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Multiple Myeloma: A Prototypic Disease Model for the Characterization and Therapeutic Targeting of Interactions Between Tumor Cells and Their Local Microenvironment

Constantine S. Mitsiades,* Nicholas S. Mitsiades, Paul G. Richardson, Nikhil C. Munshi, and Kenneth C. Anderson**

Jerome Lipper Multiple Myeloma Center, Department of Medical Oncology, Dana-Farber Cancer Institute and Department of Medicine, Harvard Medical School, Boston, MA

Abstract The interaction between tumor cells and the local milieu where are homing has recently become the focus of extensive research in a broad range of malignancies. Among them, multiple myeloma (MM) is now recognized as a prototypical tumor model for the characterization of these interactions. This is due not only to the propensity of MM cells to target the skeleton and form lytic bone lesions, but because interactions of MM cells with normal cells of the bone milieu can attenuate the anti-tumor activity of conventional therapies, such as glucocorticoids and standard cytotoxic agents, including alkylators. Herein, we highlight the recent advances in our understanding of cellular and molecular mechanisms of interactions between MM cells and their milieu. Particular emphasis is placed on the interface between MM cells and normal cell compartments of the BM, especially bone marrow stromal cells (BMSCs), and on the development of a series of new classes of therapeutic agents, including the proteasome inhibitor bortezomib, thalidomide and lenalidomide, which counteract specific aspects of those MM–BM interactions. The significant clinical activity of these novel therapies has not only led to a new era in the therapeutic management of this disease, but also underscored the importance of comprehensively characterizing the role of the local microenvironment in the pathophysiology of human neoplasias. J. Cell. Biochem. 101: 950–968, 2007.

Key words: multiple myeloma; bone microenvironment; cytokines

Multiple myeloma (MM) is the second most commonly diagnosed hematologic neoplasia in the Western World [Bataille and Harousseau, 1997; Jemal et al., 2004]. It is characterized by the accumulation in the bone marrow of a population of malignant plasma cells, which typically secrete a monoclonal immunoglobulin (M-protein) and can cause anemia due to suppression of normal erythropoiesis; lytic bone lesions and hypercalcemia due to excessive bone resorption and suppression of new bone formation; renal insufficiency due to toxic effects of light chains of the monoclonal myeloma immunoglobulin; and a multifactorial increase in risk of infections, for example, due to decrease in levels of uninvolved immunoglobulins and suppressed function of several components of normal cellular immunity. Typically MM is responsive to conventional chemotherapy followed by myeloablative doses of alkylating agents and autologous stem cell transplantation [Bataille and Harousseau, 1997]. However, cytotoxic chemotherapy-based treatment of MM is not curative, since disease recurrence eventually occurs [Mitsiades et al., 2004a]. The need to develop novel therapies for MM provided an incentive for the rapid bench-to-bedside translation of a series of new classes

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*Correspondence to: Constantine S. Mitsiades, Department of Medical Oncology, Dana Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.
E-mail: constantine_mitsiades@dfci.harvard.edu

**Correspondence to: Kenneth C. Anderson, Department of Medicine, Harvard Medical School, 44 Binney Street, Boston, MA 02115.
E-mail: kenneth_anderson@dfci.harvard.edu

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of agents for the treatment of this disease, including thalidomide, its immunomodulatory derivatives, and proteasome inhibitors. An interesting feature of these classes of drugs is that they not only possess direct anti-tumor activity, but also have the capacity to overcome protective effects that bone marrow stromal cells (BMSCs) confer to MM cells against glucocorticoids and cytotoxic chemotherapeutics [Hideshima et al., 2004, 2005; Mitsiades et al., 2004a]. While there are several potential explanations as to why these aforementioned new classes of anti-MM agents can be active even in steroid- and chemo-resistant/refractory cases, the fact that these agents overcome protective effects of the BM microenvironment on MM cells against conventional therapies has suggested that the interplay between MM cells and their local milieu has direct implications for the design of novel therapeutics that can overcome resistance to conventional agents and improve patient outcome.

The close interaction between neoplastic cells and the microenvironment(s) where they are located is not a feature limited only to MM. On the contrary, data in both hematologic malignancies and solid tumors [Mitsiades and Koutsilieris, 2001; van Kempen et al., 2003; Munk Pedersen and Reed, 2004; Zhou et al., 2005] indicate that proliferation, survival and drug resistance of tumor cells can be influenced by their local surroundings. The interplay between tumor cells and their milieu, at the primary site, local recurrence and distant metastases of solid tumors, as well as at sites of diffuse lesions in hematologic neoplasias, may have variations between different tumor types, but probably reflect an underlying biological principle which is relevant to many, if not most, human neoplasias. As early as 1889, the "seed and soil" hypothesis proposed by Stephen Paget postulated that the development of distant metastases is determined by the nature of the interaction between selected tumor cells (the "seed") and the specific organ microenvironments (the "soil") where these cells are deposited [Paget, 1889]: if the "soil" of a particular organ provides a fertile ground for the growth of tumor cells, they will form metastatic sites that can eventually lead to clinical symptoms and macroscopic detection. In contrast, if the "seed" does not have the capacity to grow optimally in the "soil" of a particular organ, tumor lesions at that site will develop slowly or not develop at all. Since the original inception of this hypothesis, extensive pre-clinical and clinical data have supported this notion. Indeed, it is now well-recognized that specific tumor types have a propensity to develop lesions in specific organs: bone is the target of lesions for multiple myeloma [Bataille and Harousseau, 1997]; prostate cancer [Smart, 1964]; and breast cancer [Clain, 1965]; liver is the target for metastases of solid tumors such as breast cancer [Nemoto and Dao, 1966] and various neoplasms of the gastrointestinal tract (e.g., colon carcinoma) [Heal and Schein, 1977]; and the brain is a frequent target of metastases of lung carcinomas [Galluzzi and Payne, 1956].

Tumor-microenvironmental interactions are therefore important for the pathophysiology of many, if not all, neoplasias, not just MM. However, at the moment MM is viewed as a prototypical disease model for characterization of these interactions [Mitsiades et al., 2004a]. This is due in part to the clinical benefit provided in MM by novel therapies which target MM cell–BM microenvironment interactions. The predilection of MM cells for lytic bone lesions underscored a biologically important link between the behavior of tumor lesions and their location. Furthermore, the paucity, up until a few years ago, of therapeutic options for MM, provided a clear impetus to develop new agents, with emphasis on those which not only target tumor cells directly but also perturb their interaction with the tumor microenvironment.

In this current article, we review recent advances in the study of tumor-microenvironment interactions in MM, as a key model for the pathophysiologic characterization and therapeutic targeting of these events. In particular, we review how normal cellular compartments of the BM, including BMSCs, osteoblasts, osteoclasts and endothelial cells, promote MM cell resistance to conventional anti-MM treatment, through cytokine- and cell adhesion-mediated mechanisms. We also describe how ongoing research into these events has identified several new molecular targets and corresponding strategies for MM treatment.

BONE MARROW STROMAL CELLS AND THEIR INTERACTION WITH MM CELLS

The interaction of MM cells with BMSCs is considered a critical component of the overall network of biological relationships established

between the malignant cells and their BM milieu in this disease. BMSCs are typically identified in the MM literature in a descriptive manner, as a heterogeneous compartment of mesenchymal cells with morphological features reminiscent of fibroblasts (as reviewed in Mitsiades et al. [2004a]). BMSCs support normal hematopoiesis in vitro [Werts et al., 1980; Greenberg et al., 1981; Kaneko et al., 1982; Reincke et al., 1982]. So the support that BMSCs provide to MM cells in terms of their proliferation, survival and drug resistance may represent an abnormal and pathophysiologically unfavorable reprise of their intrinsic ability for providing support of normal hematopoiesis. While normal hematopoietic progenitor cells residing in the marrow utilize their adhesion to BMSC and the cytokines produced by them to differentiate into mature blood cells, MM cells capitalize on these same stimuli, not for the purpose of promoting cell differentiation, but to enhance their proliferation and resistance the effects of various pro-apoptotic stimuli [Mitsiades et al., 2004a]. For instance, adhesion of MM cells to BMSCs via adhesion molecules such as VLA-4 and ICAM-1 [Uchiyama et al., 1992, 1993] enhances MM cell proliferation and viability through several complementary, mechanisms, which include cell adhesion-mediated stimulation of intracellular signaling pathways in MM cells and increased paracrine (BMSC-derived) and/or autocrine (MM cell-derived) release of cytokines/growth factors in the BM milieu [Uchiyama et al., 1992, 1993; Mitsiades et al., 2004a,b]. During in vitro interactions of MM cells with BMSCs, the adhesion-stimulated anti-apoptotic signaling precedes cytokine secretion, but ultimately the effects of cell adhesion act in concert with cytokine-triggered signaling [Nefedova et al., 2003]. It remains unclear whether BMSCs comprise of subpopulations with different functional roles in terms of their capacity to support MM cell proliferation and resistance to cell death. Moreover, the hypothesis that BMSCs cooperate with other normal cellular compartments of the BM in stimulating MM cell proliferation and drug resistance appears plausible, but has not been formally examined. A more comprehensive characterization of the biological and phenotypic features of BMSCs is required both to delineate their role in the pathophysiology of MM and to validate potential new therapeutic targets.

THE ROLE OF CYTOKINES/MITOGENS AND THEIR RECEPTORS IN THE INTERACTIONS BETWEEN TUMOR AND STROMA IN MM LESIONS

The chromosomal amplifications, deletions, and rearrangements, as well as mutations of individual genes in tumor cells are a major determinant of their biological behavior. However, the genetics of MM cells are not the sole factor affecting its pathophysiology. In fact, MM is influenced by a intricate nexus of interactions between MM cells and their local bone microenvironment: MM cells disturb the process of normal bone remodeling, and contribute to development of lytic bone [Roodman, 2002; Ashcroft et al., 2003], while the BM microenvironment, in turn, provides MM cells with an adhesive platform with access to vascular networks and a diverse array of locally produced cytokines and growth factors, all of which contribute to increased resistance of MM tumor cells against pro-apoptotic stimuli [Mitsiades et al., 2004b], such as conventional therapies, including steroids, DNA-damaging agents and irradiation.

The normal skeleton undergoes continuous remodeling, in which osteoclast-mediated resorption of old bone is followed by activation of osteoblasts for local deposition of new bone tissue [Tanaka et al., 2005]. This process is critical for normal bone physiology because it allows for local skeletal architecture to respond to changes in applied pressures to bone and thus improve its weight-bearing capacity. Under normal conditions, bone remodeling creates no net change in bone mass, because the activity of osteoblasts in depositing new bone is functionally linked with the activity of osteoclasts, in order to match the quantity of resorbed bone. However, in the MM bone milieu, bone resorption by osteoclasts and new bone formation by osteoblasts are uncoupled [Bataille et al., 1989; Taube et al., 1992]. Specifically, there is increase in osteoclast activity which is not matched by osteoblast-mediated bone deposition. This mismatch is due to activation of several pathways which trigger formation and function of mature osteoclasts; as well as suppression of several negative regulators of bone resorption and/or positive regulators of bone formation [Ashcroft et al., 2003]. For instance, it has been proposed that MM cells can stimulate RANKL expression in BMSCs

[Roux et al., 2002]; suppress osteoprotegerin (OPG), an endogenous antagonist of RANKL activity [Lacey et al., 1998]; and stimulate production of multiple pro-osteoclastogenic cytokines in the BM milieu. This large constellation of osteoclastogenic stimuli includes interleukin-6 (IL-6), IL-1α, IL-1β, and IL-11; chemokines such as MIP-1α; TNF superfamily members such as TNF-α, TNF-β (lymphotoxin-α); and other soluble mediators, including M-CSF, PTHrP, or VEGF [Mundy, 1989; Nakamura et al., 1989; Kawano et al., 1989b; Bataille et al., 1992; Uchiyama et al., 1993; Niida et al., 1999; Choi et al., 2000; Nakagawa et al., 2000; Callander and Roodman, 2001; Han et al., 2001; Ashcroft et al., 2003].

These cytokines/growth factors directly or indirectly stimulate osteoclast maturation and increased resorptive activity. They are produced by MM cells themselves and/or by normal cells of the BM milieu (including BMSCs) as a consequence of paracrine/cell adhesion-mediated stimulation by MM cells. It has also been reported that, in a subset of MM patients with extensive bone lesions, MM cells express increased levels of transcript for DKK-1 (Dickkopf-1) [Tian et al., 2003], an inhibitor of Wnt signaling, which blocks differentiation of osteoblast precursors to mature osteoblasts. A sizeable sub-population of MM patients is reported to have increased levels of DKK-1 protein in their serum (and presumably in the BM microenvironment), suggesting that this cascade may cause uncoupling of bone formation from excessive resorption in MM [Tian et al., 2003]. However, further research will be needed to delineate which other proteins can complement or substitute for the activity of DKK-1 and so account for the increased bone resorption in those MM patients without significant increases in DKK-1 levels.

The functional decoupling of bone formation from resorption has a dual role in the pathophysiology of MM. On the one hand, the decrease in bone density at the sites of MM involvement in the skeleton compromises the stability and weight-bearing capacity of the bone and can lead to spontaneous fractures and/or hypercalcemia (due to increased release of calcium from resorbed bone), which are clinical characteristics of the disease. On the other hand, the process of bone resorption results in more than mechanical consequences and is also associated with release at the site of osteoclast activity of increased levels of cytokines such as IL-6. The bone milieu thus becomes a favorable niche for proliferation, survival and drug resistance of MM cells, but also several other types of malignant cells in general. This may account at least in part for the tropism that many neoplasias exhibit for bone, including epithelial tumors such as breast or prostate cancer [Koutsilieris et al., 2000]. Several of the cytokines/growth factors which are produced within the context of the process of bone remodeling (including IL-6 and IGFs), also function as mitogens and survival factors for MM cells [Mitsiades et al., 2004a]. Therefore the BM milieu becomes a fertile ground for homing, survival and proliferation of MM cells, which in turn trigger more bone resorption because, thereby creating a vicious circle where bone resorption and tumor growth stimulate each other.

The notion that non-malignant cellular compartments in the local milieu of tumors can render malignant cells less sensitive to anticancer treatments is not restricted to MM. Extensive data suggest similar mechanisms of microenvironmentally determined drug resistance are also pertinent to other neoplasias (as reported in [Sethi et al., 1999; Taylor et al., 1999, 2000; Mudry et al., 2000; Song et al., 2000; Sausville, 2001; Sherman-Baust et al., 2003; Mougel et al., 2004; Hazlehurst et al., 2006]. Drug resistance conferred by the local microenvironment does not exclude other potential mechanisms, which have been proposed to account for de novo, or acquired drug-resistance of tumors [Huff et al., 2006; Matsui et al., 2004]. For instance, while more research may be necessary to clarify the role of the proposed "MM stem cell," data from other fields, such as the study of normal hematopoietic stem cells [Lemischka, 1997], indicate that stem cells, whether malignant or non-malignant, do not function independently of their local microenvironment. In fact, they do respond to its cues and significantly depend on the microenvironment for their biological behavior [Bissell and Labarge, 2005]. In addition, the local microenvironment of tumors can potentiate the intrinsic genetically driven potential of MM cells for drug resistance. Indeed, there are several mechanisms whereby the microenvironment and the genetic features of MM cells cooperate. For example, certain cytogenetic abnormalities can influence the pattern of

expression of adhesion molecules in MM cells, thus further influencing the MM-stromal interaction (e.g., as shown by the effect of c-maf overexpression on integrin expression in MM cells) [Hurt et al., 2004].

SIGNALING PATHWAYS STIMULATED IN MM CELLS DURING THEIR INTERACTION WITH THEIR LOCAL MICROENVIRONMENT

In the local microenvironment of bone, MM cells adhere to extracellular matrix (ECM) proteins, BMSCs and other cells of the BM milieu (including osteoblasts, endothelial cells, and hematopoietic cells). This direct contact [Damiano et al., 1999; Shain et al., 2002; Landowski et al., 2003], as well as the resulting stimulation of autocrine/paracrine production of cytokines [Uchiyama et al., 1993; Chauhan et al., 1996; Mitsiades et al., 2004b], triggers proliferative/anti-apoptotic signaling cascades in MM cells, including PI-3K/Akt/mTOR/p70S6K [Hideshima et al., 2001b; Mitsiades et al., 2004b]; IKK-α/NF-κB [Hideshima et al., 2002; Mitsiades et al., 2004b]; Ras/Raf/MAPK [Mitsiades et al., 2004b]; and JAK/STAT3 [Berger et al., 1994; Ogata et al., 1997; De Vos et al., 2000] signal transduction pathways. These cascades can be activated by binding of various cytokines and growth factors to their respective receptors and/or by direct initiation of these cascades as a result of binding of cell adhesion molecules on the surface of MM cells with other cell adhesion molecules on the surface of accessory cells of the BM milieu or with molecules of the ECM. For example, MM cells can respond to various soluble mediators present in their local microenvironment, including IL-6, insulin-like growth factors (IGFs), interleukin-1 (IL-1), interleukin-21 (IL-21), stromal-derived factor-1 (SDF-1) and hepatocyte growth factor (HGF), and respond to these factors via signaling pathways stimulated by their cognate receptors (IL-6R, IGF-1R, IL-1R, IL-21R, CXCR-4, c-met, etc) expressed on the surface of MM cells (Fig. 1). In addition, proliferative/anti-apoptotic signaling can also be directly stimulated by cell adhesion molecules, which can in turn activate kinases such as integrin-linked kinase (ILK) and focal adhesion kinase

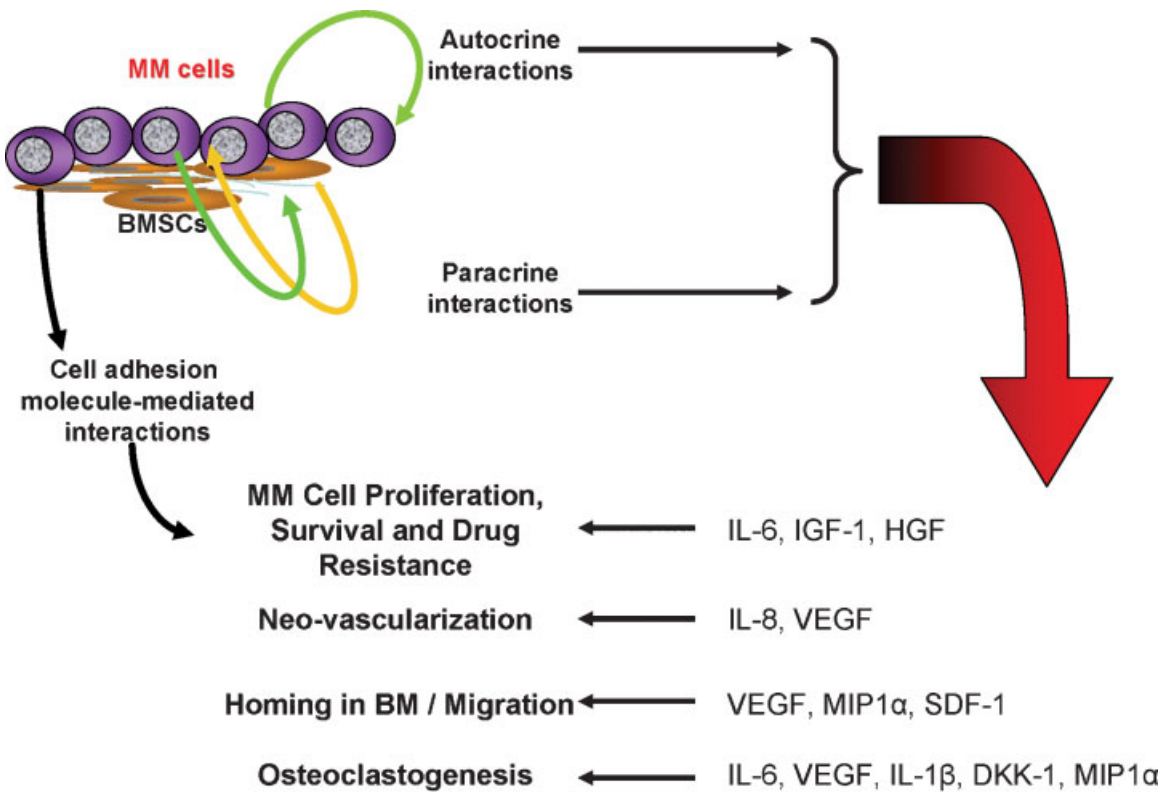

Fig. 1. Schematic representation of the type of autocrine, paracrine and cell-adhesion mediated interactions triggered in the bone milieu when MM cells interact with BMSCs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(FAK). Interestingly, these different stimuli (growth factors vs. adhesion-molecules) lead to similar functional consequences, because they converge to similar downstream cascades, including the PI-3K/Akt pathway, the mTOR/p70S6K axis, the Ras/Raf/MAPK cascade and the IKK-α/NF-κB pathway. For instance, activation of PI-3K/Akt leads to phosphorylation and cytoplasmic sequestration of pro-apoptotic members of the Forkhead family of transcription factors [Mitsiades et al., 2004b]; increased levels of D-type cyclins [Mitsiades et al., 2004b]; increased expression of caspase inhibitors, including FLIP or cIAP-2, as well as upregulation of anti-apoptotic members of the Bcl-2 family, such as A1/Bfl-1 [Mitsiades et al., 2004b]. In addition, cytokine/adhesion-triggered cascades can further stimulate the activity of telomerase (thus enhancing the replicative capacity of tumor cells) or the chymotryptic-like activity of 20S proteasome (which regulates the degradation of negative cell cycle regulators and of pro-apoptotic molecules) and stimulate the potential of tumor cells to recruit new blood vessels (e.g., HIF-1α) [Hideshima et al., 2001b; Mitsiades et al., 2002a, 2004b].

The large constellation of mitogens/survival factors released in the bone milieu in MM includes IL-6, IGFs, HGF, SDF-1 vascular endothelial growth factor (VEGF), IL-1α, IL-1β, TNF-α, and various members of the Notch family [Kawano et al., 1988, 1989a; Chauhan et al., 1996; Mitsiades et al., 2002a, 2004b; Hideshima et al., 2004; Nefedova et al., 2004]. These cytokines/growth factors can stimulate multiple signaling pathways, which often overlap. However, their biologic sequelae can be distinct because of, among other reasons, differential targeting of accessory cells in the tumor microenvironment. For example, IL-6, is not only a potent mitogen for MM cells, but also a stimulator of bone resorption, a feature shared as well by VEGF, and IL-1 [Kawano et al., 1989b; Derenne et al., 1999; Niida et al., 1999; Nakagawa et al., 2000; Callander and Roodman, 2001; Roodman, 2002]. TNF-α has been reported to directly stimulate MM cell proliferation and survival [Jourdan et al., 1999], but can also modify, in an NF-κB-dependent manner, the expression of cell adhesion molecules on the surface of MM cells, as well as BMSCs [Hideshima et al., 2001a], thereby enhancing the MM-BMSC adhesion and related production of cytokines [Hideshima et al., 2001a].

The chemokine SDF-1/CXCL12 promotes MM cell homing to the BM milieu [Hideshima et al., 2004]. Even though SDF-1 and its receptor CXCR4 have only modest, if any, direct effect on MM cell proliferation/survival [Menu et al., 2006b], they play important in directing MM cells towards the BM niche, which is more conducive to increased viability and expansion of the tumor cell population. Clinical trials have shown that the CXCR4 inhibitor AMD3100 in MM patents increases the number of mobilized CD34+ cells, thereby facilitating prompt and durable engraftment of mobilized cells, without mobilizing tumor cells from the BM [Flomenberg et al., 2005]. Although this latter feature is a favorable one in terms of potential uses of CXCR4 inhibitors to improve mobilization of normal hematopoietic stem cells for the purposes of autologous stem cell transplantation in MM, it also suggests that CXCR4 inhibition per se may not be sufficient to expel the MM cells from the BM microenvironment, for reasons that remain to be determined. One plausible hypothesis is that CXCR4 function in MM cells may be important for the establishment of their homing to the BM, but not thereafter. If this hypothesis is proven, inhibition of other chemokine-induced signaling cascades may be important for mobilizing MM cells out of the BM milieu.

IN VIVO MODELS FOR CHARACTERIZATION OF TUMOR-STROMAL INTERACTIONS IN MM AND IDENTIFICATION OF THERAPEUTIC STRATEGIES TO TARGET THEM

Tumor-microenvironmental interactions in MM involve complex networks of interactions between multiple cell types (MM cells, BMSCs, osteoblasts, osteoclasts, endothelial cells, etc), which communicate with each other through diverse cell adhesion molecules, as well as multiple cytokines and growth/survival factors. The biological outcome of these interactions is unlikely to be determined by how MM cells interact with any single cell type from the BM milieu or how they respond to any individual cytokine. Instead, it is conceivable that the behavior of MM cells is influenced by the composite effect of all these interactions simultaneously. This suggests that it may be difficult to appreciate the full effects of these interac-

tions in classical in vitro models, where reductionist approaches (e.g., interaction of MM cells with only one other cell compartment, such as BMSCs, or stimulation with individual cytokines) are typically applied. Therefore, the study of MM tumor-microenvironment interactions in pathophysiologically relevant in vivo models is imperative.

For most neoplasias, particularly solid tumors, the development and application of animal models focus primarily on subcutaneous xenografts of human tumor cell lines or on models where tumors spontaneously develop in an orthotopic manner. In contrast, substantial effort has been made, in the MM field, to generate in vivo models where malignant cells interact with the bone microenvironment. Several groups have attempted to establish transgenic models of MM [Cheung et al., 2004; Linden et al., 2004, 2005; Carrasco et al., 2005] and the recent progress achieved in that direction will hopefully allow applications of these models in an effort to better characterize not only the genetics of MM, but also how MM cells interact with their microenvironment.

So far, however, most of the research efforts studying the MM-microenvironment interactions in vivo have involved models where mouse MM cells have been injected into syngeneic immunocompetent mice, such as the 5T series (e.g., 5T2MM, 5T33MM) of mouse models, where MM cells which spontaneously develop in the ageing C57BL/KaLwRij mice can be reinjected in other mice of the same strain and recapitulated several biological features of MM, including the formation of bone lesions [Radl et al., 1990; Asosingh et al., 2001a, 2001b, 2002, 2003; Menu et al., 2002; Van Valckenborgh et al., 2002; Vanderkerken et al., 2002; Asosingh, 2003] or models where human MM cells are xenografted in immunocompromised mice [Urashima et al., 1997a; Yaccoby et al., 1998; Mitsiades et al., 2001; LeBlanc et al., 2002; Lentzsch et al., 2002; Catley et al., 2003; Sordillo and Pearse, 2003; Rousselot et al., 2004; Tassone et al., 2004a,b; Mitsiades et al., 2004b; Shi et al., 2005; Trudel et al., 2005].

Key advantages of the 5T models include their immunocompetent state, as well as the fact that the interaction of mouse MM cells with a syngeneic bone microenvironment allows for full compatibility between the cell adhesion molecules and cytokines derived of the tumor versus host compartment. In contrast, in mouse models where human MM cells (either primary tumor cells or cell lines) are xenografted in immunodeficient mice, it is theoretically possible that some cytokines or adhesion molecules from one of the two xenogeneic compartments may not necessarily interact in a fully compatible fashion with their cognate receptors or partner adhesion molecules in the other compartment. This concern can be addressed in part by the development of in vivo models in which human MM cells are injected directly into human bone grafts (SCID-hu model) xenografted into inmmunodeficient rodent recipients [Urashima et al., 1997a; Yaccoby et al., 1998; Pearse et al., 2001; Sordillo and Pearse, 2003; Tassone et al., 2004a]. In those models, the interaction of human MM cells with a human bone microenvironment can bypass the concerns about potential species-specificities of growth factor–receptor interactions and provide valuable insight into not only how human MM cells influence their milieu (e.g., in terms of bone resorption) [Pearse et al., 2001; Yaccoby et al., 2002; Sordillo and Pearse, 2003], but also assess the ability of some of novel therapies to overcome the protective effect of the local bone microenvironment on MM cells [Tassone et al., 2005; Hideshima et al., 2006].

THE RELATIVE IMPORTANCE OF IL-6 VERSUS OTHER CYTOKINES IN THE PATHOPHYSIOLOGY OF MM

Historically, IL-6 has been viewed as a major, if not the major, cytokine for proliferation and survival of MM cells. IL-6 stimulates, via its gp130 receptor IL-6R, the activation of PI-3K/Akt, MAPK and JAK/STAT3 signaling [Berger et al., 1994; Ogata et al., 1997; Chauhan et al., 1997a; Hideshima et al., 2000a, 2001b]. The sequelae triggered by the activation of these pathways allow MM cells to proliferate and resist the induction of apoptosis by dexamethasone and potentially other conventional therapeutics [Juge-Morineau et al., 1995; Chauhan et al., 1997a, 1997b, 1999, 2000; Urashima et al., 1997b]. In contrast, IL-6 is known to promote the differentiation of normal B-lineage cells to normal plasma cells [Sehgal et al., 1987]. IL-6 is also a potent stimulator of osteoclastogenesis [Lowik et al., 1989], linking the expansion of MM cell population with bone resorption, further supporting the notion of IL-6 signaling

as a prime therapeutic target for MM. However, only a subset of MM cell lines responds to IL-6 stimulation in vitro, and even fewer of these cells lines depend on exogenous IL-6 stimulation for their sustained survival in culture [Mitsiades et al., 2004b; Mitsiades (unpublished observation)]. Moreover, Moreover, among MM cell lines which respond to in vitro stimulation with IL-6, most require IL-6 levels considerably higher than those detected in peripheral blood samples of patients with MM [Mitsiades et al., 2004b]. Anti-IL-6 neutralizing antibodies (Abs) significantly suppressed, in clinical trials of MM patients, the circulating levels of C-reactive protein (CRP), a surrogate marker for IL-6 bioactivity [Bataille et al., 1995]. However, this decrease in CRP levels was not associated with clinical responses of the magnitude that was originally anticipated on the basis of the preclinical data on the importance of IL-6 for MM cells [Klein et al., 1991]. Several hypotheses were proposed to explain these clinical results, for example, that MM cells may have produced IL-6 levels too high to neutralize [Lu et al., 1995]; that administration of a single anti-IL-6 Ab may not be sufficient, due to pharmacokinetic reasons, to effectively clear IL-6 [Montero-Julian et al., 1995]; or that patients enrolled in those trials had very advanced MM, which is independent of IL-6 stimulation. Indeed, MM cell lines are generally derived from samples of patients with plasma cell leukemia and/or extramedullary plasmacytomas. In these aggressive late-stage forms of MM, the malignant plasma cells can be independent of cytokines or other cues of the BM microenvironment [Kuehl and Bergsagel, 2002]. On the other hand, in the early stages of the disease, for example newly diagnosed patients, MM cells may be more responsive to IL-6 inhibition, since their tumor cells remain responsive to, or even dependent upon, IL-6 stimulation [Kuehl and Bergsagel, 2002]. It should be noted, however, that MM cells purified from BM aspirates of newly diagnosed patients can be cultured in vitro for only brief durations of time, even when exposed to high concentrations of IL-6 and/to co-cultures with BMSCs, which secrete high levels of IL-6 (Mitsiades CS, unpublished results). This suggests that IL-6 alone may not be sufficient to stimulate long-term proliferation and survival of early stage MM cells and that the results of the first clinical trials of anti-IL-6 Abs may not be attributed exclusively to disease stage issues, but also to the fact that other signaling cascades may need to be inhibited concomitantly with the IL-6 pathway to produce major suppression of MM burden.

Another important consideration in respect to the potential role of IL-6 inhibitory strategies in MM treatment is the emergence of new classes of drugs for this disease. These are first tested in advanced disease (e.g., relapsed/refractory MM) and then moved to the newly diagnosed setting. Indeed, bortezomib, thalidomide, and lenalidomide are clinically active in the treatment of not only MM patients with relapsed/refractory disease [Singhal et al., 1999; Richardson et al., 2002, 2003, 2005], but also in the newly diagnosed setting [Rajkumar et al., 2002, 2005, 2006; Weber et al., 2003]. The recent development of these novel classes of drugs has changed for the landscape of how anti-IL-6 targeted therapies can be developed clinically: on the one hand, early stage MM, the clinical setting where anti-IL-6 strategies would be expected to have a higher probability of achieving clinical responses, is increasingly being treated with potent novel classes of drugs (thalidomide and derivatives and proteasome inhibitors), which are already known to inhibit, at least in part, the IL-6 production in the bone milieu and/or the signaling through IL-6R in MM cells [Hideshima et al., 2003]. One the other hand, clinical settings where resistance to these novel therapies has developed are too advanced for MM cells to remain IL-6-responsive and the probability of clinical responsiveness to anti-IL-6 strategies is decreased. It therefore remains to be seen what optimizations in IL-6 inhibition strategies may be needed in order for this class of agents to offer additional clinical benefit to MM patients treated with existing therapies.

THE ROLE OF IGF/IGF-1R SIGNALING IN REGULATING MM CELL BEHAVIOR: THERAPEUTIC IMPLICATIONS

These considerations have fueled further interest in the search for other cytokines/growth factors which can enhance the effects of IL-6 in IL-6-responsive MM cells and/or drive proliferation and survival of IL-6-independent MM cells in advanced disease. One pathway which fulfils these features is the signaling cascade of IGFs and their receptor IGF-1R

(CD221) [Mitsiades et al., 2004b]. This pathway has been the focus of extensive, yet sporadic, research studies in diverse neoplasias which indicated that IGFs and IGF-1R are involved in the pathophysiology of a broad spectrum of solid tumors. For example, in prospective epidemiological studies, individuals with increased levels of circulating IGF-1 are reported to have a higher risk for diverse forms of epithelial malignancies [Chan et al., 1998; Hankinson et al., 1998; LeRoith and Roberts, 2003]. Several pre-clinical studies have documented that IGF-1R expression is necessary for normal cells to undergo malignant transformation by a series of oncogenes [Porcu et al., 1992; Coppola et al., 1994; Sell et al., 1994]. In addition, extensive studies (reviewed in Mitsiades and Mitsiades [2005]), including studies with human MM cell lines, have reported that IGF-1 can stimulate increased in vitro proliferation of neoplastic cell lines, including MM. Nonetheless, this cascade was for several years not deemed to constitute an attractive target for anti-cancer drug development, in part because of concerns that inhibition of this pathway might prove too toxic. For example, the high degree of sequence homology of IGF-1R to the insulin receptor (IR) [Adams et al., 2000] originally led to the notion that selective small molecule inhibitors of IGF-1R kinase domain could not be developed for therapeutic applications. Furthermore, IGF-1R is widely expression in a broad spectrum of normal cells [Adams et al., 2000], at levels often similar to those expressed in their neoplastic counterparts. This suggested that even those strategies which can selectively target IGF-1R and spare IR, for instance monoclonal antibodies (mAbs) specific for IGF-1R [Kull et al., 1983; Poretsky et al., 1985; Chaiken et al., 1986; Flier et al., 1986], could theoretically lead to catastrophic toxicities because of blockade of IGF-1R function in a broad range of normal tissues. Such specific anti-human IGF-1R neutralizing mAbs had shown variable degrees of in vivo anti-tumor activity against various types of human solid tumors xenografted in rodents [Arteaga and Osborne, 1989; Arteaga et al., 1989]. However, these favorable results did not abate the concerns about potential toxicities of IGF-1R inhibition in humans, since anti-human IGF-1R mouse mAbs do not typically cross-react with endogenous IGF-1R in normal rodent cells. Therefore, these in vivo models conceivably provided only limited insight on possible toxicities of anti-IGF-1R mAbs in humans, and could have overestimated the maximum tolerated dose that might be achievable in human patients, particularly when the target of the mAb is so broadly expressed in normal tissues [Mitsiades and Mitsiades, 2005].

These obstacles for clinical translation of anti-IGF-1R therapeutic strategies were overcome by studies focusing initially on MM cells, as a tumor model responsive to anti-IGF-1R blockade. The generation of small-molecule selective inhibitors of the IGF-1R kinase domain, for example, the aminopyrrolopyrimidine class of IGF-1R inhibitors [Garcia-Echeverria et al., 2004; Mitsiades et al., 2004b] was also a very important step towards that direction, since these compounds can inhibit both human and rodent IGF-1R (Garcia-Echeverria C., personal communication). The safety and efficacy of these IGF-1R kinase inhibitors were examined in the SCID/NOD model of diffuse bone lesions of MM. In that model, IGF-1R kinase inhibitors (e.g., NVP-ADW742) inhibit IGF-1R function in both xenografted human MM cells and in normal mouse cells, thereby allowing for informative assessment of any potential side effects. Importantly, in this model MM tumor cells develop bone lesions, therefore allowing for evaluation of the ability of the inhibitors to overcome protective effects of the bone microenvironment on MM cells [Mitsiades et al., 2004b].

Despite the highly homologous sequences of IGF-1R and IR, particularly in their kinase domains, pyrrolo-[2,3-d]-pyrimidine compounds (including NVP-ADW742 and NVP-AEW541) [Garcia-Echeverria et al., 2004] selectively target IGF-1R [Garcia-Echeverria et al., 2004; Mitsiades et al., 2004b]. These compounds are also orally bioavailable; do not present major side effects (for instance no significant hyperglycemia was observed in the studies of these inhibitors); and, importantly, have anti-tumor activity in animal models where the diffusely distributed MM bone lesions simulate the multifocal nature of anatomic localization of these lesions in patients with MM [Mitsiades et al., 2004b]. This anti-MM activity included decreased MM tumor burden, as assessed by whole-body bioluminescence imaging, as well as prolongation of overall survival in MM-bearing mice treated with IGF-1R inhibitor. Furthermore, in vivo anti-MM activity was

achieved with parenteral as well as with oral administration of these compounds [Mitsiades et al., 2004b]. These observations confirmed that small-molecule kinase inhibitors with sufficient degree of selectivity against IGF-1R versus IR can lead to in vivo anti-tumor responses with an acceptable profile of side effects [Garcia-Echeverria et al., 2004; Mitsiades et al., 2004b]. Importantly, these results provided further validation for IGF-1R inhibition in general, providing the framework for clinical applications of other approaches to selectively target this pathway, for example, for mAbs against IGF-1R.

Although IGF-1R inhibitors trigger dose-dependent inhibition of viability in cell lines from a broad spectrum of hematologic neoplasias and solid tumors, MM cell lines were among the most sensitive to this treatment [Mitsiades et al., 2004b], suggesting that MM represents a disease model where sustained IGF-1R function may be more important for tumor cell viability, compared to many other malignancies. In addition, IGF-1R inhibitors showed anti-tumor activity not only as single agents, but also in combination with other anti-neoplastic agents, including cytotoxic chemotherapy [Mitsiades et al., 2004b]. These observations suggest that for MM cells, as for other malignant cells, IGF-1R functions as a pleiotropic regulator of a multitude of anti-apoptotic pathways. Indeed, IGF-1R activation stimulates the activity of telomerase and of the proteasome [Mitsiades et al., 2004b]; increases the expression of anti-apoptotic caspase inhibitors [Mitsiades et al., 2004b], thus contributing to resistance against agents such as dexamethasone, cytotoxic chemotherapeutics and, in part, proteasome inhibitors; primes MM cells to respond to other cytokines (such as IL-6); and stimulates the production of pro-angiogenic cytokines (e.g., VEGF) [Mitsiades et al., 2004b], thereby recruiting new vessels and further supporting the growth of the tumor cells.

The supra-additive anti-MM effects of IGF-1R inhibitors when combined with other anti-tumor agents, such as cytotoxic chemotherapeutics, can be achieved with subtherapeutic doses of each drug. This suggests that IGF-1R inhibition may both enhance anti-tumor activity of other agents and allow for lower drug doses with potentially fewer adverse events. Finally, it is important to note that concurrently with the development of pyrrolo-[2,3-d]-pyrimidine inhibitors for IGF-1R, other chemical entities with activity as IGF-1R kinase inhibitors were described. For instance, members of the chemical class of cyclolignans compounds (e.g., picropodophyllin, PPP) emerged as another group of small molecules with selective inhibitory effect against IGF-1R versus IR. As with pyrrolo-[2,3-d]-pyrimidines, PPP inhibited tumor cell growth both in vitro and in vivo in subcutaneous plasmacytoma xenograft models of solid tumors [Girnita et al., 2004] and MM [Menu et al., 2006b; Stromberg et al., 2006].

The IGF-1R signaling cascade is not the sole cytokine-driven pathway that can support the viability of MM cells or trigger their proliferation. Instead, many signaling pathways downstream of IGF-1R are also activated by other mitogens/survival factors (for instance, the PI-3K/Akt pathway can also be activated by IL-6 and HGF) [Hideshima et al., 2001b; Hov et al., 2004]. It is notable that primary MM cells from patients with advanced forms of the disease (e.g., plasma cell leukemia or extramedullary plasmacytomas) can survive and proliferate in short term in vitro cultures independently of certain BM-derived cytokines such as IL-6, but are responsive to IGF-1R inhibition [Mitsiades et al., 2004b]. This may reflect, at least in part, the fact that, compared to several other cytokines implicated in the pathophysiology of IGFs are present at high levels in the serum and locally in the BM milieu, where IGF-1 is produced by various types of normal cells including osteoblasts and BMSCs [Mitsiades et al., 2004b]. The high levels of IGFs in the BM milieu influence the biological behavior of medullary MM, while the high circulating levels of IGFs may perhaps continue to influence MM cells even at extramedullary lesions. Although there is overlap in terms of signaling pathways activated by IGFs versus other mitogens/survival factors, differences in functional sequelae may be attributed to the ability of IGFs to trigger more potent and/or sustained activation of these cascades (e.g., PI-3K/Akt, IKK/NF-κB) than some other BM-derived cytokines [Mitsiades et al., 2004b]. This finding, coupled with the high concentrations of IGFs in vivo may account, at least in part, for different functional consequences of signaling events triggered in MM cells by IGF-1R versus other cytokines/growth factors.

TUMOR-STROMAL INTERACTIONS AS TARGETS FOR THERAPEUTIC INTERVENTION IN MM: FUTURE DIRECTIONS

The interactions between MM cells and their BM milieu are pathophysiologically unfavorable because they direct interfere with bone remodeling; lead to lytic skeletal lesions; and because of the constellation of locally released cytokines/growth factors which stimulate MM cells to proliferate and resist the effects of conventional treatment [Hideshima et al., 2001b; Mitsiades et al., 2004b]. Even in early stage MM, when tumors cells may not necessarily harbor the genetic lesions that may be responsible for establishment of a constitutive drug resistant phenotype, the protection conferred by stromal cells, other normal cells of the local BM microenvironment or by soluble cytokines, may conceivably allow some of the MM cells to survive long enough to acquire those additional genetic lesions that may lead them to development of clinical resistance. Recently developed therapies for MM such as thalidomide, lenalidomide, and the proteasome inhibitor bortezomib, can overcome the protective effects of BMSCs on MM cells [Hideshima et al., 2000b, 2003], which may explain the clinical activity of these agents can exhibit even in steroid- and/or chemo-refractory MM patients, further supporting the notion that the interaction of MM cells with the BM milieu constitutes a legitimate target for future treatment.

These therapeutic interventions may be targeted at many cellular and/or molecular levels. BMSCs and other accessory cells of the bone milieu which support MM cell survival are bone fide targets for therapeutic interventions, and are already, in part at least, targeted in clinical practice with novel agents such thalidomide, lenalidomide, and proteasome inhibitors. Specifically, the tumor-associated endothelium can targeted by various anti-angiogenic therapies, while thalidomide and lenalidomide show anti-angiogenic effects in vivo [D'Amato et al., 1994, 2001; Kenyon et al., 1997; Stirling, 2000]. Bisphosphonates have been reported to exhibit direct anti-neoplastic effects [Shipman et al., 1997; Aparicio et al., 1998], but their major effect is to block osteoclast-mediated bone resorption and therefore, indirectly suppress other sequelae of osteoclastic activity, including the increased local production of cytokines [Derenne et al., 1999]. BMSCs are also viewed as bone fide targets for therapeutic intervention, especially because they are key regulators of MM cell growth in the BM. One theoretical limitation of directly targeting BMSCs is their supportive tole on normal hematopoiesis [Uhlman et al., 1991]. It may therefore be more feasible to target the nexus of cytokine/growth factors which mediate the supportive effect of BMSCs on MM cells. This could be achieved for instance by suppressing the production of these mediators by BMSCs, other cells of the host microenvironment, or even MM cells; or by decreasing the local bioavailability of these factors in the milieu of the BM. For example, histone deacetylase (HDAC) inhibitors, aside from their direct pro-apoptotic activity against MM cells [Mitsiades et al., 2003, 2004c], can also suppress the release of IL-6 by BMSCs in co-culture models of MM with stroma [Mitsiades et al., 2003]. OPG or RANK-Fc can suppress osteoclastogenesis [Sordillo and Pearse, 2003; Vanderkerken et al., 2003]. Abs against cytokines such as VEGF, HGF, DKK-1; soluble forms of their respective receptors; as well as small-molecule inhibitors of downstream signaling cascades can inhibit tumor-associated neovascularization, tumor cell proliferation, and bone resorption, respectively [Holash et al., 2002; Tian et al., 2003; Hov et al., 2004].

Another set of strategies to target tumor-microenvironmental interactions is related to perturbation of the ability of MM cells to communicate with their milieu, either through altering of the adhesive interactions of MM cells with the ECM or other cells in the BM milieu; or by inhibiting the ability of MM cells to receive the signals provided to them in the form of growth and survival factors by their local milieu [Mitsiades et al., 2006]. For instance, mAbs against cell adhesion molecules mediating MM cell binding to BMSCs have been studied in pre-clinical MM models. In such studies, mice bearing murine MM cells were treated with anti-α4 integrin mAb, which suppressed the MM tumor burden; decreased the number of osteoclasts and the degree of bone destruction; and prolonged the survival of MM-bearing mice [Mori et al., 2004]. Targeting the ability of MM cells to respond to survival cues from their milieu can also include a wide spectrum of other therapeutic strategies against cytokine/growth factor signaling cascades, either at the level of the pertinent cell surface receptors (including IGF-1R inhibitors

[Mitsiades et al., 2004b] and FGF-R3 inhibitors [Trudel et al., 2004, 2005; Zhu et al., 2005]); or at downstream molecules. Several studies have already addressed diverse approaches targeting downstream signaling effectors, including inhibition of Ras farnesylation [Sebti and Hamilton, 2000; Le Gouill et al., 2002; Bolick et al., 2003; Alsina et al., 2004]; blockade of IKK/NF-κB signaling by small molecule inhibitors of the kinase activity of IKK [Hideshima et al., 2002] or by cell permeable peptide inhibitor of the nuclear translocation of NF-κB [Mitsiades et al., 2002b]; inhibition of the PI-3K-Akt-mTOR axis (for instance with the use of small molecule mTOR inhibitors, including rapamucin or RAD-001) [Frost et al., 2004; Raje et al., 2004; Stromberg et al., 2004; Mitsiades et al., 2004d; Shi et al., 2005]; inhibition of telomerase activity [Shammas et al., 2003, 2004]; or with the use of agents that can concurrently inhibit many of these aforementioned signaling pathways at multiple molecular levels thereof. A class of agents, which can achieve this multi-targeted effect, is the heat shock protein 90 (hsp90) inhibitors, including the geldanamycin analog 17-AAG. Hsp90 inhibitors bind to the ATP-binding domain located in the aminoterminal domain of hsp90. This ATP-binding pocket is critical for the ability of hsp90 to properly regulate the three-dimensional structure of its client proteins. These hsp90 inhibitors therefore concurrently perturb the proper folding and thus inhibit the function of many signaling pathways which involve hsp90 client proteins, including the PI-3K/Akt, Ras/Raf/MAPK, mTOR/p70S6K, and IKK/NF-κB cascades.

THE GENETIC SUBSTRATE OF TUMOR-MICROENVIRONMENT INTERACTIONS IN MM

The genetic makeup of MM cells is highly complex and quite variable across different subpopulations of MM patients [Fonseca et al., 2004]. As previously mentioned, these differences in the genetic composition of MM cells can have significant impact of the nature of the interaction of MM cells with their milieu. For example, different cytogenetic abnormalities in MM cells can lead to different patterns of expression of cell adhesion molecules, which can in turn modify the affinity of MM cells for interaction with their local stroma, as shown by the ability of cells with the t(14;16) chromosomal translocation, which causes overexpression of the transcription factor c-maf, to increase the expression of β7 integrin on MM cells, leading to enhanced MM cell adhesion to stroma/ECM and ensuing cytokine production [Hurt et al., 2004]. It is important, however, to emphasize that these tumor-microenvironment interactions can be genetically influenced not only at the level of their tumor cell compartment, but also in terms of differences of the somatic DNA of the normal host cells of the microenvironment. For instance, single nucleotide polymorphisms (SNPs) have been identified in the promoters of genes encoding for diverse cytokines implicated in the pathogenesis of hematologic neoplasias and some of them are relevant to the pathophysiology of MM. A single base substitution at position −308 in the promoter of the TNFα gene results in two allelic forms, the more common G (guanine) variant (or TNF1) and the less common A adenine) variant (or TNF2) [Wilson et al., 1992]. Other SNPs have been identified in intronic sequences of genes encoding for cytokines/growth factors, for example, the polymorphism where guanine is replaced by adenine at position +252 of the first intron of the lymphotoxin-α (LT-α), giving rise to the LT10.5 and LT5.5 variants, respectively [Webb and Chaplin, 1990]. Davies and coworkers characterized the frequency of TNFα/LTα haplotypes in cases of MM versus healthy controls and observed that individuals which carry in their genomic DNA polymorphisms associated with high production of TNFα/LTα have increased risk of developing MM [Davies et al., 2000]. Neben et al. further extended these findings and reported that the adenine variant of a SNP at position −238 of the TNF-α gene promoter (TNF-238A allele) correlated with higher peripheral blood levels of TNF-α; a statistically significant advantage in terms of progression-free at 1-year of follow-up after initiation of thalidomide treatment; as well as a trend for higher overall survival rates compared to patients harboring the TNF-238G allele [Neben et al., 2002]. These results suggest that the biological behavior of MM, and the differences that it exhibits in different patients, may not be exclusively attributed to genetic variability of the MM tumor cells, but also on how genetic differences in the normal host cells of the different patients influence the properties of the bone microenvironment and how its cells can support MM cells. It has been proposed that

similar polymorphisms may contribute to the pronounced variability, between different MM patient subpopulations, in terms of other clinicopathological manifestations of the disease, for example, the degree of severity of MM bone lesions [Mitsiades et al., 2004a].

CONCLUSIONS

A key component of the recent progress in the therapeutic management of MM has been the introduction in our therapeutic armamentarium of novel classes of agents which can, at least in part, abrogate the ability of the bone microenvironment to protect MM cells from conventional therapies, such cytotoxic chemotherapy and glucocorticoids. This success has led to the realization that a better characterization of tumor-microenvironment interactions in MM will help to uncover more novel target for therapies and hopefully further improve patient outcome. A possible barrier to the rapid translation of target identification into more effective therapies is the pleiotropic nature of cellular partners with which MM cells interact in the BM milieu, as well as the multitude of molecular mediators of these interactions. Indeed, it appears unlikely that targeting any individual cellular or molecular interaction of MM cells with their milieu will be able to confer curative clinical outcomes, because of the potential for redundancy in the pathways which stimulate MM cell survival and drug resistance in the BM niche. Optimizations of currently available pre-clinical models of MM homing and proliferation in the BM, will hopefully allow us to further dissect the complexity of MM–BM interactions and the functional hierarchy of its mediators, and allow for rational development of combinatorial strategies that will hopefully inhibit selectively and comprehensively all the major tumor-microenvironment interactions between MM and their milieu.

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