

A Unique Pathway in the Homing of Murine Multiple Myeloma Cells: CD44v10 Mediates Binding to Bone Marrow Endothelium¹

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Abstract

Our group recently reported that multiple myeloma (MM) cells preferentially adhere to bone marrow (BM) endothelial cells and selectively home to the BM, suggesting the involvement of specific adhesive interactions in this process. The highly regulated expression of CD44 variant isoforms (CD44v) on the MM cells makes them good candidate adhesion molecules involved in this homing. We addressed this in the 5T experimental mouse model of myeloma. Fluorescence-activated cell sorting analysis demonstrated expression of CD44v6, CD44v7, and CD44v10 on the *in vivo* growing 5T2MM and 5T33MM myeloma lines. Antibody blocking experiments revealed the involvement of CD44v10 in the adhesion of 5T2MM and 5T33MM cells to BM endothelial cells. Coinjection of anti-CD44v10 antibodies with the myeloma cells into syngeneic mice demonstrated a selective blocking of their BM homing which resulted in a decreased BM tumor load and serum paraprotein at the end stage of the disease.

The highly restricted expression of CD44v10 on MM cells, the blocking of MM adhesion to BM endothelial cells and of homing to BM by anti-CD44v10, and the decreased BM tumor load suggest that myeloma cells home to the BM via interactions mediated by this specific region of the adhesion molecule CD44.

Introduction

MM³ is an incurable plasma cell malignancy specifically localized in the BM, in which the cancer cells produce high amounts of monoclonal immunoglobulins and induce osteolytic bone lesions by the activation of osteoclasts. Because MM cells have a post-germinal origin (1) and are diffusely spread throughout the whole BM (re)circulation and (re)entrance of the cells into (distant) BM sites is necessary. The migration of the MM cells into the extravascular compartment of the BM is referred to as “homing.” This homing of MM cells into the BM remains a poorly understood process. Knowledge of the mechanisms involved in this process is important for the development of new therapeutical strategies for this disease. In analogy to the homing of lymphocytes (2), it is assumed that the homing of MM cells is a multistep process. Initially, there is a reversible rolling on the vascular endothelium, which is proceeded by an activation-dependent firm adhesion and a subsequent transendothelial migration. Adhesion molecules and chemoattractants are the key molecules involved in this process. In different organs, different adhesion molecules and chemotactic factors are involved, making this homing highly organ specific.

CD44 is a family of adhesion molecules known to be involved in many processes, including extravasation of lymphocytes (3, 4) and cancer cell metastasis (5–7). The many isoforms of these cell surface glycoproteins are encoded by a single gene that consists of standard exons (1s to 10s) and variant exons (1v to 10v). The standard exons encode for the common part of the CD44 family members (CD44s, which is widely distributed), and the variant exons are alternatively spliced, giving rise to the different members of the family (8). The expression of the individual CD44 splice variants (CD44v) is highly restricted and is correlated with specific processes, such as leukocyte activation and malignant transformation (9). Although the expression of CD44v isoforms on human (10, 11) and murine 5T myeloma (12) cells has been reported, little is known about their specific functions in the biology of this malignancy. We recently analyzed the involvement of CD44v in the adhesive interactions between 5TMM cells and BM stromal cells and found the involvement of CD44v6 (12). *In vivo* up-regulation of this variant conferred adhesion to one of the 5TMM cell lines, which was initially CD44v6 negative (12).

As previously reported by our group, MM cells selectively home to the BM (13). We also showed that this selective homing is preceded by a preferential adhesion to BM endothelial cells (13). This restricted adhesion pattern implies the involvement of specific adhesion molecules in the interaction between MM cells and BM endothelial cells. The restricted expression of CD44v on myeloma cells (10, 11) makes them good candidate adhesion molecules involved in this process.

We herein analyzed the involvement of CD44v in the homing of MM cells to the BM in the 5T experimental mouse model of myeloma. The clinical, biological, and genetic characteristics of this model are similar to the human disease, and it is used as a model for human MM by several groups, including ours (14). We demonstrate in this report the involvement of CD44v10 in the adhesion of 5TMM cells to BM endothelial cells. In addition, CD44v10 monoclonal antibodies were able to inhibit the *in vivo* homing of 5TMM cells to the BM. This decreased BM homing resulted in decreased tumor load and serum immunoglobulin levels at the end stage of the disease.

Materials and Methods

Animals. C57BL/KalwRij mice were purchased from Harlan CPB (the Netherlands). Male mice were 6–10 weeks old when used. They were housed under conventional conditions and had free access to tap water and food. They were killed by CO₂ asphyxiation.

Myeloma Lines. The *in vivo* growing cell lines 5T2MM and 5T33MM (5T33MMvv) originate from elderly C57BL/KalwRij mice that spontaneously developed MM (15). Although the 5T2MM represents a model situation of the most common forms of human MM, with a moderate growth, the 5T33MM is a more aggressive form, with a rapid progress of the disease (16). These MM lines have been expanded into young syngeneic recipients by i.v. transfer of the diseased BM. Progression of MM in diseased animals was followed up by electrophoretic quantification of serum paraprotein (monoclonal immunoglobulins; 16). Animals were killed when a paraprotein concentration of 10 mg/ml was reached. MM cells were isolated and purified from the BM as

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³ The abbreviations used are: MM, multiple myeloma; BM, bone marrow; FACS, fluorescence-activated cell sorter/sorting.

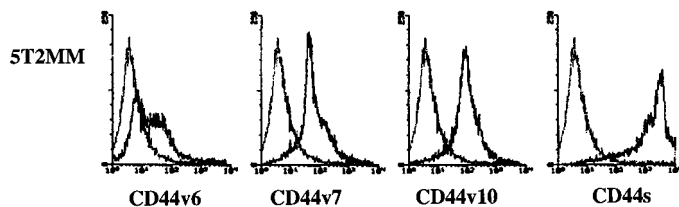


Fig. 1. Expression of CD44v and panCD44 on 5T2MM cells. BM cells were isolated from terminally diseased 5T2MM-bearing animals, purified for MM cells and stained with biotinylated anti-CD44(v) antibodies (12). 5T2MM cells expressed CD44v6, CD44v7, CD44v10, and CD44s isoforms. 5T33MM cells had a similar expression pattern (not shown, not for v6, when grown *in vitro*). Histograms from one experiment, representative of three, are illustrated.

described previously (12). Cell suspensions with at least 90% 5T2MM or 95% 5T33MM cells, as determined by FACS analysis, were obtained.

CD44v Monoclonal Antibodies. The following monoclonal antibodies against CD44v were used: CD44v6 (mouse IgG2a; LN6.1), CD44v7 (mouse IgG1; LN7.1), and CD44v10 (rat IgG1; LN10.1); 17). Pan-CD44 (CD44s) monoclonal antibodies, IM7.8.1 (rat IgG2b), and KM114 (rat IgG1) were obtained from American Type Culture Collection (Rockville, MD) and Becton Dickinson (Mountain View, CA), respectively.

Flow Cytometry. The purity of 5T2MM and 5T33MM cells after isolation and purification was determined by staining with anti-idiotypic-specific monoclonal antibodies (16). For some experiments, the tumor load was assessed by measurement of the percentage idiotype-positive cells after RBC lysis with ammonium chloride. Expression of CD44v was also determined by FACS analysis as described previously (12). For each staining, isotype-matched irrelevant antibodies were used as negative controls. All of the samples were analyzed on a FACSort (Becton Dickinson) flow cytometer.

Adhesion Assays. Quantification of the percentage adhesion of 5T2MM and 5T33MM cells to the mouse BM endothelial cell line STR-4 (18) was performed by microscopic cell counting as described previously for the adhesion of 5TMM cells to BM stromal cells (12).

***In Vivo* Migration Assays.** To study the *in vivo* homing of 5T2MM and 5T33MM cells, 0.5×10^6 ^{51}Cr -labeled cells were injected into a lateral vein of naive mice (13). When indicated, the cells were preincubated with 200 μg antibodies (30 min, room temperature) that were then coinjected into the mice. Eighteen h after injection, the mice were killed, EDTA-blood was collected, and all of the organs were removed. The radioactivity of each organ and 100 μl of blood were measured in a gamma counter (MR480C/TP ITM; Van Hopplynus, Brussels, Belgium).

Preliminary experiments indicated that the coating of the 5TMM cells with antibodies does not have any effect on the *in vivo* migration of the cells and on their capacity to induce myeloma disease.

Statistical Analyses. For statistical analyses, Student's *t* test was used. $P_s \leq 0.05$ were considered significant.

Results

Expression of CD44v on 5TMM Cells. The expression of CD44v and CD44s on 5TMM cells was analyzed by flow cytometry. 5T2MM cells expressed CD44v6, CD44v7, CD44v10, and CD44s (Fig. 1). 5T33MM cells have a similar expression pattern, as described previously (12).

CD44v10 Is Involved in the Adhesion of 5TMM Cells to BM Endothelial Cells. The adhesion of 5T2MM and 5T33MM cells to the BM endothelial cell line STR-4 was analyzed by microscopic cell counting. 5T2MM cells had a spontaneous adhesion of about 40% (Fig. 2), which was inhibited (30% inhibition, mean value) by CD44v10 monoclonal antibodies. 5T33MM cells showed a spontaneous adhesion of about 35% (Fig. 2) that was also inhibited, (38% inhibition, mean value) by CD44v10 antibodies. The other CD44v specific antibodies, as well as both pan-CD44 antibodies IM7.8.1 and KM114 (the latter not illustrated), had no significant effect on the adhesion to the BM endothelial cells. These data indicate that CD44v10 is involved in the adhesive interactions between 5TMM cells and BM endothelial cells.

The ligands of CD44 include hyaluronan (mainly), laminin, fibronectin, collagen type I and type IV, chondroitin sulfate, and possibly other yet-unidentified ligands (9). We tested these extracellular matrix structures, and in line with the literature (6, 19), none of these ligands of CD44s appeared to influence CD44v10-mediated adhesion of 5TMM cells to BM endothelial cells (results not shown).

CD44v10 Is Involved in the *in Vivo* Homing of Myeloma Cells. Because CD44v10 antibodies were able to inhibit the interaction between 5TMM cells and BM endothelial cells, we analyzed whether this inhibition resulted in a decreased BM homing *in vivo*. For this purpose, ^{51}Cr -labeled cells in medium alone, in medium with isotype-matched control antibodies, or in medium with CD44v10 antibodies were (co)injected into naive mice. As reported previously, the 5TMM cells initially homed to the BM, liver, and spleen only (12, 13). About 10% of the injected 5T2MM and 5T33MM cells homed to the BM. Pretreatment and coinjection with irrelevant isotype-matched control antibody did not inhibit homing of the cells as compared with the medium control (Fig. 3). However, CD44v10 antibodies significantly inhibited homing of the 5TMM cells to the BM, but not to the liver or the spleen. Pretreatment and coinjection with CD44v6 antibodies did not result in any inhibition (not shown), which emphasized the specificity of the inhibition observed with CD44v10 antibodies. These

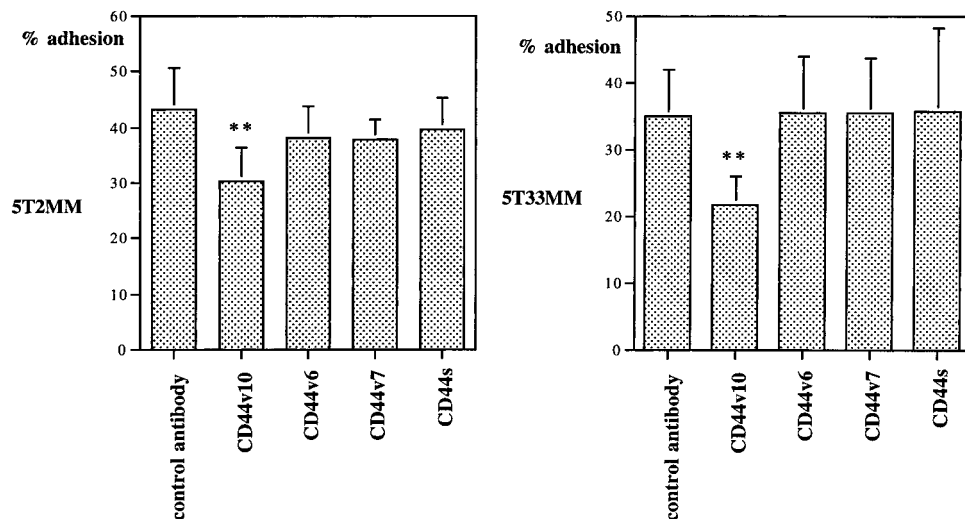


Fig. 2. Involvement of CD44v10 in the adhesion of 5TMM cells to BM endothelial cells. 5TMM cells were preincubated with different CD44v or CD44s or isotype-matched control antibodies for 30 min and subsequently seeded on confluent BM endothelial cell monolayers for 2 h. After the washing of nonadherent cells, the percentage of adherent 5TMM cells was determined by microscopic cell counting. Mean values of quadruplets of three independent experiments are shown. Bars, positive SD; **, significant difference ($P < 0.001$) compared with the control.

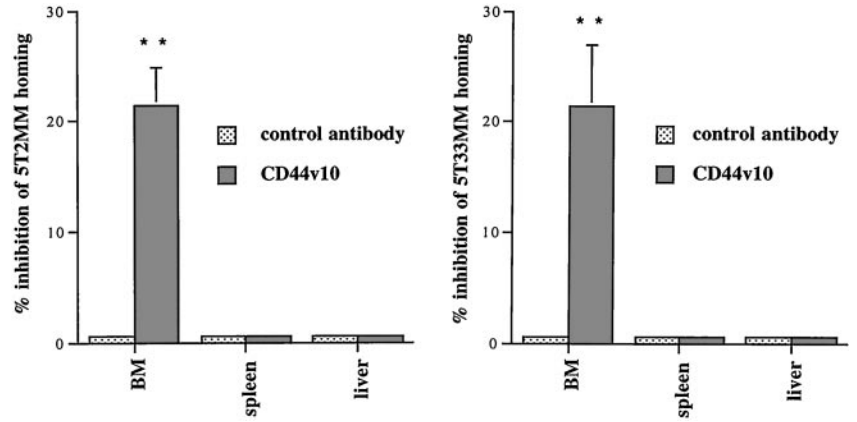


Fig. 3. Involvement of CD44v10 in the BM homing of 5TMM cells. Naive animals were (co)injected with ^{51}Cr -labeled 5TMM cells in medium alone, or in medium with isotype-matched control antibodies, or in medium with CD44v10 antibodies. After 18 h, radioactivity in the organs was measured and the percentage inhibition of homing, was calculated. The values are expressed in relation to the values obtained by injection of medium alone into the 5TMM cell. BM represents the sum of ribs, vertebrae, and fore and hind legs. Mean values of percentage inhibition are illustrated. Bars, positive SD; **, significant difference ($P < 0.001$) compared with the control. Each group contained four animals.

results demonstrate that treatment of mice with CD44v10 antibodies specifically inhibits the BM homing of 5TMM cells.

Decreased BM Homing Attributable to CD44v10 Blocking Results in Decreased Tumor Load in Terminally Diseased Animals.

The previous experiments demonstrated that CD44v10 antibodies reduced the adhesion of 5TMM cells to BM endothelial cells, resulting in a decreased *in vivo* BM homing. Does this decreased BM homing of 5TMM cells result in a decreased tumor load at the end stage of the disease? We investigated this by injecting 5TMM cells together with control or CD44v10 antibodies into naive mice, as described for the homing experiments. Animals were monitored for disease progression, and the tumor load in BM at the end stage of the disease was quantified by flowcytometry. Anti-CD44v10-treated animals had a lower BM tumor load as well as decreased serum paraprotein concentrations (Table 1). Histological examination of the BM (12) also demonstrated a decreased myeloma infiltration in the anti-CD44v10-treated group, although this was less pronounced for the more aggressive 5T33MM line (not illustrated). As reported and discussed previously, the 5T2MM cells also grow in the spleen, and 5T33MM cells also grow in the spleen and in the liver (16), inducing splenomegaly and hepatomegaly. No significant differences were observed in the weight increase (not shown) of these organs in the 5TMM animals between the control and CD44v10 groups, in line with the lack of effect of CD44v10 on the homing (Fig. 2) of the 5TMM cells to the spleen and the liver. These data indicate that the decreased BM tumor load resulted from CD44v10 blockage of BM homing. This reduced tumor load correlated well with decreased serum paraprotein levels.

Discussion

In spite of extensive research and the development of novel therapies, MM remains a deadly malignancy. One of the important characteristics of this disease is the restricted localization of the malignant cells in the BM. The mechanisms by which these malignant plasma cells enter into the BM are still not understood. Our group reported that this restricted localization is attributable to a selective rather than

a random migration of the myeloma cells (13). Analysis of their adhesive potential to different types of endothelial cells revealed a selective adhesion to BM endothelial cells and not to lung endothelial cells (13). These data indicated that myeloma cells adhere to BM endothelial cells via specific adhesion molecules. CD44 is a family of different CD44v molecules, and their restricted expression on myeloma cells (10–12) led us to investigate their involvement in the selective *in vivo* migration of myeloma cells to the BM. For this purpose, we used the 5T experimental mouse model of myeloma. In analogy to human myeloma cells (10, 11), CD44v6, CD44v7, and CD44v10 are expressed on the 5TMM lines. *In vitro* adhesion assays revealed the involvement of CD44v10 in the adhesion of both 5T2MM and 5T33MM cells to BM endothelial cells. This observation led us to analyze whether the blocking of CD44v10 by monoclonal antibodies has any effect on the *in vivo* homing. We found an inhibition of the homing to the BM for both 5T2MM and 5T33MM cells. The CD44v10 region of the CD44 molecule is specifically involved here because treatment of the mice with other CD44v such as CD44v6 did not result in any inhibition (latter results not illustrated), in keeping with the results from the *in vitro* adhesion assays. Moreover, the involvement of CD44v10 in the homing is specific for the BM because the homing to spleen and liver was not effected by the treatment with anti-CD44v10 monoclonal antibodies. Analysis of animals at the end stage of the disease demonstrated that the decreased initial BM homing was considerable, because it resulted in a decreased myeloma tumorload in the BM and serum paraprotein levels. We recently analyzed the involvement of CD44v in the adhesion of 5TMM cells to BM stroma and observed that CD44v6 and not the other variants, including CD44v10, mediates direct contact between the 5TMM cells and BM fibroblasts (12). The results presented in this work demonstrate the involvement of only CD44v10 and not the other variants in the interactions with BM endothelial cells. These data strongly indicate that certain CD44v have specific functions in the myeloma cell biology. Considered together, these results clearly indicate that CD44v10 is specifically involved in the selective *in vivo* homing of 5TMM cells to the BM.

Table 1 BM tumor load and paraprotein concentration in control and anti-CD44v10-treated animals at the end stage of the disease

BM tumor load was assessed by FACS staining for 5TMM idiotype positive cells of isolated BM cells after red blood cell lysis, and serum paraprotein levels were quantified by electrophoresis.

	5T2MM recipients		5T33MM recipients	
	Control antibody	CD44v10	Control antibody	CD44v10
Tumor load BM (%)	67.9 ± 8.6	45 ± 11 ^a	81.6 ± 6.6	61.7 ± 8 ^a
Serum paraprotein (g/dl)	0.32 ± 0.03	0.21 ± 0.06 ^b	1.3 ± 0.19	0.62 ± 0.09 ^b

^a $P < 0.03$ indicating significant difference with control animals.

^b $P < 0.05$ indicating significant difference with control animals. Each group contained 4 animals.

CD44 is involved in the *in vivo* extravasation of activated lymphocytes (3, 4). Their involvement in normal lymphocyte (20) and hematopoietic stem cell (21) homing is, however, controversial (4, 22). CD44 is also involved in the dissemination of cancer cells. Transfection of nonmetastatic rat carcinoma cells with CD44v6 conferred full metastatic behavior on these cells (5). Treatment of mice with CD44 antibodies prevented the formation of metastasis of melanoma (6) and adenocarcinoma cells (7). Although correlations have been reported between the prognosis of specific cancers and alterations in the expression pattern of CD44v (9), little is known about the functions of these different variants. This also holds true for myeloma. The expression of CD44v6 and CD44v10 are associated with stable disease (10, 11), and, as already mentioned in a previous work (12), we were able to demonstrate the involvement of CD44v6 in the adhesion of 5TMM cells to BM fibroblasts. *In vivo* blocking studies in murine models have shown that CD44v10 is involved in the metastasis of melanoma cells (6) and in the homing of activated lymphocytes in several autoimmune skin diseases (19, 23, 24). Our observation is in line with the findings of these different reports that CD44v10 mediates the process of transendothelial migration.

Myeloma cells are one of the few cell types on which CD44v in general, and CD44v10 in particular, are expressed. In mice, expression of CD44v10 was found only on the basal layers of the epidermis, in the epithelium of the upper gastrointestinal tract, and on subsets of BM cells and activated lymphocytes, as revealed by immunohistochemistry and flow cytometric examination of the total body (19). This restricted expression pattern makes CD44v10 a suitable target for therapeutical interference.

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