Wnt3/RhoA/ROCK signaling pathway is involved in adhesion-mediated drug resistance of multiple myeloma in an autocrine mechanism

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Abstract

Adhesion of myeloma cells to bone marrow stromal cells is now considered to play a critical role in chemoresistance. However, little is known about the molecular mechanism governing cell adhesion–mediated drug resistance (CAM-DR) of myeloma cells. In this study, we focused our interests on the implication of the Wnt signal in CAM-DR. We first screened the expression of Wnt family in myeloma cell lines and found that Wnt3 was overexpressed in all the myeloma cells examined. KMS-5 and ARH77, which highly expressed Wnt3 protein, tightly adhered to human bone marrow stromal cells, and accumulation of β-catenin and GTP-bounded RhoA was observed in these myeloma cell lines. Conversely, RPMI8226 and MM1S, which modestly expressed Wnt3 protein, rather weakly adhered to human bone marrow stromal. We then examined the relevance of Wnt3 expression to adhesive property to stromal cells and to CAM-DR of myeloma cells. KMS-5 and ARH-77 exhibited apparent CAM-DR against doxorubicin. This CAM-DR was significantly reduced by anti-integrin β_1 antibody, anti-integrin α_6 antibody and a Wnt-receptor competitor, secreted Frizzled-related protein-1, and Rho kinase inhibitor Y27632, but not by the specific inhibitor of canonical signaling (Dickkopf-1), indicating that Wnt-mediated CAM-DR that is dependent on integrin α_6/β_1 (VLA-6)–mediated attachment to stromal cells is induced by the Wnt/RhoA/Rho

kinase pathway signal. This CAM-DR was also significantly reduced by Wnt3 small interfering RNA transfer to KMS-5. These results indicate that Wnt3 contributes to VLA-6–mediated CAM-DR via the Wnt/RhoA/ROCK pathway of myeloma cells in an autocrine manner. Thus, the Wnt3 signaling pathway could be a promising molecular target to overcome CAM-DR of myeloma cells. [Mol Cancer Ther 2007;6(6):1774–84]

Introduction

Multiple myeloma is an end-stage B-cell neoplasia characterized by localization of malignant plasma cells in the bone marrow and not in other locations. Despite tremendous efforts in developing effective treatment modalities such as autologous stem cell transplantation, exploring new molecular targeting drugs, etc., the disease still remains incurable, mainly due to the ultimate acquisition of drug resistance (1–7).

One of the main mechanisms underlying the drug resistance has been ascribed to the adhesive property of myeloma cells to the extracellular matrix or stromal cells in bone marrow (8–11). We and others have reported that in various hematologic malignancies, including multiple myeloma, molecules responsible for the adhesive nature of tumor cells were integrins such as VLA-4 and VLA-5 (12–14). Through the interaction of integrins with their ligands, up-regulation of antiapoptotic bcl-2 family members and/or overexpression of *multidrug resistant gene 1* are induced, thereby causing tumor cells to become unresponsive to anticancer drugs (12, 13, 15). This cell adhesion–mediated drug resistance (CAM-DR) has been overcome *in vitro* and *in vivo* by a combination of chemotherapy with anti-integrin antibodies or inhibitors to drug resistance–related intracellular signal pathways (16). Furthermore, recent studies have revealed that various humoral factors from tumor cells and/or stromal cells were implicated in the adhesion and motility of myeloma plasma cells by their autocrine or paracrine action (17–21). Among the humoral factors, Wnt family members, which play a crucial role in differentiation and proliferation of normal and malignant cells in addition to the motility or adhesion mentioned above, are often activated in both bone marrow stromal cells and B-cell neoplasia including multiple myeloma (4, 22–26). On the basis of these notions, in the present study, we elucidated if any Wnt family members are involved in CAM-DR of multiple myeloma.

Materials and Methods

Cell Lines and Human Stromal Cells

Human myeloma cell lines RPMI8226, EBV-transformed ARH-77 (27, 28), MM1S, and KMS-5 (29, 30); lymphoma cell

lines Ramos, PEER, and Molt-4; and leukemia cell lines KG-1, HL-60, and U937 were cultured in RPMI 1640 containing 10% heat-inactivated FCS (Life Technologies), 2 mmol/L L-glutamine, 0.1% penicillin (100 units/mL), and streptomycin (100 mg/mL). *hTERT* gene–transduced human stromal cells (hTERT-stromal cells) were generated as previously described (31, 32) and were cultured in long-term culture medium containing MEM-α, 12.5% horse serum (Life Technologies), 12.5% FCS, 1×10^{-6} mol/L hydrocortisone (Sigma), 1×10^{-4} mol/L β-mercaptoethanol (Sigma), 2 mmol/L L-glutamine, 0.1% penicillin (100 units/mL), and streptomycin (100 mg/mL).

Antibodies, Wnt Antagonists, and Chemical Reagents

The specific antibody for β-catenin (clone 14) was purchased from BD. The specific antibodies for Wnt3 (sc-5212), RhoA (sc-418), and Cdc42 (sc-8401) were from Santa Cruz Biotechnology. The specific antibody for Rac (clone 23A8) was from Upstate, and the specific antibody for Wnt5a was purchased from R&D Systems. Function-blocking anti-integrin β_1 antibody 4B4, anti-integrin α_4 antibody HP2/1, and anti-integrin α_5 antibody SAM1 were obtained from Coulter, and anti-integrin α_v AMF7, anti-integrin α_2 Gi9, anti-integrin α_3 C3, and anti-integrin α_6 GoH3 (33) were obtained from Immunotech. Isotype control mouse IgG1 antibody MOPC21 (Sigma) and rat IgG2a antibody DD13 (Chemicon) was used in the control experiments. Recombinant secreted Frizzled-related protein-1 (sFRP-1) and Dickkopf-1 (DKK-1) were obtained from R&D Systems. Rho kinase (ROCK) inhibitor Y-27632 and GRGDS (H-Gly-Arg-Gly-Asp-Ser-OH) peptide were purchased from Calbiochem. Doxorubicin was obtained from Sigma and dissolved in sterile double-distilled water before use.

Reverse Transcription-PCR

One microgram of total RNA was reverse transcribed by SuperScript II (Invitrogen) and amplified using TaKaRa Ex Taq (TAKARA BIO, Inc.), with the primers described below. All PCR reactions were done using a PTC-200 Peltier Thermal Cycler (MJ Japan). PCR amplification using specific primers for *Wnt1* (5-GCTCCTGCTCAGAAGGTTCCAT-3, 5-GTCCGCTGTACGTGCAGAAGTT-3), *Wnt2B* (5-GTAGCCAGGGTGAACTGAGTGT-3, 5-CTAGGGAAC-CTGCAGCCTTGT-3), *Wnt3* (5-ATCATAAGGGG-CCGCCTGGCGAA-3, 5-CTTGCAGGTGTGCACGTCG-TAGA-3), *Wnt3A* (5-CTGGACAAAGCTACCAGGGAGT-3, 5-GTGGGCACCTTGAAGTAGGTGT-3), *Wnt5A* (5-CAAGGTGGGTGATGCCCTGAA-3, 5-GCACGGTCTT-GAACTGGTCGTA-3), *Wnt7A* (5-CCTGGACGAGTGT-CAGTTTCAG-3, 5-GGCCTCGTTGTACTTGTCCTTG-3), *Wnt10B* (5-GCAAGAGTTTCCCCCACTCTCT-3, 5-GTC-TCGCTCACAGAAGTCAGGA-3), *Wnt11* (5-CTCTGCG-CGCCTCTCTGGAAA-3, 5-GTGGGAGCCCACCTTCTC-ATT-3), or *glyceraldehyde phosphate dehydrogenase* (5-ACAT-CAAGAAGGTGGTGAAGCAGG-3, 5-CTCTTCCTCTT-GTGCTCTTTGCTGG-3) was done under the following conditions: one cycle of 98°C for 30 s; 35 cycles (33 cycles for Wnt3) of 98°C for 10 s, 62°C for 20 s, 72°C for 60 s; and one cycle of 72°C for 10 min. The PCR products were electrophoresed on an agarose gel and visualized by ethidium bromide staining.

Transfection of Small Interfering RNA

Dicer substrate RNA duplexes for use in RNA interference against *Wnt3* (NM_ 030753) were obtained from iGENE Therapeutics. The sequences of dsRNA synthesized newly were as follows: sense, 5′-ACGGAGAAGCGGAAG-GAAAAAUGCCAG-3′; antisense, 5′-UAUGCCUCUUCG-CCUUCCUUUUUACGG-3′. The non-silencing control small interfering RNA (siRNA) for human was purchased from iGENE Therapeutics.

RNA duplexes specific for the human *Wnt3* were transfected into KMS-5 or ARH-77 cells using the DeliverX transfection kit (Panomics) according to provided protocols. Briefly, 5 μmol/L working stocks of siRNA were diluted with Buffer-1, and siRNA Transfection Reagent was diluted with Buffer-2. Both solutions were mixed well and incubated at 37°C for 20 min to form working siRNA/siRNA transfection reagent complex. Cells were pelleted in a microtube (Falcon) by spinning for 5 min at $400 \times g$. Cells were washed thrice by PBS and pelleted again at $400 \times g$ for 5 min. After aspiration of the PBS, cells were resuspended with 300 μL of working siRNA/siRNA transfection reagent complex and incubated at room temperature for 5 min. After adding 300 μL of Opti-MEM (Invitrogen) to the microtubes, 600 μL of cells/transfection complex were transferred into one well of a six-well plate. Final concentration of Wnt3 siRNA in the sample well was 30 nmol/L. Cells/transfection complex was incubated for 4 h at 37°C and 5% CO_2. Subsequently, 1 mL of complete growth media was added and incubated overnight. Next day, after cells were washed once, and cells were washed to remove transfection complex and cultured in complete growth media at 37°C and 5% CO_2 before use.

Plasmid Vectors, Gene Transduction, and Wnt3 Conditioned Medium

The CA promoter (chicken β-actin promoter with cytomegalovirus enhancer)–based plasmid vector pCAcc was employed in this study. Human *Wnt3* cDNA containing plasmid, pCR®2.1-hWnt3, was previously described (34). To construct pCAcc-hWnt3, pCR®2.1-hWnt3 was digested with *Eco*RI and ligated in pCAcc. The integrity of the pCAcc-Wnt3 was confirmed by restriction endonuclease digestion and DNA sequencing. pCAcc-Wnt3 and pcDNA3-neo were cotransfected into L cells (American Type Culture Collection) using LipofectAMINE 2000 (Invitrogen) according to manufacturer's instructions. G418-resistant colonies were cultured using 600 μg/mL G418 for 2 weeks, and stably transfected clones that revealed β-catenin stabilizing activity were selected. Wnt3 conditioned medium (CM) or control CM was prepared from Wnt3-producing L cells (L-Wnt3) or control L cells grown to confluency in DMEM containing 10% FCS, 72 h after which culture supernatants were collected and designated Wnt3 CM and control CM, respectively.

Contact and Non-Contact Culture System

Stromal contact culture systems were constructed using BD Falcon Cell Culture Inserts and Companion Plates (BD;

pore size: 0.4 μm, pore density: $1 \times 10^8/cm^2$, 12 wells) to avoid contamination of stromal cells as reported previously (13, 35). Briefly, 1×10^4 or 5×10^4 hTERT-stromal cells were plated on the back side of the insert membrane. After obtaining a confluent stromal layer, stromal cells were washed twice and were cultured in companion plates for 2 days. For the adhesion assay, myeloma cells were washed once and resuspended in RPMI 1640 supplemented with 10% FCS, and 2×10^5 cells were added to each culture insert. In some experiments, 30 μg/mL of function-blocking anti-integrin antibodies were added to each culture insert at the time of cell seeding. Following 24 h of incubation at 37°C and 5%CO_2, suspended cells were collected, and attached cells were harvested by three trypsin-EDTA treatments. The percentage of adhesion was calculated by the ratio between the number of attached cells and the total number of collected cells. In non-contact culture, 5×10^4 hTERT-stromal cells were plated on companion plates; thereby, myeloma cells were physically separated from the stromal layer by a membrane of culture insert.

Immunofluorescent Staining for Assessment of Cell Morphology

Myeloma cells were serum starved for 12 h and incubated with control or Wnt3 CM with or without DKK-1 or sFRP-1 for 6 h. After two washings with PBS, the cells were fixed in 4% paraformaldehyde in PBS for 30 min. The fixed cells were permeabilized with PBS containing 0.2% Triton X-100 for 15 min. After the cells were soaked in 10% goat serum (Life Technologies) with PBS for 30 min, they were treated with the anti-vinculin antibody (clone hVIN-1; Sigma) in 10% goat serum with PBS for 2 h. The cells were then washed with PBS thrice followed by incubation with Alexa Fluor 488 (Molecular Probes) in 10% goat serum with PBS for 30 min. For the detection of actin filaments, Texas Red phalloidin (Molecular Probe) was mixed with the second antibody solution. After the cells were washed with PBS thrice, they were covered with an anti-fade medium containing the nuclear stain 4′,6-diamidino-2-phenylindole (VECTASHIELD DAPI, Vector Laboratories). Subsequently, they were examined using an R2100AG2 confocal laser scanning microscope (Bio-Rad, Laboratories).

Drug Cytotoxicity Assay

For drug cytotoxicity assays, myeloma cells were washed once and resuspended in RPMI 1640 containing 10% FCS, and 1×10^5 cells were added to each culture insert and cultured in the presence or absence of function-blocking anti-integrin antibodies or the Wnt inhibitors sFRP-1 or DKK-1. After 24 h, 0.03 to 3.0 μmol/L doxorubicin or vehicle control was added to each well and incubated for 1 h, then medium containing doxorubicin was removed and replaced by drug-free medium with or without the blocking antibodies, or the Wnt inhibitors sFRP-1 or DKK-1. After 24 h of incubation, suspended and attached myeloma cells were collected by three treatments of trypsin-EDTA, and cells was pelleted at $400 \times g$ for 5 min, resuspended by 200 μL of complete culture medium, and placed into 96-well palates. The survival cells were assessed by Annexin V-FITC Apoptosis Detection kit (Medical & Biological Labo-

ratories) and Premix WST-1 assay Cell Proliferation Assay System (Takara). The WST-1 assay is based on mitochondrial conversion of WST-1 to yellowish formazan, being indicative of the number of viable cells. The number of viable cells was evaluated by absorbance $A_{450\ nm}$ using Model 680 microplate reader (Bio-Rad Laboratories).

Western Blotting

For immunoblot analysis of Wnts, total protein lysates from myeloma cells were extracted with lysis buffer containing 50 mmol/L Tris, 150 mmol/L NaCl, 1.0% NP40, 0.5% sodium deoxycholic acid, and 0.1% SDS and inhibitor cocktails (Roche). Alternatively, for immunoblot analysis of β-catenin, cells were serum starved for 12 h with or without 10 μg/mL sFRP-1 or 1 μg/mL DKK-1. Subsequently, the cytosolic proteins of the cells were prepared with the Subcellular Proteome Extraction kit (Calbiochem) according to provided protocols. After protein contents were determined using a protein assay kit (Bio-Rad), protein lysates were separated on 4% to 20% SDS-polyacrylamide gradient gel and transferred to a polyvinylidene fluoride membrane using a semidry transfer apparatus (Bio-Rad). Immunoblots were probed with primary antibodies then with horseradish peroxidase–conjugated anti-IgG (Zymed) as a secondary antibody and visualized using the enhanced chemiluminescence method (ECL plus, Amersham Pharmacia Biotech) according to the supplier's instructions.

Rho Family GTPase Activity Assay

Activity of Rho, Rac, and Cdc42 was examined with a pull-down assay using a Rho Activation Assay kit and Rac Activation Assay kit (Upstate). Briefly, cells were serum starved for 12 h with or without 10 μg/mL sFRP-1 or 1 μg/mL DKK-1. The cells were washed and lysed, and lysates were incubated with glutathione S-transferase–tagged Rhotekin Rho-binding domain or glutathione S-transferase p21-binding domain bound to glutathione-agarose for 1 h at 4°C in a rotary shaker. The beads were pelleted and washed. Protein recovered was analyzed for immunoblotting with anti-RhoA, anti-Rac, or anti-Cdc42 antibodies as described above. Whole-cell lysates were similarly immunoblotted for comparison.

Statistical Analysis

Each data set was first evaluated for normality of distribution by the Komolgorov-Smirnov test to decide whether a nonparametric rank-based analysis or a parametric analysis should be used. Two groups were compared by either the Wilcoxon signed-rank test or the Student's t test (two-tailed test). $P < 0.05$ was considered statistically significant.

Results

Relevance of Adhesive Property of Myeloma Cells onto Stromal Cells to CAM-DR

To gain insight into the relationship between the adhesive property and drug resistance of multiple myeloma, we first examined the adhesive potential of four myeloma cell lines, including ARH-77, KMS-5, RPMI8226,

and MM1S, to hTERT-stromal cells. As shown in Fig. 1A, ARH-77, KMS-5 showed high adhesive potential onto hTERT-stroma cells, and RPMI8226 showed moderate adhesive potential, whereas MM1S exhibited relatively low adhesive potential (Fig. 1A). Next, we assessed the drug resistance of these myeloma cell lines in both contact and non-contact culture systems with hTERT-stromal cells. High adhesive cell lines ARH-77 and KMS-5, moderate adhesive cell line RPMI8226, and low adhesive cell line MM1S were used in WST-1 assay (Fig. 1B) and Annexin V-propidium iodide assay (Fig. 1C). In adhesive cell lines, sensitivity for doxorubicin in contact culture condition was significantly lower than that seen in non-contact culture condition or stroma-free culture (Fig. 1B and C). However, no significant difference of doxorubicin sensitivity among stroma-free culture, non-contact culture, and contact culture was observed in low adhesive MM1S (Fig. 1B and C). These results suggest that direct adhesive interaction between myeloma cells and human stromal cells are relevant to induce drug resistance.
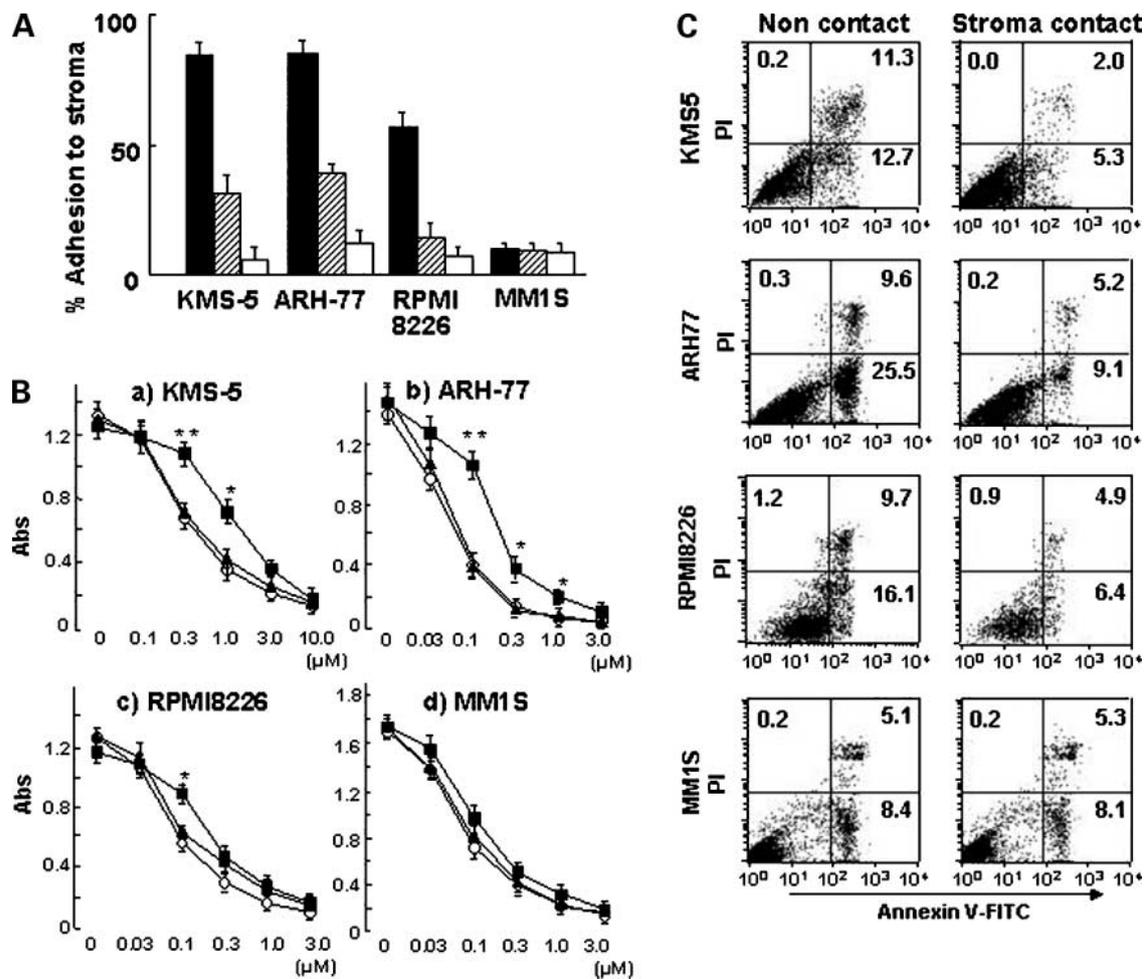
Figure 1. Adhesion rate of myeloma cell lines onto human stromal cells and comparison of survival advantage between low and high adhesive cells following doxorubicin exposure. **A,** screening for adhesion rate of myeloma cell lines onto human stromal cells. Five hundred thousand myeloma cells were allowed to adhere on stroma-free condition (*open columns*), 1×10^4 (*diagonal columns*), or 5×10^4 (*closed columns*) hTERT-stromal cells. The number of non-adherent myeloma cells and adherent myeloma cells were counted 24 h after incubation. *Y*-axis, percentage of attached cells for total number of cells. Represent three independent experiments, each done in triplicate. *Columns,* % adhesion to stroma; *bars,* SD. **B,** cytotoxic assay of myeloma cell lines by analyzing mitochondrial conversion of WST-1 to yellowish formazan. Each cell line was adhered onto 5×10^4 hTERT-stromal cells and then exposed to doxorubicin. *X*-axis, dose of doxorubicin; *Y*-axis, absorbance $A_{450\,nm}$ (*Abs*). ○, stroma-free culture; ▲, non-contact culture; ■, stromal contact culture. *a,* KMS-5 (LD_{50}: stroma-free, 0.305 μmol/L; non-contact, 0.360 μmol/L; contact, 1.439 μmol/L); *b,* ARH77 (LD_{50}: stroma-free, 0.053 μmol/L; non-contact, 0.052 μmol/L; contact, 0.265 μmol/L); *c,* RPMI8226 (LD_{50}: stroma-free, 0.106 μmol/L; non-contact, 0.139 μmol/L; contact, 0.265 μmol/L); *d,* MM1S (LD_{50}: stroma-free, 0.096 μmol/L; non-contact, 0.099 μmol/L; contact, 0.139 μmol/L). Represent three independent experiments, each done in quadruplicate. *, $P < 0.05$, non-contact culture versus contact culture; **, $P < 0.01$, non-contact culture versus contact culture (Student's *t* test). **C,** annexin assay of myeloma cell lines. *Y*-axis, propidium iodide (*PI*). KMS-5 myeloma cells were analyzed 24 h after exposure of 0.3 μmol/L doxorubicin, and other myeloma cells were analyzed 24 h after exposure of 0.1 μmol/L doxorubicin. One representative experiment of five showing similar results. The propidium iodide – positive KMS-5, ARH77, RPMI8226, or MM1S myeloma cells before doxorubicin exposure were $0.47 \pm 0.10\%$, $0.29 \pm 0.09\%$, $0.74 \pm 0.12\%$, or $0.04 \pm 0.02\%$ in non-contact culture and $0.37 \pm 0.12\%$, $0.12 \pm 0.09\%$, $1.82 \pm 0.59\%$, or $0.10 \pm 0.09\%$ in contact culture, respectively.
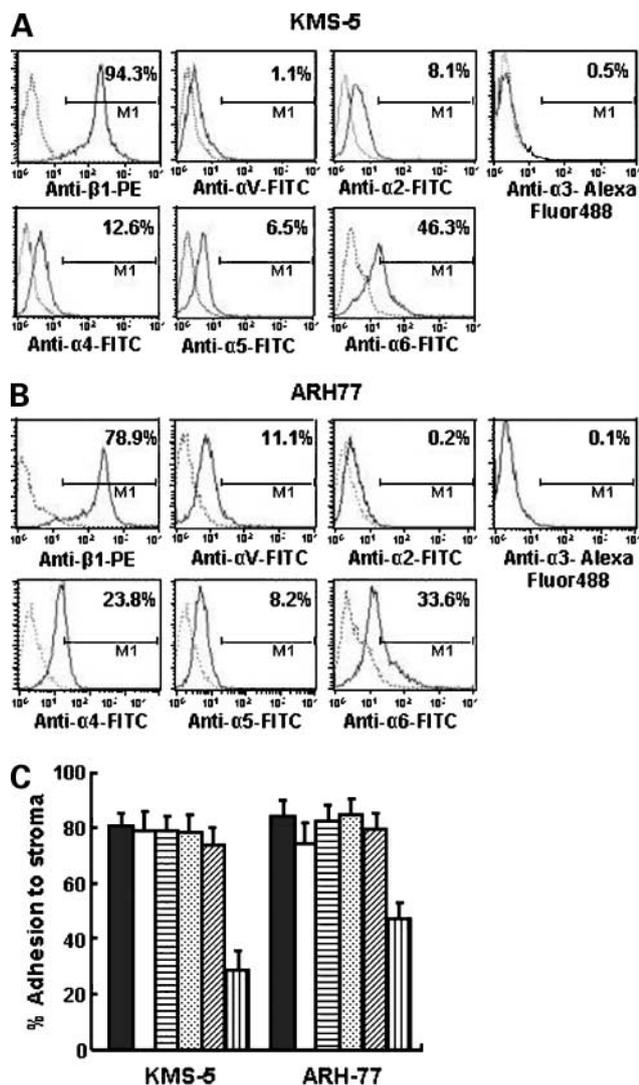
Figure 2. Flowcytometric analysis of integrin expression in myeloma cells and contribution of α_4, α_5, α_6, and β_1 integrin subunits to stromal adhesion. **A,** expression of integrins on KMS-5. **B,** expression of integrins on ARH-77. **C,** percentage adhesion of myeloma cells with 5×10^4 hTERT-stromal cells in the absence (*closed columns*) or presence of anti-β_1 (*open columns*), anti-α_4 (*horizontal columns*), anti-α_5 integrin antibody (*stippled columns*), or anti-α_6 integrin antibody (*diagonal columns*). The concentration of each anti-integrin antibody was 30 μg/mL. *Vertical column,* positive control by using 2.5 mg/mL GRGDS peptide of cell adhesion inhibitor. Represent three independent experiments, each done in triplicate. *Columns,* % adhesion to stroma; *bars,* SD.

Inhibition of CAM-DR of Myeloma Cells by Neutralizing Anti-Integrin Antibodies

It was shown that attachment of myeloma cells with extracellular matrix, including fibronectin, is inhibited by anti-integrin antibodies (27, 36). We first screened the expression of integrin on high adhesive myeloma cell line. Consistent with previous reports, KMS-5 and ARH-77 expressed various integrins (27, 37, 38). Among them, α_4, α_5, α_5, α_6, or β_1 were commonly expressed on both high adhesive KMS-5 and ARH-77 (Fig. 2A and B). Hence, we

next investigated whether the adhesion of myeloma cells to hTERT-stromal cells could be inhibited by antibodies for integrins α_4, α_5, α_6, or β_1. However, these antibodies did not affect the adhesion rates of ARH-77, KMS-5, or RPMI8226 onto stromal cells (Fig. 2C), although GRGDS peptide partially inhibited adhesion between myeloma cells and stromal cells, suggesting that the adhesive property of these myeloma cell lines to stromal cells is determined not only by α_4, α_5, α_6, or β_1 integrins but also by other unknown adhesion molecules (15, 17). We further assessed whether CAM-DR of multiple myeloma was inhibited by these anti-integrin antibodies. As shown in Fig. 3A, CAM-DR of KMS-5 and ARH-77 were substantially reduced by neutralizing anti-integrin β_1 4B4 in a dose-dependent manner (Fig. 3A). However, the reduction level did not reach that seen in the stroma free condition, and it leveled out at concentrations of 10 to 30 μg/mL of the 4B4 antibody (Fig. 3A and B). The reduction rate of CAM-DR in the presence of anti-integrin α_4 HP2/1 was not significantly different from that in the absence of the neutralizing antibody, although a similar tendency as that seen in the anti-integrin β_1 antibody was observed ($P = 0.10$; Supplementary Fig. S1A).[3] In addition, anti-integrin α_5 SAM1 did not affect CAM-DR (Supplementary Fig. S1B).[3] Interestingly, CAM-DR of KMS-5 and ARH-77 were significantly reduced by neutralizing anti-integrin α_6 GoH3 (ref. 33; Fig. 3C and D; Supplementary Fig. S2).[3] Collectively, substantial inhibition of CAM-DR was observed by blocking the integrin β_1/integrin α_6 molecule (VLA-6) without changing the adhesive property of myeloma cells to stromal cells. These findings are consistent with previous reports showing that integrin β_1 was a key molecule of CAM-DR. (12, 36) However, these results also suggested that some additional but minor molecular mechanisms should be involved in CAM-DR because the inhibitory effect of neutralizing anti-integrin β_1 on CAM-DR was not complete.

Relationship between Adhesive Property and Wnt3 Expression in Myeloma Cells

It was reported that Wnt family members contribute to modulation of the adhesive property of tumor cells onto extracellular matrix and tumor-associated fibroblasts (25, 26). More recently, it has been shown that Wnt3a enhanced the adhesive and invasive properties of myeloma cells to SV40 large T antigen–transduced human stromal cells (22, 39). We and others have disclosed that a series of Wnt family members (Wnt1, Wnt2b, Wnt3, Wnt5a, Wnt7a, Wnt10b, and Wnt11 mRNA) were expressed in human fetal and postnatal bone marrow as evaluated by reverse transcription-PCR (24, 40, 41). Based on these findings, we screened the expression of these Wnt family members in leukemia, lymphoma, and myeloma cell lines. As shown in Fig. 4A, except for Wnt3a, they were all detected with

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[3] Supplementary material for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

variable intensities in the hematologic malignant cells examined. Among these, only *Wnt3* and *Wnt5a* mRNA were detected in four of four myeloma cell lines. Accordingly, we analyzed the expression of Wnt3 and Wnt5a protein in these myeloma cell lines by Western blotting (Fig. 4B). Interestingly, highly and moderately adhesive cells (ARH-77, KMS-5, and RPMI8226) showed apparently higher expression levels of Wnt3 than low adhesive cells (MM1S). These results suggested a close relationship between adhesive potential and Wnt3 expression in myeloma cells.

### Functional Analysis of Wnt Signaling in Myeloma Cells

It has been reported that two Wnt signaling pathways, the Wnt/β-catenin pathway (canonical pathway) and the Wnt/RhoA pathway (non-canonical pathway), are involved in the regulation of the biological behavior of myeloma cells (22, 39). Hence, we focused our attention on

these pathways in our cell lines. High levels of β-catenin accumulation and RhoA activation were observed in ARH-77, KMS-5, and RPMI8226, which were higher adhesive to stromal cells than those in the low adhesive cells (MM1S; Fig. 4B). To test if the canonical and non-canonical signals are transduced by Wnt secreted from tumor cells in an autocrine manner, we treated KMS-5 cells with a Wnt inhibitor (sFRP-1), which blocks all Wnt signaling by binding directly to Wnts (DKK-1), which specifically blocks Wnt/β-catenin signaling by binding to the LRP coreceptor, and analyzed the changes of β-catenin accumulation and RhoA activation. Accumulation of β-catenin in KMS-5 was reduced by addition of either DKK-1 or sFRP-1. Furthermore, activation of RhoA was reduced by addition of sFRP-1, whereas it was not changed by DKK-1 (Fig. 4C). Incidentally, neither DKK-1 nor sFRP-1 affected activation of Rac or Cdc42 (Fig. 4C), consistent results with previous reports showing that Wnt signaling plays an important
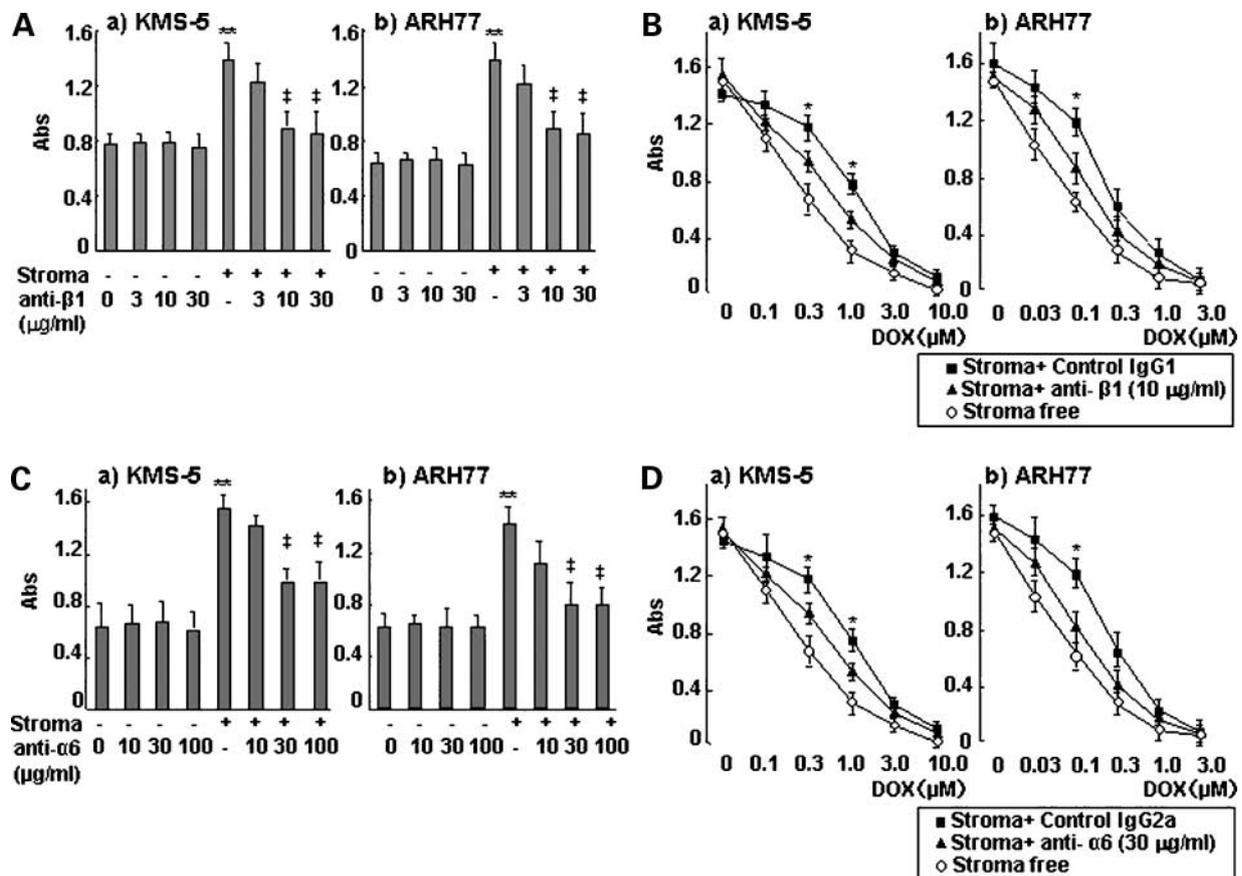
Figure 3.   Contribution of α_6 and β_1 integrin subunits to CAM-DR. KMS-5 or ARH-77 myeloma cells were incubated in a stroma-free (−) or stromal contact (+) culture system and subsequently exposed to doxorubicin for 1 h. *Y*-axis, absorbance $A_{450\ nm}$. **A,** survival of myeloma cells after exposure to 0.3 μmol/L (KMS-5) or 0.1 μmol/L (ARH-77) of doxorubicin by the treatment of 0 to 30 μg/mL anti-integrin β_1 neutralizing antibody (4B4). For control study, 10 μg/mL of isotype control antibody was used as indicated (−). **B,** dose-response curve of myeloma cell lines following multiple doses of doxorubicin (*DOX*) exposure in the presence of 30 μg/mL anti-β_1 antibody. **C,** survival of myeloma cells after exposure to 0.3 μmol/L (KMS-5) or 0.1 μmol/L (ARH-77) of doxorubicin by the treatment of 0 to 30 μg/mL anti-α_6 (GoH3). **D,** dose-response curve of myeloma cell lines in the presence of 30 μg/mL anti-α_6 antibody. *a,* KMS-5; *b,* ARH77. ****,** $P < 0.01$, stroma-free with isotype control versus contact culture with isotype control antibody; ‡, $P < 0.01$, contact culture with isotype control versus contact culture with neutralizing antibody (Student's *t* test; **A** and **C**). *, $P < 0.05$, non-contact culture versus contact culture (**B** and **D**). Represent three independent experiments, each done in quadruplicate. *Columns/points,* absorbance $A_{450\ nm}$; *bars,* SD.
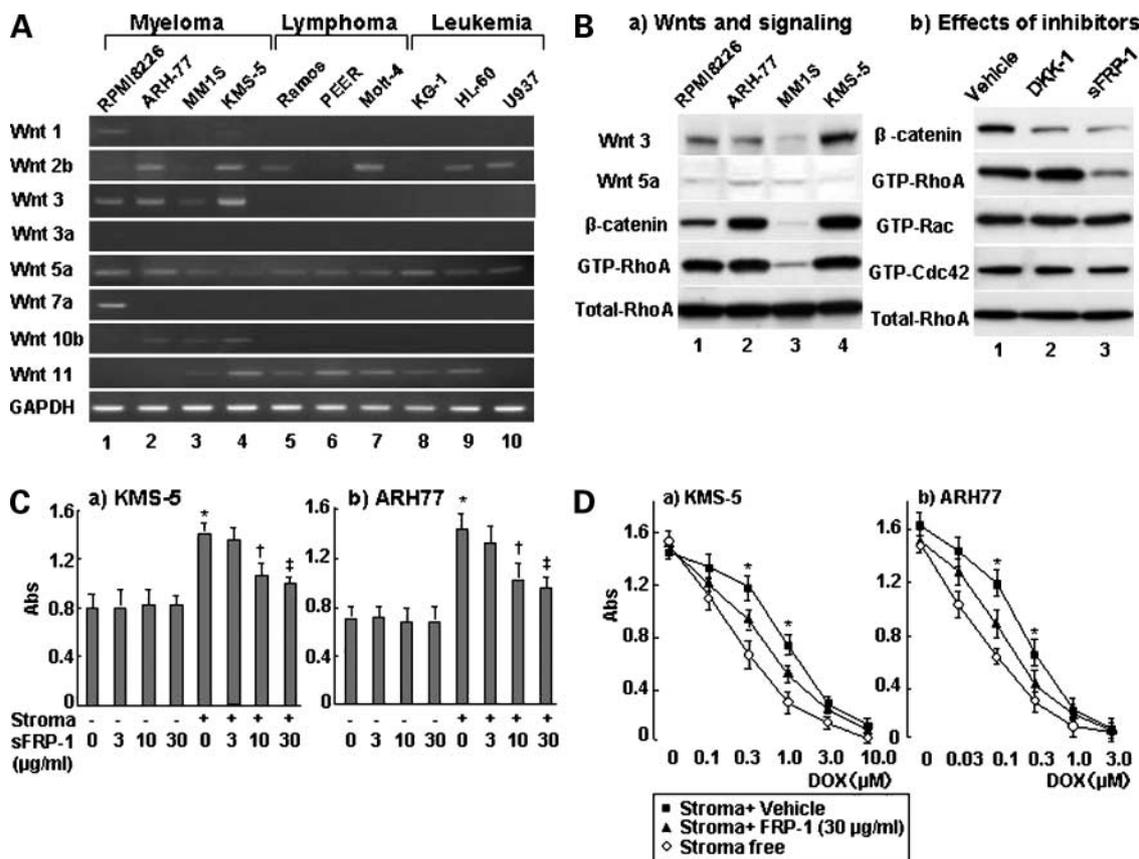
Figure 4. Screening for Wnts expression in myeloma cells and the effect of Wnt antagonists. **A,** reverse transcription-PCR analysis for myeloma, lymphoma, and leukemia cell lines. **B,** *a,* immunoblot analysis of the Wnt3 and Wnt5a expression, accumulation of β-catenin, and activation of RhoA. *b,* effect of sFRP-1 or DKK-1 on downstream of Wnt signaling in KMS-5 cells. *Lane 1,* treatment with vehicle; *lane 2,* 1 μg/mL DKK-1; *lane 3,* 10 μg/mL sFRP-1. **C,** effect of sFRP-1 on CAM-DR. KMS-5 and ARH-77 myeloma cells were incubated in a stroma-free (−) or stromal contact (+) culture system and subsequently exposed to 0.3 μmol/L (KMS-5) or 0.1 μmol/L (ARH-77) doxorubicin for 1 h. *Y*-axis, absorbance $A_{450 nm}$; *X*-axis, dose of sFRP-1. *, $P < 0.01$, stroma-free culture with vehicle versus contact culture with vehicle; †, $P < 0.05$, contact culture without inhibitor versus contact culture with inhibitor; ‡, $P < 0.01$, contact culture without inhibitor versus contact culture with inhibitor (Student's *t* test). **D,** dose-response curve after multiple doses of doxorubicin exposure in the presence of 30 μg/mL sFRP-1. *Y*-axis, absorbance $A_{450 nm}$; *X*-axis, dose of doxorubicin. Represent three independent experiments, each done in quadruplicate. *, $P < 0.01$, contact culture without FRP-1 (*vehicle*) versus contact culture with FRP-1 (Student's *t* test).

role in modulation of cell motility via RhoA activation, but not Rac1 (22, 39, 42). These results indicate that both the Wnt/β-catenin pathway and the Wnt/RhoA pathway are functional in KMS-5 myeloma cells.

**Reduction of CAM-DR of Multiple Myeloma by Inhibition of Wnt/RhoA Pathway**

We next investigated whether the Wnt signal pathway is involved in induction of CAM-DR of multiple myeloma (43, 44). To evaluate this possibility, we did a cytotoxic assay using a stroma-free or stromal contact culture system in the presence or absence of Wnt inhibitors. As the results show in Fig. 4C and D, sFRP-1 significantly inhibited drug resistance evoked by contact culture with hTERT-stromal cells and KMS-5 or ARH-77 myeloma cell lines, whereas DKK-1 did not (Supplementary Fig. S3),[3] indicating that RhoA signaling is the main pathway in modulation of CAM-DR of multiple myeloma. To examine whether Wnt3 produced by myeloma cells directly induced CAM-DR, we conducted knockdown study by using Wnt3 siRNA. As shown in Fig. 5A, dicer substrate Wnt3 siRNA could

decrease expression of Wnt3 protein (Fig. 5A). Furthermore, CAM-DR elicited by coculture with hTERT-stroma cells and KMS-5 or ARH-77 cells was significantly reduced by transfer of Wnt3 siRNA into these myeloma cells (Fig. 5B). These results suggested that Wnt3 was involved in CAM-DR in autocrine manner. However, the degree of reduction of CAM-DR by sFRP-1 or Wnt3 siRNA was not complete. On the contrary, complete reduction of CAM-DR to a stroma-free level was attained by addition of Y27632, which is an inhibitor of Rho kinase, downstream of RhoA (Fig. 5C). Collectively, in multiple myeloma, activation of RhoA signaling is responsible for generation of CAM-DR, and the main stimulant of this pathway (Wnt3), secreted from tumor cells through some other minor, non-Wnt stimulants to RhoA, is involved in induction of CAM-DR (45).

Effect of Wnt3 CM on CAM-DR of KMS-5 and ARH-77 Cells

As has been described above, Wnt3 is a most plausible candidate for the main RhoA stimulant from tumor cells. Hence, we further examined whether conditioned medium

from L-Wnt3 enhances CAM-DR of KMS-5 and ARH-77 cells. As shown in Fig. 6A and Supplementary Fig. S3,[3] some significant increment of CAM-DR was observed by addition of Wnt3 CM, and this increment was negated by addition of sFRP-1. We then verified this observation by the changes in RhoA and β-catenin expression revealed by
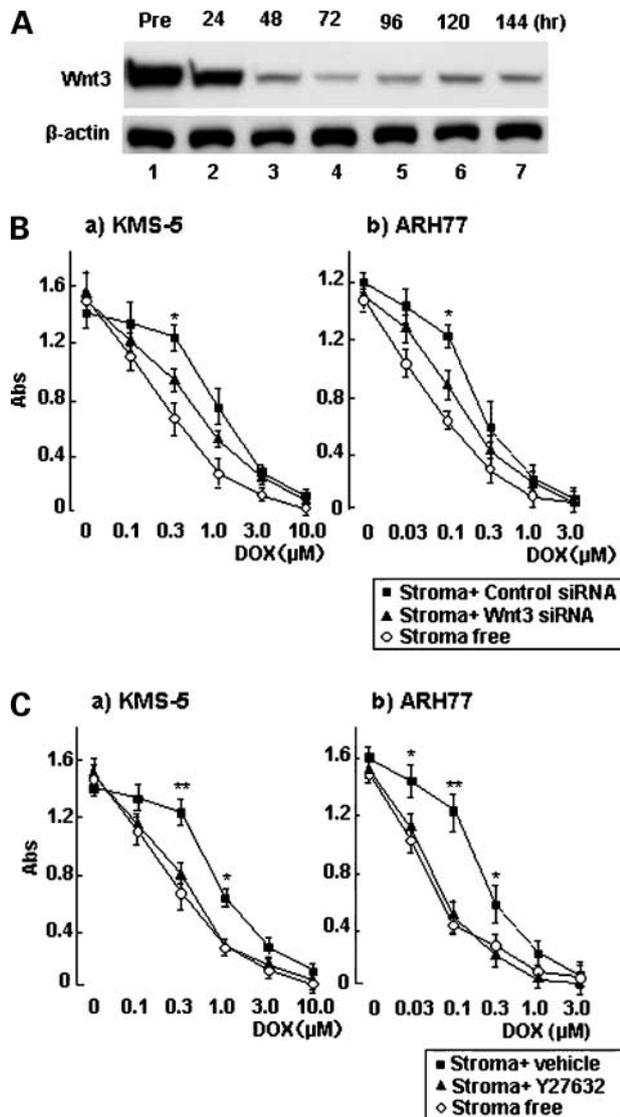
Figure 5. The effect of Wnt3 siRNA and Rho kinase inhibitor on CAM-DR. **A,** Western blot analysis of Wnt3 expression in KMS-5 cells after Wnt3 siRNA transfer. *Pre,* non-treatment of cells. β-Actin was used as internal standard. **B,** effect of Wnt3 siRNA transfer on CAM-DR. KMS-5 and ARH-77 myeloma cells were incubated in a stroma-free (−) or stromal contact (+) culture system and subsequently exposed to 0.3 μmol/L (KMS-5) or 0.1 μmol/L (ARH-77) doxorubicin for 1 h. *Y*-axis, absorbance $A_{450 nm}$; *X*-axis, Wnt3 siRNA or control siRNA. *, $P < 0.05$, contact culture with control siRNA versus contact culture with Wnt3 siRNA. **C,** effect of Rho kinase inhibitor on CAM-DR. *Y*-axis, absorbance $A_{450 nm}$; *X*-axis, dose of Rho kinase inhibitor Y27632 (10 μmol/L). Represent three independent experiments, each done in quadruplicate. *Columns,* absorbance $A_{450 nm}$; *bars,* SD. *, $P < 0.05$, contact culture without inhibitor versus contact culture with inhibitor; **, $P < 0.01$, contact culture without inhibitor versus contact culture with inhibitor (Student's *t* test).

Western blotting (Fig. 6B). By addition of Wnt3 CM, both β-catenin and active RhoA substantially increased, and the effect on β-catenin was negated by DKK-1 and sFRP-1 and that on RhoA by sFRP-1.

Rearrangement of Stress Fiber Associated with Vinculin Localization in Wnt3 CM – Treated KMS-5 Cells

The results described above suggested that the Wnt3/RhoA signal brings cells into a primed state by which cells adhere to stromal cells via integrin β1 and subsequently become refractory to drugs. However, the association between Wnt3 and integrin is unclear. We therefore explored the morphologic and immunohistochemical findings of KMS-5 cells treated with Wnt3 CM. When KMS-5 cells were incubated for >12 h, not only Wnt3 CM–treated cells but also control CM–treated cells showed similar morphologic changes (data not shown) as described below in detail; thus, we incubated them for 6 h so that control cells did not exhibit any drastic changes in morphology. Treatment with Wnt3 CM significantly accelerated a rearrangement of filamentous-actin that was detectable with phalloidin staining compared with control CM (% morphologic change, $18 \pm 5\%$ versus $61 \pm 2\%$, $P < 0.01$), and this effect of Wnt3 CM was negated by sFRP-1 but not DKK-1 treatment (% morphologic change, Wnt3 CM + sFRP-1, $14 \pm 3\%$; Wnt3 CM + DKK-1, $67 \pm 7\%$, respectively; Fig. 6C). The localization of vinculin, which is a cytoskeletal protein associated with the cytoplasmic faces of cell/extracellular matrix in integrin-mediated cellular junction (46), was similar to that of the re-assembly of the stress fibers.

Discussion

In the present study, we first confirmed a linkage of refractoriness to doxorubicin with adhesiveness to stromal cells using four different cell lines of multiple myeloma; three adhesive cell lines (ARH-77, KMS-5, and RPMI8226) showed CAM-DR, whereas the non-adhesive cell line (MM1S) was sensitive to doxorubicin. When the effect of anti-integrin antibodies on CAM-DR of the adhesive cell lines (KMS-5 and ARH-77) was examined, the anti-integrin β1 and the anti-integrin α6, but neither anti-integrin α4 nor anti-integrin α5 antibody, largely restored sensitivity to doxorubicin (Fig. 3; Supplementary Fig. S1).[3] Our findings and the previous reports that the laminin receptor VLA6 was expressed on most myeloma cell lines (27, 37, 38) suggest a pertinence of strategy to use anti-VLA6 antibody to overcome CAM-DR of multiple myeloma. However, this notion is not commonly valid for all multiple myeloma cell types but contradicts previous reports showing involvement of VLA-4 (47) or VLA-4 plus LFA-1 (45) in CAM-DR of myeloma cell lines NCI-H929 and 5TGM1. The discrepancy may be due to the difference in myeloma cell lines and the epitope of the anti-integrin α4 monoclonal antibody (27) in each experiment because extracellular matrix on stromal cells consists of quite heterogeneous molecules, and each myeloma cell line could express diverse adhesive molecule(s) relevant to CAM-DR (45, 48). Likewise, the observation that none of the antibodies against integrin β1, α4, α5,
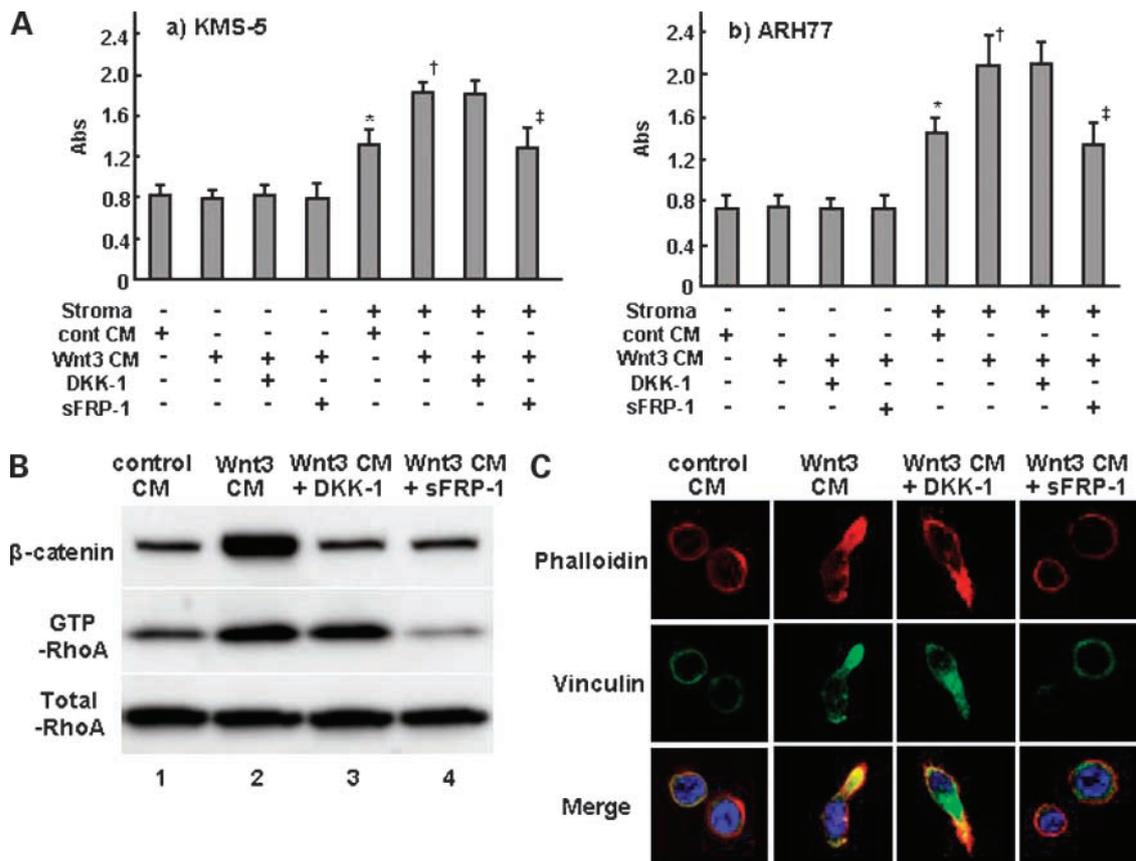
Figure 6. Effect of Wnt3 CM and Wnt antagonist on CAM-DR of KMS-5 and ARH-77 myeloma cells, Wnt signaling and adhesive property. **A,** KMS-5 (*a*) and ARH-77 (*b*) myeloma cells were incubated in a stroma free (−) or stromal contact (+) culture system and subsequently exposed to 1.0 μmol/L (KMS-5) or 0.3 μmol/L (ARH-77) doxorubicin for 1 h. *X*-axis, control CM or Wnt3 CM with or without 10 μg/mL sFRP-1 or 1 μg/mL DKK-1; *Y*-axis, absorbance $A_{450\ nm}$. Represent three independent experiments, each done in quadruplicate. *, $P < 0.01$, stroma-free with control CM versus contact culture with control CM; †, $P < 0.05$, contact culture with control CM versus contact culture with Wnt3 CM; ‡, $P < 0.01$, contact culture with Wnt3 CM versus contact culture with Wnt3 CM and Wnt inhibitor (sFRP-1; Student's *t* test). **B,** effect of Wnt3 CM on accumulation of β-catenin and activation of RhoA. KMS-5 cells were starved for 12 h and exposed to control CM or Wnt3 CM. β-Catenin accumulation was examined 6 h after treatment, and activation of RhoA was analyzed 2 h after treatment. **C,** effect of Wnt3 CM and Wnt inhibitors on adhesive property of KMS-5. KMS-5 was exposed to control CM, Wnt3 CM, Wnt3 CM + DKK-1, or Wnt3 CM + sFRP-1. The cells were fixed and double stained with Texas Red-phalloidin and the anti-vinculin monoclonal antibody. One representative experiment of three showing similar results.

or α_6 effectively detached myeloma cells from stroma (Fig. 2C) may be rationalized by heterogeneous constituents of stromal extracellular matrix, each of which imparts some adhesive nature of stromal cells to myeloma cells. In other words, adhesion of myeloma cells to stroma via interaction of certain adhesive molecules, such as integrin β_1 or VLA-6 with extracellular matrix, is necessary to bring about CAM-DR because adhesion and CAM-DR were closely related (Fig. 2B), but the molecule(s) relevant to CAM-DR alone may not be sufficient to cause tight adhesion. Nevertheless, the fact that integrin β_1 irrespective of its counterpart subunits (integrin α_4, α_5, or α_6) is commonly involved in CAM-DR of various multiple myeloma cells is indicative of future use of anti-integrin β_1 in combination with anticancer drug for treatment of multiple myeloma.

Having confirmed the close linkage of CAM-DR to adhesiveness of multiple myeloma cell lines, we then extended our exploration to examine the implication of

Wnt(s), a large family of secreted glycoproteins involved in cell proliferation, differentiation, and oncogenesis, of which certain family members, such as Wnt1 and Wnt3a, have been shown to stimulate migration and invasion of multiple myeloma cells (22). We chose Wnt1, Wnt2b, Wnt3, Wnt3a, Wnt5a, Wnt7a, Wnt10b, and Wnt11 in the present study to analyze because we and others have previously found these proteins were highly expressed in human bone marrow cells (24, 40). The results of reverse transcription-PCR and Western blotting revealed prominent expression of Wnt3 with a concomitantly increased level of β-catenin and GTP-RhoA in CAM-DR relevant cells (ARH-77, KMS-5, and RPMI8226) but not in CAM-DR non-relevant cells (MM1S; Fig. 4A and B), suggesting on autocrine signaling of a canonical and a non-canonical pathway by Wnt3. To directly prove the autocrine activation of the Wnt signaling pathway in the CAM-DR relevant cell line (KMS-5 and ARH-77), sFRP-1, which binds a wide variety Wnt family

member, including Wnt3, significantly inhibits both canonical and non-canonical pathways. Furthermore, sFRP-1 significantly inhibited CAM-DR of KMS-5 and ARH-77, whereas DKK-1, which binds to the co-receptor for the β-catenin canonical signal, did not inhibit CAM-DR. Collectively, Wnt, secreted from KMS-5 or ARH-77, is considered to be indeed involved in induction of CAM-DR by autocrine manner through the Wnt/RhoA pathway. As to the Wnt(s) that is actually functioning in the culture medium of KMS-5 and ARH-77, Wnt3 was speculated to be one of the most plausible candidates because the close correlation of Wnt3 expression with the increase of β-catenin and the GTP-RhoA in CAM-DR relevant cells was observed (Fig. 4A and B). To support the notion that Wnt3 indeed potently augments CAM-DR, we then conducted Wnt3 siRNA transfer into CAM-DR relevant cell lines and found significant reduction of CAM-DR (Fig. 5B). Moreover, when we added Wnt3 CM to KMS-5 and ARH-77 cells, there was a significant increase in CAM-DR of KMS-5 and ARH-77 (Fig. 6A; Supplementary Fig. S4).[3] Regarding the incompleteness of inhibition by sFRP-1, there may be two possibilities: (*a*) stromal cell–derived Wnt5a (24), which is not interfered with by sFRP-1 (49, 50), transduced the Wnt/RhoA signal to evoke CAM-DR in a paracrine manner; (*b*) stromal cell–derived factor-1α and/or insulin-like growth factor-1, which are both secreted from stromal cells and potently activate the RhoA, participated in paracrine induction of CAM-DR in addition to the Wnt effect (17–21). The possibility that signals other than the RhoA pathway is responsible for the Wnt unrelated CAM-DR of ARH-77 is not likely because the inhibitor of Rho kinase almost completely negated the CAM-DR (Fig. 5C). Thus, the activation of RhoA is considered to play a crucial role in induction of CAM-DR, and molecular targeting to this signal pathway may be an option in overcoming CAM-DR in multiple myeloma. With respect to the molecular mechanism by which CAM-DR is induced by the Wnt/RhoA signal, although direct evidence was not elucidated in the present investigation, it is conceivable that activated RhoA stimulates integrin clustering, thereby inducing CAM-DR, because a Wnt3 CM actually caused morphologic change with a concomitant rearrangement of the actin filament and accumulation of vinculin in the present study (Fig. 6C), which is known to associate with the β subunit of integrin in the adhesion plaques (51, 52), and activation of RhoA has been reportedly proven to stimulate integrin-mediated adhesion in peripheral T cells and thymocytes (53). Thus, in our cell lines, Wnt3 was considered to stimulate RhoA signal to bring inactive cells into a primed state for subsequent activation by integrin-mediated interaction.

In conclusion, the present study disclosed that Wnt3, which myeloma cells themselves produce, stimulate the Wnt/RhoA signal, thereby bringing cells into a primed state by which cells adhere to extracellular matrix of stromal cells, resulting in the induction of CAM-DR. Hence, targeting the RhoA signal would be a promising strategy to overcome CAM-DR.

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