

Figure 2 Establishment of the *in vitro* culture system for the assessment of cell adhesion-mediated drug resistance (CAM-DR) of multiple myeloma (MM) cells. (a) Green fluorescent protein (GFP)-transduced KMS-12BM cells were treated with either 5 nM bortezomib (Bort), 1 nM vincristine (VCR), 100 nM doxorubicin (Doxo) or 50 nM dexamethasone (Dexa) in the presence (co-culture) or absence (stroma free) of UBE6T-7 stromal cells for 48 h. Cell death/apoptosis was determined by reactivity with phycoerythrin (PE)-conjugated annexin-V (annexin-V/PE) in GFP-positive fractions on flow cytometry. Representative histogram plots are shown. Annexin-V positivity is indicated as a percentage in each histogram. (b) The Y axis shows the proportion of annexin-V-positive cells under co-culture condition with that under stroma-free condition setting at 100% in KMS-12BM cells treated with each drug. The means \pm s.d. (bars) of three independent experiments are shown. The same experiments were carried out in RPMI8226 (c) and U266 (d). Drug concentrations were 2 nM for bortezomib, 1 nM for vincristine, 100 and 70 nM for doxorubicin, and 50 and 20 nM for dexamethasone in RPMI8226 and U266 cells, respectively. The *P*-values were calculated by Student's *t*-test. **P* < 0.05.

of cell death is apoptosis (Supplementary Figure S2A). The cytotoxic effects were markedly diminished under the co-culture condition, suggesting that CAM-DR was successfully reproduced in our system (Figure 2a; Supplementary Figure S2). DR was not acquired in KMS-12BM cells cultured with stroma cells in transwells, which preclude direct cell-to-cell interactions, indicating that direct contact is indispensable for

CAM-DR of MM (data not shown). As shown in Figures 2b–d and Supplementary Table S1, CAM-DR was similarly observed in all three myeloma cell lines treated with all four drugs tested, although the extent of CAM-DR was relatively low for bortezomib (discussed later). Furthermore, CAM-DR was reproduced with different concentrations of the drugs (Supplementary Figure S2b). Using this system, we attempted to

determine which adhesion molecule(s) is important for CAM-DR in MM cells.

Reversal of CAM-DR by shRNA/siRNA- and blocking antibody-mediated knockdown of VLA-4 in MM cells

To investigate which adhesion molecule(s) is critical for the acquisition of CAM-DR in MM cells, we performed loss-of-function analyses for CD44 (HCAM), CD49d ($\alpha 4$ -integrin), CD54 (ICAM-1), CD138 (syndecan-1) and CD184 (CXCR4) using the shRNA/siRNA lentivirus system (Kikuchi *et al.*, 2007). Because CD29 ($\beta 1$ -integrin) is heterodimerized with CD49d and functions as VLA-4 ($\alpha 4\beta 1$ -integrin) on MM cells, we could achieve loss of function of VLA-4 by solely targeting CD49d. As shown in Figure 3a, shRNA/siRNA expression vectors were constructed by inserting chemically synthesized oligonucleotides containing target sequences (Supplementary Table S2) into pLL3.7 vector, and their inhibitory activities were checked in KMS-12BM cells (data not shown). Constructs with the strongest activities were transfected into three MM cell lines along with sh controls, and a specific reduction of target expression was confirmed (Figure 3b; Supplementary Figure S3). Overall, we established 15 sublines in which the expression of individual adhesion molecules was markedly downregulated, and examined the levels of CAM-DR to four anti-myeloma drugs. To quantitatively assess the contribution of each molecule to CAM-DR, we defined the ratio of annexin-V reactivity of GFP-positive cells under the co-culture condition to that under the stroma-free condition as a reversal of CAM-DR. The reversal of CAM-DR to bortezomib was detectable in CD49d-knockdown sublines of all three cell lines, whereas no reversal was observed in sublines carrying shRNA/siRNA against other four adhesion molecules and inactive sh controls (Figure 3c; Supplementary Table S3). In addition, we performed the same experiments using vincristine and dexamethasone in KMS-12BM sublines. The CAM-DR to vincristine and dexamethasone was also reversed by knockdown of CD49d but not other four molecules (Figure 3d; Supplementary Table S4). It is of note that the reversal of CAM-DR to either vincristine or dexamethasone was at the almost equal level to that to bortezomib in CD49d-knockdown sublines (compare Figures 3c and d). In view of the fact that bortezomib is relatively resistant to CAM-DR (see Figure 2 and Supplementary Table S1), this implies that bortezomib modulates the expression of CD49d in MM cells (discussed later).

Furthermore, we confirmed the importance of CD49d in CAM-DR using adhesion-blocking antibodies instead of shRNA/siRNA introduction. We used specific antibodies against CD44, CD49d, CD54 and CD184 to revert CAM-DR, but were not able to test an anti-CD138 antibody because it is not commercially available. MM cells were pretreated with these antibodies, cultured with or without stromal cells in the presence of bortezomib, and subjected to flow cytometric analysis for annexin-V reactivity. As shown in Figure 4 and Supplementary Table S5, significant reversal of CAM-DR was achieved by treatment with anti-CD49d

($\alpha 4$ -integrin) and anti-CD54 (ICAM-1) antibodies in KMS-12BM and U266 cells, albeit the effect of the former was much stronger. The effectiveness of anti-CD54 may stem from its effects on stromal cells, because previous studies revealed that CD54 was expressed on BM stromal cells (Corso *et al.*, 2005). In contrast, the other antibodies failed to revert CAM-DR in KMS-12BM and U266 cells. Unexpectedly, CAM-DR was not affected by any antibodies in RPMI8226 cells, probably due to the relatively low expression of CD49d. Although there was slight discrepancy between the results obtained with shRNA/siRNA and adhesion-blocking antibodies, our data clearly indicate that VLA-4, a heterodimer of CD49d and CD29, is the most important adhesion molecule for CAM-DR in MM cells.

Downregulation of CD49d expression by bortezomib

It is tempting to speculate that bortezomib modulates the expression of CD49d in MM cells from our two findings: the relative resistance of bortezomib to CAM-DR (see Figure 2) and equal reversal of CAM-DR to all three drugs on disruption of VLA-4 signaling (see Figure 3). In support of this view, Duechler *et al.* (2005) reported that bortezomib decreased the surface expression of CD23 in chronic lymphocytic leukemia. Therefore, we investigated the effect of bortezomib and other anti-myeloma drugs on the expression of adhesion molecules on MM cell lines. Figure 5a and Supplementary Figure S4 show the representative data of flow cytometric analysis of viable KMS-12BM cells before and after treatment with bortezomib. Untreated KMS-12BM cells strongly expressed CD29, CD44, CD49d, CD54, CD138 and CD184. Bortezomib did not affect the expression levels of CD29, CD44, CD54, CD138 and CD184, but decreased the expression of CD49d from 99.4 ± 0.1 to $34.5 \pm 0.9\%$ ($n = 3$, $P < 0.05$). Bortezomib-induced downregulation of CD49d expression was similarly observed in RPMI8226 and U266 cells: from 33.2 ± 0.6 to $12.4 \pm 1.9\%$ in RPMI8226 cells ($n = 3$, $P < 0.05$) and from 99.4 ± 0.04 to $74.3 \pm 4.1\%$ in U266 cells ($n = 3$, $P < 0.05$). Furthermore, we confirmed bortezomib-mediated reduction of CD49d expression by immunoblotting using whole-cell lysates (Figure 5b), semiquantitative reverse transcription (RT)-PCR (Figure 5c) and real-time quantitative RT-PCR (Figure 5d), suggesting that this phenomenon takes place at mRNA levels. In striking contrast, other anti-myeloma drugs, such as vincristine, doxorubicin and dexamethasone, did not affect the expression of CD49d (Figure 5e; Supplementary Figure S4) and other adhesion molecules (Supplementary Figure S5) in any cell lines examined. The specific reduction of CD49d expression by bortezomib may underlie the relative resistance of the drug to CAM-DR in MM cells.

Next, we investigated the mechanisms by which bortezomib suppresses the expression of CD49d mRNA. For this purpose, we directly inhibited NF- κ B activity in KMS-12BM cells using SN-50 peptide, which interferes with nuclear translocation of p50 by binding to its nuclear localization sequence (Lin *et al.*, 1995). Surface expression of CD49d was not affected by the

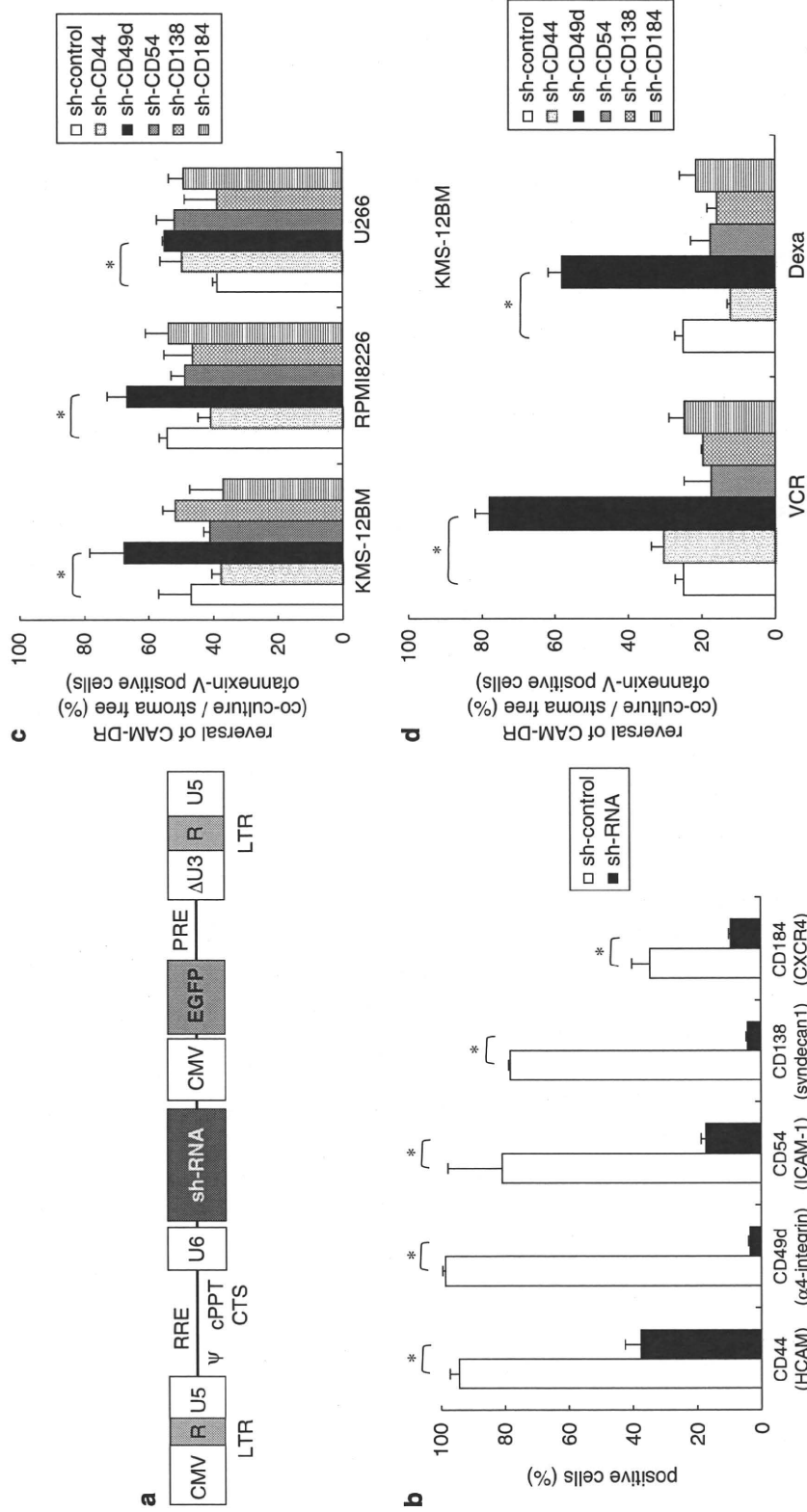


Figure 3 Effects of short hairpin RNA (shRNA)-mediated knockdown of adhesion molecules on adhesion-mediated drug resistance (CAM-DR) in multiple myeloma (MM) cells. (a) Schematic representation of a pLL3.7 lentiviral shRNA expression vector: U5 and U6 indicate U5 and U6 promoters, respectively; EGFP, enhanced green fluorescent protein; ψ, a packaging signal; RRE, responsive element; cPPT, central polypurine tract; CTS, central termination sequence; CMV, cytomegalovirus promoter; PRE, wood-chuck hepatitis virus post-transcriptional regulatory element and LTR, long terminal repeat. See Materials and Methods for details of construction. (b) KMS-12BM cells were transfected with either pLL3.7-sh-CD44, sh-CD49d, sh-CD54, sh-CD138, sh-CD184 or sh control vector. GFP-positive cells were collected by FACSaria flow cytometer, and stained with phycoerythrin (PE)-conjugated anti-CD44, anti-CD49d, anti-CD54, anti-CD138 and anti-CD184 antibodies, or PE-conjugated mouse and rat IgG isotype-matched controls. The means ± s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test. **P* < 0.05 against the sh control. (c) MM cell lines stably transfected with shRNA vectors were cultured with 2 nM bortezomib in the presence (co-culture) or absence (stroma free) of stromal cells. After 48 h, MM cells were harvested by pipetting, stained with annexin-V/PE, and subjected to flow cytometric analysis. The *Y* axis shows the reversal of CAM-DR as a ratio (%) of annexin-V positivity under co-culture vs stroma-free conditions. When annexin-V reactivity under co-culture condition is equal to that of stroma-free condition, the reversal of CAM-DR is 100%. The means ± s.d. (bars) of three independent experiments are shown. (d) KMS-12BM sublines stably transfected with shRNA vectors were cultured with 1 nM vincristine (VCR) or 50 nM dexamethasone (Dexa) in the presence (co-culture) or absence (stroma free) of stromal cells. After 48 h, the reversal of CAM-DR was examined as described above. **P* < 0.05 against the sh control.

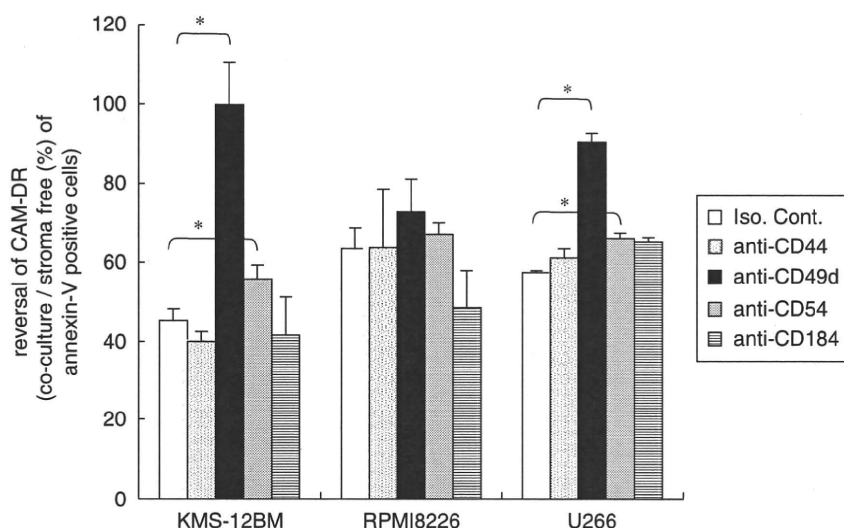


Figure 4 Effects of blocking antibodies against adhesion molecules on adhesion-mediated drug resistance (CAM-DR) in multiple myeloma (MM) cells. MM cell lines were treated with either antibodies against CD44, CD49d, CD54 and CD184 or isotype-matched controls (iso. cont.) at 10 μ g/ml at 37 $^{\circ}$ C for 1 h. After treatment, cells were cultured with 2 nM bortezomib in the presence (co-culture) or absence (stroma free) of stromal cells for 48 h. The reversal of CAM-DR was determined as described in the legend of Figure 3. The means \pm s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test. **P* < 0.05 against isotype-matched controls.

p50 inhibitory peptide, suggesting that bortezomib-mediated downregulation of CD49d is not a direct consequence of the inhibition of NF- κ B activity (Supplementary Figure S6).

The reversal of CAM-DR to conventional anti-myeloma drugs by pretreatment with bortezomib

Given that bortezomib decreases the expression of CD49d, which plays a crucial role in CAM-DR, pretreatment of MM cells with bortezomib could overcome CAM-DR to conventional anti-myeloma drugs. Finally, we tested this hypothesis using the co-culture system for CAM-DR. MM cells were pretreated with bortezomib for 24 h, followed by exposure to either vincristine or dexamethasone for additional 24 h in the presence or absence of stroma cells. Pretreatment with bortezomib significantly reversed CAM-DR to both vincristine and dexamethasone in all three cell lines tested (Figure 6a), which coincided with the detachment of myeloma cells (Figure 6b). In particular, CAM-DR to VCR was almost completely inhibited in KMS-12BM and RPMI8226 cells: reversal ratios were 87.4 ± 3.2 and $104.6 \pm 19.4\%$, respectively. In U266 cells, the effects of bortezomib were moderate but significant. This may be attributable to the relatively weak effects of bortezomib on CD49d expression in U266 cells (see Figure 5d). It should be emphasized that the combination of bortezomib with either vincristine or dexamethasone at the doses used in this experiment did not show additive effects under stroma-free condition (data not shown).

Discussion

In this study, we have clearly demonstrated that VLA-4, a heterodimer of CD49d/CD29, plays a critical role in

CAM-DR of MM using a unique strategy involving myeloma cell lines in which individual adhesion molecules were stably knocked down by the aid of shRNA. In support of our finding, several studies described the roles of VLA-4 in the pathophysiology of MM. For example, CD29-mediated adhesion of MM cells to fibronectin upregulated the expression of the CDK inhibitor p27 and induced NF- κ B activation, both of which confer CAM-DR to MM (Chauhan *et al.*, 1996; Hazlehurst *et al.*, 2000; Landowski *et al.*, 2003). Two independent groups reported that administration of anti-CD49d antibody suppressed the growth of MM cells in murine xenograft models (Mori *et al.*, 2004; Olson *et al.*, 2005). In line with these experimental findings, Schmidmaier *et al.* (2006) found that MM patients with primary multidrug resistance showed significantly higher concentrations of serum VLA-4 and ICAM-1 than responders. The involvement of VLA-4 in CAM-DR was also demonstrated in AML by the seminal study of Matsunaga *et al.* (2003), in which the combination of cytosine arabinoside and anti-CD49d antibody achieved a 100% survival rate in mice transplanted with AML cells. These findings strongly suggest that VLA-4-mediated signaling is important for the development of DR in MM and AML cells both *in vitro* and *in vivo*.

In addition, we obtained evidence indicating that bortezomib can overcome CAM-DR by selectively downregulating CD49d expression in myeloma cells. This ability was specific for bortezomib and was not observed in other commonly used anti-myeloma drugs such as vincristine, doxorubicin and dexamethasone. Moreover, we have found that bortezomib represses the expression of CD49d at mRNA levels. Regarding the mechanisms of this phenomenon, the direct involvement of NF- κ B is unlikely because the p50 inhibitory peptide

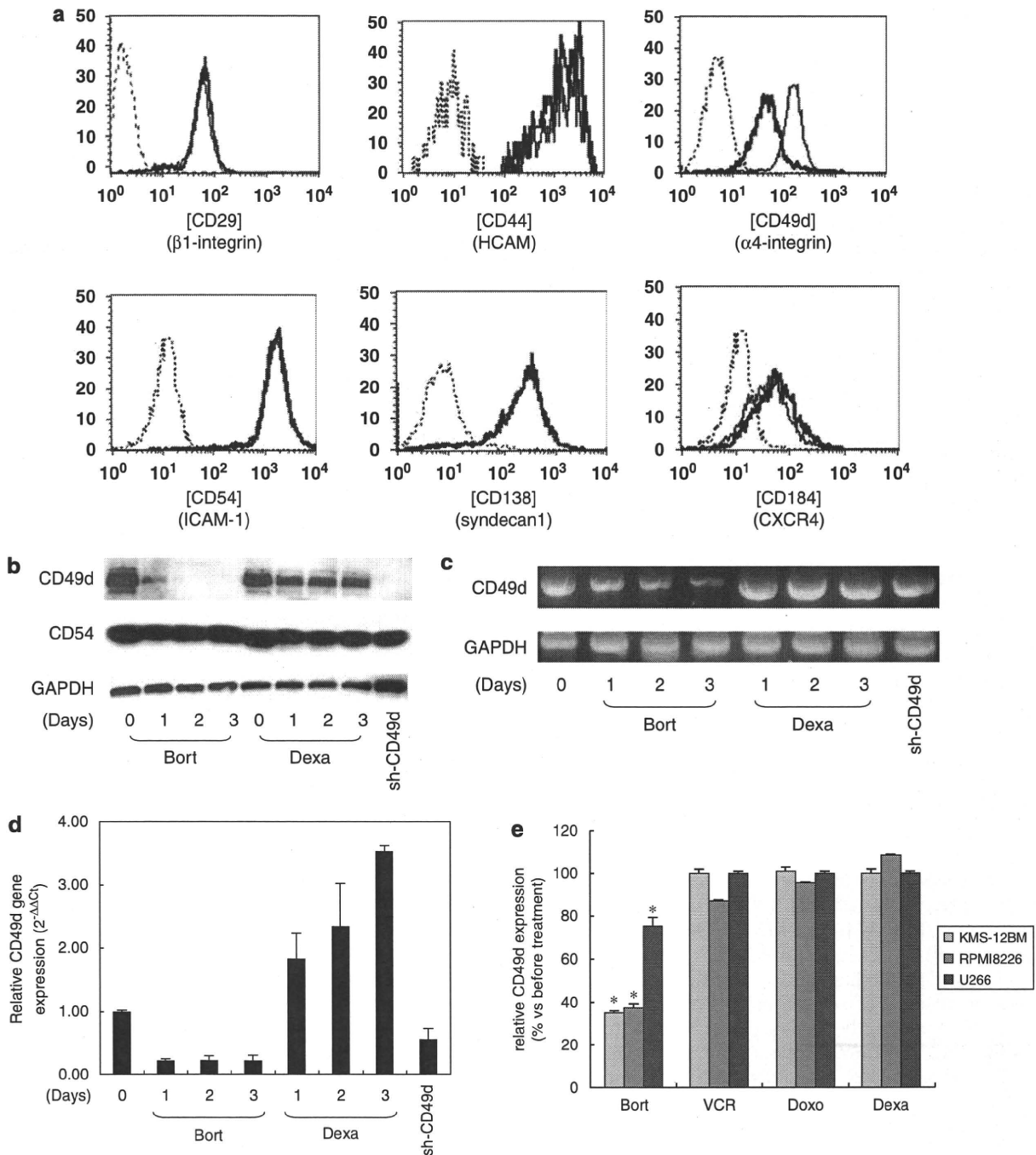


Figure 5 Effects of anti-myeloma drugs on the expression of adhesion molecules in multiple myeloma (MM) cells. **(a)** Surface expression of CD29, CD44, CD49d, CD54, CD138 and CD184 was detected by flow cytometry on KMS-12BM cells before and after treatment with 5 nM bortezomib for 48 h. Thin lines, bold lines and dotted lines show plots before treatment, after treatment and of isotype-matched controls, respectively. Representative histograms of three independent experiments are shown. **(b)** KMS12-BM cells were treated with either 5 nM bortezomib (Bort) or 50 nM dexamethasone (Dexta) for up to 3 days. We used a KMS12-BM subline transfected with pLL3.7-sh-CD49d (sh-CD49d) as a control. Cells were harvested at the indicated time points, and subjected to immunoblot analysis for the expression of CD49d, CD54 and GAPDH (loading control). **(c)** Total cellular RNA was isolated simultaneously at the experiments described in **(b)**, and subjected to semiquantitative reverse transcription (RT)-PCR for the expression of CD49d and GAPDH (loading control). **(d)** Total cellular RNA was isolated simultaneously at the experiments described in **(b)**, and subjected to real-time quantitative reverse transcription (RT)-PCR. The expression of CD49d was normalized to that of GAPDH and quantified by the 2^{-ΔΔC_t} method. **(e)** The expression of CD49d was detected before and after bortezomib treatment in KMS-12BM, RPMI8226 and U266 MM cells by flow cytometry. The concentrations of bortezomib were 5, 2 and 2 nM for KMS-12BM, RPMI8226 and U266 cells, respectively. Data shown are the means ± s.d. (bars) of relative CD49d expression (ratio (%) of CD49d positivity after vs before treatment) of three independent experiments. The *P*-values were calculated by Student's *t*-test. **P* < 0.05 against the values obtained before treatment.

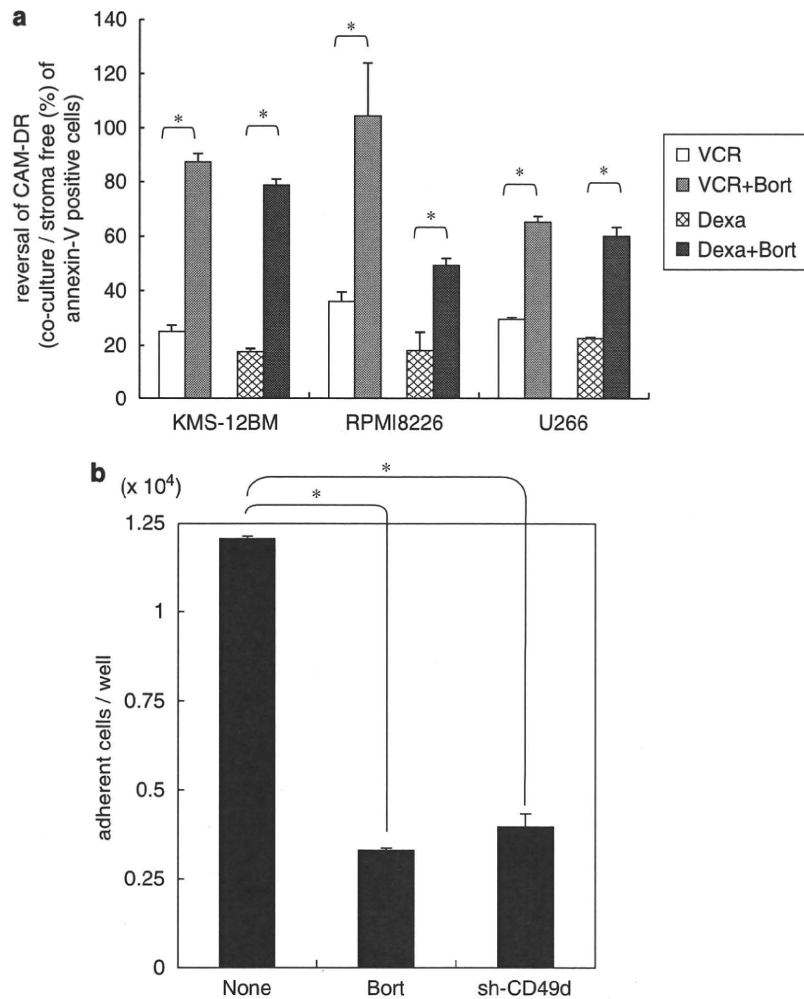


Figure 6 Bortezomib overcomes adhesion-mediated drug resistance (CAM-DR) to vincristine and dexamethasone in multiple myeloma (MM) cells. **(a)** Three myeloma cell lines were treated with 2 nM bortezomib for 24 h. After washing, we cultured cells with either vincristine (Bort + VCR) or dexamethasone (Bort + Dexa) in the absence or presence of stromal cells for additional 24 h. As controls, we carried out the same experiments without pretreatment of bortezomib (VCR and Dexa). The reversal of CAM-DR was determined as described in the legend of Figure 3. **(b)** Green fluorescent protein (GFP)-transduced KMS12-BM cells were cultured in the absence (None) or presence (Bort) of 5 nM bortezomib for 3 days. We used a KMS12-BM subline transfected with pLL3.7-sh-CD49d (sh-CD49d) as a control. Cells were then co-cultured with UBE6T-7 cells at 37 °C for 15 min. Non-adherent cells were washed off with phosphate-buffered saline (PBS), and resultant adherent cells were harvested with trypsin-EDTA. We determined GFP positivity (%) in non-adherent cells using flow cytometer. The means \pm s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test (**P* < 0.05).

did not downregulate the expression of CD49d in KMS-12BM cells, in which NF- κ B is constitutively activated through the classical pathway (Keats *et al.*, 2007). The CD49d promoter contains canonical binding sites for Sp1, Ets and WT1 (Rosen *et al.*, 1994; Zutter *et al.*, 1997; Kirschner *et al.*, 2006). We are currently investigating whether these factors are implicated in bortezomib-mediated downregulation of CD49d mRNA expression.

The reversal of CAM-DR by bortezomib may underlie its broad range of synergy with other anticancer drugs as recently described (Horton *et al.*, 2006; Noborio-Hatano *et al.*, 2007). Unfortunately, severe pulmonary complications have been reported in Japanese patients treated with bortezomib (Miyakoshi *et al.*, 2006). Dose reduction by drug combination is one

method to minimize side effects of bortezomib. Furthermore, it has been shown that the contact of MM cells with BM stromal cells through VLA-4/vascular cell adhesion molecule-1 (VCAM-1) interactions enhanced the production of osteoclastogenesis factors and that disruption of this cell-to-cell contact suppressed the development of osteoclastic osteolysis associated with MM (Michigami *et al.*, 2000; Pearse *et al.*, 2001). As bortezomib can suppress CD49d expression, this drug may also be effective for the amelioration of bone lesions in MM.

Although our findings provide rationale for safe and effective treatment strategies for refractory myeloma, further investigation is required to define suitable combinations involving not only conventional anti-myeloma drugs but also new drugs such as lenalidomide.

Previous studies revealed that both bortezomib and dexamethasone induced apoptosis primarily through caspase-9, whereas lenalidomide did so through caspase-8 (Mitsiades *et al.*, 2002). Indeed, we found that caspase-8 was not activated in MM cells by the drugs used in this study (data not shown). On the basis of these findings, the combination of lenalidomide with either bortezomib or dexamethasone is expected to trigger dual apoptotic signaling pathways and reverse DR, which results in the achievement of clinical responses in patients resistant to single agents. In support of this view, we have reported that bortezomib is effective for myeloma patients refractory to thalidomide therapy (Takatoku *et al.*, 2004). The combination of bortezomib with other anti-myeloma drugs, including thalidomide and its derivatives, is effective for overcoming CAM-DR and may improve the prognosis of MM patients.

Materials and methods

Cells and cell culture

We used four *bone fide* human MM cell lines, KHM-1B, KMS-12BM, RPMI8226 and U266, in this study (Drexler *et al.*, 2003). These cell lines were purchased from the Health Science Research Resources Bank (Osaka, Japan), and maintained in RPMI1640 medium (Sigma Co., St Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma Co.) and antibiotics. Human BM-derived stromal cell line UBE6T-7 (Mori *et al.*, 2005), which was transduced with a human telomerase catalytic protein subunit, was kindly provided by Dr Akihiro Umezawa (National Research Institute for Child Health and Development, Tokyo, Japan), and used as stromal cells in co-culture experiments. Primary MM cells were isolated from the BM of patients at the time of diagnostic procedure. CD38-positive/CD45-low or negative (CD38⁺/CD45^{low/neg}) cells were purified from the BM of healthy volunteers, and used as normal plasma cells. Informed consent was obtained in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board.

Drugs and adhesion-blocking antibodies

Bortezomib (Velcade) was provided by Millennium Pharmaceuticals (Cambridge, MA, USA). We used vincristine (Shionogi Co. Ltd., Tokyo, Japan), doxorubicin (Meiji Co. Ltd., Tokyo, Japan) and dexamethasone (Sigma Co.) as conventional anti-myeloma drugs. All drugs were dissolved in RPMI1640 medium at appropriate concentrations and stored at -80°C until use. We used the following adhesion-blocking antibodies in co-culture experiments: anti-CD44 (clone Hermes-1; Endogen, Rockford, IL, USA), anti-CD49d (2B4; R&D Systems, Minneapolis, MN, USA), anti-CD54 (Ab-2; Labvision Corp., Fremont, CA, USA), anti-CD184 (44717.111; R&D Systems), and mouse or rat IgG isotype controls (eBioscience, San Diego, CA, USA). Blocking experiments were performed as described earlier. In brief, 1×10^6 of MM cells were incubated with $10 \mu\text{g/ml}$ antibodies or isotype-matched controls in $100 \mu\text{l}$ of RPMI1640 medium for 1 h, then diluted to 2×10^5 cells/ml in complete medium, and added into culture wells preseeded with stromal cells (Matsunaga *et al.*, 2003).

Flow cytometric analysis of adhesion molecules

Cells were stained with specific monoclonal antibodies and analysed using BD-LSR or FACSaria flow cytometer

(Becton Dickinson, Oxford, UK) as described earlier (Kikuchi *et al.*, 2005). The antibodies used were phycoerythrin (PE)-conjugated antibodies against CD11a (clone HI 111; BioLegend, San Diego, CA, USA), CD29 (4B4; Beckman Coulter, Fullerton, CA, USA), CD18 (6.7), CD22 (HIB22), CD29 (HUTS-21), CD49e (IIA1), CD56 (B159), CD138 (MI15) and CD184 (12G5) (all from Becton Dickinson), CD40 (5C3), CD44 (G44-26), CD49d (9F10) and CD54 (HA58) (all from eBioscience), allophycocyanine-conjugated antibodies against CD38 (HB7; Becton Dickinson) and PE-Cy7-conjugated antibodies against CD45 (2D1; Becton Dickinson).

Cell proliferation assay

Cell proliferation was monitored using Cell Counting Kit-8 (Dojin Chemical, Tokyo, Japan). In brief, cells were seeded in 96-well flat-bottomed microplates at a density of 5×10^4 per well and incubated with or without anti-myeloma drugs for 48 h at 37°C . After incubation, $10 \mu\text{l}$ of WST-8 solution was added to each well at a final concentration of $1 \mu\text{g/ml}$. Cells grown in complete medium alone were used as controls. After incubation at 37°C for 4 h, absorbance was measured at a wavelength of 450 nm using a microplate reader.

Co-culture of MM cells with stromal cells and the assessment of cell death

For co-culture experiments, we established GFP-expressing MM cell lines by transfecting GFP expression plasmids to distinguish MM cells from stromal cells. UBE6T-7 stromal cells were seeded in 48-well plates to near confluence. After stromal cells were washed once with fresh medium, GFP-transduced MM cells were added to the plates and cultured in the absence or presence of anti-myeloma drugs. After 48 h, the cells were harvested by pipetting, washed with phosphate-buffered saline, and stained with PE-conjugated annexin-V (annexin-V/PE) (Biovision, Mountain View, CA, USA). Cell death/apoptosis was judged by annexin-V reactivity in GFP-positive populations using flow cytometer (Yanamandra *et al.*, 2006).

Construction and production of lentiviral shRNA/siRNA expression vectors

We used a lentiviral shRNA/siRNA expression vector pLL3.7 for knockdown of adhesion molecules. siRNA target sequences were designed to be homologous to wild-type cDNA sequences. Oligonucleotides were chemically synthesized, annealed, terminally phosphorylated and inserted into pLL3.7 vector. Oligonucleotides containing siRNA target sequences were shown in Supplementary Table S1. Ineffective sequences were used as sh controls. These vectors were co-transfected into 293FT cells with packaging plasmids (purchased from Invitrogen, Carlsbad, CA, USA). Infectious lentiviruses in culture supernatants were harvested, concentrated and infected as described earlier (Kikuchi *et al.*, 2007). Lentiviruses were then added into cell suspensions in the presence of $8 \mu\text{g/ml}$ polybrene, and transduced for 24 h. Transduction efficiencies were monitored by GFP expression using a flow cytometer.

Immunoblotting

Immunoblotting was carried out according to the standard method using the following antibodies: anti-CD49d (Novus Biologicals, Littleton, CO, USA), anti-CD54 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase-8, anti-caspase-9 (Becton Dickinson) and anti-GAPDH (Santa Cruz Biotechnology) (Odgerel *et al.*, 2008).

PCR

We performed semiquantitative RT-PCR and real-time quantitative RT-PCR to estimate the expression of CD49d at mRNA levels. For the former, PCR amplification was carried out with 1 μ l of cDNA solution (corresponding to 50 ng total RNA) in a 50- μ l reaction mixture containing 5 U of *Taq* polymerase, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM MgCl₂ and 100 mM dNTPs in the presence of specific primer pairs (200 nM each) as follows; CD49d, forward: 5'-GGATGTGAACAGAAAGGCAGA-3', reverse: 5'-GCCAGTGTGTAACATGGAAA-3'; GAPDH (internal control), forward: 5'-CCACCCATGGCAAATTCATGGCA-3', reverse: 5'-TCTAGACGGCAGGTCAGGTCCACC-3'. PCR products were resolved on 2% agarose gels, and visualized by staining

with ethidium bromide. The results of 40 amplification cycles are shown. For the latter, we used the same primer pairs in the SYBR Green PCR system (Applied Biosystems, Foster City, CA, USA). Data quantification was carried out by the 2^{- $\Delta\Delta$ C_t} method (Pfaffl, 2001).

Acknowledgements

This study was supported in part by grants from the Ministry of Health, Welfare, and Labor of Japan, and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Technology of Japan. KN-H and JK are winners of the Jichi Medical School Young Investigator Award.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)



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Journal of Autoimmunity 30 (2008) 121–127

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Review

Cell and gene therapy using mesenchymal stem cells (MSCs)

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Abstract

Mesenchymal stem cells (MSCs) are considered to be a promising platform for cell and gene therapy for a variety of diseases. First, in the field of hematopoietic stem cell transplantation, there are two applications of MSCs: 1) the improvement of stem cell engrafting and the acceleration of hematopoietic reconstitution based on the hematopoiesis-supporting ability; and 2) the treatment of severe graft-versus-host disease (GVHD) based on the immunomodulatory ability. Regarding the immunosuppressive ability, we found that nitric oxide (NO) is involved in the MSC-mediated suppression of T cell proliferation. Second, tumor-bearing nude mice were injected with luciferase-expressing MSCs. An *in vivo* imaging analysis showed the significant accumulation of the MSCs at the site of tumors. The findings suggest that MSCs can be utilized to target metastatic tumors and to deliver anti-cancer molecules locally. As the third application, MSCs may be utilized as a cellular vehicle for protein-supplement gene therapy. When long-term transgene expression is needed, a therapeutic gene should be introduced with a minimal risk of insertional mutagenesis. To this end, site-specific integration into the AAVS1 locus on the chromosome 19 (19q13.4) by using the integration machinery of adeno-associated virus (AAV) would be particularly valuable. There will be wide-ranging applications of MSCs to frontier medical treatments in the near future.

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Keywords: Cancer gene therapy; GVHD; Mesenchymal stem cells; Site-specific integration; Tumor targeting

1. Introduction

In bone marrow, there are different types of tissue stem cells (adult stem cells); i.e. hematopoietic stem cells and mesenchymal stem cells (MSCs). MSCs account for a small population of cells in bone marrow as a non-hematopoietic component with the capacity to differentiate into a variety of cell lineages, including adipocytes, osteocytes, chondrocytes, muscles, and stromal cells [1]. Recent studies demonstrated that MSCs are capable of supporting hematopoiesis and of

regulating immune response [2]. In addition, since MSCs can be readily isolated and expanded *in vitro*, they are expected to be a source of cell therapy. Interestingly, MSCs have the ability to accumulate at the site of: i) tissue/organ damage; ii) inflammation; and iii) cancer when administered *in vivo*. Therefore, MSCs can be utilized for: i) regenerative therapy; ii) treatment of graft-versus-host disease (GVHD) and Crohn disease; and iii) platform of cancer gene therapy (targeted delivery of anti-cancer agents). Another unique feature of MSCs is little or low immunogenicity due to the lack of expression of co-stimulatory molecules. This phenomenon makes it possible to administer MSCs without HLA matching for cell therapy. A single lot of expanded MSCs from one healthy donor can be utilized for treatment of many patients. Although clinical applications of MSCs have been conducted for the suppression of severe acute GVHD in allogeneic stem cell transplantation [3,4] and for regenerative therapy [5,6], molecular mechanisms underlying the biological effects of

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MSCs remains obscure. Finding key molecules for differentiation, immunosuppression, and hematopoietic support of MSCs would be valuable for further augmenting the efficacy of MSCs in a wide range of clinical applications. In this regard, development of the technology for genetic manipulation of MSCs is also important research project. Site-specific integration of a therapeutic gene into a safe locus in the genome should be investigated from the safety standpoint.

2. Microarray analysis of genes responsible for differentiation of mesenchymal stem cells

Genes regulating the differentiation of MSCs remain obscure and it is technically difficult to do high-throughput analysis using primary MSCs, because such cells contain heterogeneous populations. To overcome the problems related to the heterogeneity of primary MSCs, we utilized MSC-like cell lines. It has been shown that 10T1/2 cells, derived from C3H mouse embryo cells, differentiate into adipocytes, osteocytes, and chondrocytes with a treatment of 5-azacytidine. We previously established two sub-lines from 10T1/2, designated as A54 for a preadipocyte cell line and M1601 for a myoblast cell line [7]. Under appropriate culture conditions, A54 and M1601 cells terminally differentiate into adipocytes and myotubes, respectively, while parental 10T1/2 cells remain undifferentiated under the same culture conditions. Therefore, 10T1/2 cells can be utilized as a model of MSCs, and A54 and M1601 are used as committed mesenchymal progenitors. Gene expression profiles of these cell lines were compared by microarray analysis before and after differentiation.

Each of parent 10T1/2, A54, and M1601 cell lines showed a distinctive and unique gene expression profile despite morphological similarity (Fig. 1) [8]. Parental 10T1/2 cells

had 105 elevated genes including ones encoding Activin, Dlk, Nov, Grb10, p15, and many functionally unknown molecules. Dlk and Nov are known to be involved in Notch signaling pathway and were reported to have the ability to inhibit differentiation into adipocytes and osteoblasts [9]. In preadipocyte A54 cells, 201 genes were up-regulated, including genes known to be involved in adipocyte differentiation such as genes encoding C/EBP α , C/EBP δ , PPAR- γ , PAI-I, and Frizzled-1 [10]. Myoblasts M1601 cells showed 137 up-regulated genes, including ones related to skeletal muscle differentiation such as genes encoding MyoD, MLC1F, α -skeletal actin, myosin heavy chain, and myosin light chain [11] as well as genes related to cardiac muscle differentiation such as genes encoding α -cardiac actin, cardiac troponin C, and troponin T2 [12].

Previous studies have shown that preadipocytes have a higher ability to support hematopoiesis than other kinds of stromal cell components *in vitro* [12,13]. Our results of gene expression profile revealed up-regulation of critical cytokines for hematopoiesis such as SCF and SDF-1 in preadipocyte A54 cells. In addition, many chemokines, such as CXCL-1 and CCL-7, were also up-regulated. Since Ang-1 was reported to be indispensable for the self-renewal of hematopoietic stem cells [14], we performed real-time PCR analysis of Ang-1 along with SCF, SDF-1, CEBP- δ , IGF-1, and CXCL-1. The expression of these genes was highest in A54 cells among the three cell lines. Moreover, protein expression of Ang-1 was only detected in A54 among three cell lines and the level of this protein decreased after adipocyte differentiation.

To examine the effects of these three lines on hematopoiesis, we co-cultured mouse hematopoietic stem cell fraction with these three stromal cell lines. The cells in Lin(-)Sca-1(+) fraction were plated on 10T1/2, A54, or M1601 cells.

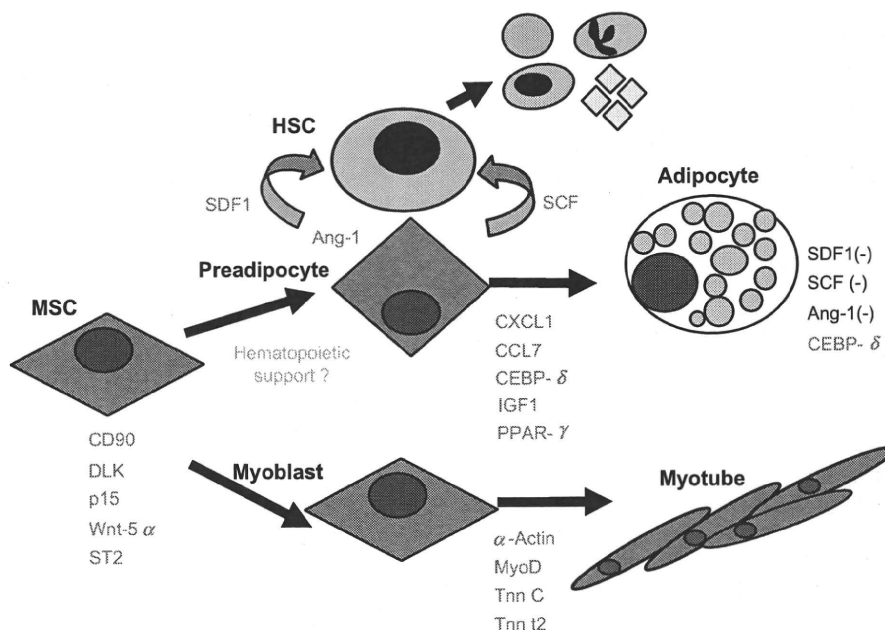


Fig. 1. Proposed model for the hierarchy of the bone marrow stromal system [8].

After 6 days of co-culture, hematopoietic progenitors were detected only on the A54 cells. These results suggest that only A54 cells have the ability to support hematopoietic cell growth among these three cell lines, consistent with the previous report. Hematopoietic cell proliferation was not observed on the layer of the terminally differentiated A54 adipocytes, suggesting that A54 cells lose the ability for hematopoietic cell support after adipocyte differentiation. To understand the molecular mechanisms of this observation, we examined the expression levels of SCF, SDF-1, and Ang-1 during adipocyte differentiation by RT real-time PCR. The expression levels of Ang-1 and SCF decreased immediately after the induction of adipocyte differentiation, and that of SDF-1 decreased gradually. In contrast to this, the level of adipocyte differentiation marker, CEBP- δ , was unchanged.

The analysis of functionally unknown molecules is currently underway. In addition, cell-to-cell contact is also believed to be crucial in the interaction between hematopoietic stem cells and MSCs. We are currently investigating the cellular and molecular events in the interactive communication between hematopoietic stem cells and MSCs.

3. Nitric oxide (NO) plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells

There is a case report of severe steroid-resistant GVHD after bone marrow transplantation, in which intravenous infusion of MSCs greatly improved clinical manifestations [3]. Moreover, multi-institutional clinical trial of MSC-treatment of severe grade III–IV acute GVHD in Europe revealed very high overall response rate (about 70%) (Le Blanc et al., ASH meeting 2006). The molecular mechanisms by which MSCs suppress T-cell proliferation are complicated, and whether a soluble factor plays a major role remains controversial. Transforming growth factor- β (TGF- β), hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO), and prostaglandin E₂ (PGE₂) have been reported to mediate T-cell suppression by MSCs [15–17]. In addition, some reports have shown that a soluble factor is the major mediator of suppression, whereas some reports have demonstrated that T-cell-MSC contact is required for this suppression.

We also investigated the molecular mechanisms using primary murine MSCs, and focused on nitric oxide (NO), because it is known to inhibit T-cell proliferation. NO is produced by NO synthases (NOSs), of which there are 3 subtypes; i.e. inducible NOS (iNOS), endothelial NOS, and neuronal NOS. It has been known that macrophages suppress T-cell proliferation, and that this suppression is caused by NO-mediated inhibition of Stat5 phosphorylation [18]. We investigated whether MSCs can also produce NO and whether NO is involved in their ability to suppress T-cell proliferation [19].

T cells proliferated in response to PMA and ionomycin, which act downstream of the T-cell-receptor complex by activating protein kinase C and inducing Ca²⁺ influx, respectively. Such T-cell proliferation was suppressed by the presence of MSC, suggesting that MSCs influence signals downstream of protein kinase C and Ca²⁺ influx. The expression of the

activation markers CD25 and CD69 on CD4 or CD8 T cells did not change even in the presence of MSCs. MSCs suppressed the production of IFN- γ but not IL-2.

Although T cells from Stat5^{-/-} mice do not proliferate upon stimulation with anti-CD3, they up-regulate CD25. Because this phenotype is similar to the status of activated T cells in the presence of MSCs, we hypothesized that MSCs suppress Stat5 phosphorylation. Indeed, Stat5 phosphorylation in activated T cells was diminished in the presence of MSCs. We found that MSCs caused a significant and cell-dose-dependent production of NO only when co-cultured with activated T cells. The induction of iNOS was readily detected in MSCs but not in T cells. RT-PCR and Western blot analysis detected iNOS expression in MSCs cocultured with activated splenocytes but not in MSCs or splenocytes when cultured alone. The immunofluorescence studies showed that iNOS was exclusively expressed in CD45⁻ adherent cells, which correspond to MSCs, but not in CD45⁺T cells. Next, we investigated the effects of *N*-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of NOS. As expected, L-NAME dose-dependently inhibited the production of NO by MSCs in the presence of activated T cells. Importantly, L-NAME restored T-cell proliferation and Stat5 phosphorylation, indicating that NO is involved in the inhibition of T-cell proliferation and Stat5 phosphorylation. Moreover, MSCs from inducible NOS^{-/-} mice had a reduced ability to suppress T-cell proliferation.

In the presence of direct interaction between T cells and MSCs, there was a high level of NO production accompanied by a strong suppression of T-cell proliferation. In contrast, both NO production and T-cell suppression were reduced in a transwell system, in which T cells were separated from MSCs by a 1- μ m-pore membrane. There are two possible explanations for the difference in T-cell suppression between the presence and absence of the transwell system. First, the amount of NO produced in the transwell system was lower than that in the presence of direct interaction. This finding suggests that direct interaction is critical for efficient production of NO as well as for strong suppression of T-cell proliferation. A second possible explanation is that, because NO is highly unstable, it can lose its activity before it reaches T cells in the transwell system.

Because TGF- β , IDO, and PGE₂ were reported as mediators of T-cell suppression by MSCs, we compared the effects of L-NAME with inhibitors of each mediator. Indomethacin (inhibitor of PGE₂ production) but not 1-methyl-DL-tryptophan (1-MT: inhibitor of IDO) or an anti-TGF- β -neutralizing antibody restored T-cell proliferation as effectively as L-NAME; however, the effects of L-NAME and indomethacin were not additive, suggesting that the NO and PGE₂ share signaling pathways leading to T-cell suppression.

In summary, our hypothesis that NO is produced by MSCs and that it suppresses T-cell proliferation in part through inhibition of Stat5 phosphorylation was supported by the following facts: (1) NO was readily detected in the medium in the co-culture of MSCs and activated T cells; (2) L-NAME restored T-cell proliferation as well as Stat5 phosphorylation; and (3) MSCs from iNOS^{-/-} mice had markedly

reduced ability to suppress T-cell proliferation. This hypothesis was further confirmed by the finding that iNOS expression was detected only in MSCs co-cultured with activated T cells.

In our scenario (Fig. 2), when MSCs are administered to the patients with severe acute GVHD, MSCs are considered to accumulate at the site of inflammation. Upon interaction with activated T cells, MSCs express iNOS and produce NO, which suppresses T-cell proliferation via inhibition of STAT5 phosphorylation. Systemic adverse effects of NO do not occur due to local production of NO with very short half-life. This is a very important point, because conventional treatment of acute GVHD causes severe systemic immunosuppression, which sometimes leads to life-threatening infections. Since MSC treatment causes just local immunosuppression, it should be much safer.

4. Interferon- γ and NF- κ B mediate nitric oxide production by mesenchymal stem cells

Human MSCs were reported to suppress Th1 differentiation and augment Th2 differentiation. Therefore, we investigated whether mouse bone-marrow-derived MSCs and the 10T1/2 cell lines have the same effect on Th1 and Th2. We found a reverse correlation between NO production and T cell proliferation in Th1/Th2 conditions, where NO production was highly induced in the presence of MSCs in Th1 but it was only minimally induced in Th2. In particular, primary MSCs and the A54 preadipocyte cell line, which induce strong T cell suppression in Th1, produce high levels of NO in Th1 condition. These results suggest that NO also plays a major

role in the preferential suppression of Th1 proliferation by MSCs.

To determine what inhibits the production of NO in Th2 condition, the two differentiation factors that support Th2 differentiation, anti-IFN- γ antibody and IL-4, were investigated. As a result, anti-IFN- γ antibody clearly inhibited the production of NO, whereas suppression by IL-4 was less evident. These results suggest that IFN- γ is a key regulator of NO production by MSCs.

Interestingly, cell supernatant collected from activated T cells had the ability to induce NO production by MSCs. IFN- γ is critical for NO production; however, in a T cell-free environment, IFN- γ alone does not induce NO production from primary MSCs. IFN- γ in combination with LPS, but not IL-2, stimulates NO secretion from primary MSCs, suggesting that both the IFN- γ and the signal from Toll-like receptor-4 (TLR4) are required for NO induction by MSCs. The addition of flagellin induced NO production in combination with IFN- γ . While, synthetic double strand RNA, poly(I:C), and CpG-oligonucleotide did not induce NO. Flagellin is a protein component of bacteria known to induce NO production from macrophages via TLR5 in the presence of either a TLR4 or IFN- γ signal. In addition to these factors, IL-1 β and TNF- α induce NO when provided in combination with IFN- γ . As NF- κ B is a downstream target of the signaling cascades activated by LPS, flagellin, IL-1 β , and TNF- α , we hypothesized that activation of NF- κ B is required for NO induction by MSCs. Bay-11-7085, a specific inhibitor of NF- κ B, suppressed induction of iNOS in MSCs, thus suggesting that NF- κ B is involved in NO production by MSCs as well as IFN- γ [20].

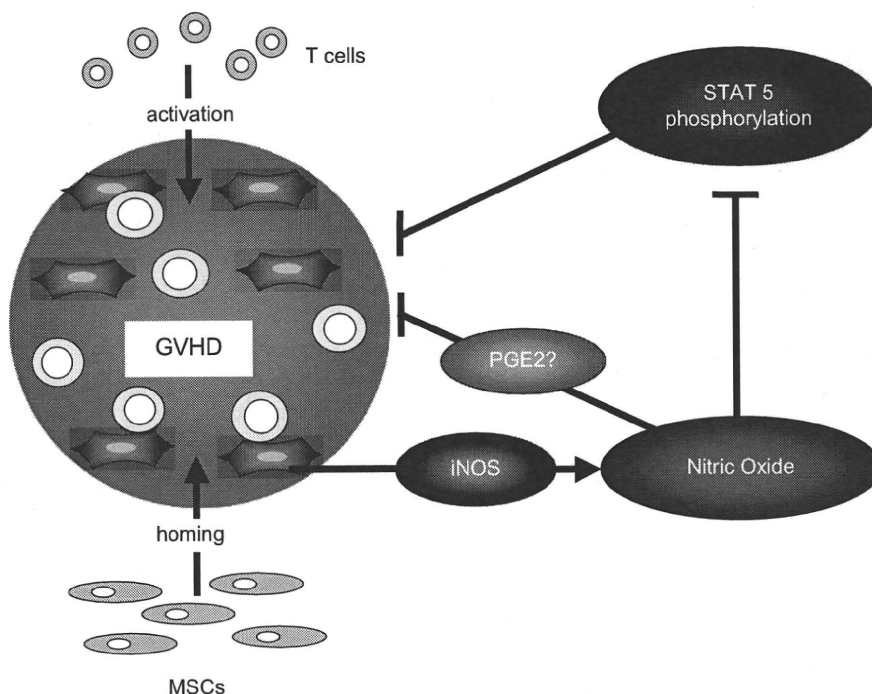


Fig. 2. MSC treatment of acute GVHD and the molecular mechanisms of T-cell suppression. MSCs are considered to accumulate at the site of inflammation and systemic adverse effects may not appear due to the local production of NO, which has very short half-life.

5. Retroviral vector-producing mesenchymal stem cells for tumor tracking and therapeutic gene amplification in suicide cancer gene therapy

MSCs are known to have a tendency to accumulate at the site of tumors, and therefore can be utilized as a platform for targeted delivery of anti-cancer agents [21–23]. The MSC-based targeted cancer gene therapy can enhance the therapeutic efficacy, because MSCs are considered to reach tumors including metastatic lesions and to deliver therapeutic molecules in a concentrated fashion. This targeted therapy can also reduce systemic adverse side effects, because the anti-cancer agents act locally at the site of tumors without elevating their systemic concentrations. We developed genetically-modified MSCs that produce retroviral vectors encoding HSVtk, aiming at augmenting therapeutic efficacy of systemic suicide cancer gene therapy (Fig. 3). The tumor tropism and anti-tumor effects of vector-producing MSCs (VP-MSCs) were examined by intravascular injection in tumor-bearing nude mice. MSCs isolated from the bone marrow of SD rats were transfected with plasmid DNA expressing luciferase alone (=non-VP-MSCs) or whole retroviral vector components (LTR-Luc or LTR-HSVtk with Gag-pol and VSV-G) (=VP-MSCs) by nucleofection. To assess tumor tropism of MSCs, nude mice were subcutaneously inoculated with 9 L rat glioma cells or Rat-1 fibroblasts, and were subsequently injected with luciferase-expressing MSCs through the left ventricular cavity. The transgene expression was periodically traced by using an *in vivo* imaging system. As a result, the transgene expression accumulated at the site of subcutaneous 9 L tumors, but undetectable at the site of Rat-1 fibroblasts. In addition, the injection of luciferase-expressing VP-MSCs caused much stronger signal of bioluminescence at the site of 9 L tumors compared with luciferase-expressing non-VP-MSCs. Immunostaining study showed that luciferase-positive cells (injected MSCs and transduced glioma cells) were detected at the periphery of tumors. To evaluate the therapeutic efficacy, tumor-bearing nude mice were treated with non-VP-MSCs or VP-MSCs combined with HSVtk/GCV system and then the size of subcutaneous tumors was periodically measured. In this model experiments, tumor growth was

more efficiently suppressed by injecting VP-MSCs compared with non-VP-MSCs (Uchibori R, et al.: manuscript in preparation). This study suggests the effectiveness of VP-MSCs in suicide cancer gene therapy. The therapeutic benefit of this strategy should be further examined in orthotopic and metastatic tumor models.

6. Site-specific insertion of a therapeutic gene into the AAVS1 locus (19q13.4) in human mesenchymal stem cells by using adeno-associated virus integration machinery

Hematopoietic stem cells, ES cells, and MSCs are attractive targets for gene therapy and regenerative medicine, since they replicate themselves and differentiate into various cell lineages. To introduce genes in these stem cells, it is especially important to utilize a system that results in a minimal risk of insertional mutagenesis. To date, only one animal virus, the adeno-associated virus (AAV), is able to integrate into a defined site in human chromosome, AAVS1 (19q13.4), which is mediated by the activity of specific replicase/integrase protein, Rep. The Rep78 or Rep68 protein recognizes the GAGC motif on the viral inverted terminal repeat (ITR) sequence and a similar motif in AAVS1, leading to the site-specific integration of the AAV genome.

We and others have reported that a plasmid transfection system utilizing AAV derived components, the *rep* gene and ITR, could integrate the gene of interest preferentially into AAVS1 in epithelial or adherent cells (e.g., 293, HeLa, Huh-7 cells) [24–26]. Our system uses two plasmids, one harboring the transgene cassette flanked by the ITR sequences, and the other for *rep* expression, allowing only plasmid DNA harboring the ITR to integrate into the AAVS1 locus. In addition, this system can deliver DNA segments larger than the 4.5-kb packaging limit of AAV. As a first step toward establishing a method capable of integrating therapeutic DNA into the AAVS1 locus in MSCs, we tested this strategy in KM-102 cells, a cell line derived from human marrow stromal cells. KM-102 cells were co-transfected with a bicistronic plasmid containing a humanized GFP gene and a blasticidin S resistance gene (*bsr*) between the ITRs and a Rep68 plasmid. After transfection, single cell clones were grown in the presence of

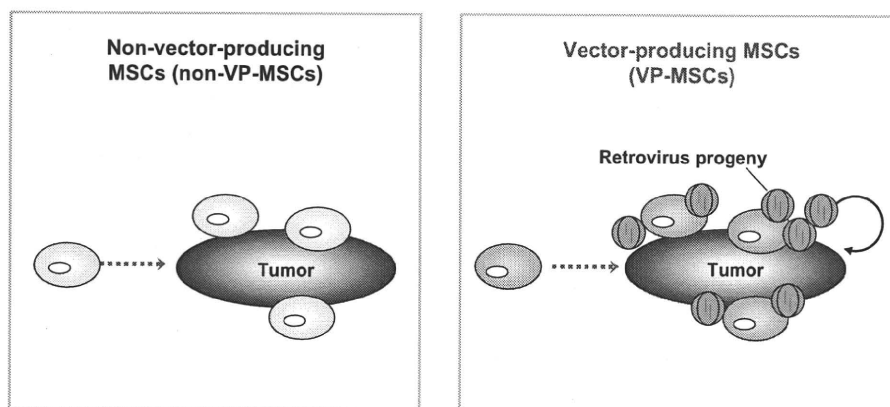


Fig. 3. Development of vector-producing tumor-tracking MSCs to augment suicide cancer gene therapy.

blasticidin S. Southern blot analysis of their genomic DNA revealed that three out of eight blasticidin S resistant clones showed site-specific integration of transgene into the AAVS1 site and that these clones had the GFP gene only at AAVS1. These results indicated that foreign DNA linked with ITR sequence could be targeted specifically into AAVS1 in KM-102 cells.

It is reported that the genome of myosin binding subunit 85 (MBS85) overlaps with the AAVS1 site [27]. To identify the junction between the transgene plasmid and the AAVS1 site, PCR was conducted using a transgene- and an AAVS1-specific primers. In two of the three clones the integration site was identified. In one clone the GFP gene was inserted at the first intron of MBS85 gene. The other clone had insertion of the GFP gene upstream of the first exon. Quantification of mRNA for MBS85 by real time PCR showed that the mRNA level decreased in these two KM-102 clones. The MBS85 is involved in the assembly of actin cytoskeleton. Although the outcome of allelic disruption of the MBS85 genome should be carefully evaluated, the system for AAVS1-specific integration of therapeutic DNA using AAV integration machinery is particularly valuable for *ex vivo* gene therapy applications for stem cells, such as ES cells and MSCs. For additional readings on the use of bone marrow cells for the treatment of autoimmunity, the reader is referred to companion papers published herein in this special issue of the Journal of Autoimmunity [28–38].

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