Research Article

The Generation and Regulation of Functional Diversity of Malignant Plasma Cells

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Abstract

Cellular diversity, which is a hallmark of malignancy, can be generated by both genetic and nongenetic mechanisms. We describe here variability in the adhesive and migratory behavior of malignant plasma cell populations, including multiple myeloma–derived lines and primary patient samples. Examination of the plasma cell lines ARH-77, CAG, and AKR revealed two distinct subpopulations of cells, one displaying highly adhesive properties (type A) and the other consisting of poorly adhesive, floating cells (type F). In the ARH-77 cell line, type A cells attach better to fibronectin and to human bone fragments and form paxillin-rich focal adhesions, whereas type F cells are highly motile and exert integrin-dependent bone marrow homing capacity in nonobese diabetic/severe combined immunodeficient mice. Flow cytometry indicated that type A cells express significantly higher levels of CD45 and CD56 and lower levels of CD138 compared with type F cells. Interestingly, culturing of either type A or type F cells under nonselective conditions resulted in the development of mixed cell population similar to the parental ARH-77 cells. Analysis of bone marrow aspirates of multiple myeloma patients revealed that spicules within the aspirates are enriched with type A–like cells. Nonadherent cells within the aspirate fluids express a marker profile similar to type F cells. This study indicates that multiple myeloma patients contain heterogeneous populations of malignant plasma cells that display distinct properties. Diverse subpopulations of malignant plasma cells may play distinct roles in the different biological and clinical manifestations of plasma cell dyscrasias, including bone dissemination and selective adhesion to bone marrow compartments. (Cancer Res 2006; 66(17): 8608-16)

Introduction

Plasma cell dyscrasias include an entire spectrum of disorders involving an aberrant proliferation of plasma cells and an excessive production of monoclonal immunoglobulins (Ig). These include, among others, disorders considered as premalignant, such as monoclonal gammopathy of undetermined significance, and malignant diseases, such as multiple myeloma and plasma cell leukemia. Multiple myeloma is a malignancy of terminally differentiated B cells with grim prognosis (1) characterized by the presence of monoclonal Ig in the blood and urine, lytic bone lesions, and infiltration of monoclonal plasma cells into the bone marrow (2). This disease is clinically viewed as a systemic disease restricted to the bone marrow. However, multiple myeloma cells can be detected in the peripheral blood of the majority of patients at presentation either by PCR analysis for the IgH chain rearrangement or by flow cytometry (3). This suggests that the malignant plasma cells can be phenotypically divergent; although most of the cells reside in the bone marrow, small subsets are able to migrate to the circulation and spread to distant sites. The bone marrow–resident subpopulations may be considered as "classic multiple myeloma cells," whereas highly motile subpopulations may be responsible for the transition between the different disease stages and the widespread distribution of the disease throughout the bone marrow system (4). This notion is supported by the observation that the absolute number of circulating multiple myeloma cells is correlated with disease stage (3). Migration of multiple myeloma cell precursors from the lymph node to bone marrow and their further spreading throughout the skeleton requires directed and active motility through and across vascular and sinusoidal channels followed by homing to the bone marrow microenvironment. Chemokines and their receptors play a key role in regulating multiple myeloma cell migration (5, 6). Hence, identification and characterization of multiple myeloma subpopulations with distinct adhesive and migratory activities may define novel therapeutic targets associated with the systemic dissemination of the disease.

Malignant plasma cells within the bone marrow attach to stromal cells and to the extracellular matrix (ECM) using several types of integrins and nonintegrin adhesive receptors (7–9). Adhesion and motility of multiple myeloma cells were reported to be mediated not only by protein-protein interactions but also via glycosaminoglycans. Multiple myeloma cells express CD44 and receptor for hyaluronan-mediated motility, whose interactions with hyaluronan modulate the adhesion, migration, proliferation, and survival of multiple myeloma cells (10–14). However, in spite of the wealth of information about molecules that might affect multiple myeloma adhesion and migration, a comprehensive understanding of these processes in multiple myeloma and their relationships to the clinical manifestations of the disease is still missing.

The characterization of multiple myeloma cells is further complicated by the apparent molecular heterogeneity of these cells. Heterogeneity in gene expression between different multiple myeloma patients according to clinical and molecular variables has been examined in several studies that included different stages of the disease (15), B-cell development (16), the Ig type (17), and chromosomal aberrations (18). Multiple myeloma shows a marked genomic instability involving both numerical and structural chromosomal aberrations, making it difficult to identify the molecular mechanisms leading to classification of distinct disease entities, prognostic risk groups, and new targeted therapies (19). We focus

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our study on intratumoral plasma cell heterogeneity. One source of tumor heterogeneity is somatic mutations (20), whereas others are nongenetic in nature, driven either by external signals or by stochastic "transcriptional noise" or different gene methylation states causing silencing of numerous tumor suppressor and proapoptotic genes (21). For example, the variations in antibiotic-resistant bacterial populations are not acquired genetically because reintroduction of antibiotic treatment showed that the population selected by the treatment is as sensitive as the original population (22).

However, despite these studies, the origin and clinical significance of malignant plasma cell heterogeneity are still unclear. In this study, we have investigated the adhesive and migratory diversity of malignant plasma cells. For this purpose, we isolated and characterized subpopulations of plasma cells that display distinct adhesive properties. The adhesive ("type A") and poorly adhesive ("type F") populations were shown to differ also in their migratory capacity, binding to bone fragments, their integrin function, surface markers, as well as in their bone marrow homing capacity in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, suggesting relevance to disease dissemination. Examination of multiple myeloma patient-derived primary samples revealed similar diversity and indicated that two types of malignant plasma cells exist in distinct microenvironmental niches within the bone marrow. Characterization of these subpopulations of malignant plasma cells and elucidation of their clinical significance may facilitate the development of novel multiple myeloma therapeutics, targeting distinct populations of malignant plasma cells.

Materials and Methods

Cells. The plasma cell leukemia-derived ARH-77 cell line was kindly provided by Hanna Ben-Bassat (Hadassah Medical School, Jerusalem, Israel; refs. 23–26). AKR and CAG multiple myeloma cell lines were kindly provided by Michael Lishner (Oncogenetic Laboratories, Sapir Medical Center, Meir Hospital, Kfar Saba, Israel). Cells were cultured in RPMI 1640 supplemented with 1 mmol/L glutamine, 50 μg/mL streptomycin, 50 units/mL penicillin, and 20% heat-inactivated bovine serum (Biological Industries, Beit-Ha'Emek, Israel) at 37°C in a humidified incubator under atmosphere of 5% CO_2 and 95% air. Flow cytometric analysis indicated that the ARH-77 cells express CD138, CD45, and CD56 as well as the κ light chain (Fig. 1*A*).

Antibodies, enzymes, and chemicals. Anti-integrin antibodies, anti-α_5 inhibitory monoclonal antibody (mAb) 16 and anti-β_1 mAb 13 (27), anti-β_1 stimulatory 12G10 (28), and anti-α_5 mAb 11 (29), were kindly provided by Kenneth M. Yamada (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD). Anti-α_4 inhibitory mAb Ab764 (anti-CD49d) was from Immunotech (Marseille, France). Monoclonal mouse anti-paxillin antibody was from Transduction Laboratories (Lexington, CA). FITC-phalloidin was purchased from Sigma (St. Louis, MO). Cy3-conjugated goat anti-mouse antibodies were from The Jackson Laboratory (Bar Harbor, ME). FITC-conjugated anti-CD138, phycoerythrin (PE)-conjugated CD56, peridinin chlorophyll protein (PerCP)–conjugated CD45, allophycocyanin (APC)-conjugated CD38, and isotype control IgG1 were all purchased from DAKO (Glostrup, Denmark) and used according to the manufacturer's instructions. Heparinase I, chondroitinase ABC, and hyaluronidase were purchased from Sigma and used according to the manufacturer's instructions.

Fractionation of plasma cell line cells. ARH-77, CAG, and AKR cells were plated on fibronectin (15 μg/mL; Sigma-Aldrich Israel Ltd., Rehovot, Israel), which coated tissue culture plates from Corning, Inc. (Acton, MA). The fibronectin was incubated for 1 hour in 37°C and then washed twice with PBS. From the original tissue culture dish, the adherent and nonadherent cells were separated. Then, the adherent cells were repeatedly transferred for 6 weeks twice weekly into new dishes, discarding cells that did not adhere, until a stable, highly adhesive (type A) phenotype was established. The floating cells were repeatedly isolated from the medium of fibronectin-coated dishes and plated on new dish without transferring cells that adhered to the previous fibronectin-coated dish. The cells were repeatedly transferred for 6 weeks twice weekly until a stable, poorly adhesive (type F) phenotype was established. Immunofuorescence staining of the subpopulations was done as described (30).

Flow cytometry. For staining with directly labeled antibodies, 50 μL (5×10^5 cells) samples were incubated with 5 μL of each of the designated mAb for 30 minutes at 4°C followed by wash with 2 mL PBS. From each sample, 3×10^4 events were acquired by FACSCalibur at a rate of 150 to 300 events per second and analyzed by the CellQuest software (Becton Dickinson, San Jose, CA).

Cell adhesion assay. The separated enriched subpopulations were plated for 30 minutes on 5-cm bacterial dishes coated with fibronectin (15 μg/mL) for 30 minutes. The dishes were washed twice with PBS to remove nonattached cells, and the remaining cells were counted microscopically. To determine the integrin dependence of the adhesion, the cells were incubated with the integrin-modulating mAbs 16, 13, 764, and 12G10 at a concentration of 20 μg/mL for 30 minutes in room temperature before adhesion, and the rest of the assay was done as described above.

Motility assay. The separated enriched subpopulations were plated on glass bottom plates (MatTek Corp., Ashland, MA) coated with fibronectin (15 μg/mL). Phase-contrast time-lapse movies were taken. The cells were maintained at 37°C on the microscope stage in medium, buffered with 10 mmol/L HEPES (pH 7.0), and kept at 37°C. Images were acquired using the DeltaVision system for 30 minutes at 30-second frame intervals. The data were analyzed using an algorithm for cell tracking statistics developed in the laboratory of Z. Kam (Weizmann Institute of Science, Rehovot, Israel).

Plasma cell adhesion to human bone fragments. The enriched subpopulations were labeled each with a different fluorescent dye linked with long aliphatic tails into the lipid regions of the cell membrane. The fluorescent dyes PKH67 (green fluorescence; Sigma) or PKH26 (red fluorescence; Sigma; ref. 31) were used according to the manufacturer's instructions. Labeled cells (5×10^5) of each of the two subpopulation were mixed 1:1, suspended in 500 μL of growth medium, and incubated at 37°C for 5 hours with six bone fragments of human bones measuring approximately $0.5 \times 0.5 \times 0.5$ cm discarded from routine orthopedic procedures. Following incubation, the bone fragments were washed thrice with PBS and the attached cells were eluted mechanically using repeated needle aspirations and analyzed by flow cytometry. The use of the discarded human bone fragments was approved by the Institutional Review Board at Tel Aviv Sourasky Medical Center (Tel Aviv, Israel).

Homing experiments in NOD/SCID mice. ARH-77 cells are often used for *in vivo* models of multiple myeloma (32–34). Homing experiments were carried out in the Veterinary Resources Unit of the Weizmann Institute of Science and approved by the local Institutional Animal Care and Use Committee. Groups of six mice received tail vein injections of a 1:1 mix of a total of 2.5×10^6 of each subpopulation ARH-77 cells labeled with different fluorophores (PKH26 or PKH67) as described above and suspended in a total volume of 200 μL PBS. Mice were sacrificed 16 hours later, and cells were suspended from internal organs, including lungs, liver, spleen, and bone marrow, and analyzed by flow cytometry. Each experiment was repeated two to three times.

Morphologic evaluation of multiple myeloma clinical samples. Bone marrow aspirates of multiple myeloma patients were subjected to routine morphologic evaluation (35) or labeled with a panel of four membrane markers (CD38, CD45, CD56, and CD138) and analyzed as described above. Samples were also stained for CD117 (the stem cell factor receptor c-kit) as an aberrant marker. Some of the bone marrow samples were sheared mechanically with repeated passages through 21-gauge needle or treated with the ECM-degrading enzymes [hyaluronidase (1 mg/mL), heparinase I (1 unit/mL), or chondroitinase ABC (0.2 unit/mL)] for 1 hour at 37°C according to the manufacturer's instructions. The use of discarded human bone marrow aspirates for this study was approved by the Institutional Review Board of Tel Aviv Sourasky Medical Center.

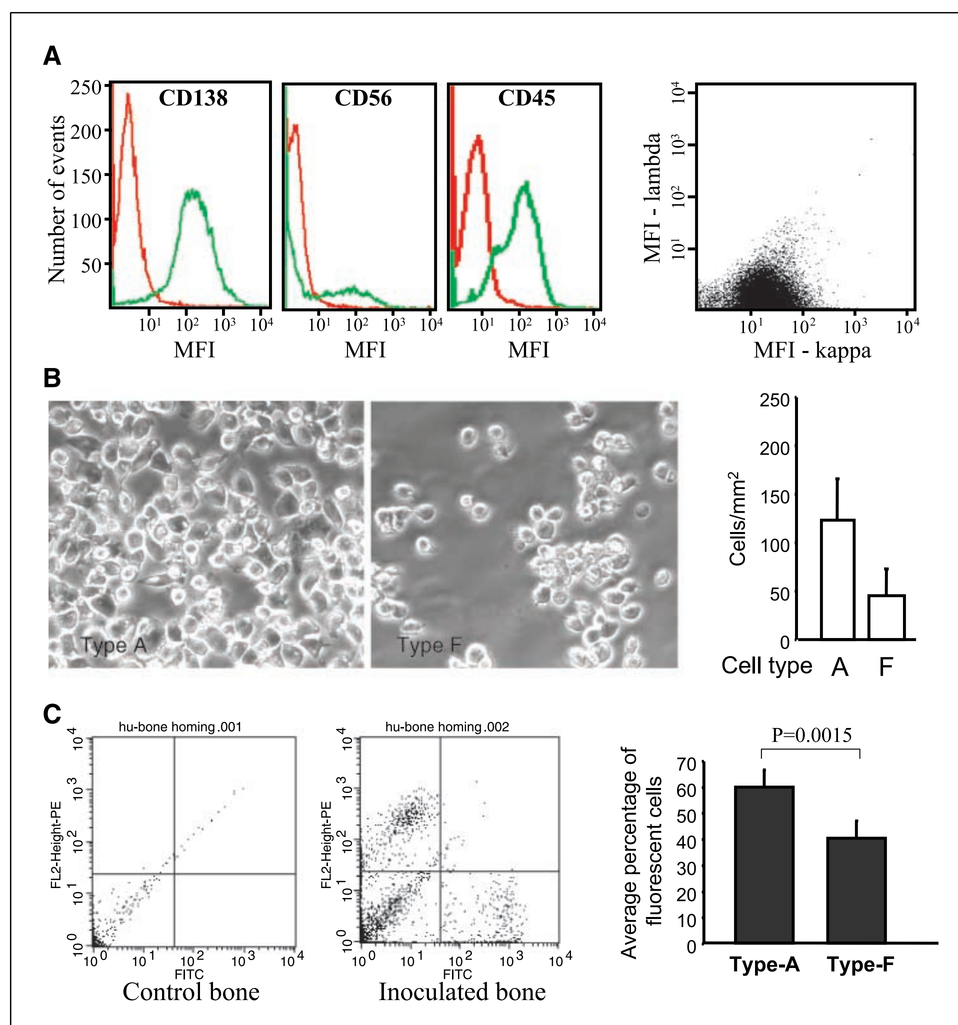

Figure 1. Isolation of adhesive variants from the ARH-77 plasma cell line. *A,* flow cytometric analysis of ARH-77 cells. Parental ARH-77 cells were labeled (*left, green lines*) for CD138 (FITC conjugated), CD56 (PE conjugated), CD45 (PerCP conjugated), or isotype controls (*left, red lines*). Alternatively, the cells were labeled (*right*) for κ light chain (FITC conjugated) and λ light chain (PE conjugated). The cells were then subjected to flow cytometric analysis. *MFI,* mean fluorescent intensity. *B,* morphologic culture presentation of plasma cell variants. *Left,* routinely passaged highly adhesive type A cells or poorly adhesive type F cells. To assess adhesion of plasma cell adhesive variants to fibronectin, type A and type F subpopulations were plated on culture dishes coated with fibronectin. After 30 minutes, the dishes were washed, 20 images were taken from the dishes, and the cells that remained after washing were counted. *Columns,* average of five independent experiments; *bars,* SD. *C,* human bone population by plasma cell adhesive variants. The two plasma cell subpopulations were labeled with the PKH67 (green fluorescence, FITC channel) or PKH26 (red fluorescence, PE channel), mixed 1:1, and incubated at 37°C for 5 hours with fragments of human bones measuring approximately 0.5 × 0.5 × 0.5 cm. Following incubation, the bones were washed and the extracted cells were analyzed by flow cytometry. *Left,* type A subpopulation attached significantly better to the bone fragments compared with type F subpopulation. *Columns,* average of five different bone fragments; *bars,* SD. One of two representative experiments.

Results

Isolation and characterization of malignant plasma cell subpopulations. ARH-77 cells, tested by flow cytometry, display heterogeneous antigen expression patterns, including variability in CD45 expression, distinct subpopulations with high and low CD56 levels, and relatively wide range of CD138 expression (Fig. 1*A*). To isolate specific subpopulations, parental ARH-77 cells were plated onto fibronectin-coated tissue culture dishes. Three days later, nonadherent cells were collected from the culture supernatant and inoculated into fibronectin-coated dishes as poorly adherent culture. The adherent cells were removed from the dishes by trypsin/EDTA treatment for few minutes and then transferred into fibronectin-coated dishes to establish a highly adherent culture. Highly adhesive and poorly adhesive subpopulations were established from the ARH-77 cell line by consecutive passages (twice weekly) on fibronectin as described above for 6 weeks. Following the isolation, stable cell populations from a frozen stock were maintained routinely under continuous selective conditions as in the original selection procedure for 1 month. As shown in Fig. 1*B*, the isolation procedure yielded two relatively stable populations: one highly adhesive to fibronectin-coated tissue culture dishes or coverslips (designated type A) and the other consisting of floating, poorly adherent cells (designated type F). The two subpopulations maintained similar surface levels of the Igκ chain as determined by flow cytometry (data not shown). Both subpopulations maintained their different adhesive capacities on fibronectin following *in vitro* passages (Fig. 1*B*). To test the adhesive interactions of the isolated adhesive variants with physiologic relevant substrates, the cells were first labeled with two different fluorescent dyes as described in Materials and Methods. Then, a total of 1×10^6 cells, a 1:1 mixture of labeled type A and type F subpopulations, was incubated with fresh human bone fragments for 5 hours. As shown in Fig. 1*C*, type A cells attached significantly better to the bone fragments compared with type F cells, indicating that the different adhesive properties of the subpopulations apply also to the physiologic bone marrow microenvironment.

Immunophenotyping of the plasma cell adhesive variants. The separated subpopulations of ARH-77 cells were labeled for some of the surface markers known to characterize multiple myeloma cells (i.e., CD38, CD138, CD45, and CD56). As shown in Fig. 2*A*, type A cells expressed higher levels of CD45 and CD56 compared with type F. In contrast, type F cells were highly positive for CD138 (Fig. 2*A*). Both ARH-77 subpopulations expressed similarly low levels of CD38. This experiment indicates that flow cytometric analysis may distinguish between these

multiple myeloma adhesive variants. As shown in Fig. 1*B*, type A subpopulation contained some nonadhesive cells and some of type F cells attached and spread transiently to the fibronectin-coated surface. However, the transient adhesive variant cells in each subpopulation maintained the surface markers of the parental subpopulations, indicating that each separated subpopulation was indeed free of cellular contaminants of the other one (Fig. 2*A*).

Subcellular organization of cytoskeletal and adhesive structures in plasma cell adhesive variants. Type A and type F cells were plated on two different substrates relevant to plasma cell physiologic adhesion: fibronectin and hyaluronan. The cells were then labeled for their actin cytoskeleton and for the focal adhesion molecule paxillin. As shown in Fig. 2*B*, type A cells readily spread on fibronectin, forming paxillin-rich adhesions, in the absence of

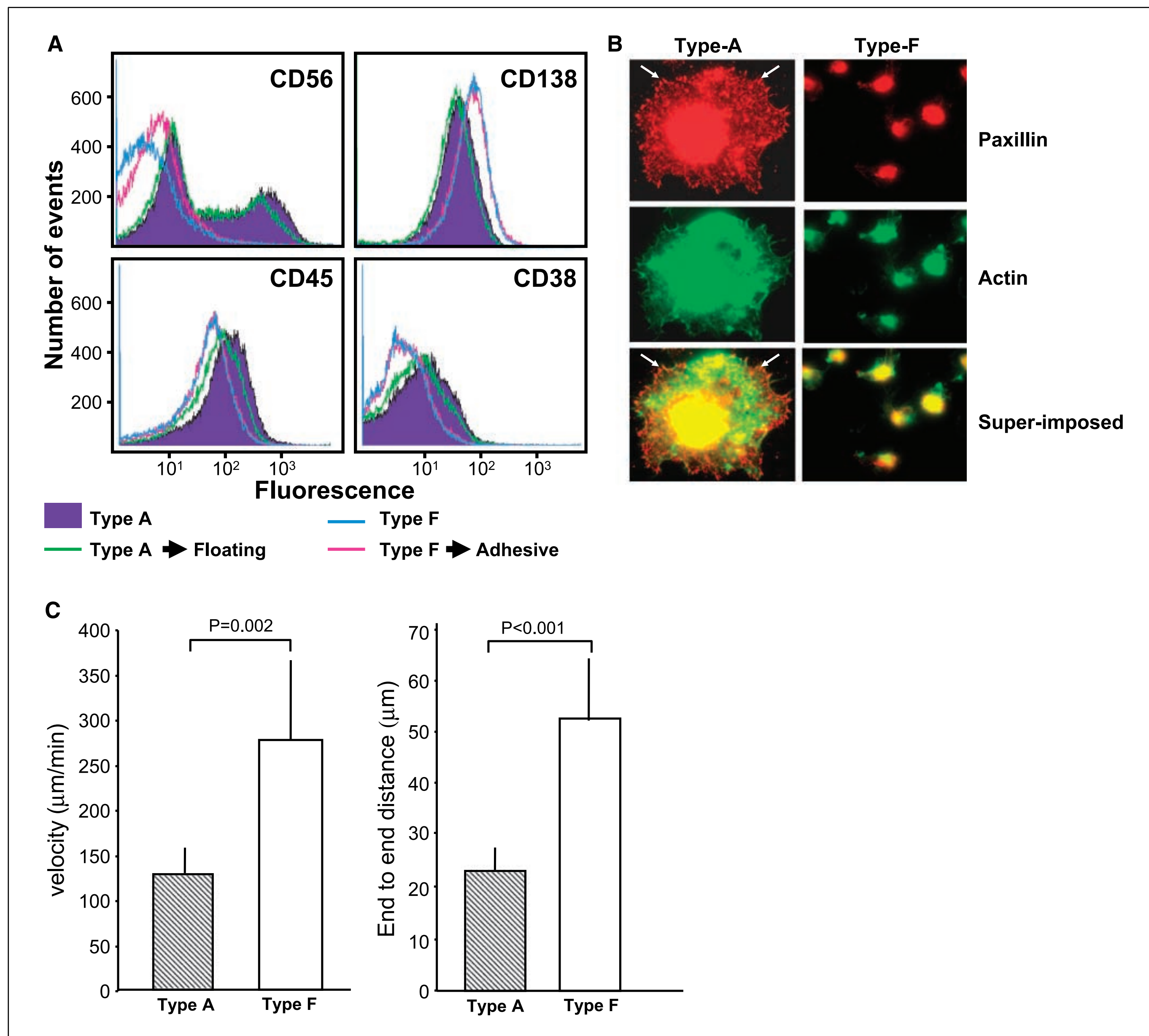

Figure 2. Flow cytometric, cytoskeletal, and motility characterization of plasma cell adhesive variants. *A,* flow cytometric analysis of type A and type F subpopulations as well as of the transient variant of each subpopulation. The cells were stained for CD138 (FITC conjugated), CD56 (PE conjugated), CD45 (PerCP conjugated), and CD38 (APC conjugated). Type A subpopulation expressed higher levels of CD45 and CD56 compared with type F subpopulation. In contrast, type F cells were highly positive for CD138. The transient variants of each subpopulation maintained the surface markers of the parental subpopulation. *B,* type A and type F differ in their cytoskeletal organization. Type A (*left*) and type F (*right*) subpopulations were plated on coverslips coated with fibronectin, then fixed, and permeabilized. The cells were then stained with mouse anti-paxillin mAb followed by Cy3-conjugated anti-mouse (*red, top*) and FITC-conjugated phalloidin (*green, middle*). *Bottom,* double-labeled images were also recorded. Type A cells (*left*) spread on fibronectin, and paxillin is organized in focal adhesion-like structures (*arrows*). In contrast, type F subpopulation (*right*) did not spread and the paxillin was not organized in adhesive structures. *C, left, in vitro* velocity of plasma cell adhesive variants. Type A and type F subpopulations were plated on culture dishes coated with fibronectin for 3 hours. Thirty-minute movies (30-second frame intervals) of the two subpopulations were then recorded. The velocity of the type F cells on fibronectin was significantly higher compared with type A cells. *Right,* end-to-end migration distance of plasma cell adhesive variants on fibronectin. The difference between the subpopulations in end-to-end distance was significantly higher than the velocity difference especially on fibronectin, indicating that the movement of the type F cells is faster and directional. *Columns,* average of three independent experiments; *bars,* SD.

actin stress fibers. In contrast, type F cells did not spread and paxillin was not organized in adhesive structures. Cells of both subpopulations did not organize adhesive structures when plated on hyaluronan (data not shown).

The migratory activity of plasma cell adhesive variants. To elucidate their motile phenotype, the two subpopulations were plated on fibronectin or hyaluronan and time-lapse movies (30 minutes total, 30-second frame intervals) were recorded. The velocity and end-to-end distance (i.e., the distance in microns between the initial and final positions of the cell) of the cells were recorded and analyzed. As shown in Fig. 2*C*, the velocity of type F cells on fibronectin was considerably higher (~2-fold) compared with that of type A cells. When the end-to-end distance was measured, the difference between the subpopulations was significantly higher than the velocity, indicating that the movement of type F cells is both fast and persistent (Fig. 2*C*). Altogether, the data suggest that type F cells are highly motile on fibronectin and that both subpopulations move more slowly on hyaluronan-coated surfaces (data not shown).

Separated type A and type F subpopulations rediversify on cultivation under nonselective conditions. The presence of phenotypically distinct subpopulations of cells within a tumor could be attributed to either genetic variations caused by mutations, to differentiation processes within the parental cell population, or to the presence of a "noisy phenotype" (e.g., variations in gene expression profile). To explore these possibilities, we have tested the long-term stability of our multiple myeloma adhesive variants by allowing the two subpopulations to grow under nonselective conditions (i.e., without applying the adhesive selective conditions). Examination of cultures of type A and type F variants indicated that, initially (within the first 2-3 weeks), the original phenotypes were well preserved. However, on longer incubation, both cultures displayed an increasing heterogeneity. Following incubation for 6 weeks, the two cultures regained heterogeneous adhesive properties indistinguishable from those of the parental ARH-77 cells (Fig. 3). Moreover, the CD138 expression profiles of these two cultures were distinctly different from those of the purified type A and type F cells and similar to that of the parental ARH-77 line. These results show that the acquisition of diverse adhesive phenotype is reversible and, thus, nongenetic in nature (see Discussion).

Adhesion mechanisms of plasma cell adhesive variants. To elucidate the molecular mechanisms underlying the differential adhesive behavior of the two subpopulations, we have examined the levels of different integrins present at their surface. As shown in Fig. 4*A*, there were no profound differences between type A and type F cells in the surface expression levels of α_4 (small difference, not statistically significant), α_5, α_V, β_1, and β_7 integrins, all of which were reported to be involved in multiple myeloma cell adhesion. The finding that the major fibronectin receptor (i.e., $\alpha_5\beta_1$) is similarly expressed on the surface of type A and type F cells was surprising, in view of the remarkable differences between the adhesion of the two cell types to fibronectin. To functionally assess integrin involvement in the adhesion of these cells, integrin-modulating antibodies were incubated with the cells before adhesion assays. Adhesion to fibronectin of type F cells was rather weak as shown above, yet it was significantly suppressed by the inhibitory antibodies against either α_4, α_5, or β_1 integrin (Fig. 4*B*). Furthermore, the adhesion of type F cells to fibronectin was enhanced by the β_1 integrin-activating antibody 12G10 (Fig. 4*B*). As shown in Fig. 4*B*, the integrin-modulating antibodies affected type A subpopulation to a much lesser extent. As expected, integrin-modulating antibodies did not affect significantly cell adhesion to hyaluronan, indicating specific activity (data not shown). Our data indicate that integrins are predominantly involved in cell adhesion of type F subpopulation, although their surface expression level is similar. Adhesion of type A cells to fibronectin may also depend on other integrin-independent adhesion mechanisms.

The *in vivo* dissemination of type A and type F cells in NOD/SCID mice. The *in vivo* behavior of the ARH-77 adhesive variants was examined by their i.v. inoculation into NOD/SCID mice followed by measurement of homing to the bone marrow, spleen, lung, and liver. The two subpopulations were labeled by fluorescent dyes as described in Materials and Methods. A total of 5×10^6 labeled cells, consisting of a 1:1 mixture of type A and type F cells, was inoculated into the tail vain of NOD/SCID mice. Sixteen hours later, the presence of labeled human plasma cells in the bone marrow, spleen, liver, and lung was examined using flow cytometric analysis. Type F cells populated preferentially the bone marrow (Fig. 5*A*) and spleen (data not shown) of the mice with

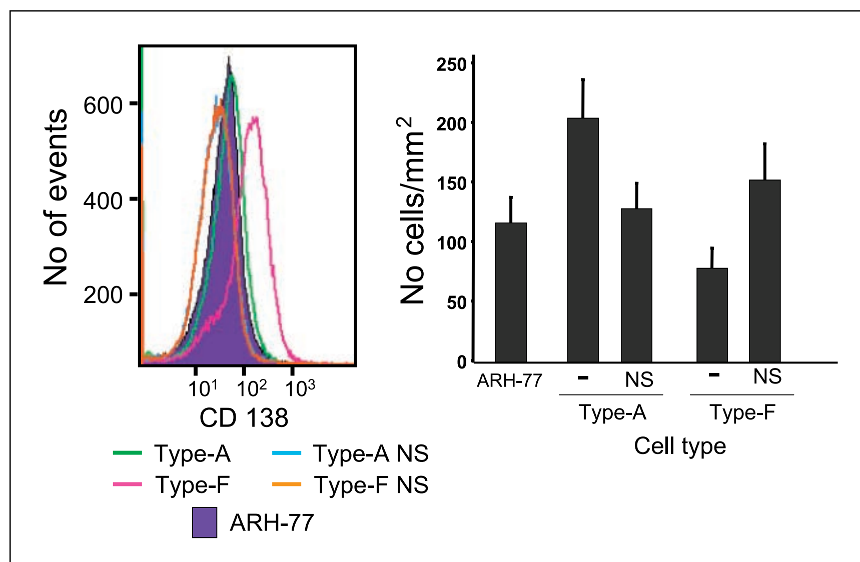

Figure 3. The maintenance of divergent adhesive phenotype depends on growth under continuous selective conditions. Type A and type F subpopulations were grown without applying the adhesive selective conditions (*NS*) for 6 weeks, and then CD138 levels (*left*, flow cytometric analysis) and adhesion to fibronectin (*right*) were compared with selected type A and type F cells as well as parental ARH-77 cells.

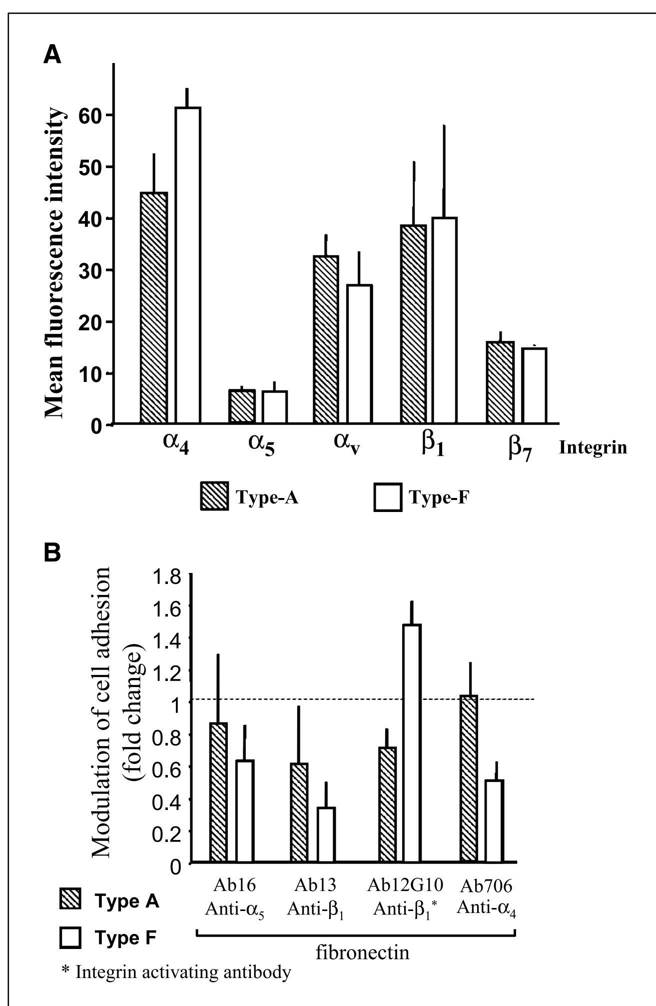

Figure 4. Integrin expression and activity in plasma cell adhesive variants. *A,* flow cytometric analysis of cell surface integrin expression. Plasma cell variant cells were stained for α_4, α_5, α_V, β_1, and β_7 integrins and measured by flow cytometry. *Columns,* mean fluorescence of two to four measurements; *bars,* SD. There were no statistically significant differences in the surface expression of the α_4, α_5, α_V, β_1, and β_7 integrins between the two adhesive variants. *B,* modulation of plasma cell adhesion by anti-integrin antibodies. Integrin-modulating antibodies were incubated with type A and type F subpopulations before adhesion assays as described in Materials and Methods. *Columns,* average of three independent experiments; *bars,* SD. Adhesion of type F subpopulation to fibronectin was significantly inhibited by the inhibitory antibodies against α_4 and α_5 and even more by the anti-β_1 integrin antibody and was significantly enhanced by the anti-β_1 integrin-activating antibody 12G10. Type A subpopulation was affected by the integrin-modulating antibodies to a much lesser extent.

only few cells detected in the liver (Fig. 5*A*) and lungs (data not shown). In contrast, type A cells were hardly detected in any of these organs (Fig. 5*A*). These data suggest that the targeting of the malignant plasma cells to the hematopoietic organs is primarily promoted by the increased motile activity of the cells rather than by their adhesive properties. In view of our *in vitro* adhesion assays, which pointed to the β_1 integrin-dependent adhesion of type F cells, we tested whether integrin modulation by inhibitory antibodies affects the dissemination of type F cells into the bone marrow. NOD/SCID mice were i.v. inoculated with 5×10^6 labeled cells, a green/red-labeled 1:1 mixture of untreated type F cells, and type F cells preincubated with inhibitory anti-β_1 integrin antibody. Sixteen hours later, the mice were sacrificed and the presence of green- and red-labeled human plasma cells was tested in the bone marrow, spleen, liver, and lung. As shown in Fig. 5*B*, treatment with anti-β_1 integrin antibody significantly reduced the dissemination of type F cells into the bone marrow.

Presence of type A and type F–like cells in other multiple myeloma cell lines and patient samples. To examine the segregation of subpopulations in multiple myeloma cell lines, we applied the adhesion enrichment procedures on the CAG and AKR multiple myeloma cell lines as described above. As shown in Fig. 6*A*, highly adhesive subpopulations of both CAG and ARK lines exhibited significantly lower CD138 levels compared with their low-adhesive/floating counterpart. Multiple myeloma cells from patient samples were examined by bone marrow morphology and flow cytometry. In a previous study, we have shown that bone marrow aspirates contain two distinct fractions of multiple myeloma cells: adhesive cells associated with the solid phase contained in the lipid-enriched spicules and their adjacent microenvironment and low-adhesive cells that reside within the fluid fraction of the bone marrow aspirate (36). To determine the flow cytometric profile of the cells in these two fractions, we have isolated bone marrow–derived spicules from aspirates of multiple myeloma patients and either sheared them mechanically with repeated passages through 21-gauge needle or treated with a cocktail of three ECM-degrading enzymes (heparinase I, chondroitinase ABC, and hyaluronidase) followed by mechanical shearing. Only a combination of shear force and ECM-degrading enzymes released the highly adhesive plasma cells from the spicules. Interestingly, the released malignant plasma cells displayed a profile very similar to type A subpopulation isolated *in vitro* with respect to its CD138 expression (Fig. 6*B*). These highly adhesive cells retained their overall multiple myeloma marker profile, including CD38 (Fig. 6*B*), and aberrant markers, such as CD117 (c-kit, aberrantly appear on some multiple myeloma cells; data not shown), and were identified morphologically as intact plasma cells. The lower levels of CD138 expression in the multiple myeloma cells extracted from the spicules were not due to the extraction procedures because these did not affect CD138 levels when applied to ARH-77 cells.

Discussion

Despite the clonal origin of most cancers, a notable characteristic feature of many primary tumors is a marked degree of cellular heterogeneity, which can be manifested at several levels. Genomic instability was documented in many cancers with one notable example in colorectal cancer, where intratumor genomic heterogeneity is generated by the accumulation of mutations during tumor progression (37). Microenvironmental conditions and clinical interventions continuously select tumor cell variants that have the capacity to proliferate and evade host defense and therapeutics (38). Biological and phenotypic variance can be found in tumors not just based on genetic alterations but also reflecting nongenetic heterogeneity (39). Epigenetic alterations (e.g., gene promoter methylation status) are considered a key factor in the generation of intratumor diversity in head and neck cancers (40) and may even obstruct cancer therapeutics (41).

Multiple myeloma is characterized by a remarkable heterogeneity in plasma cell characteristics, including morphology, maturation status, immunophenotyping, and genetic abnormalities, which are partly account for the variable disease outcome (42). The complex genetic alterations in multiple myeloma include translocations of the Ig heavy chain gene locus, which result in dysregulation of

various cellular proteins and deletions of 13q14 chromosome (43). Although genetic alterations in multiple myeloma have been studied extensively, recent studies indicate that epigenetic mechanisms may affect the physiology of plasma cells (43). Although nongenetic regulation of multiple myeloma cells by cytokines is well documented (21, 44), the role of microenvironmental adhesive interactions in generation of malignant plasma cell heterogeneity is still unknown.

In this study, we report a phenotypic diversity within a plasma cell line, manifested by major differences in cell adhesion and migration, linked to *in vivo* homing capacities and expression of cell surface markers (e.g., CD138). Multiple myeloma cell lines CAG and ARK also exhibited significantly lower CD138 levels in the adhesive cells compared with their low-adhesive/floating counterpart.

This diversity may have notable physiologic relevance because careful examination of bone marrow samples from multiple myeloma patients reveals similar heterogeneity in the expression of the same surface markers, predominantly CD138. Although we have no direct evidence about how this diversity was generated and how it is stably maintained, it seems likely that the process is attributable to nongenetic phenotypic diversification. This view is supported by the fact that, on culturing each of the separated cell populations (type A and type F) under nonselective conditions, rediversification of cells takes place, producing populations of cells that are similar to the parental ARH-77 line. This observation indicated that the transformation between adhesive phenotypes is bidirectional. Whether these transitions are driven by specific external cues or are generated due to a stochastic noise in the transcriptional program is still not clear. Yet, it seems that both states are sufficiently robust so that the two adhesive variants are stably expressed in comparable proportions under different growth conditions both *in vitro* and *in vivo*.

The basic phenotypic characterization of the two subpopulations of plasma cells sheds light on the mechanism of their adhesion, migration, and bone marrow homing. We show here that the strong adhesion of type A cells to fibronectin is associated with low migration capacity, and these cells poorly migrate to the bone marrow or other organs after i.v. injection. The generation and low turnover of adhesive structures (e.g., focal adhesions) are often associated with reduced cell motility (45). Type F cells, on the other hand, bind weakly to fibronectin via $\alpha_5\beta_1$ and α_4 integrin and readily home to the bone marrow. Although we have identified highly motile cells within the type F subpopulation, one cannot exclude that other cells, which belong to the same subpopulation, may account for its *in vivo* dissemination capacity. Interestingly, both cell types express similar integrin profile, including $\alpha_5\beta_1$. This observation suggests that the adhesion of plasma cells to fibronectin or bone fragments is not necessarily the primary factor driving bone marrow colonization *in vivo* but, apparently, increased motile capacity. The high motility rates of type F cells are driven by intrinsic mechanism independent of extracellular stimulus (e.g., SDF-1 and interleukin-6). Moreover, the adhesion of type A cells to fibronectin was less affected by $\alpha_5\beta_1$ inhibitory antibodies, whereas the weak but significant binding of type F cells to the same surfaces was effectively blocked by anti-$\alpha_5\beta_1$ and α_4 and further modulated by activating anti-β_1 antibody. These data indicate that type A plasma cells may depend on nonintegrin mechanisms for their adhesion to the substrate, and indeed, we found that carbohydrate-degrading enzymes can release highly adherent malignant plasma cells from the bone marrow spicules.

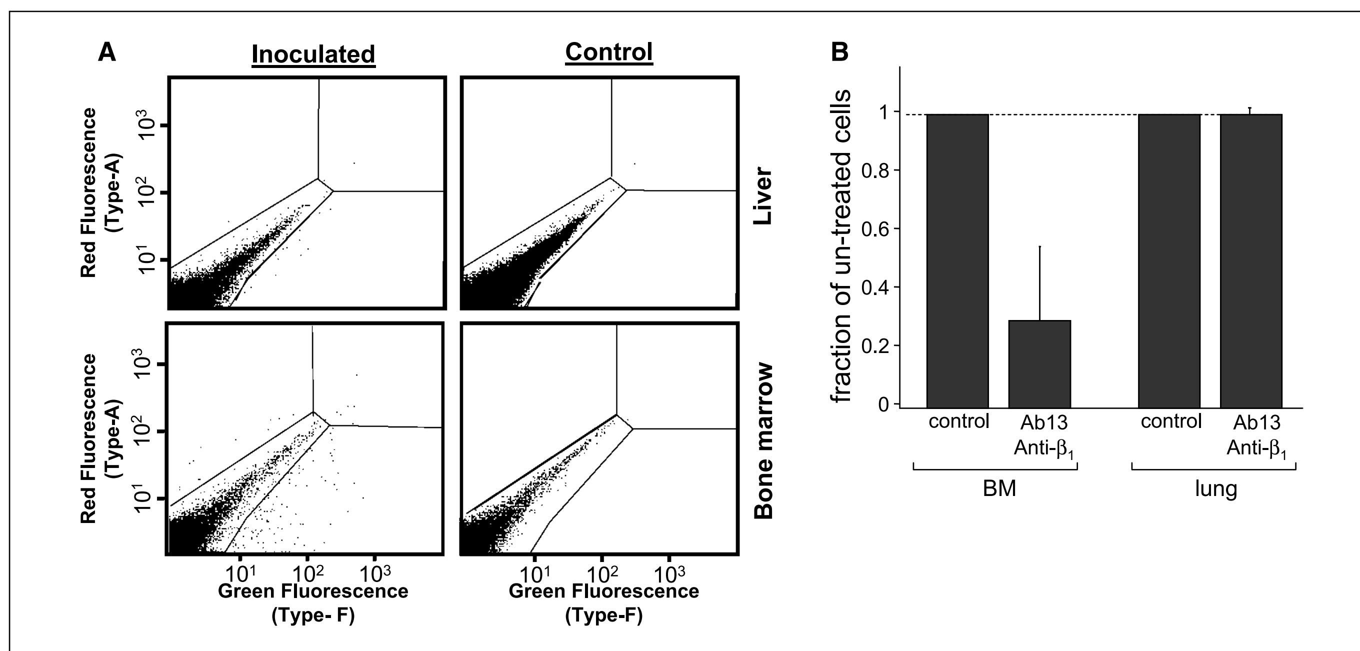

Figure 5. *In vivo* homing of plasma cell adhesive variants. *A,* homing of the plasma cell adhesive variants in NOD/SCID mice. The enriched subpopulations were labeled with the PKH67 (green fluorescence) or PKH26 (red fluorescence), mixed 1:1 in a total sum of 5×10^6, and injected to the tail vein of NOD/SCID mice. Sixteen hours later, the mice were sacrificed and the presence of human plasma cells was tested in the bone marrow and liver using flow cytometric analysis. Type F cells populated specifically to the bone marrow, and only few cells were detected in the liver. In contrast, type A subpopulation was hardly detected in any of the organs. Results are a representative experiment of three. Each experiment included at least five mice per group. *B,* modulation of plasma cell dissemination by anti-β_1 integrin antibody. Type F control cells (labeled with PKH67, green fluorescence) and type F cells preincubated with anti-β_1 integrin inhibitory antibody 13 (*Ab13*; labeled with PKH26, red fluorescence) were mixed 1:1 in a total sum of 5×10^6 and injected to the tail vein of NOD/SCID mice. Sixteen hours later, the mice were sacrificed and the presence of human plasma cells was tested in the bone marrow and lung using flow cytometric analysis. Note that the anti-β_1 integrin antibody treatment significantly inhibited the dissemination of type F cells only to the bone marrow.

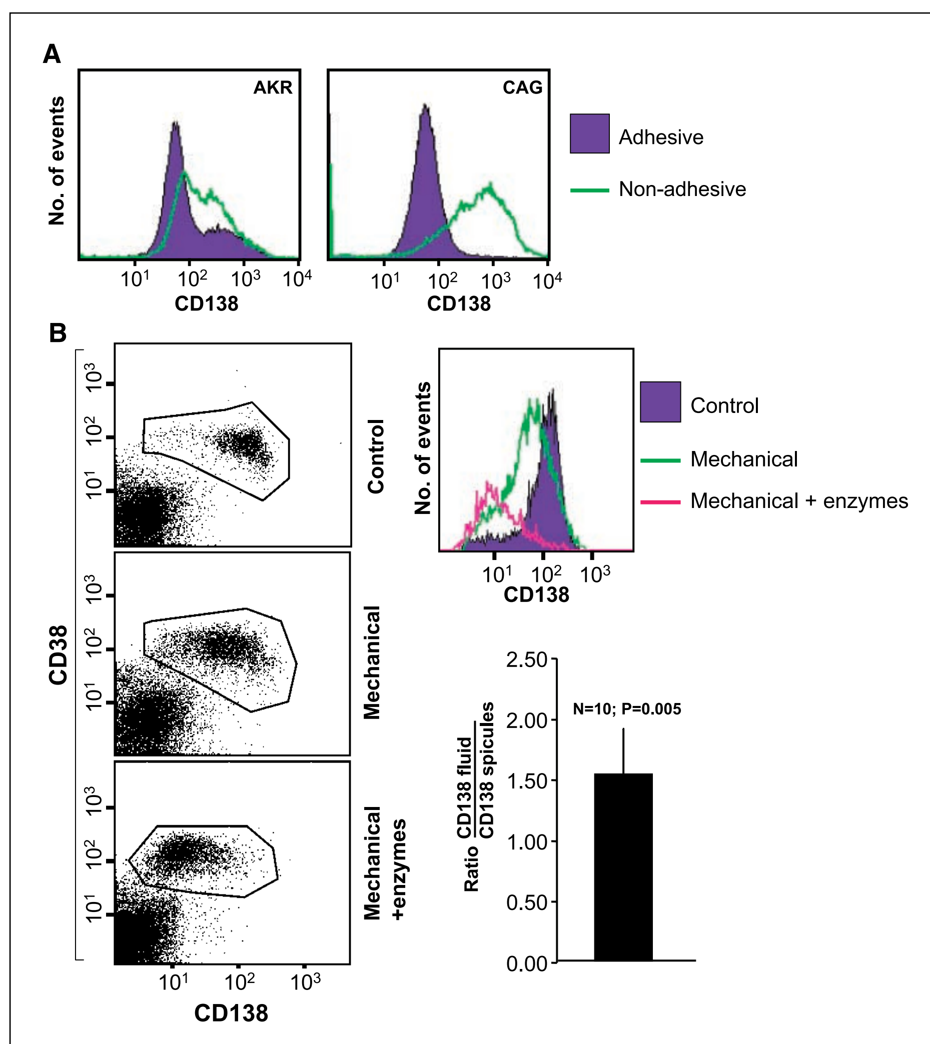

Figure 6. Adhesive variants in multiple myeloma cell lines and primary multiple myeloma samples. *A,* CD138 expression in subpopulations of multiple myeloma cell lines. CAG and AKR multiple myeloma cell lines were subjected to adhesion-based selection of subpopulations in a similar manner to the ARH-77 cells. Following 6 weeks of growth under selection conditions, the cells were harvested and subjected to flow cytometric analysis. *B,* characterization of patient-derived multiple myeloma cells. A bone marrow aspirate was obtained from a newly diagnosed multiple myeloma patient. *Right* and *left,* flow cytometry analysis of the malignant plasma cells following different cell extraction techniques. Note the decline in CD138 expression when the cells were mechanically extracted from the bone marrow spicule and even more pronounced decline when the spicules were treated with a mixture of ECM-degrading enzymes (hyaluronidase + heparinase I + chondroitinase ABC). Note the equal level of CD38 expression in all samples. *Bottom right,* average ratio of CD138 expression (mean fluorescence intensity) between the adhesive (spicule derived) or floating plasma cells derived from the same bone marrow aspirates of 10 independent multiple myeloma patients.

Alternatively, other integrins, unidentified yet, may account for type A cell adhesion to fibronectin.

We found that adhesive plasma cells, both *in vitro*–derived cells from one plasma cell line, two multiple myeloma cell lines, and cells extracted from patient bone marrow spicules express significantly less CD138. Variability in CD138 expression has been described in a recent report that questioned the role of clonogenic B cells in the peripheral blood and bone marrow of multiple myeloma patients (46). It was found that these cells are highly proliferative, contrary to multiple myeloma plasma cells known to have low proliferate index (47), and they lack CD138 expression that makes them a putative multiple myeloma stem cell (46). In our study, we found that both subpopulations are morphologically plasma cells but type A cells express lower CD138 levels compared with type F cells. The high levels of CD138 expression in type F cells *in vitro* and in the low-adherent multiple myeloma cells in the patient bone marrow are in line with previous studies, where it has been shown that, in polarized, highly motile primary multiple myeloma cells, CD138 is localized specifically on their uropod surface (48).

In summary, we found close correlation between the adhesive phenotype of plasma cells, both *in vitro* and from patient samples, and CD138 expression levels. Moreover, CD138 levels on type A and type F subpopulations were modulated in parallel to the change of their adhesive properties during growth under nonselective conditions. These data point either to causal link between CD138 and plasma cell adhesion or, at least, to a tight regulatory connection between these two characteristics. Interestingly, it was recently found that highly adhesive subpopulation of the U266 and MCC-2 multiple myeloma cell lines (49) and primary multiple myeloma cells after coculturing with osteoclasts (50) reduced the levels of CD138. This indicates that CD138 may not only serve as a general marker of plasma cells but can rather identify specific subpopulations of plasma cells according to its level of expression.

Our study points to a key process that may account for malignant plasma cell heterogeneity. The cells segregate into two predominant microenvironments within the bone marrow: a fluid compartment within the sinusoids and a variety of solid support surfaces, which include spicules and bone surfaces. The generation of diversity within multiple myeloma cell populations may be therefore continuously affected by the distinct microenvironments within the bone marrow via nongenetic mechanisms. This process yields at least two types of malignant plasma cells, which differ fundamentally in a variety of properties. Therefore, the development of effective novel anti–multiple myeloma therapy may require the understanding and managing of at least two major types of

malignant plasma cells, which are driven by fundamentally different biochemical machineries.

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