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Overexpression of DAN Causes a Growth Suppression in p53-Deficient SAOS-2 Cells

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It has been shown that the expression of *DAN* as well as *Drm/Gremlin,* a member of *DAN/Cerberus* family, is significantly down-regulated in rodent fibroblasts transformed with various oncogenes and overexpression of DAN results in the phenotypic reversion of the transformed phenotypes. In the present study, we examined the expression levels of *DAN, BMP-2, BMP-4,* and *BMPRs* (BMP receptors) in five human cell lines derived from bone and soft tissue tumors. Northern blot analysis revealed that *DAN* mRNA was detected in OS-KH and RMS-NK cells, but was not detectable in SAOS-2, NOS-1, and ASPS-KY cells. Transient overexpression of DAN in SAOS-2 cells, which lack functional p53 and pRB, resulted in a remarkable growth suppression without the induction of $p21^{Waf1}$. Interestingly, overexpression of DAN was associated with a reduction of alkaline phosphatase activity in SAOS-2 cells. Stable transfection of *DAN* in SAOS-2 cells caused a significant reduction of numbers of drug-resistant colonies, whereas the truncated form of DAN which lacked a possible signal peptide, completely lost this capability. Our results suggest that the secreted form of DAN exerts its growth-suppressive function in SAOS-2 cells in a p53-independent manner. © 2000 Academic Press

Key Words: **BMP; DAN; growth suppression; p53; SAOS-2.**

DAN was originally identified in normal rat fibroblast 3Y1 cells (1). The expression of *DAN* was significantly reduced in various transformed 3Y1 cells and the ectopic overexpression of *DAN* in v-*src*-transformed 3Y1 cells (SR-3Y1) resulted in a remarkable suppression of malignant phenotypes (2). Additionally, the forced expression of *DAN* in 3Y1 cells caused a retardation of the entry into the S phase of the cell cycle (3). Human *DAN* was mapped at chromosome 1p36.11–p36.13 which resides within the candidate tumor suppressor locus for neuroblastoma (4). Although some of primary neuroblastomas with N-*myc* gene amplification showed the structural aberrations within or close to *DAN* gene locus and DAN was able to enhance the retinoic acid-induced differentiation of neuroblastoma-derived cell lines, it is still unclear whether *DAN* is involved in the genesis and/or the progression of neuroblastoma (4, 5).

DAN encodes a 27-kDa secreted glycoprotein with a characteristic cysteine-knot structure common to a DAN/Cerberus family which includes Gremlin/Drm (6–8) and the DNA synthesis of SR-3Y1 cells was partially inhibited by the conditioned medium from *DAN*-overexpressing 3Y1 cells (6). Like *DAN*, the *Drm* expression was suppressed in normal rat fibroblast REF-1 cells transformed with various oncogenes and the high level of *Drm* expression induced apoptotic cell death in recipient cells (9). Human *Drm* maps to 15q13–q15, which is a candidate locus for metastatic breast cancer and other metastatic carcinomas (10). Recently, Hsu *et al.* demonstrated that, like noggin and chordin, DAN/Cerberus family members can interact directly with BMP-2 (bone morphogenetic protein) and block BMP signaling in early frog embryos (7). Similar results were also obtained in mouse embryonal carcinoma cells (11).

In malignant bone tumor, overexpression of BMP was associated with the poor prognosis and the local concentration of BMP was highest in giant cell tumor tissues compared with those in chondrosarcoma, osteosarcoma and benign bone (12–14). In addition, the expression level of type II receptor for BMP was correlated with metastasis in osteosarcoma (15). In the present study, we examined the expression levels of *DAN, BMP-2, BMP-4* and *BMPR*s in five human cell lines derived from bone and soft tissue tumors. We

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found that *DAN* mRNA was detected in OS-KH and RMS-NK cells, but was not detectable in SAOS-2, NOS-1 and ASPS-KY cells. Forced expression of *DAN* in p53-deficient SAOS-2 cells resulted in a remarkable growth suppression associated with a down-regulation of alkaline phosphatase activity.

MATERIALS AND METHODS

Cell lines. SAOS-2 and S138V21 were kindly supplied by Dr. R. Takahashi (Kyoto University) and Dr. N. Tsuchida (Tokyo Medical and Dental university), respectively. OS-KH, ASPS-KY and RMS-NK cells were gifts from Dr. K. Kushida (Kanagawa Cancer Center). NOS-1 cells were purchased from RIKEN Cell Bank (Tsukuba, Japan). SAOS-2 and S138V21 cells were maintained in Dulbecco's modified Eagle medium (DMEM, Nissui) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics. OS-KH, ASPS-KY, RMS-NK and NOS-1 cells were grown in RPMI 1640 (Nissui) containing 10% FCS. They were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Northern blot analysis. Total RNA was extracted from each cell line by using Trizol reagent (Life Technologies, Inc.) and chloroform extraction according to the vendor's RNA isolation protocol. Total RNA (10 μg) was fractionated by electrophoresis on 1% agarose gel containing formaldehyde. After staining with ethidium bromide to confirm the presence of equal amounts of 18S and 28S rRNA in each lane, RNA was transferred onto Nylon membranes and hybridized under the standard condition (50% formamide, 6× SSC, 5× Denhardt's solution, 0.1% SDS and 200 μg/ml of heat-denatured salmon sperm DNA) with [α^{32}P]dCTP-labeled cDNA probe at 42°C. The blot was washed twice for 30 min each at room temperature in 2× SSC containing 0.1% SDS, twice for 30 min each at 50°C in 0.1× SSC containing 0.1% SDS and exposed to an X-ray film with an intensifying screen at −70°C (16).

RT-PCR analysis. Five μg of total RNA served as template for single strand cDNA synthesis in a reaction using SuperScript reverse transcriptase (Life Technologies, Inc.) under the condition indicated by manufacturer. The sequences for the following PCR primers used were: BMPR-IA sense, 5′-GCAATTGCTCATCGAGACC-3′ and BMPR-IA antisense, 5′-CGAAGGTGTAGATGTCAGCC-3′; BMPR-IB sense, 5′-TCTTCACCACAGAGGAAGCC-3′ and BMPR-IB antisense, 5′-AAGCCACTGACAGAAGAGTAGG-3′; BMPR-II sense, 5′-AATGCAGCCATAAGCGAGG-3′ and BMPR-II antisense, TGGTACTCTGGTACGGATTCC-3′. cDNA samples were amplified for 30 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s followed by a final extension at 72°C for 10 min. PCR products were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide.

Construct. For the mammalian expression of myc-tagged DAN, the full-coding region of rat *DAN* with myc-tag at the C-terminus was prepared by using polymerase chain reaction (PCR)-based strategy and the obtained construct was verified by DNA sequencing.

β-Galactosidase assay. SAOS-2 cells (at a density of 5×10^5 cells/dish) were transfected with either an empty vector (pcDNA3-myc, Invitrogen) or an expression vector encoding myc-tagged DAN (pcDNA3-DAN-myc) together with the reporter plasmid (pCH110), which encodes β-galactosidase by using the standard calcium phosphate-DNA precipitation method (17). Twelve, 24 or 48 h after transfection, cells were washed twice with 1× PBS (10 mM sodium phosphate, pH 7.2, 150 mM NaCl) and fixed with 0.25% glutaraldehyde for 10 min at 4°C. Cells were then washed extensively with 1× PBS and soaked in the solution containing 5 mM $K_4[Fe(CN)_6]3H_2O$, 5 mM $K_3Fe(CN)_6$, 200 μM $MgCl_2$, 10 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for 12 h at 37°C. Cells were washed four times with 1× PBS, fixed with 3.7% formaldehyde for 30 min at room temperature and numbers of β-galactosidase-positive cells were counted.

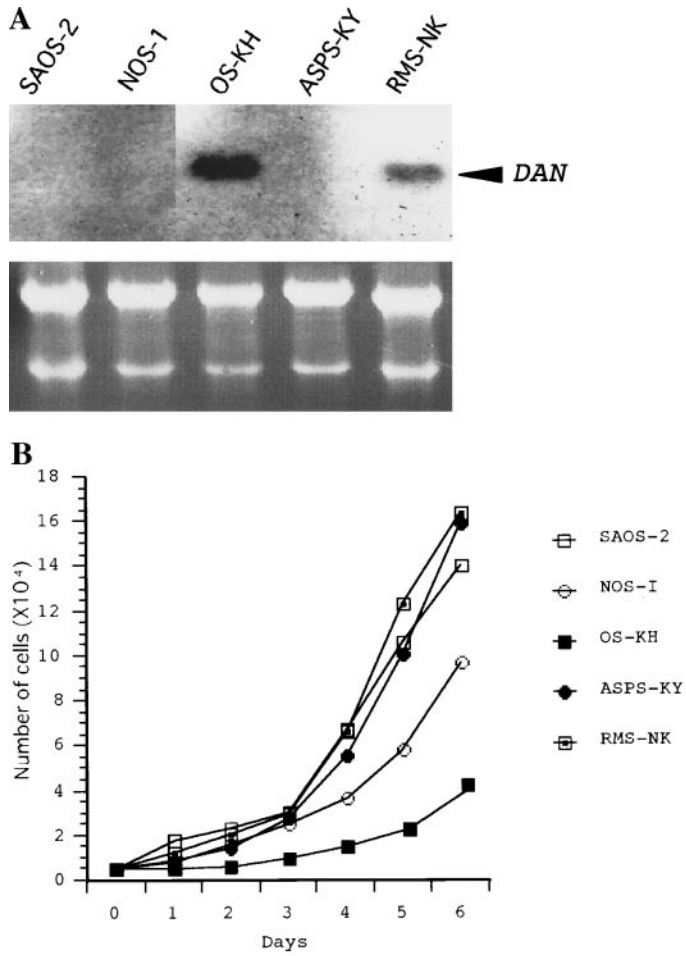

FIG. 1. Northern blot analysis of *DAN* mRNA in bone and soft tissue tumor cell lines and their growth curves. (A) Total RNA (10 μg) obtained from each cell line was hybridized with the radiolabeled human *DAN* cDNA. Ethidium bromide staining of 18S and 28S rRNAs in the agarose gel before blotting is shown in the lower panel. (B) The cells were seeded at 1×10^4 cells/35-mm dish and counted daily.

Transient transfection. After trypsinization, COS cells were plated on polylysine-coated glass coverslips in a 35-mm plastic dish at 50% confluency. One day after passage, cells were transfected with 2 μg of pcDNA3, pcDNA3-*DAN*-myc or pcDNA3-*p53* using Lipofectamine (Gibco-BRL). The cultures were maintained at 37°C for 48 h. Cells were then washed with 1× PBS twice, fixed with 3.7% formaldehyde solution, permeabilized with 0.2% Triton X-100 in PBS for 5 min, blocked by 3% bovine serum albumin in PBS and incubated with anti-myc monoclonal antibody (Invitrogen) or anti-p53 monoclonal antibody (CALBIOCHEM) at room temperature for 1 h. After washing, they were incubated with rhodamine-conjugated secondary antibody at room temperature for 1 h (Life Technologies, Inc.). They were examined by a confocal laser scanning microscope (Olympus).

Western analysis. Forty-eight hours after transfection, cells were washed twice with 1× PBS and lysed by 1× SDS sample buffer (18). Whole cell lysate or conditioned medium was subjected to 12.5% SDS–PAGE and proteins were transferred to nitrocellulose mem-

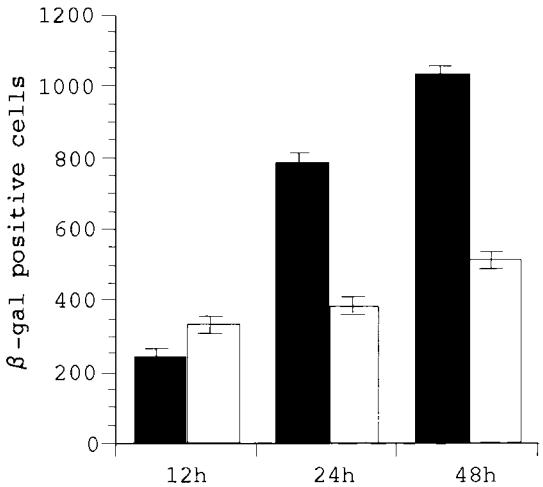

FIG. 2. Growth-suppressive function of DAN in SAOS-2 cells. SAOS-2 cells were transfected either with an empty vector (pcDNA3-myc) or with an expression vector encoding myc-tagged DAN together with the reporter plasmid pCH110 which carries *E. coli β-galactosidase* gene. At the times indicated after the transfection, cells were fixed with 0.25% glutaraldehyde and detected by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The numbers of stained cells were counted. Solid and open bars indicate the number of β-galactosidase-positive cells transfected with pcDNA3-myc and myc-DAN expression vector, respectively.

brane. The membrane was blocked at room temperature for 1 h with TBS-T buffer (150 mM NaCl, 50 mM Tris–Cl, pH 7.4 and 0.05% Tween 20) containing 5% dry milk. The blot was then incubated at room temperature for 1 h with anti-myc monoclonal antibody, followed by incubation with secondary antibody (horseradish peroxidase conjugated, Jackson ImmunoResearch Laboratories). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Colony formation assays. SAOS-2 cells (5×10^5 cells) were transfected with 20 μg of an empty vector, pcDNA-*p53*, pcDNA3-*DAN*-myc or pcDNA3-*DAN*(17–178)-myc by using the Lipofectamine. Forty-eight hours after transfection, cells were washed twice with 1× PBS, transferred to the medium containing G418 (Sigma, final concentration: 400 μg/ml) and maintained for 2 weeks. Then the numbers of G418-resistant colonies were scored after staining with Giemsa's solution.

Alkaline phosphatase activity. Forty-eight hours after transfection, cells were washed twice with 1× PBS and lysed with 200 mM sodium carbonate, 100 mM glycine, 1 mM $MgCl_2$ and 0.1% Triton X-100, followed by brief sonication. Cell lysates were centrifuged at 9000*g* (4°C for 10 min) and protein concentration was determined using the Bradford protein assay (Bio-Rad). Alkaline phosphatase (AP) activity was measured according to the manufacturer's instructions using *p*-nitrophenylphosphate as substrate (Wako).

RESULTS

Expression level of DAN in cell lines derived from human bone and soft tissue tumor. Northern blot analysis of *DAN* mRNA level in SAOS-2, NOS-1, OS-KH, ASPS-KY, and RMS-NK cells revealed that a single *DAN* mRNA of approximately 2 kb was detected in OS-KH and RMS-NK cells, whereas it was not visible in the remaining three cell lines (SAOS-2, NOS-1, and ASPS-KY) (Fig. 1A). It is interesting to note that OS-KH cells which showed the highest amount of *DAN* mRNA grew much slower than the other cells (Fig. 1B).

Transient overexpression of DAN in SAOS-2 cells. To determine the potential function(s) of DAN, we constructed an expression vector encoding full-length rat

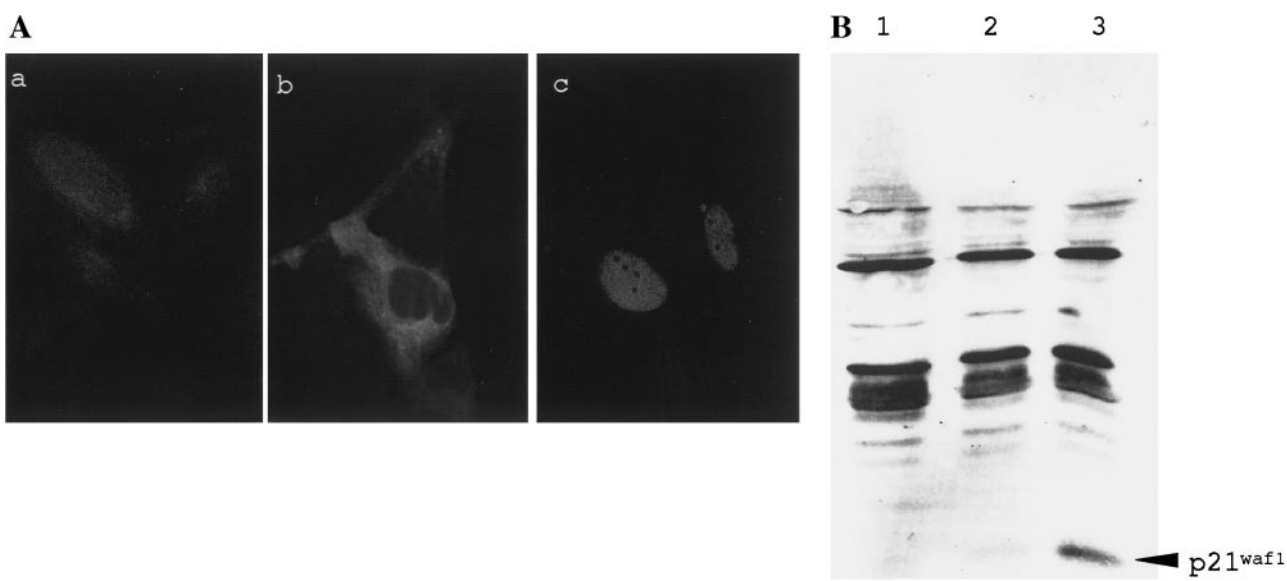

FIG. 3. Transient expression of myc-DAN or wild-type p53 in SAOS-2 cells. (A) SAOS-2 cells were transfected transiently with an empty vector (a), pcDNA3-*DAN*-myc (b), or pcDNA3-*p53* (c). Forty-eight hours after transfection, cells were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and stained with anti-myc monoclonal antibody (a and b) or anti-p53 monoclonal antibody. (B) Forty-eight hours after transfection, total cell extract was prepared from SAOS-2 cells transfected with an empty vector (lane 1), pcDNA3-*DAN*-myc (lane 2), or pcDNA3-*p53* (lane 3) and resolved by 10% SDS–PAGE. The filter was immunoblotted by anti-$p21^{Waf1}$ antibody.

DAN with myc-tag at its C-terminus (pcDNA3-*DAN*-myc) and SAOS-2 cells were transfected with pcDNA3-*DAN*-myc together with the reporter plasmid (pCH110) to identify transfected cells. Proliferation of β-galactosidase-positive cells was measured 12–48 h posttransfection. As shown in Fig. 2, transient overexpression of DAN resulted in the apparent growth-suppression in SAOS-2 cells.

Growth-suppressive activity of DAN in SAOS-2 cells. To corroborate the results obtained from the transient assays, SAOS-2 cells were transfected with an empty vector, pcDNA3-*p53* or pcDNA3-*DAN*-myc. The expression of transgenes was confirmed by immunostaining using anti-myc or anti-p53 antibody. As shown in Fig. 3A, myc-tagged DAN and p53 were expressed in cytoplasm and nucleus, respectively. Additionally, overexpression of p53 induced the $p21^{Waf1}$ production, whereas the expression of $p21^{Waf1}$ was not induced by exogenously expressed DAN (Fig. 3B). Many G418-resistant colonies were formed following transfection with the naked vector, whereas very few stable transformants were visible in plates of cells transfected with pcDNA3-*p53* or pcDNA3-myc-*DAN* (Figs. 4A and 4B).

As described previously, the N-terminal region of DAN consisted of the hydrophobic residues and the majority of DAN was secreted into the culture medium (6). We have purified DAN from conditioned medium of *DAN*-transfected 3Y1 cells and determined its N-terminal amino acid sequence. Consistent with the results reported by Stanley *et al.* (8), DAN was cleaved between Ala16 and Ala17 during secretion (data not shown). The myc-tagged DAN mutant [DAN(17–178)-myc], which lacked the N-terminal hydrophobic residues, was not secreted into the culture medium (Fig. 5A) and was unable to reduce the number of drug-resistant transformants (Figs. 5B and 5C).

p53-independent growth suppression. As described above, ectopic overproduction of DAN induced the significant growth suppression in p53-deficient SAOS-2 cells. These results suggest that DAN acts in a p53-independent manner. To test this notion using a different strategy, we used S138V21 cells which express $p53val^{138}$ and G418 resistant gene (19, 20). $p53val^{138}$ exhibited temperature-sensitive behaviors. $p53val^{138}$ acts as the mutant p53 and as the wild-type p53 at 37.5°C and at 32.5°C, respectively. S138V21 cells were transfected with an empty vector, pcDNA3-*p53* or pcDNA3-*DAN*-myc together with the selection vector (pTK-Hyg), which carried the hygromycin resistant gene. Transfected cells were grown in the presence of G418 and hygromycin B for two weeks at 37.5°C and the number of drug-resistant colonies was scored. As shown in Fig. 6, no stable colonies were recovered from cells transfected with pcDNA3-*p53* or pcDNA3-*DAN*-myc, indicating that DAN can inhibit cell growth in the absence of wild-type p53.

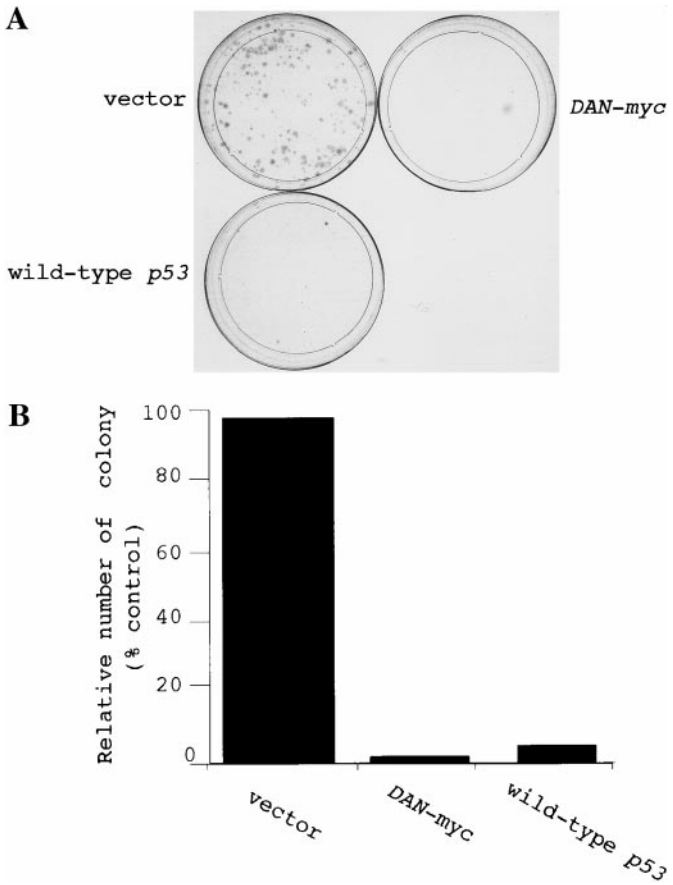

FIG. 4. Colony formation assays. (A) SAOS-2 cells were transfected with either an empty vector, pcDNA3-*DAN*-myc or pcDNA3-*p53*. Stably transfected cells were selected in the presence of G418 (400 μg/ml) for two weeks and numbers of drug-resistant colonies were scored after staining with Giemsa's solution. Representative plates are shown. (B) Summarized data. Efficiency of colony formation is represented by colony numbers counted after 2 weeks of G418 selection.

Effect of DAN on alkaline phosphatase activity. To examine a possible involvement(s) of DAN on BMP signaling, the expression levels of *BMP-2*, *BMP-4* and BMP receptors (*BMPR*s) were analyzed. Northern hybridization revealed that abundant expression of *BMP-2* and *BMP-4* was detected in RMS-NK and SAOS-2 cells, respectively (Fig. 7A). NOS-1 cells expressed a moderate level of *BMP-4*. The expression of *BMPR*s was examined by RT-PCR. As shown in Fig. 7B, SAOS-2, OS-KH and RMS-NK cells expressed three types of *BMPR*s, whereas NOS-1 and ASPS-KY cells lacked the expression of BMP receptor type IB. A substantial level of alkaline phosphatase (AP) activity was observed in SAOS-2 and NOS-1 cells, which expressed *BMP-4*, whereas AP activity was undetectable in RMS-NK cells, which expressed a large amount of *BMP-2* mRNA (data not shown). Transient overproduction of myc-tagged DAN in SAOS-2 cells significantly reduced the AP activity (about 30% inhibition),

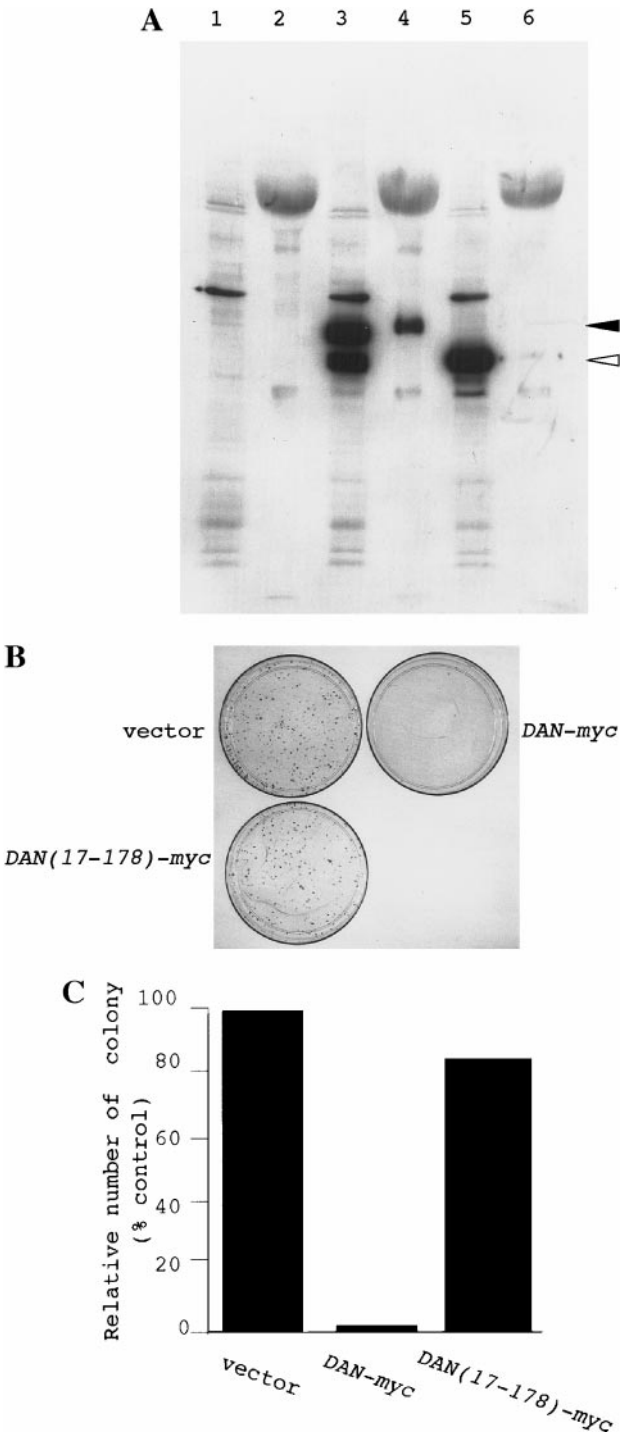

FIG. 5. Secreted form of DAN is capable of suppressing cell growth. (A) COS cells were transfected transiently with an empty vector (lanes 1 and 2), pcDNA3-*DAN*-myc (lanes 3 and 4), or pcDNA3-*DAN*(17–178)-myc (lanes 5 and 6). Forty-eight hours after transfection, total cell extract (lanes 1, 3, and 5) and conditioned medium (lanes 2, 4, and 6) were prepared from each transfection, fractionated by 12.5% SDS–PAGE, transferred onto nitrocellulose membrane, and Western blotted with anti-myc monoclonal antibody. Solid and open arrowheads indicate the position of secreted form of myc-tagged DAN and truncated form of DAN, respectively. (B) SAOS-2 cells were transfected with an empty vector, pcDNA3-*DAN*-myc, or pcDNA3-*DAN*(17–178)-myc. Transfected cells were grown in the presence of G418 for 2 weeks and numbers of drug-resistant colonies remaining on plates were measured after staining with Giemsa's solution. Representative plates are shown. (C) Summarized data.

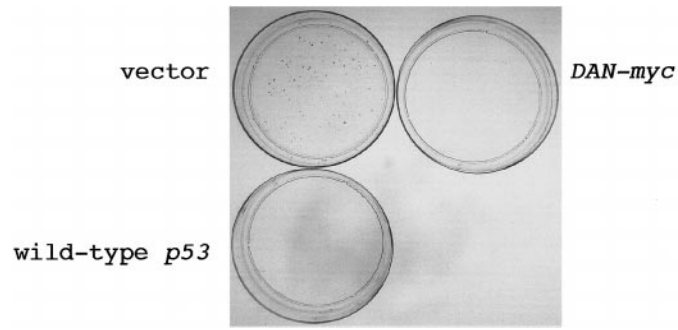

FIG. 6. p53-independent growth suppression. S138V21 (SAOS-2/p53val^{138}) cells were transfected with an empty vector, pcDNA3-*p53*, or pcDNA3-*myc-DAN* together with pTK-Hyg. Transfected cells were grown in the presence of G418 (400 μg/ml) and hygromycin B (300 μg/ml) at 37.5°C. Two weeks after transfection, numbers of drug-resistant colonies were counted after staining with Giemsa's solution. Photographs of representative plates are shown.

whereas cells transfected with the empty vector showed the similar level of AP activity to that in the control cultures (Fig. 8).

DISCUSSION

As described previously, the expression level of *DAN* is significantly reduced in various transformed cells derived from rodent fibroblasts (1, 21) and overproduction of DAN suppresses malignant phenotypes of v-*src*-transformed 3Y1 cells (2). We have shown that the retinoic acid-induced differentiation of human neuroblastoma was enhanced by the overexpression of DAN (5). Recently, Pearce *et al.* reported that DAN family members (DAN, Drm and Cerberus) repressed BMP-4 signaling in mouse embryonal carcinoma P19 cells (11). In the present work, we have examined the expression patterns of *DAN*, *BMP*, and *BMPR*s in human cell lines derived from bone and soft tissue tumors and also investigated a biological significance(s) of DAN in p53-deficient osteosarcoma cell line (SAOS-2).

Northern hybridization showed that OS-KH cells expressed a large amount of *DAN* mRNA and the growth rate of OS-KH cells was lower than those of the other cell lines that we examined. Transient overexpression and stable colony formation assays indicated that DAN can act as a potent growth suppressor in p53 deficient SAOS-2 cells. Unlike p53, DAN-induced growth suppression was not associated with the up-regulation of p21^{Waf1} expression. In addition, DAN was able to inhibit cell growth of S138V21 cells carrying the temperature sensitive *p53* mutation. As described previously,

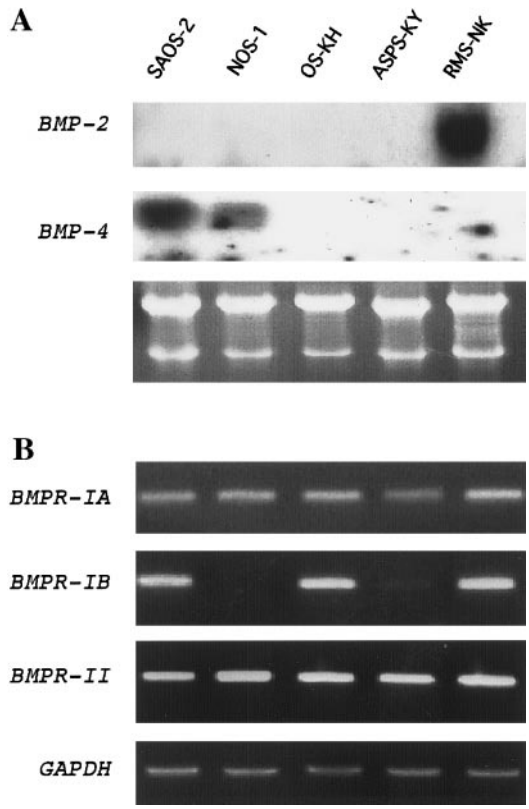

FIG. 7. Expression of *BMP-2, BMP-4,* and BMP receptors (*BMPR*s) in bone and soft tissue tumor cell lines. (A) Levels of *BMP-2* and *BMP-4* mRNAs were evaluated by Northern blot analysis. Total RNA (10 μg) derived from each cell line was hybridized with ^{32}P-labeled human *BMP-2* or *BMP-4* cDNA. Ethidium bromide staining of 18S and 28S rRNAs in the agarose gel before blotting is shown in the lower panel. (B) Detection by RT-PCR of *BMPR-IA, BMPR-IB,* and *BMPR-II* transcripts. Total RNA (5 μg) from each cell line was reverse-transcribed and amplified as described under Materials and Methods. RT-PCR of *GAPDH* is also shown as a positive control.

overexpression of DAN in normal rat fibroblast 3Y1 caused a cell cycle retardation (3). Ushijima *et al.* found that 3Y1 cell lines contain p53 point mutation in one of the mutational hot spots (22). These observations strongly suggest that DAN-mediated growth suppression occurs in a p53-independent manner. p53-induced growth suppression of SAOS-2 cells is due largely to apoptosis (23). Our preliminary results showed that the transient overexpression of DAN increased the number of SAOS-2 cells with DNA content less than 2N, however, we failed to detect the DNA fragmentation in *DAN*-transfected cells (data not shown). It remains unclear whether DAN-mediated growth suppression results from apoptosis or not.

Truncated form of DAN which lacked the N-terminal signal peptide and hence was not secreted, lost the growth-suppressive capability, suggesting that DAN exerts its growth regulatory function extracellularly. Recently, DAN has been shown to be a member of the cysteine knot superfamily (8, 11), which includes TGF-β superfamily and a number of growth factors (24, 25). Among DAN family, the homology was restricted to the cysteine-rich region (cysteine knot) (8). Cysteine residues within this region might be involved in intramolecular or intermolecular disulfide bonds. Stanley *et al.* found that DAN was secreted as a homodimer (8). It should be clarified whether the homodimer formation of DAN is mediated by the cysteine knot region and is responsible for the growth-suppressive function of DAN.

Like noggin and chordin which bind and inhibit the activities of BMP-2 and BMP-4 (26, 27), DAN has been shown to limit the actions of BMP-2 and BMP-4 in early frog embryos and mouse embryonal carcinoma cells, respectively (7, 11). In osteosarcoma, high level of BMP expression is associated with poor prognosis (12, 13). Additionally, BMP-2 and BMP-4 can stimulate the growth of osteosarcoma cells (28, 29). BMPs have been shown to stimulate the alkaline phosphatase activity (30) and SAOS-2 cells exhibited the substantial level of alkaline phosphatase activity, indicating that BMP-4 signaling might be involved in the growth of SAOS-2 cells which express both BMP-4 and BMPRs. Transient overexpression of DAN suppressed the growth of SAOS-2 cells and reduced the alkaline phosphatase activity. Our observations suggest that DAN is associated with BMP-4 and inhibits its activity to suppress the growth of SAOS-2 cells. Noggin and chordin interact directly with BMP-2 and BMP-4 and block their activities to prevent receptor binding (26, 27), however, the precise molecular mechanism how DAN inhibits the activities of BMP-2 and BMP-4 remains unknown.

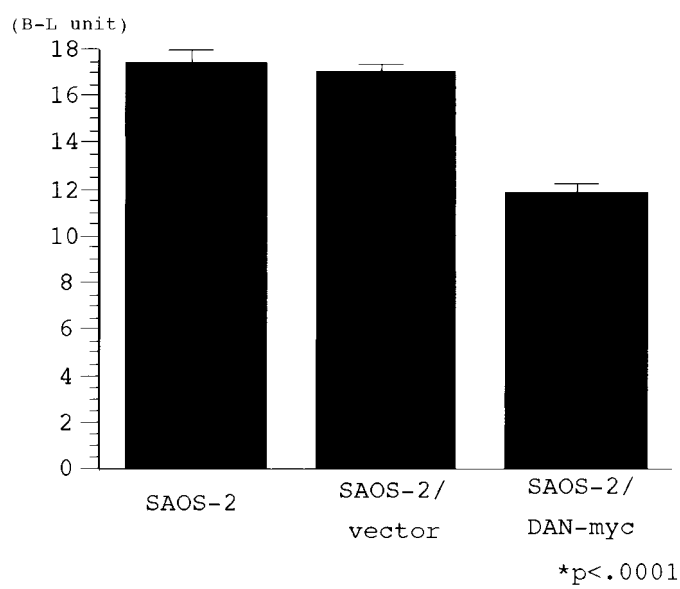

FIG. 8. Effect of overexpression of DAN on alkaline phosphatase activity in SAOS-2 cells. Forty-eight hours after transfection, alkaline phosphatase (AP) activity was measured. *$P < 0.0001$ versus cells transfected with an empty vector.

Recently, Topol *et al.* have reported that a significant amount of secreted Drm was associated with cell surface and the cell-associated form of Drm was able to interact with BMP-4 (31).

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