CD99 Acts as an Oncosuppressor in Osteosarcoma

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CD99 was recently reported to be under control of the osteoblast-specific transcription factor Cbfa1 (RUNX2) in osteoblasts, suggesting a role in the phato-physiology of these cells. No extensive information is available on the role(s) of this molecule in malignant phenotype, and osteosarcoma, in particular, has never been studied. We report that in 11 different cell lines and 17 clinical samples CD99 expression is either undetectable or very low. Being expressed in the normal counterpart, we tested the hypothesis that CD99 down-regulation may have a role in osteosarcoma development and progression. CD99-forced expression in two osteosarcoma cell lines significantly reduced resistance to anoikis, inhibited growth in anchorage independence as well as cell migration, and led to abrogation of tumorigenic and metastatic ability. Therefore, the molecule acts as a potent suppressor of malignancy in osteosarcoma. CD99 gene transfection induces caveolin-1 up-regulation and the two molecules were found to colocalize on the cell surface. Treatment with antisense oligonucleotides to caveolin-1 abrogates the effects of CD99 on migration. The findings point to an antioncogenic role for CD99 in osteosarcoma, likely through the regulation of caveolin-1 and inhibition of c-Src kinase activity.

INTRODUCTION

CD99 is a transmembrane glycoprotein encoded by the MIC2 gene (Levy *et al.*, 1979) with no homology with other known molecules except the Xga protein (Fouchet and Gane, 2000). The gene encoding CD99 is located in the human pseudoautosomal region in the distal short arms of the X and Y chromosomes (Petit *et al.*, 1988). Sequence analysis of CD99 cDNA indicates that the CD99 protein is formed by an extracellular domain, which is glycosylated with O-linked sugar residues, followed by a transmembrane domain and a 36-amino acid intracytoplasmic domain (Banting *et al.*, 1989). In normal tissues, the expression level of CD99 is particularly high in cortical thymocytes, pancreatic islet cells, granulosa cells of ovary, and Sertoli cells of testis. In addition, CD99 was found to be expressed at high levels in CD34⁺ cells of bone marrow and in all leukocyte lineages, with the highest expression in the most immature lymphocytes and granulocytes (Dworzak *et al.*, 1994). CD99 has been involved in various steps of T- and B-cell development and differentiation (Dworzak *et al.*, 1999) and a role in neural precursor cell differentiation has also been suggested (Lee *et al.*, 2003). Although its functions are not understood clearly, CD99 has

been implicated in several cellular processes, such as the cell adhesion, apoptosis, differentiation of T-cells and thymocytes (Bernard *et al.*, 1997, 2000; Pettersen *et al.*, 2001; Alberti *et al.*, 2002), migration of monocytes (Schenkel *et al.*, 2002), and intercellular adhesion between lymphocytes and endothelial cells (Bernard *et al.*, 2000).

In pathological conditions, a role for CD99 has been indicated in Ewing's sarcoma cells where it delivers cell-cell adhesion and apoptotic signals (Sohn *et al.*, 1998; Scotlandi *et al.*, 2000; Cerisano *et al.*, 2004). Very little is known about the biological significance of CD99 expression in other malignancies. In addition to Ewing's sarcoma, in which intense membranous expression of CD99 has a diagnostic relevance (Ambros *et al.*, 1991; Scotlandi *et al.*, 1996), high CD99 expression has been shown in lymphoblastic lymphoma/leukemia (Dworzak *et al.*, 2004), and sporadically in synovial sarcoma (Fisher, 1998), mesenchymal chondrosarcoma (Brown and Boyle, 2003), and rhabdomyosarcoma (Ramani *et al.*, 1993). However, there is an emerging group of neoplasia, such as pancreatic endocrine neoplasm and gastric adenocarcinoma (Jung *et al.*, 2002; Maitra *et al.*, 2003), in which CD99 expression is diffuse in benign diseases and lacking in the malignant counterparts. Because a recent study (Bertaux *et al.*, 2005) provides evidence for a link between CD99 and osteoblastic lineage by indicating MIC2 under control of the transcription factor Cbfa-1 (RUNX2), which is essential for osteoblast differentiation, we explored whether the expression of CD99 may have a specific role(s) in osteosarcoma, a bone tumor generally derived from ab-

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Figure 1. Expression of CD99 in osteoblasts and osteosarcoma. (A) CD99 was particularly evident in the cell adhesion structures (arrowheads) of osteoblasts maintained in cell culture for 21 d. (B) Bone callus tissue sample was used to check the expression of CD99 in osteoblasts. The level of expression was higher when osteoblasts adhered to each other and lined the bone surface (arrowheads) compared with less differentiated osteoblasts included in immature bone matrix, as shown in the inset. (C) Cytofluorometric expression of CD99 in a panel of osteosarcoma cell lines. The Ewing's sarcoma cell line SK-ES-1 was used as positive control. (D) Representative osteosarcoma sample that shows positivity to CD99. Note the low level of expression.

errant proliferating osteoblasts. The weak or null expression of CD99 in osteosarcoma prompted us to test its functional role by forcing its expression in two human osteosarcoma cell lines. The in vitro and in vivo behavior of several CD99 transfectants is reported here. Significant reversal of osteosarcoma malignancy was observed and molecular mechanisms underlying CD99 effects were explored.

MATERIALS AND METHODS

Cell Lines and Transfection

The osteosarcoma cell lines U-2 OS and Saos-2 were obtained from American Type Culture Collection (Manassas, VA). Cells were routinely cultured in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Paisley, Scotland), supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% inactivated fetal bovine serum (FBS) (Cambrex Bio Science Verviers, Verviers, Belgium) and maintained at 37°C in a humidified 5% CO₂ atmosphere. Stable transfectants expressing CD99 were obtained from the two osteosarcoma cell lines by using calcium-phosphate transfection method. Cells transfected with the empty vector pcDNA3 were also used as negative controls. Transfectants were selected in IMDM containing 10% FBS and 500 μ g/ml neomycin (Sigma-

Aldrich, St. Louis, MO) and maintained in selective medium for a maximum of eight in vitro passages before the in vitro and in vivo characterization.

Cytofluorometric Analysis of CD99 and Integrins

The expression of CD99 and integrins was analyzed by indirect immunofluorescence using the following primary antibodies: anti-CD99 O13 monoclonal antibody (MAb) (Signet, Dedham, MA), 1: 80 dilution; anti-vitronectin receptor ($\alpha_v\beta_3$ clone LM609; Chemicon International, Temecula, CA), 1:50 dilution; CDw49b VLA2 (anti- α_2 chain, $\alpha_2\beta_1$; Immunotech SA, Marseille, France), 1:10 dilution; P1B5 (anti- α_3 chain, $\alpha_3\beta_1$; Calbiochem-Novabiochem, San Diego, CA) 1:20 dilution; CDw49d VLA4 (anti- α_4 chain, $\alpha_4\beta_1$; Immunotech), 1:10 dilution; CDw49e VLA5 (anti- α_5 chain, $\alpha_5\beta_1$; Immunotech), 1:10 dilution; and CDw49f VLA6 (anti- α_6 chain, $\alpha_6\beta_1$; Immunotech), 1:10 dilution.

Analysis of Growth Features in Monolayer Conditions

Doubling time was determined by daily harvesting of cells after seeding of 20,000 cell/cm² in IMDM 10% FBS. Cell viability was determined by trypan blue dye exclusion.

Soft Agar Assay

Anchorage-independent growth was determined in 0.33% agarose (Sea-Plaque; FMC BioProducts, Rockland, ME) with a 0.5% agarose underlay. Cell

Figure 2. Expression of CD99 isoforms in U-2 OS- and Saos-2-transfected cells. Relative expression of CD99 in U-2 OS and Saos-2 clones by cytofluorometry. Open profile represents cells stained with secondary antibody alone; solid profile represents cells stained with the anti-CD99 antibody. In each panel, the ordinate represents the number of cells. Similar extracellular expression of CD99 was observed in all the transfectants. 6647 Ewing's sarcoma cells were also included as positive control.

suspensions (10,000–33,000 cells/60-mm dish) were plated in semisolid medium (IMDM 10% FBS plus agar 0.33%) and incubated at 37°C in a humidified 5% CO₂ atmosphere. Colonies were counted after 10–14 d.

Cell Cycle Analysis

For the evaluation of BrdUrd labeling index, cell cultures were incubated with 10 μ M bromodeoxyuridine (BrdUrd) (Sigma-Aldrich) for 1 h in a CO₂ atmosphere at 37°C. Harvested cells were fixed in 70% ethanol for 30 min. After DNA denaturation with 2 N HCl for 30 min at room temperature, cells were washed with 0.1 M Na₂B₄O₇, pH 8.5, processed for indirect immunofluorescence staining, using α -BrdUrd (Euro-Diagnostics, Milan, Italy) diluted 1:4 as a primary MAb, and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, Milan, Italy). For the cell cycle analysis, 70% ethanol-fixed cells were pretreated with 100 μ g/ml RNase for 30 min at 37°C and stained with 20 μ g/ml propidium iodide before flow cytometric analysis.

Analysis of Apoptosis

Detection and quantification of apoptotic cells was obtained by the flow cytometric analysis (FACSCalibur; Becton Dickinson) of Annexin-V-fluorescein isothiocyanate (FITC)-labeled cells. This test was performed according to the manufacturer's instructions (Medical & Biological Laboratories, Naka-ku Nagaya, Japan). Propidium iodide incorporation was evaluated in association with the fluorescent signal intensity to allow the discrimination of necrotic and apoptotic cells.

Poly-2 Hydroxyethylmethacrylate (Poly-HEMA) Assay

Six-well plates were treated with poly-HEMA (Sigma-Aldrich) following the Folkman and Moscona method (Folkman and Moscona, 1978). Briefly, wells were treated with a 1-ml solution of poly-HEMA diluted in ethanol 95% (12 mg/ml) and left to dry at room temperature. Then, 250,000 cells/well were seeded in IMDM 10% FBS and incubated at 37°C in a humidified 5% CO₂ atmosphere. Viable and dead cells were counted after 24, 48, and 72 h. Detection and quantification of apoptotic cells as well as of cell cycle phases were obtained according to the procedures described above.

Motility Assay

Motility assay was performed using Transwell chambers (Costar, Cambridge, MA) with 8- μ m pore size, polyvinylpyrrolidone-free polycarbonate filters (Nucleopore, Pleasanton, CA). Cells (10⁵) in IMDM plus 1% FBS were seeded in the upper compartment, whereas IMDM plus 10% FBS was placed in the lower compartment of the chamber as chemoattractants. Cells were incubated for 18 h at 37°C in the presence or not of c-Src inhibitors (PP1 and PP2 analogs and 2.5–5 μ M herbimycin) (Calbiochem, San Diego, CA), and the number of cells that migrated toward the filter to reach the lower chamber base was counted after fixation and Giemsa staining. All the experiments were performed in triplicate.

Extracellular Matrix (ECM) Adhesion Assay

The adhesive ability of U-2 OS- and CD99-transfected cells was analyzed by using the CytoMatrix cell adhesion strips coated with human collagen type IV, vitronectin, fibronectin, laminin, or collagen type I (Chemicon International). Cells (40,000 cells/well) were seeded and incubated for 1 h at 37°C. Adherent cells were fixed and stained with 0.2% crystal violet 10% ethanol. After washing and solubilization with 5 mM NaH₂PO₄, pH 4.5, relative attachment was determined using absorbance readings at 550 nm on a microplate reader. All the experiments were performed in triplicate.

Fluorescence on Adherent Fixed Cells

U-2 OS and Saos-2 osteosarcoma cells were seeded in IMDM 10% FBS and grown on coverslips for 48 h before being fixed in 4% paraformaldehyde at room temperature and permeabilized with 0.15% Triton X-100 in phosphate-buffered saline (PBS). All preparations were incubated with PBS containing 4% bovine serum albumin to saturate nonspecific binding. Immunofluorescence staining for caveolin-1, phosphoY416 c-Src, or β -catenin was performed with the primary α -caveolin-1 MAb (BD Transduction Laboratories, Lexington, KY) (diluted 1:100), or the anti-phospho-Src tyr416 (Cell Signaling Technology, Beverly, MA) (diluted 1:10) or the anti- β -catenin MAb (Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:50).

Immunoprecipitation Analysis

Cell lysates from U-2 OS and U-2/CD99 wild-type (wt) clones were prepared with a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1% deoxycholate, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, and 10 μ g/ml aprotinin). Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA), and equivalent amounts of total cell lysates (500 μ g) were immunoprecipitated with appropriate antibodies by overnight incubation at 4°C. Protein G plus/protein A agarose beads (40 μ l; Calbiochem) were then added to each lysate and incubation continued for 1 h. After collecting, the beads were washed three times with lysis buffer, resuspended in 20 μ l of SDS-gel sample buffer, and boiled for 5 min. Western blotting (WB) was then performed. The following antibodies were used: anti-CD99 12E7 MAb (Dako Cytomation Denmark, Glostrup, Denmark; 1.5 μ g for immunoprecipitation, 1:10,000 dilution for WB); anti-caveolin-1 antibody (Transduction Laboratories, 1 μ g for immunoprecipitation, 1: 5000 dilution for WB); anti-Src antibody (Cell Signaling Technology; 1:1000 dilution for WB).

Western Blotting

Equivalent amounts of total cell lysates from U-2 OS and U-2/CD99 wt clones were separated by 10% SDS-PAGE under denaturing conditions and transferred onto nitrocellulose membrane. Membranes were incubated overnight with the following primary antibodies: anti-phospho-Src tyr416 (Cell Signaling Technology; 1:1000 dilution) and anti-phospho Akt Ser473 (Cell Signaling Technology; 1:1000 dilution). Analysis of Akt and c-Src was also performed to verify the total proteins, as control (primary antibodies for Akt or c-Src diluted 1:1000; New England Biolabs, Cell Signaling Technology). After washes with 1 $\times$ Tris-buffered saline/Tween 20, the membranes were incubated with secondary anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (Dako Cytomation Denmark) (1:1500 dilution), and revealed by ECL Western blotting detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Tumorigenic and Metastatic Ability in Athymic Mice

Female athymic 5-wk-old Crl:nu/nu (CD-1) BR mice (Charles River Italia, Como, Italy) were used. Tumorigenicity was determined after s.c. injection of 5 $\times$ 10⁶ cells, whereas metastasization was evaluated after i.v. injection of 2.5 $\times$ 10⁶ cells. Tumor growth was assessed once a week by measuring tumor volume. Negative mice were checked for 6 mo after cell injection. The number of pulmonary metastases was determined 2 mo after cell inoculation by counting with a stereomicroscope after staining with black India ink. The experimental procedures were approved by the local ethical committee.

Antisense Oligonucleotides

U-2/CD99wt57 cells (20,000 cell/cm²) were seeded in complete medium. On day 1, cells were incubated with or without scramble or antisense oligonu-

Figure 3. In vitro growth features of U-2 OS and Saos-2 cells and CD99 derived clones. (A) Cell growth curves in IMDM 10% FBS. (B) Growth in soft agar of parental osteosarcoma cells and CD99 derived clones. Cells were seeded at a concentration of 10,000–33,000 cells, and the number of colonies in triplicate plates was determined after 10 d of growth in 10% FBS. Data are expressed as means of six plates $\pm$ SE (* p < 0.05, Student's t test) with respect to U-2 OS parental cells. (C) Survival of U-2 OS cells and derived clones on poly-HEMA-coated dishes. Cells (250,000) were seeded on poly-HEMA-coated dishes in IMDM 10% FBS. The number of live cells per plate was determined by trypan blue vital cell count at the times indicated on the abscissa. Data represent the mean $\pm$ SE of duplicate experiments. (D) Cytofluorometric

cleotides (Biognostik, Göttingen, Germany) at a concentration of 3 μ M. Every 24 h, cells were washed and resuspended in fresh medium containing 3 μ M oligonucleotides. Cells were harvested after 72 h of treatment and seeded for evaluation of migration. Repression of caveolin-1 expression was checked by real-time polymerase chain reaction PCR.

RNA Isolation

Total RNA was extracted using the TRIzol extraction kit (Invitrogen). The quality of RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide; the 18S and 28S RNA bands were visualized under UV light. To perform microarray hybridization, two independent extractions of RNAs were obtained from U-2/CD99wt57, U-2/CD99wt136, and U-2 OS cells.

cDNA Microarray Hybridization

Hybridizations were performed on Human 1 cDNA microarray slides (Agilent Technologies, Palo Alto, CA) containing 16,281 cDNA sequences. Total RNA was used to obtain labeled cDNA, according to the manufacturer's instructions (Agilent Direct-Label cDNA synthesis kit protocol; Agilent Technologies). U-2/CD99wt57 and U-2/CD99wt136 cDNAs were labeled with cyanine 5-dCTP (Cy5), whereas U-2 OS was labeled with cyanine 3-dCTP (Cy3). In an additional experiment U-2/Empty cDNA, labeled with Cy5 was hybridized with U-2 OS Cy3-cDNA. In brief, 20 μ g of total RNA was reverse-transcribed with a specific primer mixture using Moloney murine leukemia virus reverse transcriptase (Agilent Direct-Label cDNA synthesis kit) in the presence of Cy5 or Cy3 (PerkinElmer Life and Analytical Sciences, Boston, MA) at 42°C for 1 h. RNaseIA (Agilent Technologies) was then added to degrade the RNA. Labeled cDNAs were purified with QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Additional washes with 35% (wt/vol) guanidine hydrochloride were performed to ensure efficient removal of the unincorporated dye-labeled nucleotides. Equivalent amounts of Cy5-cDNA and Cy3-cDNA were combined, vacuum-dried, resuspended in deposition hybridization buffer (Agilent Technologies), with Human Cot-1 (Invitrogen) and deposition control targets (QIAGEN) and hybridized to microarray slides for ~17 h, according to the manufacturer's instructions. The slides were washed with 0.5 $\times$ standard saline citrate (SSC) + 0.01% SDS for 10 min and with 0.06 $\times$ SSC for 5 min.

cDNA Microarray Analysis

Hybridized slides were scanned using ScanArray LITE confocal laser scanner (PerkinElmer Life and Analytical Sciences) minimizing the total number of saturated spots for both channels. Image analysis was performed with QuantArray software (PerkinElmer Life and Analytical Sciences). Spots showing evident blemishes were flagged and excluded from analysis. For each spot signal intensity for both channels was calculated by subtracting local background. For each array, a normalization factor was calculated dividing the total signal intensity of all spots in Cy3 channel by the signal of all spots in Cy5 channel. Spots whose measured area was higher than 50% of the average element size on the array in at least one channel were kept for further analysis. Ratios between mean net fluorescence from Cy5 channel and mean net fluorescence from Cy3 channel were calculated for each spot (representing ratios between RNA expression values detected in either U-2/CD99wt57 or U-2/CD99wt136 cells and those obtained in the parental U-2 OS cell line. Cy5/Cy3 expression ratios were log-transformed (base 2). A cut-off filtering criterion was used: only those genes with greater than twofold induction or repression in both comparisons were considered. Functional cluster result analysis was also performed by using the MAPPFinder and Fatigo program.

Quantitative Real-Time PCR

Real-time PCR was performed on the panel of CD99 osteosarcoma-transfected cells for caveolin-1. In particular, 1 μ g of total RNA was denatured at 65°C for 10 min and then reverse-transcribed in a 100- μ l reaction mixture containing 500 μ M of each dNTP, 125 U of MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA), 40 U of RNase inhibitor (Applied Biosystems), 2.5 μ M oligo(dT), 1 $\times$ TaqMan reverse transcriptase buffer, and 5 mM MgCl₂ at 48°C for 40 min. Reactions performed in the absence of enzyme or RNA were used as negative controls. Gene-specific primers were designed using Primer Express software (Applied Biosystems): caveolin-1 (#A1878826), forward 5'-CGA GAA GCA AGT GTA CGA CGC-3' and reverse 5'-ACC ACG TCA TCG TTG AGG TG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward GAAGGT GAAGGTCGGAGTC and reverse GAAGAT-GGTGATGGGATTTC. Amplification was performed at the following cycling

conditions: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. A SYBR Green PCR Master Mix (Applied Biosystems) was used with 1 ng of cDNA and with 100–400 nM primers. A negative control without any cDNA template was run with every assay. All PCR reactions were performed by using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated passes the baseline threshold. The target gene mRNA is quantified by measuring C_T to determine the relative expression. Data were normalized to GAPDH. The relative expression of the different mRNAs was also normalized to a calibrator, consisting of the parental cell line mRNA, and was expressed as 2^{− $\Delta\Delta$ C_T}, where Δ C_T is C_T target genes − C_T GAPDH, and $\Delta\Delta$ C_T is Δ C_T sample − Δ C_T calibrator. All samples were resolved in a 2% agarose gel to confirm the PCR specificity.

Statistical Analysis

Differences among means were analyzed using Student's *t* test. Fisher's exact test was used for frequency data.

RESULTS

Expression of CD99 in Osteoblasts and Osteosarcoma

Expression of CD99 was observed in osteoblastic cell cultures derived from tibial plate and expressing the differentiative markers bone/liver alkaline phosphatase and osteocalcin as well as in tissue samples (Figure 1, A and B). In vitro, the expression of CD99 was particularly high on the lamellipodia-like structures (Figure 1A, arrows), whereas in tissue samples it is intense in the osteoblasts adhering to each other and lining the bone surface (Figure 1B, arrows), in keeping with the cell adhesion properties of CD99 (Bernard *et al.*, 1995; Hahn *et al.*, 1997). Osteoblasts in an earlier phase of differentiation show weak expression of CD99, which may be in accord with their need to proliferate and migrate within immature bone matrix (Figure 1B, inset). In osteosarcoma cell lines, the expression of CD99 is generally very low (Figure 1C) and only six of 17 primary osteosarcomas show barely detectable CD99 by immunohistochemistry (Figure 1D).

Selection and Characterization of CD99-transfected Clones

To analyze the role of CD99 in osteosarcoma malignancy, we transfected the U-2 OS and SaoS-2 osteosarcoma cell lines with the CD99 cDNA. Stable transfectants were selected in G418 (500 μ g/ml) and characterized for CD99 expression. Among the clones obtained, three transfectants from each cell line were chosen on the basis of CD99 surface expression level, in comparison with Ewing's sarcoma cells used as positive control (Figure 2).

CD99 Expression Reduces Anchorage-independent Growth and Migration

CD99 reduces the growth ability of osteosarcoma cells. This effect, although modest in monolayer (Figure 3A), is dramatic in anchorage-independent conditions, where CD99 overexpression becomes lethal for osteosarcoma cells. Indeed, CD99-forced expression strikingly reduced the size and the number of colonies of U-2 OS and Saos-2 transfectants (Figure 3B). Empty vector-transfected cells did not show any changes in soft agar colony formation. Nontransfected, anchorage-dependent cells undergo anoikis (i.e., detachment-induced apoptosis) when deprived of contact with the extracellular matrix. To test whether the reduced growth in soft agar of CD99 transfected cells is caused by a greater sensitivity to anoikis, we examined cell survival after cultures in dishes coated with poly-HEMA, in which adherence of cells is prevented (Folkman and Moscona, 1978). Under these conditions, the survival of U-2/CD99 cells was severely reduced (Figure 3C), and the occurrence of apoptosis was significantly higher, as documented by Annexin test

Figure 3 (cont). analysis of apoptotic U-2 OS cells and derivatives by Annexin-V and propidium iodide after 24 h. Data represent the mean of triplicate experiments. (E) Cell cycle analysis of U-2 OS cells and CD99-derived clones in basal conditions. Data are from an experiment representative of two independent experiments.

Figure 4. Adhesive and migratory features of parental osteosarcoma cells and CD99-derived clones. (A) Migration ability of U-2 OS and Saos-2 cells and CD99-transfected cells. Cells were seeded in the upper compartment of a transwell chamber; in the lower compartment, IMDM 10% FBS was used as the source of chemoattractant. Each column represents the mean $\pm$ SE of three independent experiments. * $p < 0.05$; Student's *t* test. (B) Effects of CD99 expression on the adhesive properties of U-2 OS and Saos-2 CD99-transfected cells. Cells were plated on wells coated with different ECM components accordingly to the manufacturer's instructions. After 1 h, the number of adherent cells was determined after incubation with crystal violet. Data, which are means of four to six plates, are expressed as percentage of parental cell line mean values.

(Figure 3D). In addition, a reduction of cells in S phase was observed (Figure 3E). Therefore, CD99 inhibition of anchorage-independent growth is due both to enhancement of anoikis and inhibition of cell proliferation. Also, transfected CD99 clones showed reduced cell migration (Figure 4A) and increased adhesion on the extracellular matrix components collagen I, laminin, and fibronectin (Figure 4B). Modulation

of adhesive properties of CD99-transfected cells is not because of integrin expression that remains substantially unchanged in CD99 transfectants (our unpublished data).

CD99 Expression and Malignancy

Consistent with the in vitro data, CD99 overexpression reduced tumorigenicity and metastasis ability of osteosarcoma

Table 1. Tumorigenic and metastatic ability of U-2 OS parental cells and U-2/CD99-transfected clones

Cell line	Tumorigenicity		Experimental metastases	
	Incidence (%)	Latency (d)	Incidence (%)	Median (range)
U-2 OS	7/10 (70)	41 ± 9	16/19 (84)	78 (0–200)
U-2/Empty	3/5 (60)	49 ± 15	9/10 (90)	5 (0–200)
U-2/CD99wt53	0/5		1/10 (10)	0 (0–3)
U-2/CD99wt57	0/5		0/10 (0)	0
U-2/CD99wt136	0/5		7/15 (47)	0 (0–24)

cells xenotransplanted in nude mice. Indeed, CD99-transfected U-2 OS cells failed to form tumors when injected s.c. in athymic mice, whereas significantly reducing the number of lung metastases upon tail injection in comparison with control cells (Table 1). These effects are not because of variations in MMP-2 and MMP-9 activity of CD99 transfected cells, which remain unchanged compared with parental cell line (our unpublished data).

Genetic Profile of CD99-transfected U-2 OS Cells

Two independently transfected clones (U-2/CD99wt57 and U-2/CD99wt136) were compared with the parental cell line U-2 OS and U-2/Empty cells for gene expression profile. Relative intensity ratios ($\log_2$ -transformed ratios) were calculated. Of the modulated genes, only those genes displaying induction or repression of their expression greater than twofold in at least 75% of the arrays (i.e., 3 of 4 independent experiments) were included in the final analysis. Four hundred eleven genes showed significantly different levels of expression in both U-2/CD99wt57 and U-2/CD99wt136 compared with controls: 326 genes were up-regulated, whereas the remaining 85 genes were down-regulated. The most up- and down-modulated genes ($\log_2$ mean ratio $\geq$ or ≤ -1.5 , respectively) are shown in Table 2. The up-modulated genes were grouped according to cellular component and biological functions, and the analysis obtained with Fatigo is shown in Figure 5. The majority of up-regulated genes encode for proteins associated to plasma membrane, nucleo/mitochondrial membranes, or to cytoskeleton, particularly the actin cytoskeleton. Among the eight most represented functional groups, three are related to cell motility and adhesion, regulation of cell growth and apoptosis, and cell-cell signaling.

CD99 Induces Up-Regulation of and Colocalizes with Caveolin-1, Which Participates in Reversal of U-2 OS Malignancy, Likely through Inhibition of Src Activity

Among up-regulated genes, we focused our attention on caveolin-1, which has been indicated as a candidate tumor suppressor gene in sarcomas (Wiechen *et al.*, 2001.). Caveolin-1 drives the formation of plasma membrane caveolae and anchors them to the actin cytoskeleton, modulates cell interaction with the extracellular matrix, pulls together and regulates signaling molecules, and transports cholesterol. Through these functions, caveolin-1 might play an important role in cell movement and growth (Navarro *et al.*, 2004). An increased expression of caveolin-1 was confirmed by quantitative PCR in all U-2 OS and Saos-2 transfectants (Figure 6A). Expression of caveolin-1 on the cell membrane was increased by CD99 gene transfection in comparison

Table 2. Mostly differentially up-regulated genes in U-2/CD99wt57 and U-2/CD99wt136 compared with the parental cell line U-2 OS and U-2/Empty cells

Gene name	Gene description	Log ratio mean
EMP1	Epithelial membrane protein	2.17
FST	Follistatin	2.14
CTGF	Connective tissue growth factor	2.03
CAV1	Caveolin-1, caveolae protein, 22 kDa	1.94
Anxa1	Annexin-A1	1.88
TBPL1	TBP-like	1.83
LYPLA1	Lysophospholipase I	1.81
SRPX	Sushi-repeat-containing protein, X-linked	1.81
CRYAA	Crystalline, α A	1.70
TCEA1	Transcription elongation factor A (SII), 1	1.69
UAP1L1	UDP-N-acetylglucosamine pyrophosphorylase1	1.65
BAP1	BRCA-associated protein-1	1.64
COX7B	Cytochrome c oxidase subunit VIIb	1.62
MASP1	Mannan-binding lectin serine peptidase 1	1.62
CAPZA2	Capping protein (actin filament) muscle Z-line	1.61
MGC12261	Nuclear DNA-binding protein	1.58
PPP1CB	Protein phosphatase 1, catalytic subunit	1.58
IFRD1	Interferon-related developmental regulator 1	1.57
ALCAM	Activated leukocyte cell adhesion molecule	1.57
GJA1P1	Gap junction protein, α 1, 43 kDa (connexin)	1.57
SYPL1	Synaptophysin-like 1	1.57
EIF3S6	Eukaryotic translation initiation factor 3	1.55
EDEM1	ER degradation enhancer, mannosidase α	1.55
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	1.54
PITPNB	Phosphatidylinositol transfer protein, β	1.54
ZNF23	Zinc finger protein 23 (KOX16)	1.52
RTN4	Reticulon 4	1.52
HIF1A	Hypoxia-inducible factor 1, α subunit	1.52
VBP1	Von Hippel-Lindau binding protein 1	1.51
CD44	CD44 antigen	1.50
RXRG	Retinoid X receptor, γ	1.50
UTRN	Utrophin (homologous to dystrophin)	1.50
KRTHB3	Keratin, hair, basic 3	-3.18
ACTG2	Actin, γ 2, smooth muscle	-2.27
CD3E	CD3E antigen, epsilon polypeptide	-2.24
ACTA2	Actin, α 2, smooth muscle	-2.22
CHST10	Carbohydrate sulfotransferase 10	-2.13
KRTHB1	Keratin, hair, basic 1	-2.08
ENO2	Enolase 2	-2.05
ID1	Inhibitor of DNA binding 1, dominant negative	-2.03
MLLT7	Myeloid/lymphoid or mixed-lineage leukemia	-2.01
ACTG1	Actin, γ 1	-1.98
CCT7	Chaperon containing TCP1, subunit 7	-1.96
EEF1D	Eukaryotic translation elongation factor 1 δ	-1.85
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein	-1.79
VCP	Valosin-containing protein	-1.79
UBTF	Upstream binding transcription factor, RNS poly	-1.74
MRPL28	Mitochondrial ribosomal protein L28	-1.73
KRT14	Homo sapiens keratin 14 (epidermolysis bullosa)	-1.68
ACTG1	Actin, γ 1	-1.66
TPM2	Tropomyosin 2	-1.64
EEF1D	Eukaryotic translation elongation factor 1 δ	-1.57
KRT6A	Keratin 6A	-1.56
TUBA3	Tubulin, α 3	-1.54
CCT7	Chaperonin containing TCP1, subunit 7	-1.53
ATP6VOB	ATPase, H ⁺ transporting, lysosomal 21 kDa	-1.52
SIAHBP1	Fuse-binding protein-interacting repressor	-1.52
HSPA1A	Heat shock 70 kDa protein 1 A	-1.50
PTK9L	PTK9L protein tyrosine kinase 9-like	-1.50

with parental osteosarcoma cells, which have caveolin-1 expression barely detectable (Figure 6B). Costaining with anti-caveolin-1 and anti-CD99 antibodies showed their par-

Figure 5. Graphical representation of up-regulated genes in U-2/CD99 transfectants compared with parental cells and U-2/Empty cells. Three hundred twenty-six genes showed significantly different higher levels of expression in both U-2/CD99wt57 and U-2/CD99wt136 compared with control cells. The up-modulated genes were grouped according to cellular component and biological functions by using Fatigo program.

tial colocalization at cell membrane and at sites of cell–cell contacts (Figure 6B). Immunoprecipitation experiments using anti-CD99 and anti-caveolin-1 antibodies confirmed that the two proteins were associated with each other (Figure 6C). To test whether such interaction has any functional mean, we treated the cells with antisense phosphorothioate oligonucleotides to caveolin-1 (Figure 6, D and E). Antisense but not scramble oligos restored the migratory ability of U-2/CD99wt136-transfected cells to the level of the parental cell line (Figure 6F).

Caveolin-1 functions as a scaffolding protein to concentrate and organize signaling molecules within caveolae membranes, frequently leading to their inactivation. We analyzed the Wnt signaling and c-Src activity in CD99 transfectant cells. Both these two pathways, whose constitutive activation can often cause cellular transformation and modulate cell cycle and migration, have been described to be negatively regulated by caveolin-1 (Li *et al.*, 1996; Galbiati *et al.*, 2000). In addition, they are involved in osteoblasts functions (Marzia *et al.*, 2000; Westendorf *et al.*, 2004), and c-Src was proposed to mediate the increased cell motility associated with a CD99 splice variant overexpressed in a breast cancer cell line (Lee *et al.*, 2002). We could not appreciate any difference in the amount of β -catenin or in its cellular localization between CD99 clones and parental cell line (our unpublished data) that may indicate this pathway as a mediator of the CD99/caveolin-1 functions. On the contrary, c-Src was found to colocalize with caveolin-1 and with CD99 (Figure 7A), indicating the existence of a complex CD99–caveolin-1–Src on the membrane of U-2/CD99-transfected cells that could act as an allosteric inhibitor of c-Src kinase activity. Consistently, three different inhibitors of c-Src kinase family (PP1 and PP2, 2 inhibitors acting by binding the ATP binding site, and herbimycin A that inhibits c-Src function by irreversibly binding to the thiol groups of the kinase) were found to significantly suppress motility of the parental but not U-2/CD99 transfectant cells (Figure 7, B and C). In addition, c-Src as well as Akt, a mediator of cellular survival pathways also involved in c-Src functions (Windham *et al.*, 2002), showed decreased phosphorylation in U-2/CD99 cells (Figure 7D). c-Src phosphorylation changed at the level of tyrosine 416 (Y416), corresponding at site of c-Src autophosphorylation, and its decreased amount of phosphorylation in CD99-transfected cells was confirmed also by fluorescent immunostaining of the cells (Figure 7E).

DISCUSSION

CD99 has been recently linked to osteoblast differentiation (Bertaux *et al.*, 2005), MIC2 gene being posed downstream the control of the transcription factor Cbfa-1(RUNX2), which drives osteoblast differentiation (Lian *et al.*, 2004). Few other sporadic findings link CD99 to osteoblast functions. Hamilton *et al.* (2001) found that the supernatants from several cancer cell lines specifically down-regulate CD99 on human trabecular osteoblasts (AHTO-7 cells), whereas inducing osteoblast activation and proliferation. Here, we show expression of CD99 in osteoblasts, varying from high at the sites of cell adhesion to low in more immature, proliferating, and migrating osteoblasts. This pattern of expression is in keeping with the role of CD99 in cell adhesion processes (Bernard *et al.*, 1995; Hahn *et al.*, 1997). Thus, it seems conceivable that osteosarcoma, a bone tumor generally derived from aberrant-proliferating osteoblasts, should down-regulate the expression of CD99, showing low, if any, CD99 positivity as we observed. Forced expression of the molecule in two osteosarcoma cell lines offers the opportunity to study whether CD99 has any functions in the pathophysiology of osteoblastic lesions.

In this article, we show that expression of CD99 significantly affected osteosarcoma cell behavior, regulating crucial biological processes required for the development of metastasis. In particular, CD99 overexpression has a dramatic negative impact on the ability of osteosarcoma cells to grow and survive in anchorage-independent conditions. Because anchorage independence is one of the earliest characteristics for the establishment and maintenance of the transformed phenotype and resistance to anoikis is a common property of tumor cells considered to be crucial to the development of metastases, CD99 overexpression might be predicted to induce substantial reversal of malignancy of osteosarcoma cells, which was exactly what we observed when U-2/CD99 cells were injected in athymic mice. The complete absence of tumor development after 6 mo from cell injection implies that U-2/CD99 cells must have died and confirms in vitro data suggesting that the effect of CD99-forced expression is not simply to slow down growth but actually to activate mechanisms able to induce cell death. The higher ability of CD99-transfected cells to adhere to collagen I, fibronectin, and laminin together with the lower in vitro migration of these cells complete the pattern of biological processes re-

Figure 6. Caveolin-1 is required for CD99 functions in modulating osteosarcoma migratory abilities. (A) Relative expression of caveolin-1 mRNA in U-2 OS and Saos-2 cells transfected with anti-CD99. The relative target gene mRNAs expression of U-2 OS parental cells was used as a calibrator ($2^{-\Delta\Delta CT} = 1$). Data expressed as mean values $\pm$ SE. (B) Immunostaining of caveolin-1 and CD99 on adherent cells. Caveolin is labeled by anti-caveolin MAb and revealed by FITC-conjugated anti-mouse IgM. CD99 is labeled by the anti-CD99 013 MAb and revealed by Cy3-conjugated anti-mouse IgG. U2OS and Saos-2 parental cells express low levels of caveolin-1 and CD99. Cells overexpressing wild-type CD99 (U2/CD99 wt57 or Sa/CD99wt22) accumulate caveolin-1 at the cell membrane. Colocalization of CD99 and caveolin-1 at the cell membrane is shown in the merged image, and it is indicated by arrows. (C) Immunoprecipitation of CD99 from U-2/CD99wt57 and U-2/Empty cells was followed by immunoblotting with anti-caveolin-1 antibody. Note that caveolin-1 coimmunoprecipitates with CD99A signal, but much weaker than in U-2/CD99wt57 cells, as was observed also in U-2/Empty cells, likely because of the cells expressing both the molecules, although at very low levels. (D) Real-time analysis of caveolin-1 expression in U-2/CD99wt57 cells after 72-h pretreatment with 3 μ M scramble or three different caveolin-1 antisense oligonucleotide sequences. (E) Immunostaining of caveolin-1 in U-2/CD99wt57 cells after 72-h pretreatment with 3 μ M scramble or caveolin-1 antisense oligonucleotide sequences. (F) Migratory ability of U-2/CD99wt57 cells after 72-h pretreatment with 3 μ M scramble or three different caveolin-1 antisense oligonucleotide sequences.

Figure 7. C-Src colocalizes with caveolin-1 and with CD99, and c-Src kinase activity is inhibited in CD99-transfected cells. (A) Immunoprecipitation of CD99 or caveolin-1 from U-2/CD99wt57 and U-2/Empty cells was followed by immunoblotting with anti-c-Src antibody. Note that c-Src coimmunoprecipitates with both the two molecules. (B and C) Migratory ability of U-2 OS and CD99-transfected cells after treatment with the c-Src inhibitor PP1 (2.5 μ M) or PP2 (5 μ M) or herbimycin (2 μ M). The PP3 compound (5 μ M) is a negative control for Src family tyrosine kinase inhibitors PP1 and PP2, despite that it inhibits the activity of epidermal growth factor receptor kinase. (D and E) Evaluation of c-Src kinase activity in CD99-transfected cells. (D) Phosphorylation of c-Src at tyrosine 416 (Y416) as well as phosphorylation of Akt was analyzed by Western blotting. Membrane were stripped and reblotted with antibodies against total c-Src and Akt as control. (E) Fluorescent immunostaining of Y416 phosphorylation in U-2 OS and U-2/CD99wt 57 cells.

quired for metastasization that are affected by CD99 over-expression. It is worth noting that all these in vitro features are strongly correlated with metastases, which can be considered a model of anchorage independence because they usually arise from single cells or small cluster of metastatic cells, and consistently, the expression of CD99 almost completely abolishes lung metastasization of U-2 OS cells. This result may have important implications for osteosarcoma, a primary malignancy of bone with a high, and almost exclusive, propensity to metastasize the lung. Despite >30% of patients with localized disease eventually develop distal metastases after intensive chemotherapy (Ferrari *et al.*, 2003),

molecular mechanisms underlying disease progression currently are indeed largely unknown. Our findings indicate that expression of CD99 significantly affected malignancy of osteosarcoma cells, pointing out for the first time that CD99 can function as an oncosuppressor. However, this condition may have been sensed by interpreting some sporadic data from the literature. In fact, the in vitro down-regulation of CD99 in B lymphocytes generates cells with Hodgkin and Reed-Sternberg phenotype, suggesting a role for CD99 loss of function in the pathogenesis of Hodgkin disease (Kim *et al.*, 2000). Moreover, CD99 has been recently described down-regulated in pancreatic endocrine neoplasms, and its

loss is interpreted as a negative prognostic marker (Maitra *et al.*, 2003; Goto *et al.*, 2004). With the main exception of some pediatric tumors, such as Ewing's sarcoma and acute lymphoblastic leukemia, which express high levels of the protein, the CD99 loss, rather than overexpression, seems to be the rule in neoplasms, indicating that its down-regulation may be required for neoplastic progression. Our observations support the view that low, if any, expression of CD99 may substantially contribute to malignancy.

Because of the largely undefined role of CD99 in general, and in osteosarcoma in particular, we performed gene expression profile of CD99 stably transfected clones in comparison with that of the parental cell line. Three hundred twenty-six genes were found to be up-regulated, indicating that the sole overexpression of CD99 is sufficient to induce remarkably changes in the osteosarcoma molecular signature. A great amount of CD99 up-regulated genes encode for proteins located to plasma membrane or to cytoskeleton, particularly the actin cytoskeleton. Because a tight and complex interplay exists between cell adhesion, cytoskeleton components, and malignancy (Brunton *et al.*, 2004), variations in the expression profile of these genes could provide a plausible explanation for the different behavior of CD99-transfected osteosarcoma cells. Among the CD99-induced genes encoding for cell adhesion receptors or cell-matrix adhesion molecules, we analyzed the functional involvement of caveolin-1, a principal component of caveolae membranes (Drab *et al.*, 2001), which has already been described as a putative oncosuppressor gene in sarcomas (Wiechen *et al.*, 2001). Expression of caveolin-1 was found to be reduced in sarcoma tissue samples and strongly down-regulated in tumorigenic fibrosarcoma cells compared with the nontumorigenic variant (Wiechen *et al.*, 2001). In our model, we demonstrate that caveolin-1 forms a stable complex with CD99. In addition, in CD99-transfected cells, caveolin-1, besides being overexpressed, specifically localizes to areas of cell-cell contact. Transient inhibition of caveolin-1 expression by antisense oligos in CD99-transfected cells restores cell migration to the level of parental cells, therefore being sufficient to abrogate at least one of the effects induced by CD99 overexpression. These observations may be related to the ability of caveolin-1 to function as a scaffolding protein to concentrate and organize signaling molecules within caveolae membranes (Navarro *et al.*, 2004). Because the interaction of caveolin-1 with several signaling molecules, whose constitutive activation can often cause cellular transformation, leads to their inactivation, caveolin-1 acts in many models as a tumor suppressor protein. Among other effects, caveolin-1 inhibits Wnt as well as c-Src signaling (Li *et al.*, 1996; Galbiati *et al.*, 2000), by recruiting β -catenin or c-Src to caveolae membrane domain. Both the two pathways are largely involved in the pathophysiology of osteoblasts (Marzia *et al.*, 2000; Westendorf *et al.*, 2004) and in crucial processes implicated in the pathogenesis and progression of tumors, such as cell cycle, migration, resistance to anoikis and anchorage independence (Parsons and Parsons, 2004; Harris and Peifer, 2005). So, we tested their role as possible mediators of the CD99/caveolin-1 functions. Although we failed to appreciate any difference in the amount of β -catenin or in its cellular localization between CD99 clones and parental cell line, we observed a reduction of Src kinase activity and Akt phosphorylation, a downstream effector involved in c-Src regulation of anoikis (Windham *et al.*, 2002), in CD99 transfectants. Because c-Src activity may be mediated by conformational changes and we found colocalization of CD99 with caveolin-1 and Src, it is possible that the complex CD99-caveolin-1-Src acts as an allosteric inhibitor of c-Src.

Caveolin-1 indeed possesses all the qualities of a classic scaffolding protein and may provide a selective framework in that binding each caveolin-interacting protein to the same cytosolic membrane region of caveolin-1 facilitates their cross-talk. Leading to subsequent inhibition of c-Src activation, CD99 expression may thus represent a mechanism by which fine-tuning of c-Src activity is achieved. Of note, c-Src inhibition enhances osteoblast differentiation (Marzia *et al.*, 2000). Expression of CD99 in more mature osteoblasts and the reversal action of the molecule against malignancy indirectly support the view of the molecule as a new mediator of bone pathophysiology.

In conclusion, our data indicate for the first time that CD99 should be down-regulated in osteosarcoma to express full malignancy. Indeed, CD99 forced expression suppressed osteosarcoma cell growth in anchorage independence, resistance to anoikis, migration, and metastasization through a mechanism requiring membrane overexpression of caveolin-1, which forms stable complexes with CD99. It is very likely that CD99 acts by enhancing caveolin-1, and recruiting c-Src to caveolae contributes to decrease c-Src kinase activity. We think that these results may open new avenue for the understanding of the mechanisms regulating osteosarcoma pathogenesis and progression.

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