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Bone marrow microenvironment in myelomagenesis: its potential role in early diagnosis

Arun Balakumaran¹, Pamela Gehron Robey¹, Neal Fedarko², and Ola Landgren^{3,†}

¹ National Institute of Dental and Craniofacial Research, National Institutes of Health, Room 222, Building 30, 9000 Rockville Pike, Bethesda, MD 20892, USA

² Johns Hopkins Bayview Medical Center, 5A64 JHAAC, MD, USA

³ National Cancer Institute, National Institutes of Health, Bldg 10, Room 13N240, 9000 Rockville Pike, Bethesda, MD 20892, USA

Abstract

Multiple myeloma (MM) is the second most common hematological malignancy, with an overall survival of 4–6 years. It is always preceded by a premalignant stage called monoclonal gammopathy of unknown significance (MGUS). Importantly, at this time we lack reliable predictors to determine who will progress from MGUS to MM, and who will remain stable. The bone marrow microenvironment plays a key role in myelomagenesis (growth, survival and migration of malignant plasma cells). In the present review, we summarize and discuss our current understanding of the bone marrow microenvironment and its compartments in relation to myelomagenesis. Although it remains to be proven, we believe that an improved characterization of the cellular constituents, the extracellular matrix components and the soluble factors of the bone marrow could open up novel avenues to better understand underlying mechanisms of the transformation from MGUS to MM. Ultimately, this will lead to the development of early treatment of high-risk precursor disease aimed to delay/prevent MM.

Keywords

angiogenesis; bone marrow microenvironment; bone turnover; extracellular matrix; monoclonal gammopathy of unknown significance; multiple myeloma; multiplex ELISA; osteoclast; proteoglycans ligand; Wnt signaling

Clinical problem

Multiple myeloma (MM) is a hematologic malignancy of postgerminal center B cells, with an incidence of approximately 20,000 in the USA, and over 50,000 patients living with the disease at any time [1]. Clinically, it is characterized by clonal expansion of plasma cells that accumulate in the bone marrow (BM), producing only a monoclonal immunoglobulin (M-protein; usually IgG or IgA), or only circulating κ - or λ -free light chains (FLC) [2]. In peripheral

[†]Author for correspondence: Tel.: +1 301 496 0670, Fax: +1 301 402 0172, landgreo@mail.nih.gov.

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blood, M-proteins can be detected by serum protein electrophoresis and immunofixation. Abnormal FLC patterns can be determined in peripheral blood as well as in urine [2].

Lytic lesions in bone are a common feature of MM, making it a painful and disabling disease. Other common complications include anemia, renal failure and infections (Box 1). Presenting signs and symptoms vary greatly, ranging from abnormalities detected on blood screening to severe manifestations, such as spinal cord compression from bone abnormalities. During the past decade, three new drugs have been approved for the treatment of MM in clinical practice (thalidomide, lenalidomide and bortezomib). In addition, a broad range of targeted small molecules and monoclonal antibodies are currently undergoing development in preclinical and early clinical trials [3]. The hope is that these new efforts will contribute to extend the historically dismal overall survival for MM patients (at the turn of the 21st Century, the median survival was approximately 3–4 years). [1].

Although it has been known for decades that individuals diagnosed with the asymptomatic condition, monoclonal gammopathy of undetermined significance (MGUS), on average, have a 1% annual risk of developing MM [4], until recently, it has not been known whether all MM cases are preceded by a premalignant stage (MGUS). Based on 77,469 healthy adults enrolled in a US nationwide population-based prospective cancer screening trial, we recently identified a total of 71 individuals who developed MM during the course of the study. Using serially collected prediagnostic serum samples obtained up to almost 10 years prior to MM diagnosis, all MM cases were found to be preceded by MGUS [5]. In accordance with recent observations, when M-protein and involved-FLC ratios were evaluated over time in individual patients, approximately half the study population showed a year-by-year increase prior to MM diagnosis, while the other half maintained largely stable abnormal serum protein level up to the diagnosis of MM. Although it remains to be confirmed, it has been speculated that ‘evolving MGUS’ could potentially be a marker for an early myeloma with a slow rate of progression. Similar to the prospective cancer screening study, another recent study evaluated 30 MM cases with available prediagnostic serum stored in the US Department of Defense Serum Repository [6]. In that study, 27 of the 30 MM cases were found to have a preceding MGUS diagnosis. Regarding the three MM patients without evidence of a prior MGUS diagnosis, two had IgD MM and one patient had only one serum sample available 5 years and above prior to MM diagnosis (i.e., the subject could not be tested for MGUS between 2 and 4 years prior to MM). This novel observation from two independent studies establishes an essential role for MGUS in the pathway to MM.

Importantly, at the present time, we lack reliable predictors to determine who will progress from MGUS to MM and who will remain stable. Both in the prospective screening trial and the study based on samples from the Department of Defense, approximately 50% of the MGUS patients who progressed to MM had evidence of increasing M-protein levels, while the remaining 50% had a stable M-protein concentration [5,6]. Consequently, key tasks for the future are to develop individualized follow-up and intervention approaches for patients diagnosed with MGUS.

Solution

Clearly, the advent of modern molecular diagnostics has enhanced our knowledge of chromosomal changes in MM [7]. However, using FISH, gene-expression profiling (GEP) or other available molecular techniques, we can still not distinguish MGUS patients from MM patients.

Importantly, there is emerging evidence that the BM microenvironment plays a key role in myelomagenesis (growth, survival and migration of malignant plasma cells) [8,9]. In our opinion, to better dissect underlying mechanisms of pathogenesis, the solution is to assess MM

tumor cells in studies that include analysis of the BM microenvironment. Although the BM microenvironment is commonly referred to as the ‘non-tumor’ entity, it has to be kept in mind that it is a complex network including a broad range of cells and factors (Figure 1). Indeed, the BM microenvironment consists of three components: the cellular component (hematopoietic and non-hematopoietic cells, including the vasculature); the extracellular matrix component (fibrous proteins, proteoglycans, glycosaminoglycans, and small integrin-binding ligand N-linked glycoproteins [SIBLING]); and the soluble component (cytokines, growth factors, adhesion molecules, and other factors) (Box 2). The three components are intertwined with multiple feedback loops within and between the compartments. In MM, the balance between the three compartments is deranged and there is a need to better clarify how the tumor cells can reprogram the microenvironment. In addition, we need to assess how predisposing susceptibilities in the BM microenvironment might play a role in the initiation of myelomagenesis and its progression from MGUS to MM. Future studies based on improved technologies are needed to characterize longitudinally collected samples from MGUS to MM in the same person.

In the present review, we summarize and discuss our current understanding of the BM microenvironment and its compartments in relation to myelomagenesis. Although it remains to be proven, we believe that an improved characterization of the cellular constituents, the extracellular matrix components and the soluble factors of the BM could open up novel avenues to better understand underlying mechanisms of the transformation from MGUS to MM. It is our thinking that such knowledge can be used for improved diagnostics and prognostics, and, ultimately, for the development of clinical trials focusing on early treatment for patients with high-risk precursor disease (MGUS and smoldering myeloma [SMM]) (Box 1).

Cellular compartment

The cellular compartment of the microenvironment consists of two major types of cells: hematopoietic and nonhematopoietic cells, including the marrow vasculature.

The hematopoietic cells include hematopoietic stem cells (HSCs), erythrocytes, megakaryocytes and platelets; immune cells, such as T lymphocytes, B lymphocytes, natural killer (NK) cells and macrophages, as well as monocytes and dendritic cells (DCs).

The nonhematopoietic cells consist of stromal cells, including pericytes, some of which are skeletal stem cells (also known as ‘mesenchymal stem cells’), marrow adipocytes, fibroblasts, osteoblasts, osteoclasts, and endothelial cells. While a great deal of work has been carried out in the last few years in elucidating the HSC microenvironment, only recently have studies focused on the B-cell microenvironment. Osteoblasts, reticular (stromal) cells, pericytes, marrow adipocytes, osteoclasts and endothelial cells are all touted as important for the HSCs [10–13]; osteoblasts are important for B-cell development [14]. Plasma cells, which are long lived, are in direct contact with CXCL12-positive stromal cells and possibly DCs; whether a direct contact exists for malignant plasma cells is unknown. However, most of these studies have been in mice, whose bone architecture is different from humans. Mouse trabecular bone is lined by osteoblasts whereas human trabecular bone is covered by lining cells, which may be stromal in nature, or ‘resting’ osteoblasts. Osteoblasts are only found at bone-remodeling sites in humans.

Alterations in many of the cellular components by MM have been summarized in the following paragraphs. In Table 1, we have listed categories of cell types in the cellular component of the BM microenvironment, markers to study and techniques that can be used to detect the cells of interest.

T & B cells

Both T and B cells show significant functional impairment in MM. T cells are hyperactive [15], whereas B-cell-driven antibody responses are deficient. Impairment of the global T-cell receptor diversity, as well as decreased numbers and function of suppressive CD25^{high}/FOXP3⁺/CD4⁺ Treg cells in MM partially accounts for the aberrant T-cell response [15,16]. Cytokines, including IL-6, abrogate the generation of Treg cells [17]. In addition to adverse immunologic effects, the aberrant T-cell response also contributes to MM bone disease.

Th17 cells predominately secrete IL-17 and IL-22. They are generated from a naive CD4 population in the presence of TGF- β and IL-6 or IL-1 β and expanded in the presence of IL-23. In MM, Th17 cells are enriched in the BM relative to the circulation [18]. Th17 cells have been shown to be immunosuppressive and to promote the growth of MM [19].

NK & NK T cells

The frequency of NK T cells determined in MM by flow cytometry in MM overlaps with that in patients with MGUS. The frequency of T cells (CD3⁺) and NK cells (CD3⁻/CD56⁺) are also comparable between these cohorts. However, progression of MGUS patients to MM is associated with an acquired, but potentially reversible, defect in IFN- γ production of NK T cell function, assessed by ELISPOT, indicating a role in controlling the malignant growth of MM cells [20].

Dendritic cells

Dendritic cells are antigen-presenting cells, which are responsible for initiating and maintaining immunity and tolerance. Interaction of MM cells with DCs has been shown to be important [21] and peripheral DCs from both MGUS and MM patients have been reported to be defective [22]. DCs of MM patients are functionally defective due to IL-6-, VEGF- or β 2-microglobulin-induced inhibition of DC maturation [22–25]. In addition, direct MM cell–DC interaction enhances MM clonogenicity [21]. Coculture of MM cells with DCs resulted in spontaneous cell–cell fusion, leading to formation of multinucleated bone resorbing giant cells. This suggests that DCs, known to be enriched in myeloma tumors, may be direct precursors for MM-associated osteoclasts [26]. Dhodapkar *et al.*, showed that uptake of apoptotic but not necrotic MM cells by DCs led to enhanced induction of Th17–1 cells, demonstrating that DCs induce expansion of polyfunctional IL17-producing T cells in MM microenvironment [18].

Macrophages

Macrophages are white blood cells produced by the division of monocytes. Macrophages are phagocytes and also stimulate lymphocytes and other immune cells to respond to pathogens. They can be identified by specific expression of a number of proteins, including CD14, CD11b, F4/80 (mice)/EMR1 (humans), lysozyme M, MAC-1/MAC-3 and CD68 identified by flow cytometry or immunohistochemical staining.

Zheng *et al.* found significantly increased numbers of CD68⁺ macrophages in BM biopsies of MM patients compared with controls [27]. Coculture of macrophages and myeloma cell lines or primary myeloma cells protected both spontaneous and chemotherapy drug-induced apoptosis by attenuating the activation and cleavage of caspase-dependent apoptotic signaling [27]. This attenuation was dependent on direct cell-to-cell contact.

Active MM macrophages exposed to VEGF and bFGF acquired endothelial cell (EC) markers and formed capillary-like structures mimicking BM ECs compared with macrophages from nonactive MM, MGUS or controls, suggesting that macrophages from MM contribute to neovessel formation through mimicry. This ability seems to proceed parallel to the progression from MGUS to MM. BM biopsies of active MM harbored ‘mosaic’ vessels, formed by ECs,

EC-like macrophages and macrophages themselves. These findings were rare in nonactive MM and absent in MGUS or controls [28].

Endothelial cells

The ECs line the interior surface of blood vessels. The malignant plasma cells and the associated BM stromal cells (BMSCs) in MM cause increased secretion of EC survival factors, such as VEGF and decreased secretion of anti-angiogenic factors [29]. The resulting imbalance of angiogenic regulators accounts for a rapid increase in tumor vessels to support tumor growth, and in abnormal structure and formation of mosaic blood vessels. These mosaic vessels consist of ECs as well as highly proliferating circulating endothelial precursors/angioblasts, HSCs, progenitor cells, monocytes, macrophages and tumor cells [28,30,31]. Moreover, recent studies suggest the existence of MM-specific ECs [32–34]. Interaction of ECs with malignant plasma cells results in increased neovascularization; microvessel density in the BM progressively increases from MGUS to SMM to MM. ECs enhance the invasion of MM cells by stimulating secretion of matrix metalloproteinases (MMP)-9 [35]. Angiogenesis also has a prognostic value in MM. For example, Rajkumar *et al.* found angiogenesis to progressively increase from MGUS to MM, indicating that angiogenesis may be related to disease progression [36]. Functionally, tumor-associated vasculature causes chaotic and variable blood flow as well as vessel leakiness, resulting in lowered drug delivery and further selection of more malignant plasma cells. A recent report comparing gene-expression profiles of MGUS and MM endothelial cells demonstrated 22 genes differentially expressed that may play an important role in MM progression. Specific pathway analysis suggests that these genes are involved in the control of apoptosis, extracellular matrix formation and bone remodeling, cell adhesion, angiogenesis and cell proliferation [37].

BM stromal cells

Bone marrow stromal cells comprise the adherent fraction of cells (in humans; in mice, macrophages are also adherent) obtained from BM aspirates and biopsy, which can be expanded *ex vivo*. A subset of these cells, are skeletal stem cells capable of giving rise to osteoblasts, marrow adipocytes and hematopoietic supporting stroma [12].

Bone marrow stromal cells isolated from MM patients produce higher levels of IL-6, DKK1 and factors associated with angiogenesis and osteogenic differentiation, compared with controls [38,39]. They also have decreased ability to inhibit T-cell proliferation [40]. The production of metalloproteinases by the BMSCs of MM patients is also distorted; Zdzisinska *et al.* reported in a recent study, that production of MMP-1, MMP-2 and TIMP-2 is significantly increased compared with BMSCs from healthy controls [41]. All of these studies have used *ex vivo* cultured BMSCs, which can result in alteration of the original properties.

Osteoclasts & osteoblasts

Defects in osteoclasts and osteoblasts were recognized early, since MM patients typically exhibit osteolytic bone ('punchout') lesions associated with bone pain, pathologic fractures and diffuse osteoporosis in the central skeleton, the skull and long bones. Increased osteoclast formation and activity in the vicinity of MM cells along with lower numbers of osteoblasts and decreased bone formation result in the lytic lesions [42,43]. A quantifiable excess of bone resorption by static histomorphometry was an early indicator of patients with MGUS who progressed to MM [44]. Since quantitative bone biopsies are not performed routinely in all centers, bone densitometry and biochemical markers in serum or urine can be of potential to determine bone remodeling in patients with MGUS methods to foretell their progression to MM (Table 2). A variety of osteoclast-activating factors, such as macrophage inflammatory protein-1 α (MIP-1 α) [43,45], receptor of NF- κ B ligand (RANKL) or osteoprotegerin (OPG) ligand, VEGF, TNF- α , IL-1 β , parathyroid hormone-related protein, HGF, and IL-6, produced

by both tumor as well as stromal cells, trigger increased osteoclast activity, which modulates MM cell growth and survival [43,45]. Conversely, lower numbers of osteoblasts and decreased bone formation in MM are associated with dysregulation of several signaling molecules, including Runt-related transcription factor 2 (Runx2)/Cbfa1, Wnt and IL-3. First, MM cells block Runx2 in human BM osteoblast progenitors triggered by VLA-4/VCAM-1-mediated contact or IL-7 secretion [42,43,45]. Second, microarray analysis showed that the Wnt-signaling antagonist, DKK1, an inhibitor of osteoblast differentiation, is significantly overexpressed in patients with MM who had lytic bone lesions [46]. DKK1-induced inhibition of osteoblastic progenitors deranges the BM micro environment required for HSC differentiation, which contributes to immunosuppression and anemia in MM patients. Myeloma-derived DKK1 also disrupts Wnt-regulated OPG and RANKL production by osteoblasts [47]. Third, IL-3 is increased in BM plasma from MM patients and triggers increased osteoclast formation, as well as blocking differentiation of preosteoblasts to mature osteoblasts. Importantly, CD3⁺ T cells have been identified as the main source of IL-3 in MM patients. A potential critical role of CD3⁺ T cells in MM bone disease is further supported both by their increased production of RANKL, and their promotion of osteoclast formation and survival [42]. Conversely, administration of a polyclonal DKK1 antibody ameliorates the bone-specific morbidity of MM and immunodepletion of secreted frizzled-related protein (SFRP)2, which prevents binding of Wnt proteins to their receptors and, thereby, inhibits osteoblast function, significantly restores bone mineralization *in vitro* [48]. Though osteoblastic inhibition has been extensively reported in MM, patients with early MM have increased bone formation rates based on dynamic histomorphometry studies suggesting that osteoblastic recruitment is a critical initial step in the progression of MGUS to MM [49].

A thorough understanding of BM in myeloma is possible by a pathologist's assessment of aspirates and biopsy, immunohistochemistry, flow cytometry from BM aspirates and, to a lesser extent, of peripheral blood. We are working out techniques to study stromal cells, including their gene expression fresh from isolation by BM aspirates. The changes in the microenvironment over the progression from precursor stage to myeloma (MGUS to SMM to MM) can be studied if samples from an individual at various stages of the disease are available. None of the described cellular changes have been observed the same individual progressing from MGUS to MM.

Extracellular matrix component

The extracellular matrix component is a dynamic 'filler' substance existing between various cells in the BM microenvironment. It has multiple functions, including its capacity to provide support and anchorage for cells, segregating tissues from one another and regulating intercellular communication. In addition, it sequesters a wide range of cellular growth factors to act as a local depot for them.

The extracellular matrix component contains the following four categories: fibrous proteins (such as collagen, laminin, fibronectin and elastin), which are the majority (>90%) of the extracellular matrix, proteoglycans (such as heparan sulfate-containing proteoglycans and small leucine-rich repeat proteoglycans [SLRPs] decorin, biglycan, fibromodulin and lumican being the best characterized), glycosaminoglycans (particularly hyaluronan), and SIBLING proteins (such as osteopontin, bone sialoprotein [BSP] and dentin matrix protein-1 [DMP-1]).

Extracellular matrix remodeling is mediated by the balance of proteases (MMPs and tissue inhibitors of metalloproteinases [TIMPs]) described in the 'soluble component' section (see below).

In Box 2 we list factors in the extracellular matrix. These factors are usually detected by immunohistochemistry, western blot and quantitative PCR in tissue sections. ELISA, western blots and quantitative PCR are used for the detection/quantification from serum.

Fibrous proteins

Tough bundles of fibrous proteins are a major component of the extracellular matrix, and support most tissues providing cells structure from the outside. In BM, there is a relative paucity of large fibrillar structures. Instead it is characterized by a fine network of reticular fibers. Interactions of human myeloma cells with the reticular fibers may explain the specific retention of the plasma cells within the BM but not much work has been done to elucidate these interactions. A recent study analyzed the expression and localization of fibrous proteins, such as fibronectin, laminin and collagens I and IV, in the core biopsies of normal volunteers, patients with MGUS or MM patients with low-, mid- and high-level plasmacytosis in the BM [50]. Expression of laminin was the same for all groups but levels of fibronectin and collagen I were reduced in the group of MM patients with high-levels of plasmacytosis. Expression of collagen IV in the BM of MGUS and MM patients was higher than in the BM of normal volunteers. Furthermore, the plasma cells isolated from the patients with high-level plasmacytosis overexpressed collagen IV.

Proteoglycans

Proteoglycans are composed of protein cores to which glycosaminoglycans (GAGs) are covalently attached. They form large complexes with other proteoglycans, hyaluronan and other matrix proteins, such as collagen. They are involved in binding water and cations, such as sodium, potassium and calcium, and in regulating the movement of molecules through the matrix. Individual functions of proteoglycans can be attributed to either the protein core or the attached GAG chain. They are classified by size and the nature of the core protein; an example of a large proteoglycan is aggrecan and the SLRPs include decorin, biglycan, fibromodulin and lumican.

Syndecan-1 is a transmembrane heparan sulfate-bearing proteoglycan known to regulate multiple biological functions at the cell surface and within the extracellular matrix. Its functional activity can be modulated by heparanase, an enzyme that cleaves heparan sulfate GAG chains and whose expression has been associated with an aggressive phenotype in many cancers. In addition to remodeling syndecan-1 by cleaving its heparan sulfate chains, heparanase influences syndecan-1 location by upregulating expression of enzymes that accelerate its shedding from the cell surface. Heparanase also alters the level of nuclear syndecan-1; the nuclear localization of syndecan-1 drops dramatically as revealed by confocal microscopy, western blotting and quantification by ELISA after addition of recombinant heparanase or upregulation of heparanase expression. Heparanase-1 is expressed at high levels in some patients with myeloma. In a severe combined immunodeficient (SCID) mouse model, enhanced expression of heparanase in a myeloma cell line dramatically increases the MM bone involvement [51] and microvessel density [52].

Syndecan-1 shed from the myeloma tumor cell surface accumulates in the BM and serum of patients. When present at high levels in the serum, syndecan-1 is an independent indicator of poor prognosis [53–55]. However, high-serum syndecan-1 is more than simply an indicator of poor prognosis. Studies in animal models have shown that high levels of soluble syndecan-1 enhance both the growth and metastasis of tumors [51]. Syndecan-1 exerts its growth-promoting effects by regulating the activity of many effector molecules important for myeloma growth and survival, including HGF and heparin-binding EGF family members, among others [56,57]. The high levels of heparan sulfate in the tumor microenvironment resulting from syndecan-1 shedding also act as positive regulators that condition the microenvironment for

robust tumor growth. For example, heparan sulfate binds to and promotes the activity of important angiogenic growth factors, such as FGF-2 and VEGF. This activity can occur in trans, indicating that shed syndecan-1 can contribute to the high level of angiogenesis seen in many patients with myeloma [58,59]. In addition, the syndecan-1 that becomes embedded within the myeloma tumor stroma can serve as a reservoir for storage and concentration of heparan sulfate-binding growth factors that can later be mobilized by cleavage of the heparan sulfate by heparanase [53,60]. This mobilization of heparan sulfate-retained growth factors in the BM is likely to contribute to the high rate of relapse among patients with myeloma.

Treatment with bacterial heparinase III (an enzyme that degrades heparan sulfate chains) and an inhibitor of human heparanase (an enzyme that synergizes with syndecan-1 in promoting myeloma progression), blocked the growth of myeloma *in vivo*; knockdown of syndecan-1 expression by RNA interference (RNAi) inhibited the growth of myeloma tumor development in the human SCID (SCID-hu) model of myeloma *in vivo*. Together these results confirm the importance of syndecan-1 in myelomopathobiology.

The SLRPs are a family of proteins that are present in extracellular matrix and share multiple repeats of a leucine-rich structural motif, flanked by cysteine residues. These proteins appear to interact in many cases with collagen, modifying the deposition and arrangement of collagen fibers in the extracellular matrix, and also with cells and with soluble growth factors. The interaction of SLRPs with cells and with growth factors, such as TGF- β , may affect the proliferation of cells in addition to modifying the extracellular environment. Post-translational modification of SLRPs with carbohydrates and sulfate-containing groups appears to modify the function of SLRPs. Changes in SLRP expression and modification in the cornea, atherosclerotic plaque, joints, bone, tendons and kidney may be associated with disease progression in those tissues. Decorin is one SLRP expressed throughout the body that stabilizes collagen fibrils and that also antagonizes the action of the cytokine TGF- β , blocking cell cycle progression and potentially playing a role in cancer and wound healing. The SLRP biglycan is expressed in bone and other connective tissues and genetic disruption of biglycan in mice causes low bone mass similar to osteoporosis.

Small leucine-rich proteoglycans, such as decorin, are involved in the antimyeloma effects of osteoblasts through direct and indirect mechanisms by interfering with the stimulatory effects of osteoclasts and BMSCs on myeloma cell growth and by inhibiting myeloma-induced angiogenesis and osteoclastogenesis [61].

Glycosaminoglycans

Glycosaminoglycans (or mucopolysaccharides) are long unbranched polysaccharides consisting of a repeating disaccharide unit and form an important component of connective tissues. GAG chains may be covalently linked to a protein to form proteoglycans.

Hyaluronan is a major glycosaminoglycan component (not bound to a protein core) of the extracellular matrix in the BM and is the principal ligand for the widely distributed cell surface glycoprotein molecule, CD44. Hyaluronan is a survival and proliferation factor for human myeloma cells. It can be an important component in the MM physiopathology *in vivo* by potentiating autocrine and/or paracrine IL-6 activities. Hyaluronan-induced survival was unchanged, even when MM cell lines were incubated in the presence of blocking anti-CD44 antibody and no detectable production of IL-6 was detected, suggesting that hyaluronan concentrates IL-6 near the plasma cells and potentiates the autocrine activity of IL-6 in a CD44-independent way [62].

The expression of CD44 is decreased on MM plasma cells and nonmalignant B cells in affected BM of MM patients [63]. An abnormally low or high concentration of hyaluronan in the serum

of patients with MM was associated with a shorter median survival than those with an intermediate hyaluronan concentration [64].

SIBLING proteins

Small integrin-binding ligand N-linked glycoproteins, a family of five proteins comprising osteopontin, BSP, DMP1, dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE), are an emerging group of molecular factors that are involved in cellular cancer progression. SIBLINGs are soluble, secreted proteins that can act as modulators of cell adhesion as well as autocrine and paracrine factors by their interaction with cell surface receptors such as integrins. SIBLINGs propagate biological signals by initiating integrin signaling and by binding and sequestering other proteins to the cell surface. Increased expression of SIBLING proteins is a consistent feature for many known human malignancies and could act as informative serum markers as was shown for prostate cancer in a recent study: BSP, DSPP and osteopontin were elevated in serum from prostate cancer subjects, with serum DSPP exhibiting the greatest difference [65]. BSP blood levels predict tumor burden, neoplastic bone involvement and prognosis in MM [66]. The role of SIBLING proteins in the BM during progression of MM can be studied by immunohistochemistry of the BM biopsies from MGUS and MM patients.

Soluble component

A variety of cytokines, growth factors, adhesion molecules and other factors are secreted by hematopoietic (both tumor and nontumor) and nonhematopoietic cells in the BM. In Box 2, we provide a summary of well-characterized factors in the literature and techniques that can be used to detect factors of interest. Below, we discuss functions and interactions between BM microenvironmental soluble factors in myelomagenesis.

Cytokines

IL-6 is a key growth and survival factor in MM [67,68], predominantly produced and secreted by BMSCs and osteoblasts [42], which correlates with MM tumor cell mass, disease stage and prognosis. Its levels have been found to be elevated in MM. It is a potent stimulator of osteoclast formation, both *in vitro* and *in vivo* [69,70]. It also enhances the effects of PTHrP, IL-1 and TNF- α , which are involved in osteoclastogenesis. Moreover, IL-6 induces expression of Xbp-1, a transcription factor involved in plasma cell/MM cell differentiation [71–75]. IL-6 also triggers activation of MEK/MAPK-, JAK/STAT3-, and PI3K/Akt-signaling pathways [76]. The IL-6 triggered JAK/STAT3 pathway induces upregulation/activation of antiapoptotic proteins, Mcl-1 and BclXL and Pim1, as well as c-Myc. Conversely, blockade of IL-6 induces upregulation of the proapoptotic BH3-only protein, Bim, concomitant with Mcl-1 downregulation and activation of Bax, thereby inducing MM cell apoptosis [77]. However, many MM cell lines grow independently of IL-6. Moreover, binding of MM cells to BMSCs trigger survival even after inhibition of the IL-6/gp130/STAT3 pathway, suggesting that MM growth mechanisms other than IL-6 may exist. These findings may also explain why therapeutic approaches targeting IL-6 have not induced responses in Phase I clinical trials. Elevated expression and levels of IL-6, a proinflammatory cytokine, are likely to contribute to extracellular matrix turnover (wound healing and extracellular matrix remodeling being a normal sequelae in inflammation). It may be that the IL-6-associated remodeling of the extracellular environment facilitates MM cell growth and progression by tilling a fertile soil for cells (disrupting containment barriers and exposing sequestered angiogenic and growth factors). Taken together, these data show that IL-6 is a crucial, but not a sole factor in MM pathogenesis.

In MM, SDF-1 α and its G-protein-linked cognate receptor, CXCR4 (CD184) are expressed in the BM of MM patients. SDF-1 α is primarily produced by BMSCs but also by MM cells. Functionally, SDF-1 α rapidly and transiently upregulates VLA-4-mediated MM cell adhesion to both fibronectin and VCAM-1 [51]. Furthermore, SDF-1 α promotes proliferation, induces migration and protects against dexamethasone-induced apoptosis in MM cells through MAPK, as well as NF- κ B and Akt-mediated pathways. In addition, SDF-1 α upregulates secretion of IL-6 and VEGF in BMSCs and is a critical regulator of MM cell migration and homing [78].

The TNF- α superfamily includes CD40L, B lymphocyte activating factor (BAFF) and proliferation-inducing ligand (APRIL). CD40L is expressed by antigen-presenting cells, T cells, as well as B-cell malignancies, including MM. Functionally, it mediates p53-dependent increases in MM cell growth, MM cell migration, triggers VEGF secretion and induces membrane translocation of Ku86 and Ku70 proteins involved in IgH class switching [70]. Moreover, CD40-activated MM cells adhere to fibronectin and are protected against apoptosis triggered by irradiation and doxorubicin [70].

TNF- α – secreted mainly by macrophages – triggers only modest MM cell proliferation, survival, and drug resistance. However, it markedly upregulates (by a fivefold increase) secretion of IL-6 in BMSCs and induces NF- κ B-dependent expression of CD11a/LFA-1, CD54/ICAM-1, CD106/VCAM-1, CD49d/VLA-4 and/or MUC-1 on MM cell lines, as well as CD106/VCAM-1 and CD54/ICAM-1 expression on BMSCs. Expression of these molecules results in increased (two- to four-fold) specific binding of MM cells to BMSCs, with related induction of IL-6 transcription and secretion, as well as cell adhesion-mediated drug resistance [76].

B lymphocyte activating factor is normally expressed by monocytes, macrophages, DCs, T cells and BMSCs. It exists as a membrane-bound and a cleaved soluble protein. MM cells, compared with normal plasma cells, showed higher expression of receptors for BAFF and APRIL. In addition, high levels of BAFF and APRIL are also produced by osteoclasts. In MM, both tumor cells and BMSCs express high levels of BAFF and APRIL, as well as levels of their receptors [79]. BAFF secretion by BMSCs is further augmented upon adhesion to MM cells [80]. Functionally, BAFF and APRIL protect MM cells from apoptosis induced by IL-6 deprivation and dexamethasone, and promote MM cell growth, as well as adhesion to BMSCs. These processes are mediated through NF- κ B, PI3K/Akt and MAPK pathways.

RANKL is a member of the TNF family and increases osteoclastogenesis by binding to the RANK receptor on osteoclast precursors to induce osteoclast differentiation. OPG, a naturally occurring decoy receptor for RANKL, acts as an inhibitor of RANKL to inhibit osteoclastogenesis. In the marrow of MM, RANKL is increased and OPG is decreased. While RANKL and OPG are expressed mainly by BMSC and osteoblasts, small amounts of RANKL are also produced by MM cells. The altered expression of RANKL and OPG by BMSCs is thought to be due to the binding of MM cells, which can be blocked by an antibody to VLA-4, the molecule on MM cells responsible for adherence to VCAM-1 receptor on BMSCs, or with the recombinant soluble form of VCAM-1.

Macrophage inflammatory protein (MIP)-1 α not only increases osteoclast differentiation and maturation [81] but also acts like a growth factor for MM cells [82]. It can also potentiate the effects of other factors involved in osteoclast maturation, such as RANKL and PTHrP [83].

Growth factors

VEGF is secreted by several MM cell lines and is present in MM BM plasma. VEGFR-1 is highly expressed by MM cells, consistent with autocrine signaling [32]. Specifically, VEGF induces MM cell migration on fibronectin [84] and survival signaling through upregulation of

Mcl-1 [32–34]. In addition to MM cells and ECs, VEGF also affects osteoblasts, NK cells, monocytes and endothelial progenitors [32].

In MM, IGF-1 induces tumor cell growth, survival and migration. Moreover, a positive relation of baseline BMI and MM was recently reported on two large prospective cohorts, the Nurses' Health Study and the Health Professionals follow-up Study [85], further supporting an important role of IGF-1 in MM pathophysiology.

In a case report, the conversion of MGUS to overt MM occurred in strict temporal relationship with elevated serum IGF-1 levels [86]. In addition, serum IGF-1 levels have been linked to survival in MM [87]. Moreover, IGF-1 receptor is the only gene significantly elevated in a comparison of microarray profiles of myeloma cells with and without chromosome 13 deletion, a dire prognostic feature [88].

Qiang *et al.* showed that IGF-1 is a potent inducer of myeloma cell migration/invasion using a transendothelial migration assay with myeloma cells lines. Local concentration gradients of IGF-1 determined whether MM cell lines will migrate or proliferate. Furthermore, IGF-1-mediated proliferation was largely regulated by the PI3K/Akt pathway, but migration required activation of PI3K/PKC and PI3K/RhoA [89].

Decreased levels of bone formation markers, such as alkaline phosphatase and osteocalcin are seen with marked osteoblastopenia in MM patients. MM cells *in vitro* have been shown to inhibit RunX2/CBFA1 activity, which, in turn, inhibits osteoblast formation. Cytokines that inhibit osteoblast activity, such as IL-7 and IL-3, have also been shown to be increased in the marrow supernatants of patients with MM [42,90].

Hypercalcemia commonly occurs in MM but not in SMM or MGUS. Calcium-sensing receptors expressed on myeloma cells can respond to locally high levels of Ca^{2+} at sites of osteoclastic bone resorption and stimulate their proliferation in an IL-6- independent manner [91]. Calcium-sensing receptor activation can possibly lead to increased PTHrP secretion by transactivating the EGF receptor as was seen in prostate cancer cells [92].

Other factors

Matrix metalloproteinases are a family of zinc-dependent endopeptidases involved in the degradation of structural components of the extracellular matrix seen in both physiological and pathological tissue degradation [93–95]. The human MMP family consists of more than 20 members and is divided into subgroups, such as the collagenases and the gelatinase and stromelysins, according to substrate specificity. MMPs are further classified based on their structural relationship into minimal-domain MMPs, simple hemopexin-domain- containing MMPs, gelatin-binding MMPs, furin-activated secreted MMPs, vitronectin-like insert MMPs, transmembrane MMPs, glycosylphosphatidylinositol (GPI)-anchored MMPs and type II transmembrane MMPs. Enzymes belonging to the first five groups are secreted while the remaining are membrane-type MMPs.

The production of MMPs is regulated both on a transcriptional level and further through an activation step where inactive zymogen are activated by proteolytic cleavage of the propeptide domain. This processing enables the cysteine residue in the conserved sequence to interact with the zinc ion in the catalytic domain. For some MMPs the activation is dependent on furin or related proprotein convertases, whereas at least one secreted MMP can be activated by the membrane-bound MMP, MMP14. Additionally, plasmin is capable of activating some MMPs *in vitro*. Once activated MMPs are inhibited by endogenous inhibitors such as the TIMPs and the kazal-type inhibitor RECK. The balance between active MMPs and TIMPs is thought to determine the net activity of MMPs.

Several MMPs are overexpressed in carcinomas, suggesting a role in malignant transformation, but the role of MMPs in disease progression is very diverse and not solely limited to a role of tumor progression [96]. The expression of MMPs in MM cells has been well studied. MMP-9 has been found in purified myeloma cells isolated from MM patients [97–99]. Vacca *et al.* were also able to detect MMP-2 in plasma cells from MM patients [98]. The expression of MMP-8 and -13 was detected in malignant plasma cells from plasmacytomas [99]. It is important to understand the production of MMPs in BMSCs and ECs since BMSCs are critical to the progression of MM. BMSCs secrete MMP-2 and MMP-1 [97]. ECs isolated from MM patients (MMECs) secreted three- to four-times more active MMP-2 and -9 than human umbilical vein endothelial cells [100].

Most of the MMPs are secreted as inactive proenzymes and are activated extracellularly by proteolytic cleavage. Interaction of MMPs with each other can lead to their activation. MMP-7, secreted by MM cells, is responsible for the activation of MMP-2 produced by BMSCs [101]. The uPA/plasmin system is also involved in MMP activation [102]. This was demonstrated with leukemia cells, which produce significant amounts of proMMP-9. Activation was achieved by adding plasminogen to the leukemia cells [103]. uPA converts plasminogen to plasmin, which in turn can activate MMPs. Recent results indicate that uPA is expressed by myeloma cells [104,105].

Matrix metalloproteinases, through their multiple actions on growth factors, enhance neovascularization and osteoclast activation contributes to MM progression. Two important growth factors in MM are IL-6 and IGF-1. Specific ligand-binding protein of the receptor for IL-6, IL-6Ra, is released from MM cells by proteolytic cleavage [106], which binds to IL-6, leading to an increased proliferation of MM cells. IGF binding proteins (IGFBPs), which are substrates of MMPs, regulate the bioavailability of IGF by binding to the growth factor. Serum levels of IGFBP-3 are decreased in MM patients, suggesting that the protein is cleaved [87]. Shedding of IGFBPs can increase the amount of bioavailable IGF-1 resulting in MM progression. The enzyme responsible for this shedding is not known, but ADAM family members may be responsible for the cleavage of growth factors in MM [87,107].

Matrix metalloproteinases may also have a role in the enhanced neovascularization seen in MM [108,109], since they are involved in the different processes of angiogenesis, such as proteolysis of the ECM, migration of ECs and the release of angiogenic factors from the ECM [110]. A larger microvessel area and a higher secretion of MMP-2 and -9 was shown in patients with active MM than in those with nonactive MM, MGUS or control subjects [98]. ECs isolated from the BM of MM patients produce a higher level of MMP-2 and -9 compared with HUVECs [100]. Treatment of MM-diseased mice with the broad-spectrum MMP inhibitor, SC-964, resulted in an almost complete inhibition of angiogenesis [111]. Furthermore, MMPs may also have a role in the homing of MM cells. Treatment of the 5TMM cells, a mouse myeloma cell line, with the gelatinase inhibitor, EGCG, resulted in the inhibition of invasion and thus demonstrated the involvement of MMP-9 in invasion, the last step of homing. The upregulation of MMP-9 after interaction of MM cells with ECs also indicates that MMP-9 might play a role in the homing process [35,111].

Matrix metalloproteinases play a role in normal bone remodeling and, thus, may have a role in the development of osteolytic lesions. MMPs are involved in osteoclast recruitment to sites of bone remodeling [112] and the enzymes can degrade mineralized bone matrix [113,114]. Treatment of 5T2MM-diseased mice with the MMP inhibitor, SC-964, resulted in a significant decrease in the number of osteolytic lesions and the prevention of cancellous bone loss induced by the presence of 5T2MM cells [111]. Other evidence suggesting the role of MMPs in osteolytic bone disease is the inhibition of MMPs by biphosphonates, used as therapy in MM

for preventing bone resorption. Zoledronate significantly inhibits MMP-1 secretion by BMSCs, but strongly upregulates MMP-2 production [115].

Tissue inhibitors of metalloproteinases are the natural inhibitors of MMPs, but may also have other functions. There have been four TIMPs described, TIMP-1, -2, -3 and -4. As with MMPs, there is some controversy over the functions of TIMPs in cancer development. Because they are able to inhibit MMPs, it was believed that they could inhibit tumor growth, invasion, angiogenesis and metastasis. Several studies confirm this hypothesis [116–118]. However, it has also been demonstrated that high TIMP levels in certain types of malignant tumors in humans are associated with poor outcome. This could be due to the multifunctional role of TIMPs. TIMP-1 and -2 are able to stimulate the growth of several cells [119–122], and TIMP-2 has been described to be involved in the activation of proMMP-2 [123]. TIMP-3 possesses proapoptotic capacity [124,125], whereas TIMP-1 has antiapoptotic effects on certain cell types [126,127]. Recently, DNA array demonstrated a higher level of TIMP-1 and the same level of TIMP-2 in the MMECs compared with the HUVECs [100]. Higher levels of serum TIMP-1 correlated with advanced disease and predicted for poor survival in patients with MM treated with novel agents [128,129]. Predominant TIMP-1 expression in tumors with plasmacytic/plasmablastic phenotypes (including MM) supports an important role for TIMP-1 in the pathogenesis of plasmacytic/plasmablastic tumors [129]. More research is necessary to find out more about the expression and role of TIMPs in MM disease.

Key integrins mediating MM cell–ECM adhesion are β_1 (CD29) and $\alpha_v\beta_3$ -integrin. MM cell adhesion to fibronectin is predominantly mediated through β_1 -integrin and directly protects tumor cells from DNA-damaging drugs (e.g., anthracyclines and alkylating agents) by induction of cell adhesion-mediated drug resistance, a reversible G1 arrest associated with increased p27kip1 (encoded by CDKN1B) levels [130,131]. MM cell adhesion to vitronectin and fibronectin is predominantly mediated through $\alpha_v\beta_3$ -integrin binding and triggers production and release of urokinase-type plasminogen activator, MMP-2 and MMP-9, thereby promoting tumor cell invasion and spreading [132].

Diagnostic techniques to study the microenvironment in MGUS to MM progression

The template for disease detection has been that one develops a test to measure the quantity of a single biological marker and that this biomarker will track with the presence of the disease. The likelihood that a single biological measure can be identified that will provide optimal diagnostic information for MM is not great. Lymphocytic proliferation, differentiation or neoplastic transformation are not internal, self-contained programmed cell processes. Rather they are dependent on receptor sensing and feedback with, as well as processing of, the extracellular matrix components discussed earlier. Developing an understanding of these complex interactions is not well served by the reductionist approach of single biomarker analysis. An alternative approach to this complexity has been the use of microarray technology to screen thousands of genes for their expression by tumors and develop a ‘molecular fingerprint’ for a given cancer. Gene arrays have drawbacks for diagnosis that include an overabundance of data to sift through as well as a requirement for isolated samples that contain genetic material (typically tissue biopsies). With recent advances in proteomics, protein arrays can be constructed to measure multiple markers simultaneously in a given sample. Unlike gene arrays, which measure the expression of thousands of genes, directed protein arrays can be made which enable the quantification of specific proteins (up to 23) in serum, plasma, urine or saliva.

The levels of potential biomarkers are traditionally screened initially by methodology that is not optimized for quantification and high throughput. For example, proteins in a complex mix,

such as in blood, can be separated by SDS PAGE and, after transfer of the resolved proteins to a membrane, antibodies for specific potential biomarkers can be used to detect the relative levels. An advantage of this screening methodology is that the relative molecular weight of the protein/biomarker is determined as well as its abundance. A disadvantage is that this method is not conducive for screening large numbers, as the number of samples and standards that can be analyzed at a given time (in one gel) is limited and further reduced by the necessity to include controls to assess potential variations in the distinct steps of the procedure (e.g., how well samples were separated in the acrylamide gel or how efficiently they were transferred to the membrane). Furthermore, efficiency of transfer of proteins varies with the size of the protein, hence the values determined are not total levels but relative levels.

For the measurement of many proteins at one time, two distinct technologies are currently in use for multiple simultaneous protein analysis. One technology is based on flow cytometry and utilizes capture antibodies conjugated to specific beads that differ in their detection signal (e.g., fluorescence emission wavelength) and a single sample is exposed to beads with a number of distinct capture antibodies. A parallel set of beads are exposed to a graded set of standards and flow cytometric methods enable quantification of signal from standards and interpolation of values for the beads reacted with test samples. The other technology represents a 'nano' technological upgrade to ELISAs, where multiple distinct capture antibodies are absorbed within each well of a 96-well plate in a predefined array. Serum samples added to wells containing the multiple capture antibodies in discrete regions (dots) within each well. Multiplexed ELISAs are processed similarly to regular ELISAs with multiple washes and incubation steps. Typically, chemiluminescent is used for the detection and quantification of dots within wells across the entire plate. Both technologies have the advantage of requiring a much smaller sample (serum, plasma, saliva or urine), typically 25–50 µl. In addition, the results from one multiplexed plate or bead-based analysis yields data much faster than running four, ten or more individual 'single-plex' immunoassays. A disadvantage of both techniques is that potential interactions between the different antibodies used on the single sample need to be assessed and taken into account. Also, for both methods, specificity of the antibodies is paramount. At least with SDS PAGE and western blotting, the relative molecular weight can be used to confirm the identity of the immunoreactive material, whereas bead-based or ELISA-based assays yield only a 'positive' immunoreactive signal. An issue with using either of these two techniques is that a standard of significant quantity and purity is necessary for each component measured. For many of the extracellular matrix components described, such standards are not readily available. A reasonable approach to developing biomarkers for MM would be to use screening methodology (SDS PAGE coupled with western blotting or SDS PAGE coupled with mass spectrometry) to determine a signal that correlates with disease. Positive targets from the screen would then be further developed in multiplexed analyses.

As discussed in this review in detail, the BM microenvironment contains a complicated network between each component and the tumor cells. Beyond this fact, one has to keep in mind that literature focusing on techniques used to identify component functions in a normal situation might be different from that in pathological situations (i.e., MM). For these reasons, one has to be cautious when extrapolating techniques/results from the normal situation to MM. Future studies are needed to better characterize variations in normal versus pathological BM.

Expert commentary

As has been described in detail, the BM microenvironment is a very complex network, including a wide range of cells, extracellular matrix proteins and soluble factors. At this time, a fair amount of research has been conducted to better understand the role of the BM microenvironment in MM. However, a major drawback in most studies is the fact that only a selected number of factors have been evaluated in any given study. In addition, it should be

cautioned that many studies have been limited to *in vitro* changes of the BM microenvironment using MM cell lines. Given their aggressive biology, most cell lines are more related to plasma cell leukemia rather than MM. Future investigations using MM patient samples need to be inclusive and incorporate a broad range of microenvironmental factors in the study of MM. Probably the reasons why such studies have not yet been conducted are costs, diverse expertise in selected cells and markers, the application of various assays and test, as well as access to tissues. Importantly, only limited information is available from studies based on successive BM samples from the same patient from MGUS to MM. Indeed, a gap in the literature is the lack of understanding regarding morphologic and molecular changes that occur from MGUS to MM. Finally, high-throughput strategies are being used extensively to characterize MM cell subtype and to differentiate malignant from benign plasma cells. However, to our knowledge, these strategies have not yet been systematically assessed in relation to BM microenvironmental patterns, as has been recently carried out for lymphoma [133].

Five-year view

At this time, we are not able to predict which patients will remain stable with MGUS or SMM, and which will progress to MM. This is owing to the lack of prospective data from MGUS progressing to MM, as well as the unavailability of serial samples. Furthermore, no studies have yet taken a systems biology approach and integrated microarray and proteomic analyses of the stromal microenvironment. Such efforts could have a major impact and contribute to early detection of high-risk precursor disease. In fact, it is very possible that changes in the serum levels of a few microenvironmental molecules could predict the MM progression.

To facilitate the development of a systems biology approach, at the National Cancer Institute, NIH, in Bethesda (MD, USA), we have designed and initiated prospective studies focusing on the trajectory from precursor disease (MGUS and SMM) to fullblown MM. Using a broad array of BM microenvironmental markers, as discussed in this review, we have developed studies that take into account both the BM microenvironment as well as MM tumor cell biology. The goals of our studies are to improve our understanding of mechanisms of progression from MGUS to MM, to provide better patient counseling for follow-up, to identify high-risk precursor patients, and to develop novel treatment trials designed to delay/prevent MM progression. We welcome patients and referring physicians to participate in our ongoing studies. In 5 years, these studies will provide the framework for improved diagnostics, prognostics, and early treatment of MM precursor disease.

Key issues

- Multiple myeloma (MM) is a hematologic malignancy of postgerminal B cells characterized by accumulation of plasma cells in bone marrow and evidence of an M-protein detectable in peripheral blood and/or in urine by serum protein electrophoresis or immunofixation.
- MM is consistently preceded by a premalignant asymptomatic disease state, known as monoclonal gammopathy of unknown significance (MGUS). However, we have no way of detecting the MGUS patients who will progress to MM and who will remain stable. There is an emerging need to diagnose the patients who will progress to MM.
- With improved diagnostics and prognostics in the future, we will be able to better define patients with high-risk precursor disease (MGUS and smoldering MM [SMM]). In parallel with the development of novel therapies, these efforts will provide new opportunities to develop clinical trials focusing on early treatment for MGUS and SMM.

- The MM microenvironment in the bone marrow consists of cellular and matrix components bathed in a fluid milieu.
- Studies of microenvironmental markers, both in plasma/serum and in bone marrow aspirate/biopsy, on successive samples from the same patient with MGUS and at MM diagnosis will be key to discovering biomarkers predicting MGUS to MM transformation.
- High-throughput strategies, in addition to well-established techniques, such as ELISA, western blot, quantitative PCR and immunohistochemistry, will greatly aid in the development of biomarkers.

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Figure 1. Microenvironment in myelomagenesis

Note the complexity of the interplay between MM cells and the various microenvironmental cells such as bone marrow stromal cells, osteoblasts, osteoclasts, dendritic cells and endothelial cells. The network of intracellular pathways as a result of release of cytokines and growth factors influences the progression of MGUS to MM. BMSC: Bone marrow stromal cell; MGUS: Monoclonal gammopathy of unknown significance; MM: Multiple myeloma; MMP: Matrix metalloproteinase.

Adapted from [135].

Table 1

Study of the cellular component of the bone marrow microenvironment.

Category/cell type	Markers to study	Techniques to detect
<i>Hematopoietic cells</i>		
Hematopoietic stem cells	CD34 ⁺ , CD117 ⁺ , CD59 ⁺ , CD90 ⁺	IHC, FC
Erythrocytes	CD71 ⁺	IHC, FC
Megakaryocytes/platelets	GPIIIa, GPIX	IHC, FC
Lymphocytes	CD3 ⁺ , CD19 ⁺ , CD20 ⁺	IHC, FC
Natural killer cells	CD3 ⁺ , CD56 ⁺	IHC, FC
Natural killer T cells	V α 24 ⁺ V β 11 ⁺	IHC, FC
Monocytes/macrophages	CD11b, CD68	IHC, FC
Dendritic cells	CD1c	IHC, FC
<i>Nonhematopoietic cells</i>		
Endothelial cells	vWF, CD34 +	IHC, FC
Pericytes	CD146 (plus stromal markers)	IHC, FC
Stromal/reticular cells	CD90, CD106, ALP, collagen 1	IHC, FC
Fibroblasts	CXCL12	IHC, FC
Osteoblasts	BSP, OCN	IHC, WB
Osteoclasts	TRAP	IHC
Marrow adipocytes	PPAR- γ , adiponectin	WB, IHC

FC: Flow cytometry; IHC: Immunohistochemistry; vWF: von Willebrand factor; WB: Western blot.

Table 2

Biochemical serum markers to study bone turnover.

Category	Bone formation or resorption	Techniques to detect
Serum type I collagen N-terminal propeptide	Formation	Radioimmunoassay
Serum type I collagen C-terminal propeptide	Formation	ELISA
Serum total osteocalcin	Formation	Two-site ELISA recognizing both the intact and the N-terminal mid-fragment
Serum bone alkaline phosphatase	Formation	Immunoenzymatic assay
Serum or urine crosslinked C-telopeptide of type I collagen	Resorption	A two-site ELISA recognizing the β -isomerized sequence of type I collagen C-telopeptide
Serum or urine crosslinked N-telopeptide of type I collagen	Resorption	Competitive ELISA
Serum tartrate resistant acid phosphatase type 5b	Resorption	ELISA
Urine hydroxyproline	Resorption	ELISA
Urine pyridinolone	Resorption	ELISA
Urine deoxypyridinolone	Resorption	ELISA

Box 1

Diagnostic criteria for monoclonal gammopathy of unknown significance, smoldering (asymptomatic) myeloma and multiple myeloma.

MGUS

- Serum M-protein <30 g/l
- Clonal bone marrow plasmacytosis <10%
- No other B cell lymphoproliferative disorder

Smoldering (asymptomatic) myeloma

- Serum M-protein >30 g/l
- Clonal bone marrow plasmacytosis >10%
- No related organ and tissue impairment

Multiple myeloma

- M-protein in the serum or urine
- Clonal bone marrow plasmacytosis or plasmacytoma
- Related organ and tissue impairment [†]

[†] Related organ and tissue impairment: 1. Hypercalcemia: serum calcium >2.75 mmol/l; 2. Renal dysfunction: serum creatinine >173 mmol/l; 3. Anemia: hemoglobin 2 g/dl below lower limit of normal; 4. Lytic bone lesions (CT and MRI may be used to identify suspicious findings on plain films); 5. Symptomatic hyperviscosity; 6. Amyloidosis; 7. Recurrent bacterial infections (more than two episodes in 1 year).

MGUS: Monoclonal gammopathy of unknown significance.

Adapted from [134].

Box 2

Bone marrow microenvironment consists of the cellular component, the extracellular matrix component, and the soluble component.

<i>Cellular component</i>
Hematopoietic cells <ul style="list-style-type: none"> • Hematopoietic stem cells • Hematopoietic progenitors • Erythrocytes • Megakaryocytes/platelets • Lymphocytes • Monocytes/macrophages • Dendritic cells • Endothelial cells
Nonhematopoietic cells <ul style="list-style-type: none"> • Vascular cells • Pericytes • Stromal/reticular cells • Fibroblasts • Osteoblasts • Osteoclasts • Marrow adipocytes
<i>Extracellular matrix component[†]</i>
Fibrous proteins <ul style="list-style-type: none"> • Collagen • Laminin • Fibronectin • Elastin
Proteoglycans <ul style="list-style-type: none"> • Heparan sulfate proteoglycans • Small leucine-rich repeat proteoglycans (such as decorin, biglycan, fibromodulin, lumican)
Glycosaminoglycans <ul style="list-style-type: none"> • Hyaluronan
SIBLING proteins <ul style="list-style-type: none"> • Such as osteopontin, bone sialoprotein and dentin matrix protein-1
Adhesion molecules <ul style="list-style-type: none"> • VLA-4 • VLA-5 • LFA-1

- VCAM-1
- ICAM-1
- Syndecan-1 (CD138)

Soluble component[†]

Cytokines

- IL-1 β
- IL-3
- IL-6
- IL-10
- IL-15
- IL-21
- TNF- α
- SDF1
- TGF- β
- bFGF
- SCF
- Ang-1
- BAFF
- RANKL
- PTHrP
- SDF-1
- MIP-1 α

Growth factors

- IGF-1
- VEGF
- TGF- β
- bFGF
- HGF
- Ang-1

Other factors

- MMPs
- TIMPs
- Calcium

Under each subcategory we have provided well-characterized cells, proteins and factors in the literature. The list is not always complete and the nomenclature is constantly under development.

[†] All of the above listed extracellular matrix and soluble components can be detected by western blot, ELISA, gene expression and quantitative PCR. In addition, some matrix metalloproteinases can be detected by zymogen assays.

MMP: Matrix metalloproteinase; SIBLING: Small integrin-binding ligand N-linked glycoprotein; TIMP: Tissue inhibitors of metalloproteinase.