Published in final edited form as:

Exp Hematol. 2008 November ; 36(11): 1524–1534. doi:10.1016/j.exphem.2008.05.009.

The tumorigenic potential and disease manifestations of malignant B-cell variants differing in their fibronectin adhesiveness

Liat Nadav^{1,4}, Vyacheslav Kalchenko², Meir Max Barak³, Elizabeth Naparstek^{4,5}, Benjamin Geiger^{1,6}, and Ben-Zion Katz^{4,5,6}

¹Departments of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel

²Veterinary Resources, Weizmann Institute of Science, Rehovot 76100, Israel

³Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel

⁴Hematology Institute, Tel Aviv Sourasky Medical Center Tel Aviv University, Tel Aviv, Israel

⁵Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Abstract

Objective—Microenvironmental interactions of malignant B-cells can modulate various *in vitro* physiological responses including their proliferation, migration, apoptosis and drug resistance. The disease manifestations of human malignant B-cell variants, isolated based on their differential interactions with fibronectin, were examined in NOD/SCID mice.

Methods—Disease manifestations were assessed by pathological examinations and skeletal imaging of NOD/SCID mice injected with malignant B-cell variants. Dissemination patterns were analyzed by whole-body real-time imaging of mice injected with fluorescence-labeled malignant cells.

Results—The initial dissemination patterns and dynamics of both high (Type-A) and low (Type-F)-adherent variants, following intravenous inoculation, were similar. Both cell types reached the spleen and liver within 30 minutes after injection, then increasingly accumulated within the bone marrow. Mice injected with Type-A cells developed multiple myeloma-like disease within the bone marrow, with multiple lytic bone lesions. In contrast, Type-F cells displayed low tumorigenic capacity in spite of their efficient homing to the bone marrow niche. In addition, Type-A cells grew as extramedullary tumors in some of the intravenous inoculated mice, and formed solid tumors following subcutaneous injection. Both cell variants retained their characteristics surface markers following *in vivo* outgrowth as tumors, indicating that at least some of their properties are relatively stable.

Conclusion—The data suggest that the differential tumorigenicity of the B-cell adhesive variants is attributable to the capacity of Type-A cells to survive and proliferate within the bone marrow, rather than to different initial dissemination of the two cell populations.

© 2010 International Society for Experimental Hematology. Published by Elsevier Inc. All rights reserved.

⁶Corresponding authors: Dr. Ben-Zion Katz, PhD, Tel: 972-3-697 3517, Fax: 972-3-697 4452, bkatz@tasmc.health.gov.il, ⁶Prof. Benjamin Geiger, PhD, Tel: 972-8-934 3910, Fax: 972-8-946 5607, benny.geiger@weizmann.ac.il.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

adhesion; B-cells; bone; fibronectin; tumor

Introduction

The B-cell lineage is the origin of various hematological malignancies including acute and chronic leukemias, different types of aggressive and indolent lymphomas and plasma cell dyscrasias (e.g. multiple myeloma). Various genetic events (e.g. chromosomal translocations) are involved in the development of B-cell malignancies. Biochemical signals that govern the generation of immunoglobulin diversity can also trigger molecular events involved in B-cell tumorigenesis [1,2]. Molecular lesions in B-cells that can lead to malignancy development may also be initiated by viruses (e.g. EBV. [3,4]). Although cancer cells in general, are considered less dependent on and/or responsive to regulatory factors and stimuli, compared with their normal counterparts, many studies indicate that microenvironmental interactions play an important role in regulating malignant B-cell physiology [5]. For example, survival-promoting microenvironments can support the growth of CLL cells, and rescue them from cytotoxic therapy [6]. In the case of multiple myeloma (MM), the bone marrow (BM) stroma supplies growth factors and cytokines that are essential for the growth of the malignant plasma cells and the progression of the disease [7–9]. Moreover, adhesive interactions between MM cells and various constituents of the extracellular matrix (ECM) are known to regulate key physiological activities of the malignant plasma cells, including proliferation and resistance to apoptosis and cytotoxic drugs [7,10–13].

A hallmark of tumorigenesis is genetic instability, resulting in generation of diverse populations of malignant cells [14]. In diffuse large B-cell lymphoma the malignant cell population is diverse, and the extent of this diversity may correlate with high risk for disease relapse and resistance to therapy [15]. The possibility that microenvironmental factors generate diversity in B-cell malignancy in clinical specimens has been suggested previously, but the clinical significance of such variants has not been evaluated yet [16,17]. We previously demonstrated that the malignant B-cell line ARH-77 cell is heterogeneous in its matrix adhesiveness and migratory activity [16]. Using selective adsorption on fibronectin-coated surface, we isolated two subpopulations of ARH-77 B-cells, one displaying highly adhesive/low motile properties (Type-A), and the other, low adhesive/highly motile phenotype (Type-F). The two subpopulations differ in several surface markers, display different cytoskeletal organization [16], as well as distinct gene expression profiles (Nadav et al., unpublished data). Moreover, variant sub-populations of MM cells have also been observed in patient samples, where cells expressing high levels of CD138 (similar to Type-F cells) were found in the liquid phase of the BM, and low CD138 expressors (similar to Type-A cells) were found firmly attached to the BM spicules [16]. In the present study, we analyzed the tumorigenic potential, *in vivo* dissemination patterns and disease manifestations of these two malignant B-cell populations. The findings indicate that while both cell types home, similarly, to the BM, the fibronectin-adhesive variant displays a much higher capacity to develop a malignant disease similar to human MM.

Materials and Methods

Cells

The ARH-77, EBV-transformed plasma-cell line was kindly provided by Prof. Hanna Ben-Bassat (Hadassah Medical School, Jerusalem, Israel), and cultured as previously described [16]. The cells were subjected to serial adhesion cycles on fibronectin, yielding Type-A and Type-F cell populations as previously described [16].

Mice, irradiation and inoculation

NOD/SCID mice, 4–6 weeks old, were obtained from Harlan laboratories Ltd. (Ein Kerem, Jerusalem) and maintained at the Veterinary Resources facility of the Weizmann Institute of Science. All the *in vivo* experiments were approved by the Weizmann Institute's Animal Care and Use Committee (IACUC). Mice were exposed to 150 cGy (rads) of radiation from a Gammacell 40 source. Twenty-four hours later, 1×10^7 cells of either Type-A or Type-F variants were injected into the tail veins of 20 female NOD/SCID mice, in two independent groups of 10. The mice were then observed daily until they developed apparent disease manifestations. For S.C. growth, 1×10^7 cells of either Type-A or Type-F variants, were each injected into the flank of 5 non-irradiated mice. These mice were also observed daily, until they developed palpable tumors.

Cell adhesion assay

Cell adhesion assay was carried out as previously described [16]. Briefly, cells were plated for 30 minutes on 5 cm bacterial dishes coated with 15 $\mu\text{g/mL}$ fibronectin (Sigma, St. Louis, MO). The dishes were then washed twice with PBS to remove nonattached cells and the remaining cells were counted microscopically.

Flow cytometry

FITC-conjugated anti-CD138 and isotype-control IgG1 were purchased from DAKO (Glostrup, Denmark) and used according to the manufacturer's instructions. For staining with directly-labeled antibodies, 50 μL samples (5×10^5 cells) were incubated with 5 μL of each of the designated mAb at 4°C for 30 min, and then washed with 2 mL PBS. From each sample, 3×10^4 events were acquired by FACS Calibur at a rate of 150 to 300 events per second, and analyzed using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

Homing experiments in NOD/SCID mice

The homing of Type-A and Type-F cells was investigated using whole Body Optical Imaging. Groups of nine anaesthetized mice were injected with Type-A or Type-F cells, labeled with near-infrared (NIR) lipophilic carbocyanine dye [1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR)] (Invitrogen, Carlsbad, CA, USA) [18]. The cells (1×10^7) were incubated in 10 ml phosphate-buffered saline (PBS) containing 3.5 $\mu\text{g/mL}$ DiR dye, and 0.5% ethanol at 37°C for 30 min. The cells were then washed twice with PBS, and the viability of the labeled cells was verified by trypan blue staining. Labeled cells were then injected intravenously into NOD/SCID mice, whose hair was previously removed. DiR has absorption and fluorescence maxima at 750 and 782 nm, respectively, which correspond to low light absorption and autofluorescence in living tissues. This property enables the harvesting of a significant signal from labeled cells, with very low tissue background levels [18]. The mice were then observed by an IVIS® 100 Imaging System (Xenogen, Cranbury, NJ). For higher magnification visualization of labeled cells in the skull, the mice were scarified and the head skin was removed. An SZX12 microscope (Olympus, Japan) coupled with Charged Coupled Device (CCD) Pixelfly (PCO, Germany) and compatible filter set for DiR were used for observation.

Histopathology

Animals were sacrificed, and tissues were excised and fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin:eosin. Histopathological examination was performed with a Nikon E600 microscope (Nikon Corporation, Tokyo, Japan). Images were recorded with a Nikon DXM1200 camera (Nikon Corporation).

Micro-CT

Mice were scanned with a GE Explore Locus Micro-CT System (GE Healthcare, Ontario, Canada). The 3D images were analyzed using a Microview 3D Volume & Analysis Tool (GE Healthcare).

Results

Disease manifestations of adhesive B-cell variants in NOD/SCID mice

The main objective of the present study was to elucidate the tumorigenic properties of malignant B-cell variants that were isolated by differential adhesive interactions with fibronectin. 1×10^7 highly adherent (Type-A) or 1×10^7 poorly adherent (Type-F) cells were injected, each, into the tail veins of 20 NOD/SCID mice. Of the 20 mice injected with Type-A cells, 11 developed tumors within 50 days (Fig. 1A), which were manifested in a variety of ways, depending on their location (Fig. 1B). Six of the 11 disease-bearing animals developed head swelling and a lateral tilt in their posture, compatible with a tumor originating in the skull, with the mass causing a lateral pressure on the brain tissue (Fig. 1B). Two of the 11 disease-bearing animals developed hind leg paralysis, due to spinal cord compression by the tumor (Fig. 1B). Three of them developed palpable tumors in the leg, in the cervical region and in the scapula. The mean latency period prior to disease onset in mice injected with Type-A cells was 40 days (Fig. 1A). In contrast, only three of the 20 mice injected with Type-F cells developed manifestations of disease: two of these displayed head swelling without lateral tilt, and one developed head swelling and hind leg paralysis. The mean latency period in mice injected with Type-F cells was 73 days (Fig. 1A). Similar results were obtained when lower numbers (2×10^6) of cells were injected (not shown). The parental ARH-77 cells exhibited intermediate level of tumorigenic activity (Fig. 1A). While intravenous injection enables cells to home into their normal physiological environment, subcutaneous inoculation is not considered orthotropic. Subcutaneous inoculations can therefore assess the ability of the cells to grow without physiological microenvironmental support or constraints. As shown in Figure 1A, 4 out of 5 non-irradiated mice injected S.C. with Type-A cells developed palpable tumors after a mean latency time of 35 days. None of the non-irradiated mice developed tumors following such injection with Type-F cells.

Dissemination patterns and dynamics of adhesive B-cell variants, as examined by whole body optical imaging

A critical stage in the generation of hematopoietic malignancies involves the homing of the malignant cells to their physiologically relevant sites. We therefore examined *in vivo* the homing patterns and dynamics of the B-cell adhesive variants in the i.v.-inoculated mice, utilizing two complementary imaging tools: Whole body optical imaging of the mice was performed at various time points following inoculation, followed by higher resolution, microscopy-based imaging, which was utilized to observe the dissemination kinetics of the cells within the BM compartment in the skull. As shown in Figure 2, rapid accumulation of both Type-A and Type-F cells was observed in the spleen and liver, reaching >50% of the maximal value within 30 min. followed by significantly slower homing of cells into the BM of three independent organs, vertebrae, skull and femur (Fig. 2, A and B). The initial dynamics of the dissemination appeared to be somewhat faster for Type-F cells, yet by 72 hours post-inoculation the total accumulated fluorescence intensity was similar for both variants (Fig. 2, A and B).

Fluorescence microscopy was further employed in order to track the dissemination of the B-cell variants within the skull. As shown in Figure 2A (lower panel), the distribution of the circulating cells was restricted to the BM of the skull, without penetrating into the brain tissue. These high-resolution images revealed cell aggregates in the BM, concentrating mainly in the

fissures and in the occipital area, where the bone is relatively thick. Here again, Type-F cells disseminated more rapidly than Type-A cells, though 72 h post-inoculation, the overall fluorescence intensity of both variants was similar (Fig. 2A).

Analysis of the osteolytic activity of the adhesive B-cell variants in NOD/SCID mice, using Micro-CT

In order to characterize the disease manifestations in NOD/SCID mice, injected with Type-A or Type-F cells, treated and control mice were scanned by micro-computed tomography (micro-CT), which enables the visualization of bone structure, density and texture. The bone areas affected by tumors in several Type-A injected mice, displaying disease manifestations, were visualized at higher resolution. Figure 3, upper panel, depicts representative examples of several bony areas, including the hind leg, skull, lumbar and cervical vertebrae of affected mice, as well as non-affected mice, either injected with Type-F cells, or untreated. The bone surface texture in mice injected with Type-A cells was rough, affecting most severely the fibula (red arrow). Marked changes in the tibia texture were also observed, with decline in mineralized bone structure. The bone mineral density (BMD) in the knee joint of this same mouse was compared to that of a normal-looking knee from a mouse injected with Type-F cells (Fig. 3, lower panel) or untreated mouse (not shown). This analysis demonstrated areas of reduced BMD in the tibia of the mouse injected with Type-A cells (red arrow), as well as foci, apparently representing lytic lesions (red arrowhead) where no minerals were detected. The skull of the mouse injected with Type-A cells was also found to be severely affected (Fig. 3 top), sustaining damage which could have been caused by a combination of several distinct effects. Changes in the bone texture along both the outer and inner aspects of the skull (red arrow) reflect bone lysis. In addition, the fissures were open due to pressure caused by intracranial tumor growth from within the skull (red asterisk, and Figure 5C). A BMD scan of the skull rim emphasized the lack of continuity, and the diminished BMD adjacent to the missing skull bone regions. In the lumbar vertebrae, the same elements of lytic lesions and altered bone texture were evident (red arrow); fractures in the cervical vertebrae were also seen (Fig. 3, red arrows).

Histopathological characteristics of disease generated by Type-A cells

Mice injected with the highly adherent Type-A cells developed MM-like disease which disseminated throughout the body, leading to destruction of appendicular and long bones and nervous tissue, but with visceral sparing. Within bone and nervous tissues, multicentric disease was a regular finding. Typically, clinical manifestations only partially reflected the full extent of the lesions, as seen in post-mortem examination. Figure 4A shows an example of massive neoplastic proliferation, which led to extensive destruction of the vertebral column and the loss of normal anatomic structures. Figure 4B depicts, at higher magnification, a view of a normal vertebra (black arrowhead) adjacent to a vertebra which has been largely obliterated by neoplastic infiltration (black arrow), with malignant cells extending beyond the vertebral body. These data indicate that though the proliferation of malignant cells may be initiated within the BM, their outgrowth is not necessarily confined to this compartment. In a similar manner, neoplastic growth was found in the subdural space of the skull, with destruction of calvaria (black arrow) (Fig. 4C); this tumor apparently originated from within the BM of the skull (Fig. 4D). In contrast, the tumors seen in mice injected with Type-F cells were usually solitary intracranial masses. One Type-F mouse developed a combination of intracranial and vertebral masses.

In mice inoculated with Type-A cells, the disease was manifested in neurological symptoms (e.g. head swelling, hind leg paralysis). Pathological examination of tissue sections taken from these mice revealed considerable damage to nerve tissues. Figure 5A shows pronounced compression of the spinal cord (black arrow) as a result of neoplastic proliferation within the vertebral canal. Figure 5B indicates neoplastic infiltration (black arrowhead) of a dorsal root

ganglion (black arrow). In our animal model, the disease produced nervous deficits associated with intracranial masses, causing mass effect and pressure on the brain. Figure 5C depicts one such example, an intracranial tumor (black arrow) pressing on and distorting the midbrain. The lateralization of the tumor clinically correlated with the head tilt which this mouse developed (Figure 5, inset). High-resolution microscopic examination of the brain tissue (Fig. 5D) showed a discrete border between the tumor and the brain (marked with black arrowheads).

The ex vivo properties of tumor-derived Type-A cells

Several tumors that grew in mice, injected with Type-A cells, were explanted and grown *in vitro*, in order to compare the tumor cells phenotype to that of the injected cell population. Adhesion assays demonstrated that the cells derived from these tumors retained their highly adhesive phenotype, regardless of the tumor site *in vivo* (Fig. 6A). In contrast, cells originating from the brain tumor isolated from mice injected with Type-F cells did not adhere at all (data not shown), indicating that the differences in adhesive properties between these two malignant plasma cell variants are retained in the developing tumor. Moreover, these results indicated that the formation of tumors, albeit in rare cases, by Type-F cells, is not attributable to inter-conversion of Type-F cells into Type-A. We previously demonstrated that Type-A and Type-F cells differ in their flow cytometric profiles, including in the expression of known MM-related antigens such as CD138 [16]. As shown in Figure 6B, flow cytometric analysis of the cells originating from these tumors also conserved their flow cytometric profile. Thus, Type-A cells, grown *ex vivo*, had lower levels of CD138 compared to Type-F cells: in fact, these levels were even lower than those of the cultured Type-A cells, prior to inoculation. Similarly, cells originating from a brain tumor found in a mouse injected with Type-F cells were characterized by CD138 expression levels that were even higher than those of the Type-F cells originally injected into the mice (data not shown).

Discussion

Various studies indicate that extracellular matrix components of the BM microenvironment may play a crucial role in the pathogenesis of B-cell malignancies. Thus, adhesion to fibronectin via $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins confers resistance to FAS mediated apoptosis to neoplastic plasma cells, and interactions of MM cells with both free and immobilized vitronectin and fibronectin stimulate their proliferation [11,19]. In B-CLL, adhesion to fibronectin can induce trans-endothelial migration and invasion of the leukemic cells [20], or protect B-CLL cells from the cytotoxic effects of fludarabine [21]. These findings, demonstrating the capacity of matrix adhesion to affect the behavior and fate of cancer cells, compelled us to isolate adhesion variants from a B-cell malignancy, and compare their properties [16]. In the present study we directly examined whether adhesion variants of malignant B-cells represent different tumorigenic potential *in vivo*, using the NOD/SCID mouse model, which was previously utilized to assess the malignancy of ARH-77 cells [22]. This assay indicated a significantly higher incidence of MM-like disease with a much shorter latency period in mice injected with the adhesive Type-A B-cells, as compared to mice injected with the poorly adhesive Type-F B-cells.

The different tumorigenic properties of Type-A and Type-F cells could, in principle be attributed to a variety of cellular features, including cell homing into the BM, long term survival in the BM niche, and capacity to proliferate in that site [23–26]. Specific homing of malignant cells to the target tissue is often considered critical for a successful establishment and growth of tumors [26]. In order to determine whether the preferential development disease by Type-A cells is attributable to the higher capacity of these cells to reach the BM, we performed a short-term (up to 72 hours) homing analysis of the two variants. We found that the homing patterns of Type-A and Type-F cells were generally similar. Both variants disseminated mainly

to the spleen, liver and BM, which are the primary organs for MM cells [27]. It is, nevertheless noteworthy that disease manifestations were detected only in the BM, without apparent involvement of the liver and spleen.

Although the general dissemination patterns of the two adhesive variants were similar, the homing of Type-F cells was maximal at 24 hours, while Type-A cells reached similar dissemination only after 72 hours. Supportive data, obtained in our previous work [16], indicated that *in vitro*, Type-F cells migrate considerably faster than Type-A cells. These data suggest that the low tumorigenic capacity of Type-F cells is not attributable to poor BM homing, but rather to their poor long-term survival in the BM, and to the capacity to proliferate in that site. Collectively, our results indicate that the establishment and development of disease is, predominantly determined by interactions of the cells with the microenvironment in the target organs, rather than by restricted dissemination routes.

Micro-CT analysis provided striking information on the similarity between the disease model in mice, inoculated with Type-A cells, and the human MM disease. Clear lines of similarity were noted, including major bone lesions, altered bone surface texture, fractures, and in some cases, complete loss of bone. We systematically measured BMD the leg, thoracic vertebrae, and the skull, and found that low BMD is confined to areas where tumor growth was apparent. Previous studies with this technique have shown that BMD of the lumbar spine is often diminished in MM patients at diagnosis, whereas BMD of the hip is rarely affected, a finding which also corroborates the local effect suggested by our data [28,29].

An intriguing question, highlighted by the present study is the possible causal relationships between the adhesive phenotype and the apparent tumorigenic properties. Two general mechanisms should be considered, namely – a direct involvement of matrix adhesion in the fine homing of the malignant B-cells into a specific BM niche, affecting their invasive growth characteristics, or an indirect mechanism, whereby Type-A cells exhibit higher survival capacity than Type-F cells (related to, or independent of integrin signaling). It is noteworthy that the increased tumorigenicity of Type-A cells (compared to Type-F) cannot be attributed to differences in the initial homing to BM, which was similar in the two adhesive variants. It should be mentioned that previous studies support the notion that highly adhesive cells have a growth advantage, which is enhanced by the cells' adhesion to the BM niche [30]. It was also found that cells with a highly adhesive phenotype characterize overt MM cells, compared with the less adhesive plasma cells derived from smoldering myeloma patients [31]. Regulation of malignancy by other extracellular matrix proteins (e.g. thrombospondin-1) [32], and malignant clonal dominance by growth conditions [33], have already been established. Such studies clearly advocate for epigenetic regulatory modes of malignancy by microenvironmental factors and conditions.

Future studies may clarify the biochemical and molecular characteristics of adhesive malignant B-cells (and in particular, MM cells), thereby generating novel therapeutic targets. In this respect, it is already known that anti-adhesion therapy can significantly inhibit the progression of MM and its clinical manifestations in murine models [34]. Moreover, anti-integrin based agents may be utilized to overcome adhesion-mediated drug resistance [35]. The development of novel therapies for the treatment of B-cell malignancies will be based on a deeper understanding of the basic molecular mechanisms driving this diversity.

Acknowledgments

This study was supported by grants from the Israel Science Foundation and the NIGMS grant for the Cell Migration Consortium (NIH Grant U54GM64346). BG holds the Erwin Neter Professorial Chair in Cell and Tumor Biology.

References

1. Ramiro AR, Nussenzweig MC, Nussenzweig A. Switching on chromosomal translocations. *Cancer Res* 2006;66:7837–7839. [PubMed: 16912154]
2. Gonzalez D, van der Burg M, Garcia-Sanz R, et al. Immunoglobulin gene rearrangements and the pathogenesis of multiple myeloma. *Blood* 2007;110:3112–3121. [PubMed: 17634408]
3. Epeldegui M, Widney DP, Martinez-Maza O. Pathogenesis of AIDS lymphoma: role of oncogenic viruses and B cell activation-associated molecular lesions. *Curr Opin Oncol* 2006;18:444–448. [PubMed: 16894291]
4. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. *Nat Rev Cancer* 2004;4:757–768. [PubMed: 15510157]
5. Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer* 2005;5:251–262. [PubMed: 15803153]
6. Munk Pedersen I, Reed J. Microenvironmental interactions and survival of CLL B-cells. *Leuk Lymphoma* 2004;45:2365–2372. [PubMed: 15621749]
7. Mitsiades CS, Mitsiades NS, Munshi NC, Richardson PG, Anderson KC. The role of the bone microenvironment in the pathophysiology and therapeutic management of multiple myeloma: interplay of growth factors, their receptors and stromal interactions. *Eur J Cancer* 2006;42:1564–1573. [PubMed: 16765041]
8. Pagnucco G, Cardinale G, Gervasi F. Targeting multiple myeloma cells and their bone marrow microenvironment. *Ann N Y Acad Sci* 2004;1028:390–399. [PubMed: 15650264]
9. Roccaro AM, Hideshima T, Raje N, et al. Bortezomib mediates antiangiogenesis in multiple myeloma via direct and indirect effects on endothelial cells. *Cancer Res* 2006;66:184–191. [PubMed: 16397231]
10. Ng MH. Death associated protein kinase: from regulation of apoptosis to tumor suppressive functions and B cell malignancies. *Apoptosis* 2002;7:261–270. [PubMed: 11997670]
11. Ria R, Vacca A, Ribatti D, Di Raimondo F, Merchionne F, Dammacco F. Alpha(v)beta(3) integrin engagement enhances cell invasiveness in human multiple myeloma. *Haematologica* 2002;87:836–845. [PubMed: 12161360]
12. Garcia-Gila M, Lopez-Martin EM, Garcia-Pardo A. Adhesion to fibronectin via alpha4 integrin (CD49d) protects B cells from apoptosis induced by serum deprivation but not via IgM or Fas/Apo-1 receptors. *Clin Exp Immunol* 2002;127:455–462. [PubMed: 11966761]
13. Hazlehurst LA, Dalton WS. Mechanisms associated with cell adhesion mediated drug resistance (CAM-DR) in hematopoietic malignancies. *Cancer Metastasis Rev* 2001;20:43–50. [PubMed: 11831646]
14. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature* 1998;396:643–649. [PubMed: 9872311]
15. Yang MH, Yen CC, Chiang SC, et al. Prognostic significance of clonal diversity of immunoglobulin gene rearrangements in patients with diffuse large B-cell lymphoma. *Oncol Rep* 2005;13:503–508. [PubMed: 15706425]
16. Nadav L, Katz BZ, Baron S, Cohen N, Naparstek E, Geiger B. The generation and regulation of functional diversity of malignant plasma cells. *Cancer Res* 2006;66:8608–8616. [PubMed: 16951174]
17. Zhu D, Orchard J, Oscier DG, Wright DH, Stevenson FK. V(H) gene analysis of splenic marginal zone lymphomas reveals diversity in mutational status and initiation of somatic mutation in vivo. *Blood* 2002;100:2659–2661. [PubMed: 12239182]
18. Kalchenko V, Shvitiel S, Malina V, et al. Use of lipophilic near-infrared dye in whole-body optical imaging of hematopoietic cell homing. *J Biomed Opt* 2006;11:050507. [PubMed: 17092148]
19. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* 1999;93:1658–1667. [PubMed: 10029595]
20. Redondo-Munoz J, Escobar-Diaz E, Samaniego R, Terol MJ, Garcia-Marco JA, Garcia-Pardo A. MMP-9 in B-cell chronic lymphocytic leukemia is up-regulated by alpha4beta1 integrin or CXCR4 engagement via distinct signaling pathways, localizes to podosomes, and is involved in cell invasion and migration. *Blood* 2006;108:3143–3151. [PubMed: 16840734]

21. de la Fuente MT, Casanova B, Moyano JV, et al. Engagement of alpha4beta1 integrin by fibronectin induces in vitro resistance of B chronic lymphocytic leukemia cells to fludarabine. *J Leukoc Biol* 2002;71:495–502. [PubMed: 11867687]
22. Huang YW, Richardson JA, Tong AW, Zhang BQ, Stone MJ, Vitetta ES. Disseminated growth of a human multiple myeloma cell line in mice with severe combined immunodeficiency disease. *Cancer Res* 1993;53:1392–1396. [PubMed: 8443818]
23. Corre J, Mahtouk K, Attal M, et al. Bone marrow mesenchymal stem cells are abnormal in multiple myeloma. *Leukemia* 2007;21:1079–1088. [PubMed: 17344918]
24. Perez LE, Parquet N, Shain K, et al. Bone Marrow Stroma Confers Resistance to Apo2 Ligand/TRAIL in Multiple Myeloma in Part by Regulating c-FLIP. *J Immunol* 2008;180:1545–1555. [PubMed: 18209050]
25. Hecht M, von Metzler I, Sack K, Kaiser M, Sezer O. Interactions of myeloma cells with osteoclasts promote tumour expansion and bone degradation through activation of a complex signalling network and upregulation of cathepsin K, matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA). *Exp Cell Res*. 2007 in press.
26. Vande Broek I, Vanderkerken K, Van Camp B, Van Riet I. Extravasation and homing mechanisms in multiple myeloma. *Clin Exp Metastasis*. 2007 in press.
27. Vanderkerken K, De Greef C, Asosingh K, et al. Selective initial in vivo homing pattern of 5T2 multiple myeloma cells in the C57BL/KalwRij mouse. *Br J Cancer* 2000;82:953–959. 2000. [PubMed: 10732771]
28. Dhodapkar MV, Weinstein R, Tricot G, et al. Biologic and therapeutic determinants of bone mineral density in multiple myeloma. *Leuk Lymphoma* 1998;32:121–127. [PubMed: 10037007]
29. Abildgaard N, Brixen K, Eriksen EF, Kristensen JE, Nielsen JL, Heickendorff L. Sequential analysis of biochemical markers of bone resorption and bone densitometry in multiple myeloma. *Haematologica* 2004;89:567–577. [PubMed: 15136220]
30. Teoh G, Anderson KC. Interaction of tumor and host cells with adhesion and extracellular matrix molecules in the development of multiple myeloma. *Hematol Oncol Clin North Am* 1997;11:27–42. [PubMed: 9081202]
31. Silvestris F, Cafforio P, Calvani N, De Matteo M, Lombardi L, Dammacco F. In-vitro functional phenotypes of plasma cell lines from patients with multiple myeloma. *Leuk Lymphoma* 2006;47:1921–1931. [PubMed: 17065007]
32. Weinstat-Saslow DL, Zabrenetzky VS, VanHoutte K, Frazier WA, Roberts DD, Steeg PS. Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis. *Cancer Res* 1994;54:6504–6511. [PubMed: 7527299]
33. Rak JW, Kerbel RS. Growth advantage ("clonal dominance") of metastatically competent tumor cell variants expressed under selective two- or three-dimensional tissue culture conditions. *In Vitro Cell Dev Biol Anim* 1993;29A:742–748. [PubMed: 8407718]
34. Mori Y, Shimizu N, Dallas M, et al. Anti-alpha4 integrin antibody suppresses the development of multiple myeloma and associated osteoclastic osteolysis. *Blood* 2004;104:2149–2154. [PubMed: 15138161]
35. Damiano JS. Integrins as novel drug targets for overcoming innate drug resistance. *Curr Cancer Drug Targets* 2002;2:37–43. [PubMed: 12188919]

Figure 1. Disease incidence and clinical symptoms in NOD/SCID mice, injected with Type-A and Type-F cell subpopulations

A. Disease incidence and latency period seen in mice injected with Type-A (closed colored squares) and Type-F (open triangles) cells. Kaplan-Meier analysis of tumor onset shows a higher incidence of tumors in mice injected with Type-A cells, as compared to those injected with Type-F cells (mice injected i.v.: 11/20 as compared to 3/20; mice injected s.c.: 4/5 as compared to 0/5), and shorter latency period to disease onset in Type-A injected mice. Also shown: Tumor incidence in mice injected with parental ARH-77 cells (dashed line) **B.** Clinical manifestations in disease-bearing mice. The pictures show the most prevalent symptoms observed: hind leg paralysis, indicating a tumor in the thoraco-lumbar area of the spine, and lateral tilt in the posture accompanied by head swelling, indicating the presence of a brain tumor. The few disease-bearing mice injected with Type-F cells displayed the same symptoms (data not shown).

Figure 2A

Figure 2B

Figure 2. *In vivo* homing patterns and dynamics of Type-A and Type-F subpopulations in NOD/SCID mice

A. Non-invasive color-coded near-infrared fluorescent (NIRF) images of Type-A and Type-F homing in NOD/SCID mice. Upper panel - Images show massive primary homing to liver and spleen, and secondary homing to the BM. Type-A and Type-F cells both home to the same organs. Images represent findings in at least three different mice. Organ designations: Sk=Skull, Sp=Spleen, L=Liver, V=Vertebrae, F=Femur. Lower panel - High-resolution NIRF microscopic analysis of the cranium of a sacrificed NOD-SCID mouse after removal of skin, at different time points. The images show that the source of the cranium fluorescence is the BM, mainly in the area of the occipital sutures, and not the brain tissue. Type-F cells accumulate in the skull more rapidly, reaching the maximal intensity after 24 hours, while signals from Type-A cells reach the same intensity only after 72 hours. The cutoff of low intensity light was adjusted to make a detected auto-fluorescent signal below the threshold. The monochrome-graduated bar and the color-graduated bar indicate fluorescence intensity in arbitrary units.

B. The kinetics of Type-A and Type-F cell homing in NOD/SCID mice (Dashed line - Type-A cells, solid line - Type-F cells). The rate of cell accumulation in the liver and spleen is higher

compared to that seen in the BM, both for Type-A and Type-F cells. In the BM, the numbers of Type-F cells reach a plateau after 48 hours and then tend to decrease, whereas the numbers of Type-A cells continuously accumulate. Type-F cells accumulate more rapidly (significant results were obtained in the skull and vertebrae) within the different organs, as shown by lower T1/2 values (Bar graph, bottom right) (White bars – Type-A cells, Black bars – Type-F cells). Each point on the graph represents the average $\pm$ S.D. of three individual measurements performed on three different mice.

Figure 3. Micro-CT skeleton imaging and bone mineral density (BMD) of mice injected with Type-A and Type-F cells

Upper panel - Images were taken from mice injected with Type-A cells, in whom disease manifestations and pathological findings were noted in the skull, the vertebral column and the leg. Images show altered bone texture in the fibula, skull and vertebrae (red arrows), lytic lesions in the tibia (red arrowhead), detachment of the temporal and occipital bones in the area of the sutures due to pressure of the growing tumor from within the skull (red asterisks), and fractures in the cervical vertebrae (red arrow). Middle panel: Mice injected with Type-F cells who were normal in appearance had no bone pathology. Lower panel: BMD decreased in areas of tumor and bone destruction, while adjacent areas are not affected. Two examples are the tibia, with areas of decreased BMD (red arrow), and areas with no bone detection at all (red arrowhead), that could represent lytic lesion. The femur in the diseased mouse resembles that of an unaffected control. In the skull of the diseased mouse, the surface of the cranium is not continuous (asterisks), and the existing bone manifests lower BMD.

Figure 4. The destructive effects of growing MM tumors on tissue

A. Massive neoplastic proliferation leading to extensive destruction of the vertebral column and of normal anatomic structures. **B.** View at high magnification of a normal vertebra (arrowhead) adjacent to a vertebra which has been largely obliterated by neoplastic infiltration (arrow). **C.** Neoplastic growth in the subdural space of the skull with destruction of calvaria (arrow). **D.** View at high magnification showing a tumor originating from the BM of the skull bone.

Figure 5. Damage to the nervous system inflicted by tumor growth

A. Pronounced compression of the spinal cord (arrow) arising from neoplastic proliferation within the vertebral canal. **B.** View at high magnification of neoplastic infiltration (arrow) of a dorsal root ganglion (arrowhead). **C.** The tumor (arrow) presses and distorts the midbrain. The lateralization of the tumor clinically manifested itself as a head tilt (insert). **D.** The malignant cells have low affinity to the neuroparenchyma. A discrete border is present between the tumor and the brain (marked by double arrowheads). The cortex is infiltrated by a low number of malignant cells that are mostly confined to the perivascular spaces of Virchow-Robbins (arrow).

Figure 6A

Figure 6B

Figure 6. Cells isolated from mice injected with Type-A cells retained their adhesive and flow-cytometry phenotype

A. Tumors isolated from mice injected with Type-A cells retained their adhesive phenotype, as demonstrated by an adhesion assay. Type-F cells (black bar) demonstrated low levels of adhesion, while Type-A cells (light gray bar) and two tumors derived from mice injected with Type-A cells (dark gray bars) were found to be highly adhesive (* $p < 0.05$, compared with Type-F cells). One brain tumor isolated from a mouse injected with Type-F cells showed a tendency to adhere that was even lower than that of the original Type-F cells (data not shown). **B.** CD138 expression levels are low in Type-A cells (light gray bar) compared to those seen in Type-F cells (black bar). This phenomenon was also observed in tumors derived from mice injected with Type-A cells (light gray bars), whether by i.v. or s.c. (even lower than Type-A cells, * $p < 0.009$) injection. The insert shows a representative analysis of a tumor isolated after i.v. injection. The bars indicate an average $\pm$ S.D of tumors isolated after i.v. or s.c. injection, as well as one brain tumor.