vessels that themselves promote progression. Increased microvascular density and high bone marrow angiogenesis are an adverse prognostic factor in MM (Sezer et al. 2000; Iwasaki et al. 2002). CD34-positive microvessel areas in the bone marrow of patients with newly diagnosed MM correlate in a multivariate analysis with bone marrow plasma cell infiltration and b2-microglobulin (Sezer et al. 2001b). Moreover, after chemotherapy, MVD decreases significantly in patients in complete or partial remission (Sezer et al. 2001b).

MM patients' bone marrow endothelial cells express and secrete higher amounts of the IL-8, interferon inducible T-cell-a chemoattractant (I-TAC), stromal cell derived factor 1 alpha (SDF-1 α), and monocyte chemoattractant protein-1 (MCP-1) than healthy umbilical vein endothelial cells (HUVECs) (Pellegrino et al. 2005). The direct production of osteopontin, the expression and activity of its major regulating gene Runx2/Cbfa1 in MM cells, and the potential role of osteopontin in plasma cell induced bone marrow angiogenesis have been elucidated (Colla et al. 2005; Cheriya et al. 2005).

Human MM cell lines express and secrete Ang-1, but not its antagonist Ang-2 (Giuliani et al. 2003). Ang-1 alone is expressed in about 47% of newly diagnosed MM patients in stages I–III. Moreover, microarray comparison of the gene expression profiles of bone marrow plasma cells and extramedullary plasmacytoma cells has shown an eightfold upregulation of Ang-1 expression in the latter (Hedvat et al. 2003). Evaluation of the density and number of vessels per field in bone marrow biopsies has revealed a significant increase of angiogenesis in Ang-1 positive as opposed to Ang-1-negative patients (Giuliani et al. 2003). A 5.8-fold increase of Ang-1 transcript was detected in MM associated with higher angiogenesis (Munshi et al. 2004). The presence of anti-Tie2 (Ang-1 receptor) blocking antibody completely abolished vessel formation induced by conditioned medium of several human MM cell lines (Giuliani et al. 2003). Multiple myeloma plasma cells influence the expression of Angs and Tie2 receptor in the bone marrow milieu. Several human MM cell lines upregulate the expression of Tie2 by endothelial cells at both the mRNA and the protein level (Giuliani et al. 2003). This *in vitro* effect has been confirmed *in vivo* by the finding of overexpression of Tie2 in isolated bone marrow MM endothelial cells as compared to HUVECs (Vacca et al. 2003a). Nakayama et al. (2004) showed that mast cells promote the growth of plasma cell tumors through secretion of Ang-1, which stimulates angiogenesis in conjunction with tumor-derived vascular endothelial growth factor (VEGF).

The angiogenic switch was owing to an increase in FGF-2 production and secretion by plasma cells, probably caused by expression of oncogenes (c-myc, c-fos, c-jun, ets-1) coding for angiogenic factors, and activated as a consequence of immunoglobulin translocations and genetic instability of plasma cells (Vacca et al. 2001a).

Accordingly, by using the 5T22 murine model for MM, Asosingh et al. (2004) showed that the switch is preceded by the expression of mRNA for VEGF and secretion of protein by plasma cells. Furthermore, they demonstrated that the angiogenic switch was preceded by an increase in the percentage of CD45 negative MM cells with high levels of VEGF secretion.

Other studies, however, showed that the expression levels of VEGF, FGF-2, and their receptors were similar among plasma cells from MGUS, smoldering MM and newly-diagnosed MM, suggesting that increasing angiogenesis from MGUS to MM is, at least in part, explained by increased tumor burden rather than increased expression of VEGF/FGF-2 by single plasma cells (Kumar et al. 2004). Another possibility is that the inhibition of angiogenesis in MGUS is lost with progression, hence that the switch from MGUS to MM may involve a loss of an anti-angiogenic activity (Kumar et al. 2004). We have demonstrated that in the chick embryo chorio-allantoic membrane (CAM) assay MM endothelial cells induced an angiogenic response comparable to that of FGF-2, while reverse transcriptase-polymerase chain reaction (RT-PCR) demonstrated that the expression of endostatin mRNA detected in MM treated CAM was significantly lower respect to control CAM (Mangieri et al. 2008). These data suggest that angiogenic switch in MM may involve loss of an endogenous angiogenesis inhibitor, such as endostatin.

Hose et al. (2009) substantiated that “angiogenesis may not be critical for MM pathogenesis, but just an epiphenomenon driven by the accumulation of malignant

plasma cells and a production of pro-angiogenic cytokines that have a dual role as growth and survival factors for MM cells”.

2.3 Angiogenic Cytokines and Multiple Myeloma Progression

Interleukin-6 is a major growth, survival factor and anti-apoptotic factor for MM plasma cells. It is mainly produced by bone marrow stromal cells and also by plasma cells themselves. It is angiogenic (Motro et al. 1990). Elevated serum IL-6 receptor concentrations are associated with a poor prognosis in MM (Pulkki et al. 1996). Contact of plasma cells with bone marrow stromal cells upregulates the latter's transcription and secretion of IL-6 which are further enhanced by cytokines (particularly TGF- β) secreted by plasma cells (Urashima et al. 1996). Interleukin-6 stimulates VEGF secretion via an IL-6 receptor expressed by plasma cells (Cohen et al. 1996; Bellamy et al. 1999; Dankbar et al. 2000; Gupta et al. 2001). Similarly, stimulation of endothelial cells and bone marrow stromal cells with VEGF induced a significant and dose-dependent increase in IL-6 secretion.

The secreted isoforms of VEGF, such as VEGF121, VEGF145 and VEGF165, as well as the ECM and surface-bound VEGF189 and VEGF206 are produced by both MM cell lines and a patient's bone marrow plasma cells (Figs. 2.1 and 2.2) (Bellamy et al. 1999; Podar et al. 2001). VEGF acts as an autocrine inducer of growth and chemotaxis via VEGF receptor-1 (VEGFR-1) (Podar et al. 2001). It increases IL-6 production by bone marrow stromal cells via VEGFR-2 and thus forming a paracrine loop for tumor growth (Dankbar et al. 2000) and angiogenesis. Moreover, adhesion of plasma cells to bone marrow stromal cells increases VEGF secretion by both cell types (Hideshima et al. 2005), and so enhances angiogenesis. Vascular endothelial growth factor production by plasma cells is also regulated by TNF- α of bone marrow stromal cells (Neufeld et al. 1999). VEGF and its receptors VEGFR-2 and VEGFR-3 are highly expressed by bone marrow MM endothelial cells and plasma cells at both mRNA and protein levels compared to MGUS endothelial cells (Figs. 2.1, 2.3 and 2.4) (Vacca et al. 2003a; Ria et al. 2004). Elevated serum VEGF in MM patients is correlated with both increased angiogenesis in MM bone marrow and higher plasma cell LI (Vacca et al. 1999a; Rajkumar et al. 2000b).

Vincent et al. (2005) have shown that VEGF released by fetal bone marrow stromal cells interacts with VEGFR-1 of MM plasma cells in a paracrine fashion. Moreover, a neutralizing anti-VEGFR-1 monoclonal antibody, but only partially, blocks plasma cell motility *in vitro*, indicating that the functional VEGF-A/VEGFR-1 interaction is essential for MM homing and migration, and that bone marrow stromal cells secrete other cytokines and/or chemokines that promote plasma cell migration irrespective of the VEGF-A/VEGFR-1 signaling. Vascular endothelial growth factor may also mimic the role of M-CSF by increasing osteoclast development (Niida et al. 1999) and stimulating resorptive activity (Nakagawa et al. 2000), so that bone destruction is mediated via more than one route. VEGF triggers a signaling cascade

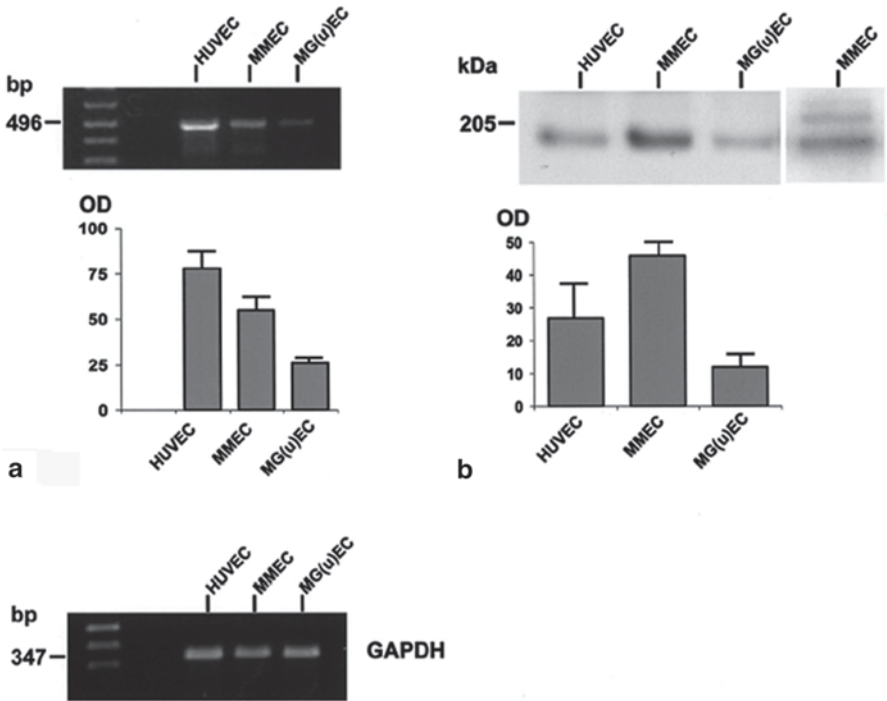


Fig. 2.1 Parallel expression of VEGF-A at mRNA (a), and protein (b), levels. Measurement by ELISA of the VEGF-A released into endothelial cell conditioned media. Histograms show mean $\pm$ 1 SD in the groups of patients and HUVEC. (Reproduced from Ria et al. 2004)

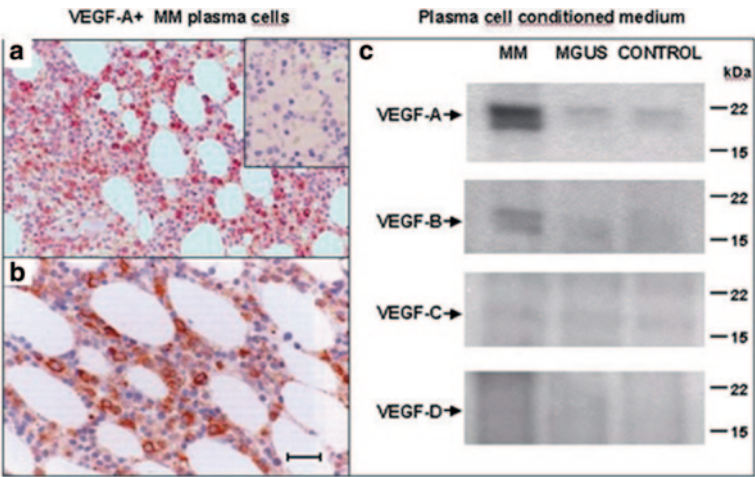


Fig. 2.2 Staining with VEGF-A of bone marrow from a patient with MM (a, b) insert: the negative control obtained with rabbit pre-immune serum replacing the VEGF-A antibody (c). Western blot analysis for VEGF homologs of plasma cell culture medium from a representative MM patient, MGUS patient and control subject. (Reproduced from Vacca et al. 2003a)

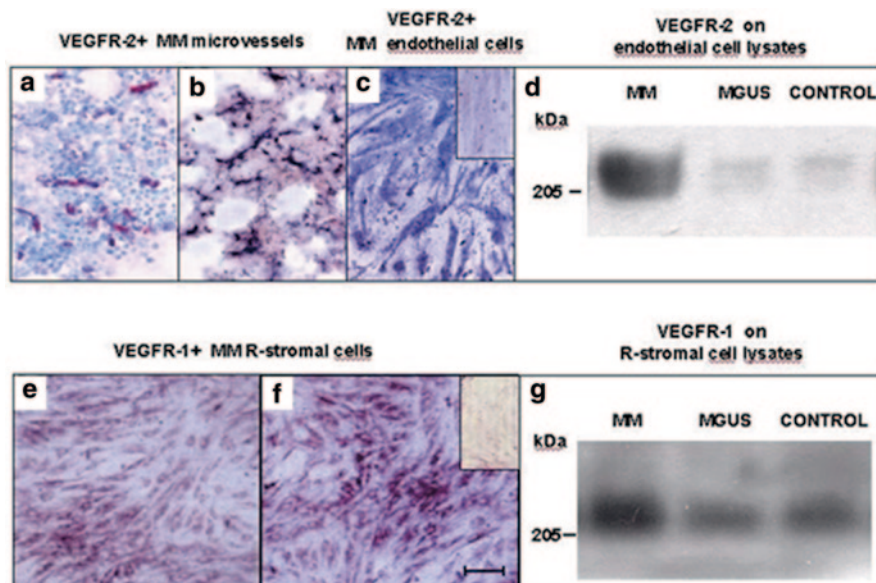


Fig. 2.3 Staining with VEGFR-2 of a bone marrow from a patient with MM showing numerous thin microvessels (a). *In situ* hybridization for VEGFR-2 mRNA highlighting neovessels (b), and enriched endothelial cells (c), of the MM patient. Insert: RNase-treated cells as the negative control. Western blot analysis of the VEGFR-2 of endothelial cell lysates from the patients with MM, MGUS, and the control subject, showing enhanced expression in MM (d). Immunocytochemical staining with VEGFR-1 (e) and *in situ* hybridization for VEGFR-1 mRNA of residual-stromal cells from the patient with MM (f). Insert: RNase-treated cells as the negative control. Western blot analysis for the VEGFR-1 of residual-stromal cell lysate from the patient with MM, and the patient with MGUS and the control subject (g). The expression of VEGFR-1 is higher in MM. (Reproduced from Vacca et al. 2003a)

in plasma cells, including the extracellular-signal regulated kinases (ERK) pathway, which mediates cell growth, and the phosphatidylinositol-3 kinase/protein kinase C (PI3 K/PKC) -dependent cascade that mediates migration (Podar et al. 2001). Le Gouill et al. (2004) have demonstrated that VEGF upregulates expression of anti-apoptotic proteins, including myeloid cell leukemia, survivin, and cellular inhibitor of apoptosis protein. Furthermore, free bovine serum (starvation)-induced apoptosis in plasma cells is partially prevented by VEGF, confirming that this is a potent anti-apoptotic cytokine in MM (Lichtenstein et al. 1995; Chauhan et al. 1997a, b; Tu et al. 2000). Bone marrow stromal cells secrete also VEGF-C and VEGF-D (Vacca et al. 2003a). VEGF is thus prevalently produced by plasma cells and stimulates the proliferation and chemotaxis of MM endothelial cells via VEGFR-2 and of bone marrow stromal cells via VEGFR-1. Activation of the latter results in VEGF-C and VEGF-D production that stimulates plasma cell growth via VEGFR-3. Increased proliferation and chemotaxis displayed by endothelial cells and bone marrow stromal cells in response to plasma cell conditioned medium is not fully abolished by addition of anti-VEGF antibody, suggesting the secretion of other angiogenic cytokines (Vacca et al. 2003a).

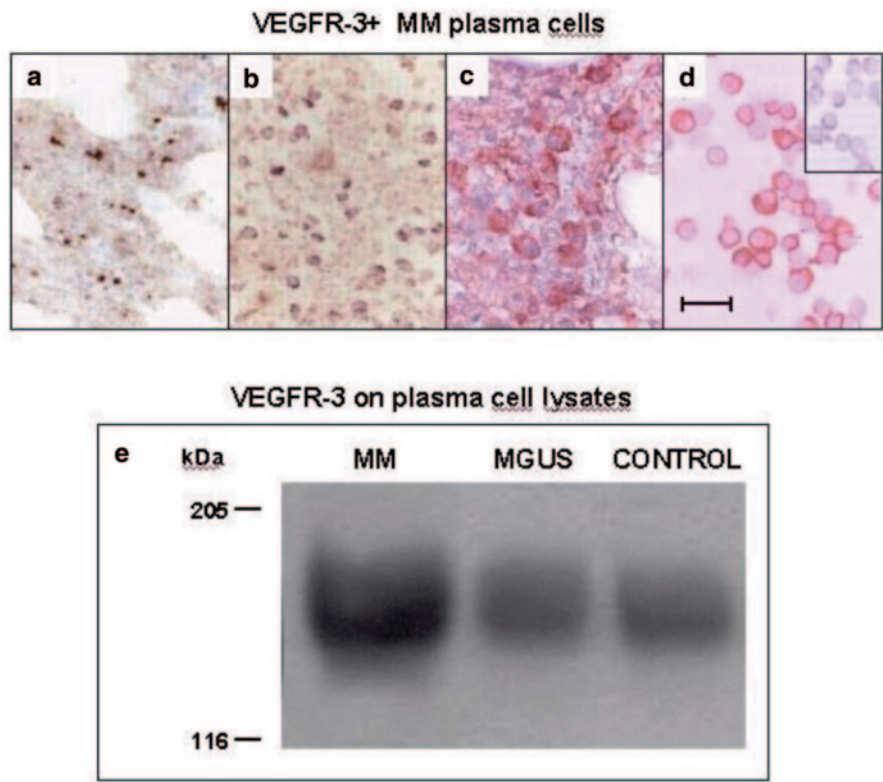


Fig. 2.4 *In situ* hybridization for VEGFR-3 mRNA of bone marrow from two patients with MM (a, b) and staining with VEGFR-3 of bone marrow (c) and enriched plasma cells (d), of the patient. Inset: the negative control obtained with rabbit preimmune serum replacing the VEGFR-3 antibody. Western blot analysis for the VEGFR-3 of plasma cell lysates from patient with MM, a patient with MGUS, and a control subject (e). The VEGFR-3 expression is higher in MM. (Reproduced from Vacca et al. 2003a)

Plasma cells are source of FGF-2 in the bone marrow of patients with active MM (Vacca et al. 1999; Bisping et al. 2003), and FGF-2 concentrations in marrow aspirates and peripheral blood correlate with MM activity (Di Raimondo et al. 2000; Sezer et al. 2001; Sato et al. 2002). MM plasma cells induced an intense angiogenic response *in vivo* the chick embryo CAM assay (Fig. 2.5) Ribatti et al. 2003a). An anti-FGF-2 antibody significantly inhibited angiogenesis induced *in vivo* in the CAM assay by MM plasma cell culture medium (Fig. 2.6), (Vacca et al. 1999a). Bisping et al. (2003) have investigated the expression of high affinity FGF receptor (FGFR-1) through R-4 in RPMI-8226 and U266 MM cell lines and patients' plasma cells, as well as in bone marrow stromal cells. Moreover, they demonstrated that FGF-2 induces a time- and dose-dependent increase in IL-6 secretion by bone marrow stromal cells, suggesting that FGF-2 is both an angiogenic growth factor in MM via IL-6 and a supporter of plasma

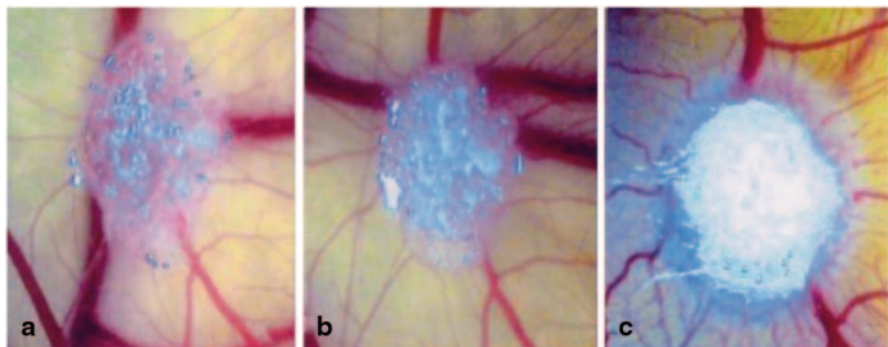


Fig. 2.5 Time-course of the macroscopic appearance of a CAM implanted at day 8 (a), with a sponge loaded with 18,000 plasma cells of an active MM patient. Note that, whereas on day 9 (b), no vascular reaction is detectable, on day 12 (c), numerous allantoic vessels develop radially towards the implant in a “spoked-wheel” pattern. (Reproduced from Ribatti et al. 2003a)

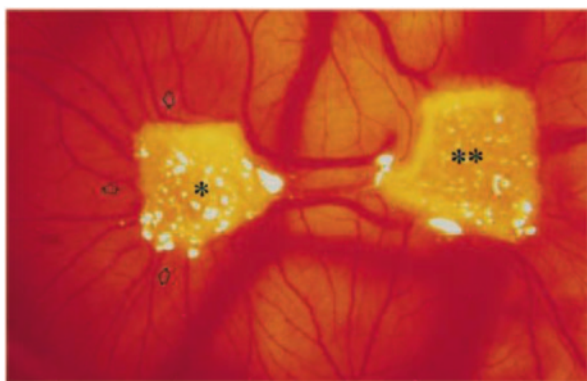


Fig. 2.6 Macroscopic picture of a CAM at day 12 of incubation implanted simultaneously at day 8 with a sponge loaded with the plasma cell culture medium of an active MM patient alone (*) and with a second sponge loaded with the same culture medium added with an anti-FGF-2 antibody (**). Note the angiogenesis toward the one-asterisk sponge (some neovessels are arrow-headed), and its inhibition by the anti-FGF-2 antibody. (Reproduced from Vacca et al. 1999a)

cell growth and survival by paracrine stimulation of the IL-6 released by bone marrow stromal cells.

TNF- α mediates upregulation of adhesion molecules of plasma cells lymphocyte function associated antigen-1 and very late antigen-4 (LFA-1 and VLA-4) and bone marrow stromal cells intercellular cell adhesion molecule-1 and vascular cell adhesion molecule-1 (ICAM-1 and VCAM-1), and thus enhances heterotypic adhesions and activates IL-6 secretion by bone marrow stromal cells (Hideshima et al. 2001a). Moreover, TNF- α secreted by plasma cells induces NF-kappa B (NF-kB)-dependent upregulation of adhesion molecules on both MM and bone marrow stromal cells (Hideshima et al. 2001b), thereby increasing the binding of MM to bone

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