The CD49d$^{+/high}$ subpopulation from isolated human breast sarcoma spheres possesses tumor-initiating ability

KYUNG-MIN LEE[1], WONSHIK HAN[1,2], JONG BIN KIM[1], INCHEOL SHIN[3], EUNYOUNG KO[1,2], IN AE PARK[4], DONG SUP LEE[5], KUENHEE OH[5] and DONG-YOUNG NOH[1,2]

[1]Cancer Research Institute and [2]Department of Surgery, Seoul National University College of Medicine; [3]Department of Life Science, College of Natural Sciences, Hanyang University; Departments of [4]Pathology and [5]Anatomy, Seoul National University College of Medicine, Seoul, Republic of Korea

Received July 18, 2011; Accepted September 12, 2011

DOI: 10.3892/ijo.2011.1289

Abstract. Primary breast sarcomas (PBSs) that arise from mammary stroma are very rare, highly aggressive and therapy-resistant tumors with a heterogeneous phenotype. In this study, we sought to identify tumor-initiating cells (TICs) in PBSs and to describe their features. We isolated long-term self-renewing sarcospheres (designated NDY-1) from primary breast carcinosarcoma tissue (sarcoma component >95%) using the anchorage-independent culture method. NDY-1 spheres expressed various mesenchymal cell markers, and their tumorigenic potential was markedly reduced in adherent culture conditions, compared to spheres. Screening for integrins revealed a marked decrease in CD49d expression in adherent culture conditions of NDY-1. The CD49d$^{+/high}$ subpopulation sorted from NDY-1 spheres displayed higher cell viability and sphere-forming ability than CD49d$^{-/low}$ population *in vitro*. Moreover, the CD49d$^{+/high}$ population displayed high tumor initiating ability in limiting dilution transplantation to NOD/SCID mice, and the xenotransplanted CD49d$^{+/high}$ population recapitulated the complexity of the original primary tumors. Greater doxorubicin resistance was exhibited by the CD49d$^{+/high}$ population, compared with the CD49d$^{-/low}$ population. Thus, our results collectively demonstrate that CD49d$^{+/high}$ cells from sarcospheres display enhanced sphere-forming, drug resistance and tumor-initiating abilities. To our knowledge, this is the first study to identify TICs from breast sarcoma.

Introduction

Cancers generally originate from a single cell by aberrant proliferation, but display heterogeneous phenotype (1). The tumor-initiating cells (TICs) or cancer stem cell hypothesis has recently been developed to explain this biologic heterogeneity of cancer. The TIC model suggests that a defined subset of cancer cells has the exclusive ability to form tumors, similar to the self-renewing ability of normal stem cells (2). These TICs are responsible for initiating and maintaining tumor growth, and may be highly resistant to radiation and chemotherapy (3,4). Consequently, relapse after remission is possibly due to a failure to eradicate TICs, which, despite bulk tumor shrinkage, can subsequently reproduce the entire malignant phenotype (4). Therefore, effective targeting of these cell populations is essential for successful therapy.

Following their initial identification in leukemia (5), TICs have been detected in several types of solid tumors (6-10). The TIC hypothesis was recently explored in mesenchymal tumors, such as bone sarcoma (11) and Ewing's sarcoma (12,13). However, the apparent existence of TICs has not been confirmed in primary breast sarcomas (PBSs) to date.

PBSs are rare, highly aggressive and therapy-resistant, accounting for >1% of all primary breast malignancies (14). These tumors may be heterogeneous neoplasms that arise from mammary mesenchymal elements (15,16). PBSs are composed not only of composite histologic subtypes that vary in terms of etiology and response to different therapies, but also differential diagnostic subtypes, including sarcomatoid carcinoma, carcinosarcoma and fibromatosis of the breast (14). Despite significant advances in medical and surgical management, these tumors have no suitable therapeutic modalities, and are associated with high risk of recurrence and poor prognosis. Therefore, clarification of their molecular features is critical in identifying the relevant targets for therapeutic intervention (17,18). However, difficulties in this task arise due to the relative rarity of these tumors and lack of suitable cell line models.

Integrins are a family of transmembrane proteins containing 18 α- and β-subunits in humans (18). Each of the several α-subunits interacts with β-subunits to form a heterodimeric receptor. Integrins play diverse roles in this context, including promotion of cell proliferation and migration (18), as well as hyperproliferation and carcinogenesis in various cell types (19,20). In addition, integrins are implicated in cancer stem cell self-renewal and regulation of their differentiation properties (21,22). In mouse models, CD24^{high}CD29^{low}CD61^{high} cancer progenitor cells display significant tumorigenic (23) and meta-

Correspondence to: Professor Dong-Young Noh, Cancer Research Institute and Department of Surgery, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu 110-744, Seoul, Republic of Korea
E-mail: dynoh@plaza.snu.ac.kr

Key words: tumor-initiating cells, breast sarcoma, CD49d

static potential (24). Overall, elucidation of the mechanism(s) underlying cancer stem cell production is crucial, in view of the high mortality rates associated with the presence of undifferentiated tumor cells.

In this study, we assumed the presence of TICs in PBS. We isolated self-renewing sarcospheres from primary human sarcomatoid carcinomas of breast specimens using an anchorage-independent culture technique (25,26), and revealed the existence of distinct populations with tumor-forming ability and drug resistance, both *in vivo* and *in vitro*.

Materials and methods

Isolation and in vitro expansion of spheres from human breast carcinosarcoma specimens. All studies were performed with the approval of the Institutional Review Board of Seoul National University. Primary human tumor specimens were obtained upon diagnostic radical surgery of breast cancer patients. Carcinosarcoma (mixed sarcoma component >95% and squamous cell carcinoma <5%) was examined in this study. Single cell suspensions from tumor specimens were prepared, as described previously (25,26). Briefly, to obtain single cell suspensions, specimens was cut into small fragments, minced with sterile scalpels, and incubated for 1-2 h at 37˚C in the presence of collagenase I (Sigma, St. Louis, MO) to allow complete digestion. At the end of the incubation, cells were filtered through a 40-μm nylon mesh (BD, Bedford, MA), and single cells plated at 1,000 cells/ml in serum-free DMEM (Dulbecco's modified Eagle's medium):F12 = 3:1 medium supplemented with 20 ng/ml epidermal growth factor (EGF; Invitrogen, Carlsbad, CA), 20 ng/ml basic fibroblast growth factor (bFGF; Millipore, Temecula, CA), 10 ng/ml leukemia inhibitory factor (LIF, Millipore), B27 supplement (Invitrogen) and antibiotic-antimycotic (Invitrogen). Cells were grown under these conditions as nonadherent spherical clusters. The medium was replenished every 3-4 days, and cells were passaged weekly.

Sphere formation assay and assessment of spheroid to adherent morphology. Primary spheres were dissociated with 0.25% Trypsin-EDTA solution (Invitrogen), and 100 cells per well plated in a 96-well ultra low attachment culture dish (BD) in 200 μl of sphere culture medium. Every 3 days, 20 μl of medium was added per well. The number of spheres was counted for each well after 7 days of plating. Adherence of sphere-derived cells was induced by culturing dissociated cells for 2 weeks on type IV collagen (Sigma)-coated dishes in DMEM supplemented with 10% fetal bovine serum (Invitrogen) in the absence of growth factors.

Flow cytometry analysis and fluorescence-activated cell sorting (FACS). Cells were dissociated from spheroids or monolayers with trypsin-EDTA solution. Suspended cells were collected by centrifugation and washed with flow cytometry buffer comprising PBS containing 0.1% bovine serum albumin (Bovogen Biological, Melbourne, Australia) and 0.05% sodium azide. Cells ($5x10^5$) were stained using concentrations of fluorochrome-conjugated monoclonal antibodies recommended by the manufacturer for 30 min at room temperature in the dark. Anti-human CD49b, CD49c, CD49d, CD49f, CD29a and CD29d antibodies were purchased from Serotec Laboratories (Oxford, UK). Antibodies against human CD24, CD44, CD54, CD90, CD49a, CD49e, CD49f and CD71 were obtained from PharMingen (Biosciences, San Jose, CA), and CD133 and CD324 from Militenyi (Bergisch Gladbach, German) and R&D systems (Minneapolis, MN), respectively. After staining, cells were washed with 3 ml of flow cytometry buffer and resuspended in the same buffer. Background staining was assessed by incubation of cells with mouse fluorochrome-isotype controls. Flow cytometric analysis was performed by analyzing 5,000 events on a FACSCalibur flow cytometer (BD Biosciences). For fluorescence-activated cell sorting, single cells were suspended in 0.5% BSA/PBS buffer labeled with anti-CD49d (FITC labeled) and isolated using a FACSAria flow cytometer (BD Biosciences). Routinely, >90% of the sorted cell population was CD49d-positive. Cell viability was assessed by staining with 7-amino-actinomycin D (7AAD, BD Biosciences, routinely >95%).

Reverse transcriptase polymerase chain reaction (RT-PCR). RNA was isolated from both spheroid and adherent cells with the RNA mini kit (Qiagen, Valencia, CA), and reverse-transcribed using MMLV Reverse transcriptase (Invitrogen). The cDNA was amplified using rTaq polymerase (Takara, Japan) for 28 cycles with the following set of primers: human CD49d (NM_000885), annealing temperature 60˚C, amplicon length 409 bp, 5'-GAGT GCAATGCAGACCTTGA-3' (forward) and 5'-GCCAGCCT TCCACATAACAT-3' (reverse); β-actin, annealing temperature 55˚C, amplicon length 47 bp, 5'-CACTGTGTTGGCGTAC AGGT-3' (forward) and 5'-TCATCACCATTGGCAATGAG-3' (reverse). Amplified products were electrophoresed on 2% agarose gels, with β-actin as the loading control.

Western blotting. Cells were washed twice with PBS, and total cell lysates prepared in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF). Protein concentrations were measured with the Bradford assay using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. Equal amounts of cell lysates were separated by 10% SDS-PAGE. Protein bands were electrotransferred to Hybond-ECL nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, UK). Blots were blocked with blocking buffer (5% non-fat dry milk in TBS-T (TBS containing 0.05% Tween-20) for 1 h, followed by incubation overnight at 4˚C with CD49d mouse monoclonal and β-actin mouse monoclonal IgG antibodies (Sigma). Blots were washed three times in TBS-T, and incubated with peroxidase-conjugated affinipure rabbit anti-mouse IgG (1:5000 dilution, Jackson ImmunoResearch) or peroxidase-conjugated affinipure mouse anti-rabbit IgG for 1 h at room temperature. After washing with TBS-T three times for 5 min each, immunocomplexes were visualized by enhanced chemiluminescence (Amersham Biosciences).

Assessment of cell proliferation and chemotherapy sensitivity. For the proliferation assay, CD49d$^{+/high}$, CD49d$^{-/low}$ and bulk unsorted cells were seeded at 100 cells per well (96-well Ultra Low plates; Falcon) in 100 μl sphere-conditioned medium. After 7 days, cell viability was determined with the CellTiter-Glo®

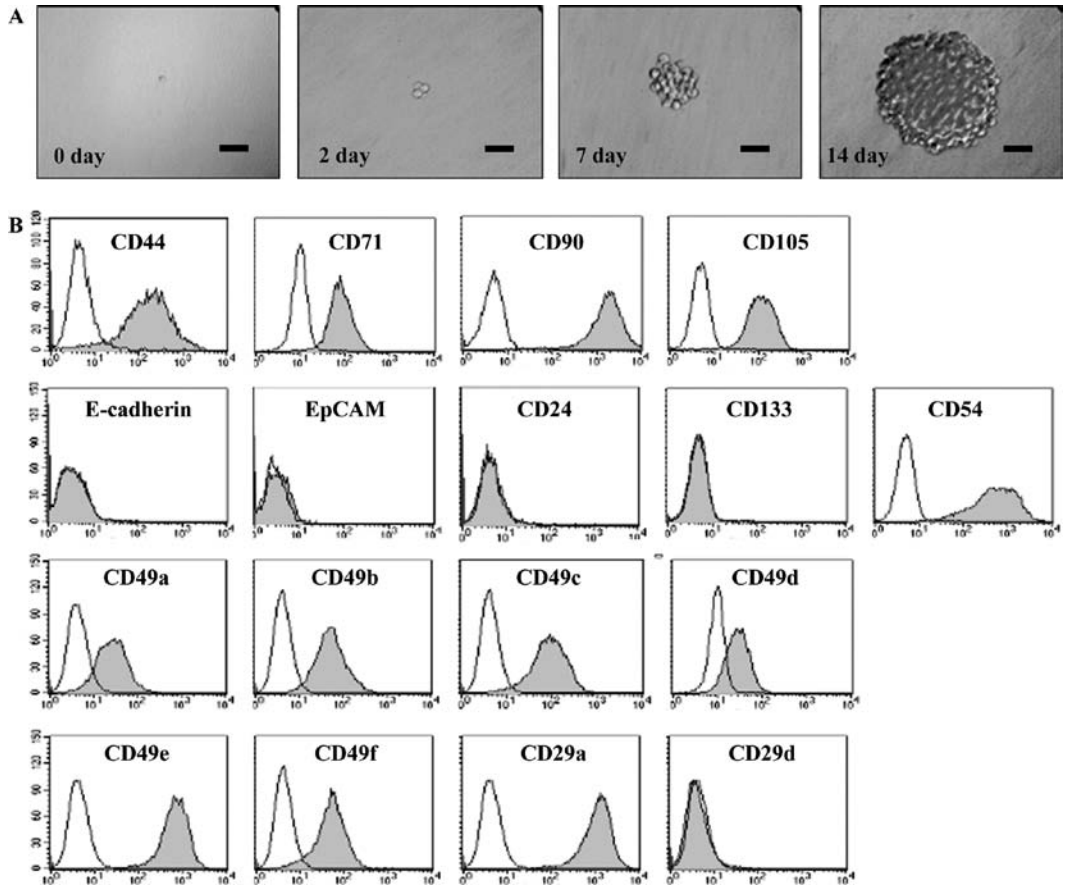

Figure 1. Self-renewal ability and expression of various cell surface markers of established NDY-1 sphere cells. (A) Single cells from NDY-1 spheres were cultured for up to 14 days. Magnification, x400; scale bar represents 50 μm. (B) Mesenchymal stem cell markers (CD44, CD71, CD90 and CD105) and various cell surface markers (integrins, E-cadherin, EpCAM, CD24, CD133 and CD54) were analyzed by flow cytometry. Each empty histogram represents the isotype control. The gray histogram represents specific binding of the indicated antigen.

Luminescent Cell Viability Assay Kit (Promega, Madison, WI). To assess cytotoxicity, unsorted and sorted ($CD49d^{+/high}$, $CD49d^{-/low}$) cells were seeded as described above, treated for 72 h with 10 nM doxorubicin (Sigma), and examined using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega).

In vivo xenograft experiments. Five- to seven-week-old female NOD/SCID mice were purchased from Jackson laboratory (Bar Harbor, ME), and maintained in accordance with the standards of the Seoul National University Hospital Animal Ethics committee (Seoul, Korea). Mice were inoculated subcutaneously with 17 β-estradiol pellets (Innovative Research of America, Sarasota, FL, USA) 1 day before injection of cells. Adherently growing cells derived from enzymatic dissociated spheres (10^6, 10^5, 10^4, 10^3, $2x10^2$, $1x10^2$ cells), $CD49d^{+/high}$ and $CD49d^{-/low}$-sorted cells (10^4, 10^3, $5x10^2$, $2x10^2$ cells) were mixed with Matrigel (BD Pharmingen) at a 1:1 ratio, and injected into the inguinal mammary fat pads of 6-8 week-old NOD/SCID mice. Engrafted mice were inspected twice a week for tumor appearance by visual observation and palpation. At a tumor diameter of 1 cm or 2 or 3-month transplantation, mice were sacrificed, and tissues immediately fixed for H&E staining.

H&E stain and immunohistochemistry (IHC). Tissue specimens were fixed in 10% formalin solution for 24 h, embedded in paraffin blocks, and 4-μm thick sections prepared. Next, specimens were subjected to slide mounting and H&E staining. For IHC, paraffin sections were deparaffinized and sequentially rehydrated. Following microwave antigen retrieval with citrate buffer (Dako, pH 6.0), endogenous peroxidase activity was blocked with 3% H_2O_2. Slides were subsequently incubated with CD49d (Abcam, Cambridge, MA) and vimentin (Calbiochem, San Diego, CA) for 1 h at room temperature, and subjected to Envision-HRP (Dako, Glostrup, Denmark) reactions. The reaction was performed by adding 3,3'-diaminobenzidine (DAB) substrate chromogen (DakoCytomation), counterstained with hematoxylin, dehydrated, and the slides coverslipped with Histomount. Images were visualized with a Leica Application Suite (LAS, Leica Microsystems Ltd., Switzerland) equipped with a Leica microscope (CH-9435 Heerbrugg, Leica Microsystems Ltd.).

Statistical analysis. Graphs were generated and quantitative results compared with the paired Student's t-test using Sigma Plot (Statistical Solutions Ltd., Cork, Ireland).

Results

Establishment and characterization of sarcospheres (NDY-1 spheres) from human breast carcinosarcoma. To enrich and

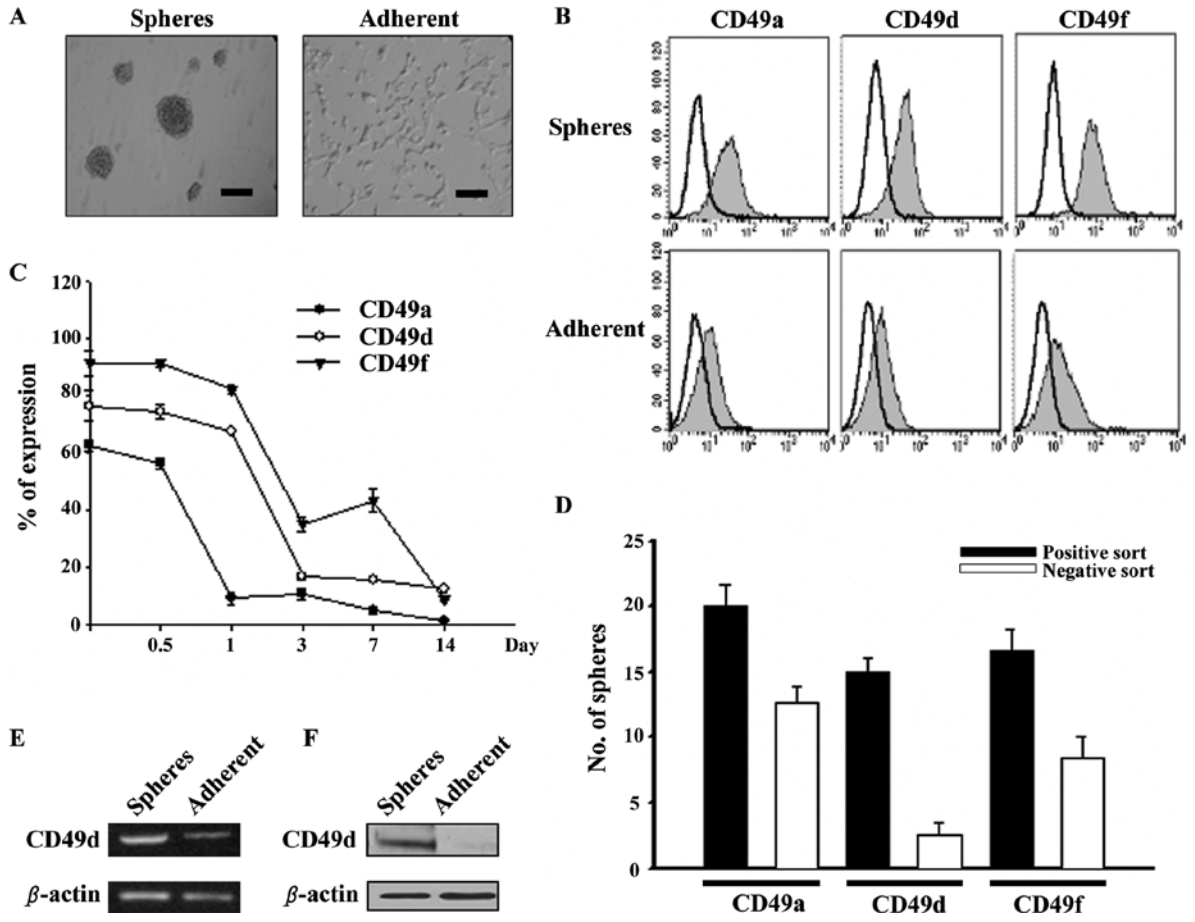

Figure 2. Expression of CD49a, CD49d and CD49f in spheres and adherent cells from NDY-1. (A) Phase-contrast images of spheres and adherent cells from NDY-1. Magnification, x100; the scale bar represents 50 μm. (B) Expression of CD49a, CD49d and CD49f in spheres and adherent cultures for 14 days. (C) Changes in expression patterns of CD49a, CD49d and CD49f in NDY-1, following the start of adherent cultures up to 14 days, analyzed using FACS. The value is presented as % positive cells. (D) Sphere formation assay in cells sorted for CD49a, CD49d and CD49f, respectively. (E) CD49d mRNA and (F) CD49d protein expression in NDY-1 sphere and adherent cultured cells for 14 days analyzed using RT-PCR and immunoblotting.

isolate tumor-initiating cells (TICs), we cultured cells from primary carcinosarcoma tissue in anchorage-independent culture conditions previously optimized for maintaining spheres, as described in Materials and methods. These cells were expanded as spheroids for >1 year *in vitro*. The established spheroid cell line was designated NDY-1 spheres.

To evaluate *in vitro* self-renewal ability, primary spheroids were enzymatically dissociated into single cell suspensions and plated onto 96-well plates at a concentration of 1 cell/well. Each cell could generate secondary spheres with increased sizes (approximately several hundred micrometers) after 1-2 weeks (Fig. 1A), providing evidence of self-renewal in NDY-1 spheres.

Next, we analyzed the expression of various cell surface markers in NDY-1 spheres. NDY-1 spheres were derived from carcinosarcoma mostly composed of sarcoma (>95%) arising from mesenchymal tissue. The expression levels of epithelial cell and mesenchymal stem cell (MSC) markers were examined. Interestingly, NDY-1 spheres did not express E-cadherin and EpCAM epithelial markers, but strongly expressed MSC markers, such as CD44 (hyaluronic acid receptor), CD71 (transferrin receptor), CD90 (Thy-1) and CD105 (endoglin) (Fig. 1B). Immunofluorescence experiments additionally disclosed vimentin expression in NDY-1 spheres (data not shown). Further characterization with flow cytometry analysis revealed that the NDY-1 spheres were negative for CD133 (prominin-1), a stem cell marker for numerous tumors (8,13), and CD24 (single chain sialoglycoprotein), but positive for CD54 (ICAM). We confirmed the absence of other cell types in the spheres using lineage markers (data not shown). Stromal sarcomas are defined solely on the basis of malignant mesenchymal components and lack of epithelial features (14). Accordingly, we propose that NDY-1 spheres are derived from non-epithelial mesenchymal cells, and designate these sarcospheres.

Recently, integrins have been directly implicated in tumorigenesis (22). CD49f and CD29a (also known as α6 and β1 integrins, respectively) are used as markers of mouse mammary stem/progenitor cells (21,27). Flow cytometry was utilized to screen for the expression of various integrins. NDY-1 spheres expressed high levels of CD49a, CD49b, CD49c, CD49d, CD49e, CD49f and CD29a (α1, α2, α3, α4, α5, α6 and β1, respectively), but not CD29d (integrin β4; Fig. 1B).

Biological differences between NDY-1 spheres and adherent cells. We employed an anchorage-independent culture model to enrich TICs (25,26). Cellular differentiation was achieved by applying adherent conditions (i.e., withdrawal of growth factors and addition of FBS) for 2-3 weeks. To determine the biological differences between NDY-1 spheres and adherent

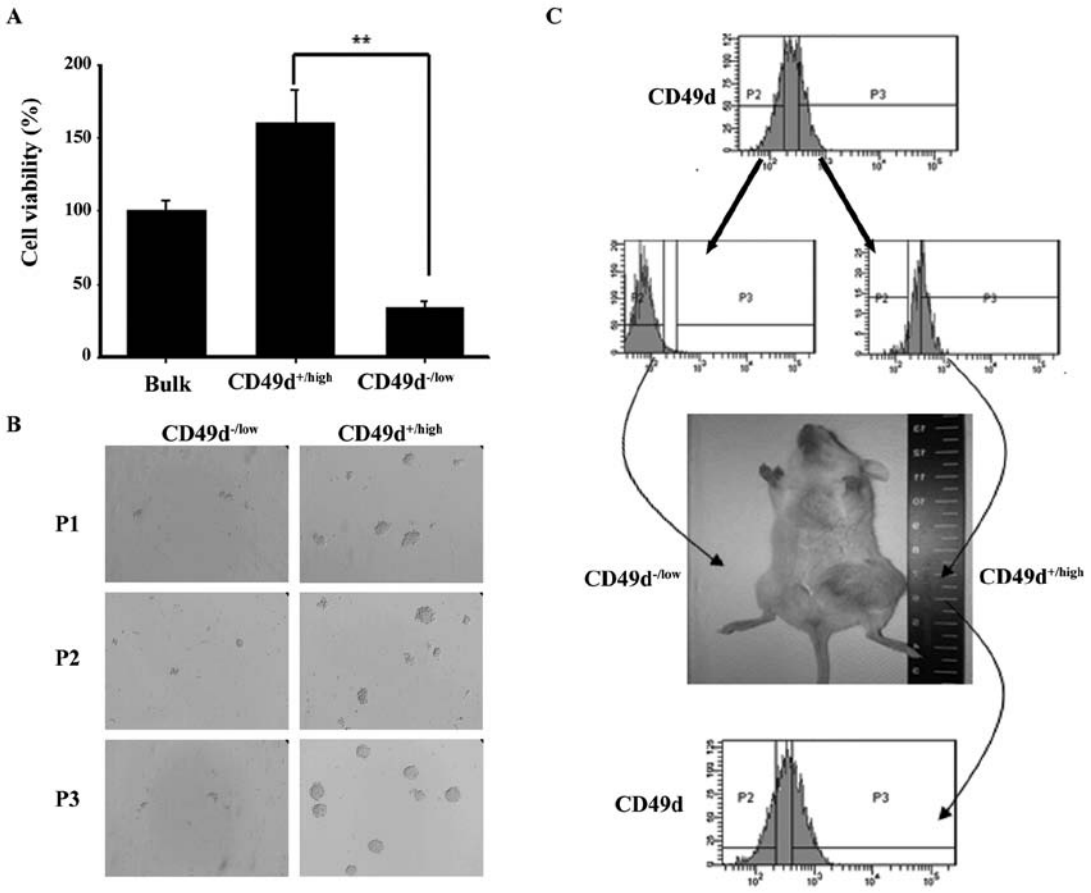

Figure 3. *In vitro* and *in vivo* characterization of CD49d$^{+/high}$ cells. (A) Unsorted (bulk) and sorted (CD49d$^{+/high}$ and CD49d$^{-/low}$) cells were cultured for 7 days, and cell viability measured. (B) Efficiency of sphere formation was measured based on the number of spheres at each serial passage of CD49d$^{+/high}$ and CD49d$^{-/low}$ populations. (C) CD49d$^{+/high}$ cells were isolated from NDY-1 spheres using FACS. The purity of the sorted population was generally >90%. Tumor formation was observed in a single mouse injected with CD49d$^{+/high}$ and CD49d$^{-/low}$ cells in the bilateral mammary fat pads. CD49d$^{+/high}$-derived tumors displayed original distribution of CD49d expression in NDY-1. **p<0.01, Student's t-test. Columns, mean; bars, SE.

cells, floating cells were adhered to the collagen-coated culture dish, which subsequently altered to spindle-shaped morphology (Fig. 2A). Comparative flow cytometry analysis of adherent cells and parental spheres revealed decreased sphere-forming ability (data not shown) and reduced expression of CD71 and CD105 in adherent cells (data not shown). We additionally observed decreased expression of the anti-apoptotic proteins, Bcl-2 and Bcl-xL, in adherent cells (data not shown). CD49a, CD49d and CD49f were significantly downregulated in adherent cells, compared with spheres (Fig. 2B). CD49a, CD49d and CD49f levels decreased from 71 to 1%, 75 to 16%, and 90 to 51%, respectively, within 2 weeks after adhesion culture (Fig. 2C).

To compare the *in vivo* tumorigenic efficacy between spheres and adherent cells, we transplanted various concentrations of cells in mammary fat pads of NOD/SCID mice. As shown in Table I, NDY-1 spheres induced tumor formation within 2 months, even when as few as 100 cells per mouse were injected, whereas adherent cells failed to induce tumor formation when <10^4 cells were injected. The results suggest that NDY-1 spheres are enriched in TICs, but lose tumor-initiating ability in adherent culture conditions.

We hypothesize that three integrins, CD49a, CD49d and CD49f, play major roles in the maintenance of anchorage-independent cell growth and tumor initiating ability. NDY-1 sphere cells were sorted according to expression via the fluorescence-activated cell sorting (FACS) technique. For all three integrins, sphere-forming abilities were significantly higher in positive-sorted cells, compared to negative-sorted cells. Differences were most significant when sorted for CD49d$^{+/high}$ and CD49d$^{-/low}$ (Fig. 2D). In view of previous reports that malignant mesenchymal tumors of the breast show a stromal reaction for fibronectin (28) and CD49d is a receptor for fibronectin (29), we focused on CD49d in subsequent experiments.

In vitro and in vivo characterization of CD49d$^{+/high}$ cells from NDY-1 spheres. To verify reduced expression of CD49d in adherent NDY-1 cells, we determined the levels of CD49d using RT-PCR and immunoblotting. Downregulation of CD49d mRNA (Fig. 2E) and protein levels in adherent NDY-1 cells was confirmed (Fig. 2F).

NDY-1 spheres were sorted into CD49d$^{+/high}$ and CD49d$^{-/low}$ cell populations, and the characteristics of the subgroups compared. In a cell viability assay, the CD49d$^{+/high}$ fraction displayed significantly increased proliferation, compared with bulk and CD49d$^{-/low}$ fractions (1.6- and 5-fold higher, respectively; Fig. 3A, p<0.01). We additionally compared the sphere-forming abilities of CD49d$^{+/high}$ and CD49d$^{-/low}$ popula-

Table I. Comparison of tumorigenic potential between NDY-1 spheres and adherent cells.

Cell dose	Incidence (%)		Termination (day)
	Sphere	Adherent	
$1x10^6$	8/8 (100)	4/8 (50.0)	60
$1x10^5$	6/6 (100)	1/6 (17.0)	60
$1x10^4$	8/8 (100)	1/8 (12.5)	60
$1x10^3$	6/6 (100)	0/6 (0)	60
$2x10^2$	2/8 (25)	0/8 (0)	60
$1x10^2$	1/8 (12.5)	0/8 (0)	60

Table II. Tumorigenic capacity of CD49d$^{+/high}$ and CD49d$^{-/low}$ cells from human breast NDY-1 spheres.

Cell dose	Incidence (%)		Termination (day)
	CD49d$^{+/high}$	CD49d$^{-/low}$	
$1x10^4$	4/4 (100)	4/4 (100)	90
$1x10^3$	5/6 (83)	0/6 (0)	90
$5x10^2$	2/8 (25)	0/8 (0)	90
$2x10^2$	2/8 (25)	0/8 (0)	90

tions after serial passages. The CD49d$^{+/high}$ fraction formed spheres efficiently in anchorage-independent conditions and generated second and third spheres, but not the CD49d$^{-/low}$ fraction (Fig. 3B). Our data clearly suggest that self-renewing cells exist in the CD49d$^{+/high}$ population.

Next, we evaluated the tumor-initiating ability of CD49d$^{+/high}$ and CD49d$^{-/low}$ populations after xenograft in NOD/SCID mice. The CD49d$^{+/high}$ and CD49d$^{-/low}$ populations displayed sufficient survival rates (regular purity >90%; Fig. 3C), as confirmed by 7-amino-actinomycin D (7-AAD) staining (both regular negative populations >96%; data not shown).

In the limiting dilution transplantation assay into NOD/SCID mice, the CD49d$^{+/high}$ population induced tumor formation, even when as few as 200 cells were injected. In contrast, the CD49d$^{-/low}$ population did not induce tumor formation in NOD/SCID mice, even up to 3 months after injection of $1x10^3$

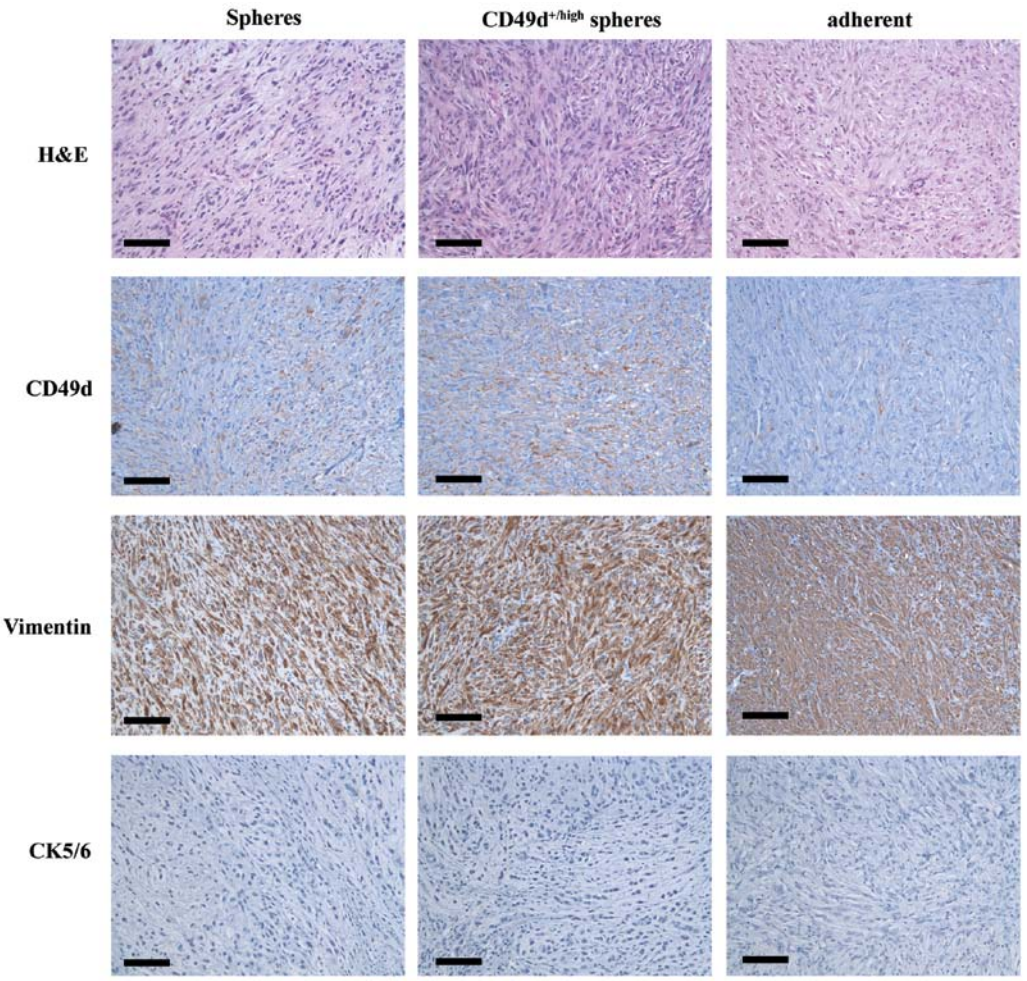

Figure 4. Histologic phenotype of tumor xenografts derived from injection of NDY-1 spheres, adherent cells, and sorted CD49d$^{+/high}$ population general histology of xenograft tumors derived from NDY-1 spheres, CD49d$^{+/high}$-sorted cells and NDY-1 adherent cells observed with H&E staining (Top). CD49d, vimentin (middle) and CK5/6 (bottom) expression are detected with immunostaining. Magnification, x200; scale bar represents 50 μm.

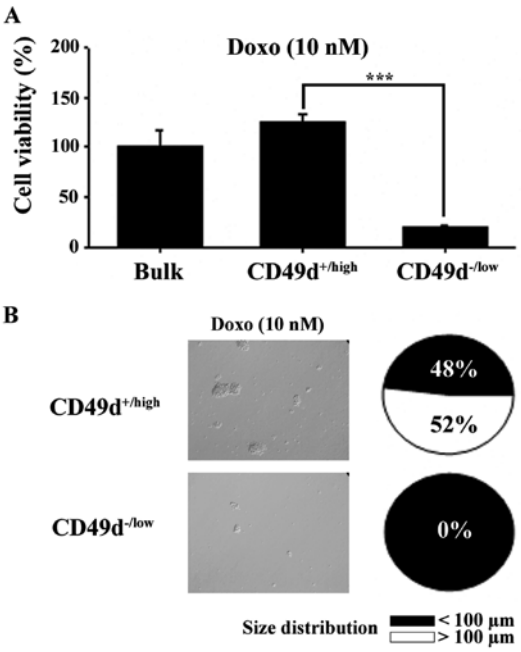

Figure 5. Doxorubicin resistance of CD49d$^{+/high}$ and CD49d$^{-/low}$ populations. (A) Cell viability was measured in each cell population after 10 nM doxorubicin treatment. (B) Phase-contrast images of sphere formation and sphere size distribution diagram after 3 days of exposure to 10 nM doxorubicin. $^{***}p<0.001$.

cells. These data indicate that CD49d$^{+/high}$ subpopulations derived from NDY-1 spheres are enriched in tumor-initiating cells (Table II).

To ascertain whether the CD49d$^{+/high}$-derived tumors retain phenotypic diversity similar to the original NDY-1 spheres, tumor cells were analyzed for CD49d expression using flow cytometry. Mouse cell contaminants were eliminated by H2K (mouse histocompatibility class I) FACS sorting. Expression of CD49d was recovered in tumors arising from CD49d$^{+/high}$ xenografts, as in NDY-1 spheres (Fig. 3C). The tumors also displayed comparable expression patterns of various cell surface markers to those in NDY-1 spheres, as shown in Fig. 1B. This finding suggests that the CD49d$^{+/high}$ populations give rise to both non-tumorigenic CD49d$^{-/low}$ and CD49d$^{+/high}$ cells, recapitulating the complexity of primary tumors from which the tumorigenic cells are derived.

Histological analysis of xenograft tissues with H&E (hematoxylin and eosin) staining revealed that CD49d$^{+/high}$ population-derived tumors resembled parent NDY-1 sphere-derived tumors exhibiting typical sarcoma morphology. In immunostaining experiments, all tumors strongly expressed vimentin and were negative for cytokeratin (CK) 5/6 (Fig. 4). NDY-1 sphere- and CD49d$^{+/high}$ population-derived tumors expressed high levels of CD49d, while NDY-1 adherent cell-derived tumors displayed weak CD49d expression (Fig. 4).

Doxorubicin resistance of CD49d$^{+/high}$ cells. Previous studies suggest that a small population of TICs plays a potential role in resistance to chemotherapy (3,4). We treated CD49d-sorted cells with 10 nM doxorubicin for 3 days, and evaluated cell viability. The CD49d$^{+/high}$ population displayed 6-fold greater resistance than the CD49d$^{-/low}$ population, as assessed on the basis of cell viability ($p<0.001$; Fig. 5A). Size distributions of spheres formed by each sorted population were analyzed using phase-contrast microscopy after doxorubicin treatment. Sphere sizes were >100 μm in 52% of spheres formed by the CD49d$^{+/high}$ population. In contrast, no spheres formed by CD49$^{-/low}$ population were larger than 100 μm (Fig. 5B). These results provide further evidence of the increased chemoresistance of TICs.

Discussion

We demonstrated that a subset of sarcoma cells have the capacity to form spheres and self-renew in a culture system previously developed to enrich stem cells from breast tumors (25,26), with the view to establishing that breast sarcomas contain tumor-initiating cells (TICs). The existence of TICs was initially confirmed in hematologic abnormalities (5) and numerous other epithelial malignancies, including breast (10), prostate (6), ovary (7), colon cancers (8) and melanoma (9).

Primary breast sarcomas (PBSs) are rare and highly malignant heterogeneous tumors arising from the mesenchymal tissue of mammary gland. The rarity of this tumor limits most studies to small retrospective case reports, and its molecular pathogenesis is poorly understood. Established NDY-1 spheres were negative for epithelial cell markers (E-cadherin and EpCAM), but strongly expressed vimentin (Figs. 1B and 4), indicating that these spheres are sarcoma cells derived solely from the mesenchymal component. To date, no adequate cell line models in human breast sarcoma have been established. Thus, NDY-1 cells may present a useful *in vitro* model for analyzing the tumor biology of PBSs.

A recent study shows that CD133^{+} Ewing's sarcoma cells have the capacity to initiate and sustain tumor growth, and retain the ability to differentiate mesenchymal lineages (13). These data support the extension of the TICs hypothesis to include tumors of mesenchymal lineage, and suggest that sarcoma arises from a primitive mesenchymal precursor through transformed mesenchymal stem cells (MSCs). Our NDY-1 spheres expressed high levels of MSC markers, specifically, CD44, CD71, CD90 and CD105 (Fig. 1B). While mesenchymal lineage differentiation was not examined in our system, diminished CD71 and CD105 expression on the surface was observed in adherent cells (data not shown). It is thought that NDY-1 spheres originate from MSC transformation.

Integrins, the major cellular receptors for extracellular-matrix components, play essential roles during cancer initiation and progression as well as cell differentiation in normal development, critical in cell migration, invasion and metastasis during tumor progression (22,30). In addition, integrins are implicated in cancer stem cell self-renewal and regulation of their differentiation properties (21). Among them, the CD49d (α-4 integrin, very late antigens VLA-4) interacts with the principal ligands, vascular cell adhesion molecule-1 (VCAM-1), fibronectin and mucosal addressin cell adhesion molecule-1 (MadCAM-1) (29) and heterodimerizes with CD29a (β-1 integrin), which mediates not only cell-cell and cell-extracellular matrix interactions. Several reports indicate that integrin-mediated adhesion influences cell survival and possibly prevents programmed cell death, eventually leading to acquisition of *de novo* drug resistance (31). CD49d-expressing cells are relatively resistant to the apoptotic effects of doxorubicin (31),

consistent with our data showing that the CD49d$^{+/high}$ population is doxorubicin-resistant, compared with the CD49d$^{-/low}$ population (Fig 5). We additionally observed decreased expression of the anti-apopototic proteins and ABCG2 drug transporter in adherent cells, low levels of CD49d expressing cells (data not shown), drug resistance are thought to be mediated by CD49d expression. There is a need to study further the mechanism of CD49d-mediated drug resistance.

Increased levels of CD49d are detected in metastatic sarcoma cells, compared to primary sarcomas, suggesting that CD49d may confer resistance to anoikis-related cell death. In a human osteosarcoma cell line model, anoikis-resistance was reduced after the addition of anti-CD49d mAb, suggesting a pivotal role of CD49d in controlling cell death (32). Our results show upregulation of CD49d in spheres compared to adherent cells, supporting a role in anoikis resistance (Fig 2C).

In addition, CD49e/CD29a plays a role in ovarian carcinoma spheroid formation (33). However, the CD49e/CD29a expression in NDY-1 cells was unchanged between spherical and adherent condition (data not shown), while CD49d expression was decreased in the adherent condition (Fig. 2B and C). Based on the observation, CD49d might play a pivotal role in NDY-1 spheroid formation.

In conclusion, we successfully isolated self-renewing sarcospheres from human breast carcinosarcoma. These cells are able to grow in suspension as floating spheres, similar to normal and tumor stem cells in the undifferentiated state (25,26). Moreover, the CD49d$^{+/high}$ sarcospheres represent a TICs population with mesenchymal characteristics. Tumorigenic CD49d$^{+/high}$ cells display phenotypic heterogeneity and greater drug resistance than the CD49d$^{-/low}$ population. CD49d and other relevant pathways may thus be effective candidate therapeutic targets for breast sarcoma.

Acknowledgments

This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A084022) and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0004148).

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