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ORIGINAL ARTICLE

Transfection of NF-κB decoy oligodeoxynucleotide suppresses pulmonary metastasis by murine osteosarcoma

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Nuclear factor-kappa B (NF-κB) has a pivotal role in the progression and distant metastasis of cancers, including malignant bone tumors. To inhibit NF-κB activation, a new molecular therapy using synthetic double-stranded oligodeoxynucleotide (ODN) as a 'decoy' *cis* element against NF-κB has been developed. To determine whether pulmonary metastasis of osteosarcoma is reduced by inhibiting the action of NF-κB, NF-κB decoy ODN was transfected into the nuclei of murine osteosarcoma cells with high pulmonary metastatic potential, the LM8 cell line, using a three-dimensional alginate spheroid culture model. An *in vitro* study demonstrated the successful transfection of LM8 cells cultured in alginate beads by 'naked' NF-κB decoy ODN and that the activation of NF-κB signaling was significantly suppressed. Tumor growth was not affected by transfection of NF-κB decoy ODN, however, the expression of vascular endothelial growth factor (VEGF) and intercellular adhesion molecule 1 (ICAM-1) mRNA was markedly decreased. Furthermore, the transfection of 'naked' NF-κB decoy ODN effectively suppressed pulmonary metastasis in an *in vivo* alginate bead transplantation model. Our results suggest that NF-κB has a central and specific role in the regulation of tumor metastasis and could be a molecular target for development of anti-metastatic treatments for osteosarcoma.
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Introduction

Osteosarcoma is the most common primary malignant tumor of bone in children. Despite multidisciplinary treatments for this tumor, a significant proportion of patients developed pulmonary metastasis and eventually succumbed to the disease. Therefore, there is an urgent need to develop new approaches to suppress the progression to pulmonary metastasis.[1]

The transcription factor nuclear factor-kappa B (NF-κB) is a heterodimeric DNA-binding protein that consists of two major polypeptides, p50 and p65.[2] In resting cells, NF-κB is sequestered in the cytoplasm by IκB proteins. Stimulus-mediated phosphorylation and subsequent proteolytic degradation of IκB allows the release and nuclear translocation of NF-κB, where it transactivates several target genes, such as vascular endothelial growth factor (VEGF), inter-cellular adhesion molecule-1 (ICAM-1), interleukin-1 (IL-1) and matrix metalloproteinases (MMPs).[3] As these NF-κB-related gene expressions are considered to be involved in a series of sequential steps, including invasion, intravasation, survival in the circulation, adhesion and growth in distant organs, it is generally thought that NF-κB has an essential role in tumor progression and metastasis.[4] Increased and aberrant NF-κB signaling activity has been extensively documented in cancer cells, with implications for cellular proliferation, antiapoptosis, promotion of angiogenesis and metastatic tumor spread.[5–8] Moreover, blocking the NF-κB signaling pathway has been reported to inhibit bone metastasis of breast cancer,[9] and the angiogenesis, invasion and metastasis of prostate cancer[10] and melanoma.[11] Importantly, an increased activation of NF-κB has also recently been identified in a human osteosarcoma cell line and is thought to contribute to the maintenance of a highly proliferative malignant phenotype.[12–17]

To inhibit NF-κB activation, a new molecular therapy using synthetic double-stranded oligodeoxynucleotide (ODN) as a 'decoy' *cis* element against NF-κB has been developed.[18] When the NF-κB decoy ODN is transfected into cells, it binds competitively to activated NF-κB and prevents transactivation of the target genes. The NF-κB decoy ODN strategy has been applied to various diseases, such as re-stenosis after angioplasty or stenting, glomerulonephritis, rheumatoid arthritis and atopic dermatitis (see review[19]). In oncology, it has been reported that the intra-tumor injection of NF-κB decoy ODN inhibited

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the cachexia induced by adenocarcinoma[20] and that intravenous treatment with NF-κB decoy ODN inhibited the hepatic metastasis of M5076 reticulosarcoma in mice.[21] Therefore, we hypothesized that transfection with NF-B decoy ODN would suppress the tumor growth and pulmonary metastases of osteosarcoma. Using murine osteosarcoma cells from two metastatic clones, the parental Dunn cell line and its derivative LM8 with greater metastatic potential to the lung,[22] we have recently established a novel alginate-encapsulated tumor spheroid model[23] to mimic the *in vivo* microenvironment. The purpose of this study was to examine the effects of NF-B decoy ODN on tumor progression and metastasis-related gene expression *in vitro*, as well as pulmonary metastasis *in vivo*, using the LM8 cell line in the alginate-encapsulated tumor spheroid model.

Materials and methods

Three-dimensional alginate spheroid culture

The LM8 murine osteosarcoma cell line, which has a high pulmonary metastatic potential, was used for this study. The LM8 cell line was derived from the original Dunn cell line[24] by *in vivo* selection.[22] In this study, LM8 cells were seeded at a density of 2.0×10^6 cells in 175 cm^2 culture flasks. When >90% confluency was reached at day 2, the cells were digested using 0.05% trypsin and encapsulated in 1.2% low-viscosity alginate (Keltone LV; Kelco, Chicago, IL) in 0.15 M sodium chloride (NaCl) at a concentration of 4.0×10^6 cells per ml.[23] Encapsulation was achieved by gently expressing drops of the cell suspension through a 21 gauge needle from a 10 ml syringe into 102 mM calcium chloride; each drop was instantly transformed into a semisolid microspheric bead. After 10 min of incubation to allow further polymerization, the newly formed beads were washed three times with normal saline, followed by one wash with Dulbecco's modified Eagle medium (DMEM: Gibco BRL, Grand Island, NY) to remove excess calcium chloride. The beads were then cultured in complete medium: DMEM supplemented with 10% fetal bovine serum (FBS: Life Technologies, Gaithersburg, MD) and 50 μg ml^{-1} gentamicin (Gibco BRL). The cultures were maintained at 37 °C in a humidified atmosphere with 5% carbon dioxide.

Animals

Five-week-old C3H male mice were purchased from Japan Oriental Yeast (Tokyo, Japan). All mice were housed under specific pathogen-free conditions with a 12-h light and dark cycle. Housing, animal care and experimental protocols were approved by the Animal Care and Use Committee of Mie University.

Transfection of NF-κB decoy ODN

Phosphorothioate double-stranded ODN, for which sequences have been reported,[18] was used:

NF-κB decoy ODN: 5′-CCTTGAAGGGATTTCCCT CC-3′ and 3′-GGAACTTCCCTAAAG GGAGG-5′.

Scrambled decoy ODN (SCD): 5′-TTGCCGTACCTG ACTTAGCC-3′ and 3′-AACGGCATGGACTGAAT CGG-5′.

The SCD was used as an ODN control. NF-κB decoy ODN and SCD were provided by AnGes MG (Osaka, Japan). After 24 h of pre-culture in serum-free medium (SFM), naked NF-κB decoy ODN or SCD were transfected for 4 h into LM8 cells encapsulated in alginate beads as follows: 1. Control (SFM); 2. Scrambled decoy ODN at 100 nM (SCD); 3. NF-κB decoy ODN at 100 nM (Decoy 100 nM); and 4. NF-κB decoy ODN at 10 μM (Decoy 10 μM). The beads of all experimental groups were then cultured in DMEM with 10% FBS.

Transfection efficiency

FITC-labeled decoy ODN (1 μM) was used to determine transfection efficiency. FITC-labeled phosphorothioate ODN labeled on the 3′ and 5′ ends of NF-κB decoy ODN were provided by AnGes MG. The intensity of fluorescence was observed using confocal microscopy (FLUOVIEW FV1000, Olympus, Tokyo, Japan). Nuclei were counter-stained with propidium iodide (Invitrogen, Carlsbad, CA).

NF-κB (p65) ELISA

LM8 cells were serum-starved for 24 h and treated with SFM, SCD, decoy 100 nM and decoy 10 μM for 4 h. The original Dunn cell line was used as a control (in SFM. To extract LM8 and Dunn cells from the alginate beads, each treatment group of 100 beads was dissolved with dissolving buffer (0.15 M NaCl, 30 mM l^{-1} ethylenediaminetetraacetic acid (EDTA) and 55 mM sodium citrate; pH 6.8) at 4 °C for 10 min with gentle shaking. The cells were recovered by centrifugation at 100 *g* for 5 min.[25] The cells were then resuspended in hypotonic buffer (Active Motif, Carlsbad, CA), and the cytoplasmic fraction was isolated by centrifugation (14 000 *g*, 4 °C). The nuclear fraction was separated by resuspending the residual pellet in complete lysis buffer (Active Motif) followed by centrifugation (14 000 *g*, 4 °C). The binding of NF-κB p65 subunits to the NF-κB binding consensus sequence 5′-GGGACTTTCC-3′ of the nuclear fraction was measured using a TransAM NF-κB enzyme-linked immunosorbent assay (ELISA) kit (Active Motif) according to manufacturer's instructions.[26] The activated NF-κB (p65 subunits) in the nuclear fraction, which was bound to 96-well microtiter plates coated with an oligonucleotide containing the NF-κB binding consensus sequence, was detected by an anti-p65 subunit antibody followed by the addition of a horseradish peroxidase conjugated secondary antibody and measured spectrophotometrically (Multiskan JX; Thermo Fisher Scientific, Waltham, MA) at 450 nm wavelength with a reference wavelength of 650 nm.

Cell proliferation assay

The bromodeoxyuridine (BrdU) assay (Cell Proliferation ELISA, BrdU (colorimetric), Roche, Penzberg, Germany) was used to evaluate the effect of NF-κB decoy ODN on

cell proliferation of LM8 osteosarcoma cells *in vitro*. Cells cultured in alginate beads following 4 h of transfection were incubated in 12-well plates (CELLSTAR, Greiner Bio-One, Tokyo, Japan) at 100 beads per 2 ml DMEM supplemented with 10% FBS and 50 μg ml^{-1} gentamicin at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h. The alginate beads were then separated into 96-well microtiter plates (CELLSTAR, Greiner Bio-One) with three beads in 200 μl of medium per well ($n=10$). BrdU labeling solution (20 μl per well) was added. After 8 h, the supernatant was discarded and the beads were dissolved with 200 μl dissolving buffer per well at 4 °C for 20 min. The plate was centrifuged at 300 *g* for 10 min and the medium was removed, and the samples were stored at −20 °C until used. This procedure was repeated daily from day 2 to day 7 of culture. According to the manufacturer's instructions, metabolic activity was measured using an ELISA plate reader at 450 nm wavelength with a reference wavelength of 650 nm.

RNA extraction and cDNA synthesis for quantitative real-time PCR

Alginate beads containing LM8 cells were treated with SFM, SCD, decoy 100 nM and decoy 10 μM for 4 h and cultured with SFM for 24 h. To extract LM8 cells from the alginate beads (each group of 100 beads), the beads were dissolved with dissolving buffer at 4 °C for 10 min with gentle shaking,[27] and the LM8 cells were separated by mild centrifugation. The pellet (containing LM8 cells) was washed three times with cold PBS. RNA was isolated using RNAqueous-4PCR kit (Ambion, Austin, TX) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was performed by oligo (dT) 15 priming from 1 μg of total RNA using a cDNA synthesis kit (Roche, Mannheim, Germany) according to the manufacturer's protocols. The cDNA was subjected to real-time PCR quantitative-PCR using the ABI PRISM 7000 (Applied Biosystems, Foster City, CA). TaqMan PCR was performed in 25 μl volumes using the TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan probe/primers specific for glyceraldehydes-3-phosphate dehydrogenase (GAPDH; code number Ma99999915_g1), VEGF (code number Ma00437304_al) and ICAM-1 (code number Ma0051 6023_al) were purchased from TaqMan Assays-on-Demand Gene Expression Products (Applied Biosystems). All PCR assays were performed in triplicate. The reaction conditions were 50 °C for 2 min; 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing and elongation). The reaction mixture without cDNA was utilized as a control. Threshold cycle numbers (CT) were determined with an ABI PRISM 7000 Sequence Detection System (version 1.1 software). GAPDH was used as the housekeeping gene for an internal control. Each mRNA level was divided by the GAPDH level of each sample.[28] The levels were expressed as an x-fold induction compared with control group. All samples were analyzed in triplicate.

Cell detachment assay

One bead containing either the Dunn or LM8 cell line was cultured in 100 μl of DMEM with 10% FBS ($n=10$) in a 96-well plate (CELLSTAR, Greiner Bio-One) for 24 h, following which the bead was transferred to a new well. The number of cells that detached from the alginate bead and adhered to the bottom of the culture plate during the next 24-h culture period was determined. The adhered cells were fixed in 4% paraformaldehyde, stained with Hematoxylin and manually counted under a light microscope. This assay was repeated daily until day 7 of culture.

In vivo *study*

Five-week-old male C3H mice ($n=40$) were used to estimate the *in vivo* pulmonary metastatic potential.[23] Mice were divided into four experimental groups with 10 mice in each group. The mice were anesthetized with ketamine (80 mg kg^{-1}) and xylazine (7 mg kg^{-1}). In each experimental group, five alginate beads containing LM8 cells ($\sim 2.0 \times 10^5$ cells) pre-transfected with NF-κB decoy ODN (100 nM, 10 μM) or SCD were transplanted subcutaneously into the dorsal skin of mice through a 5 mm longitudinal skin incision. After transplantation of the beads, the skin was closed with No. 5-0 vicryl sutures. Weight and tumor volume, calculated as (minor axis)^2 × (major axis)/2 mm^3 were measured every other day. After killing the mice on day 35, the lungs were removed, fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm) were cut and stained with Hematoxylin-Eosin. On the maximal area of thin sections of each tissue, the number of metastatic tumor nodules was counted microscopically.

Statistical analysis

All data are expressed as mean ± s.d. The association among the variables was determined by one way ANOVA with Fisher's PLSD *post hoc* test using Stat-view software (Abacus Concepts, Berkeley, CA). A P value <0.05 was considered statistically significant.

Results

Distribution of FITC-labeled decoy ODN

We first examined the presence and distribution of 'naked' NF-κB decoy ODN in murine osteosarcoma. Fluorescein isothiocianate (FITC)-labeled decoy ODN was used to evaluate the transfection efficiency of 'naked' NF-κB decoy ODN in the three-dimensional alginate culture system. After a 4-h transfection of FITC-labeled decoy ODN, confocal analysis revealed that the FITC-fluorescence was distributed in a punctuate pattern throughout the nuclei of LM8 cells (Figure 1). FITC-fluorescence was seen in the nuclei of all cells within the alginate beads, including those of dividing cells (Figures 1d–f). FITC-fluorescence was equally distributed to all LM8 cells within the alginate beads.

NF-κB DNA-binding capacity

To evaluate the inhibitory effect of NF-κB decoy ODN on activation of NF-κB in the LM8 cell line, the

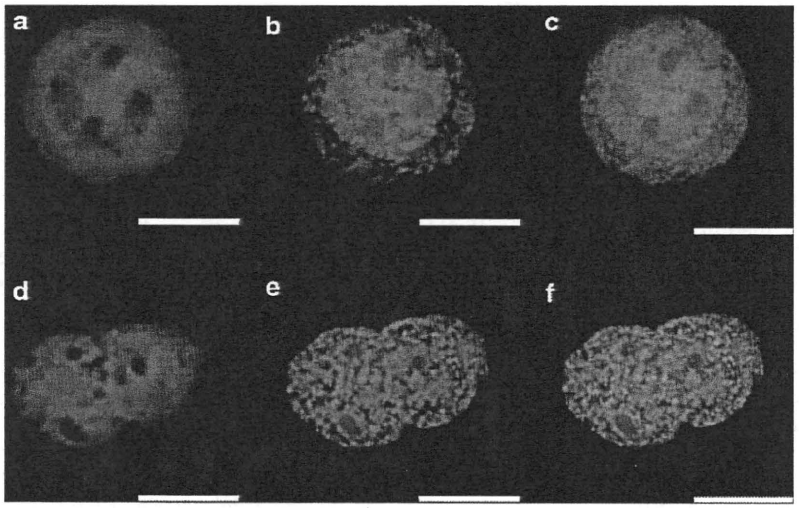

Figure 1 Distribution of FITC-labled NF-κB decoy oligodeoxynucleotide (ODN) by LM8 cells cultured in alginate beads. LM8 cells cultured in alginate beads were transfected with 'naked' fluorescein isothiocianate (FITC)-labeled NF-κB decoy ODN (green) for 4 h. Nuclei were stained with propidium iodide (red). The transfection efficiency was ~100%. FITC-fluorescence was also confirmed in the nuclei of dividing LM8 cells. (**a, d**): nuclear stain; (**b, e**): FITC-labeled decoy; (**c, f**): overlay. Scale bar: 10 μm. The color reproduction of this figure is available on the html full text version of the manuscript.

DNA-binding capacity of NF-κB was evaluated using a p65 enzyme-linked immunosorbent assay (ELISA).[26] The LM8 parent Dunn cell line[22] was also evaluated. The NF-κB (p65) signaling activity of untreated LM8 cells (control) was significantly higher ($P<0.01$), about threefold, than that of the Dunn cells (Figure 2). Treatment with NF-κB decoy ODN suppressed the NF-κB signaling activity of LM8 cells in a dose-dependent manner (percentage of control; SCD: $-14.0\pm4.9\%$, $P<0.05$; decoy (100 nM): $-27.7\pm10.3\%$, $P<0.01$; decoy (10 μM): $-74.5\pm3.1\%$, $P<0.01$). Importantly, compared with the parent Dunn cell line, the activation of NF-κB (p65) by the LM8 cell line was significantly ($P<0.05$) suppressed by transfection of 10 μM NF-κB decoy ODN (decoy (10 μM): $-74.5\pm3.1\%$; Dunn: $-61.7\pm2.8\%$; $P<0.05$; percentage of control).

Cell proliferation

The ELISA-based bromodeoxyuridine (BrdU) assay was used to evaluate the effect of NF-κB decoy ODN on the cell proliferation of LM8 osteosarcoma cells *in vitro*. The growth of LM8 osteosarcoma cells cultured in alginate beads was not affected by 2–7 days incubation with NF-κB decoy ODN or SCD (Figure 3). No significant differences were observed among the experimental groups throughout the experimental period.

mRNA expression of VEGF and ICAM-1

To examine the molecular mechanisms underlying the inhibitory action of NF-κB decoy ODN on metastasis by LM8 cells, VEGF and ICAM-1 mRNA was quantified by real-time PCR *in vitro*. At 24 h after inoculation of LM8 cells, the transfection of decoy (10 μM) induced a

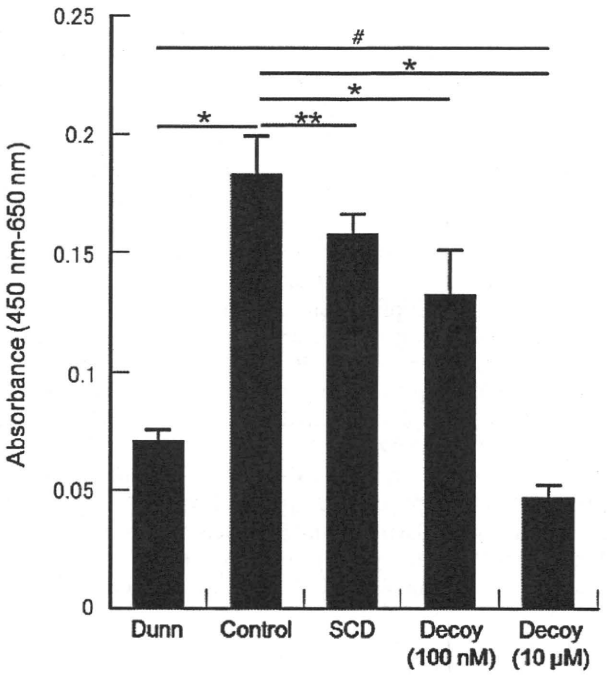

Figure 2 Effect of NF-κB decoy oligodeoxynucleotide (ODN) on the DNA binding capacity of NF-κB. The NF-κB DNA binding activity of each experimental group was evaluated using an NF-κB (p65) enzyme-linked immunosorbent assay (ELISA). (1) Dunn (Dunn cell line) (serum-free medium), (2) LM8 cells (serum-free medium), (3) Scrambled decoy ODN at 100 nM (SCD), (4) NF-κB decoy ODN at 100 nM (Decoy 100 nM) and (5) NF-κB decoy ODN at 10 μM (Decoy 10 μM). $^{**}P<0.05$ and $^{*}P<0.01$, compared with the untreated LM8 cells. $^{\#}P<0.01$, compared with the 10 μM group.

significant reduction of mRNA levels of *VEGF* genes compared with the other groups (Figure 4a) (percentage of control; SCD: $+12.7 \pm 2.1\%$; Decoy (100 nM): $+0.7 \pm 2.7\%$; Decoy (10 μM): $-31.2 \pm 3.1\%$, $P<0.01$). Similarly, the expression of the *ICAM-1* gene was markedly decreased in the 10 μM group compared with the other groups (Figure 4b) (percentage of control; SCD: $-0.3 \pm 9.1\%$; Decoy (100 nM): $-5.9 \pm 10.8\%$; Decoy (10 μM): $-34.3 \pm 2.6\%$, $P<0.01$).

Cell detachment assay

The parental Dunn cell line and its derivative LM8 encapsulated in an alginate bead culture system were able to grow in a three-dimensional structure with cells detaching from the alginate environment. The number of detached cells was greater in the LM8 cell line than in the Dunn cell line, suggesting that the cell kinetics in this culture system reflect the *in vivo* malignancy potential of the cells.[23] To evaluate the effect of the NF-κB decoy ODN on the metastatic potential *in vitro* using this culture system, the number of cells detaching from the alginate bead and adhering to the bottom of the culture plate was quantified. On day 1 of culture, detached cells were found only in the control group. The number of cells detaching from alginate beads increased with time in all experimental groups, however, the number of detached cells for the Decoy (10 μM) group was significantly ($P<0.05$) lower than that of the control group on day 7 (Figure 5a). Representative images on day 7 of culture show that clumps of LM8 cells were found in the control group (Figure 5b), whereas only solitary cells were identified in the decoy (10 μM) group (Figure 5c).

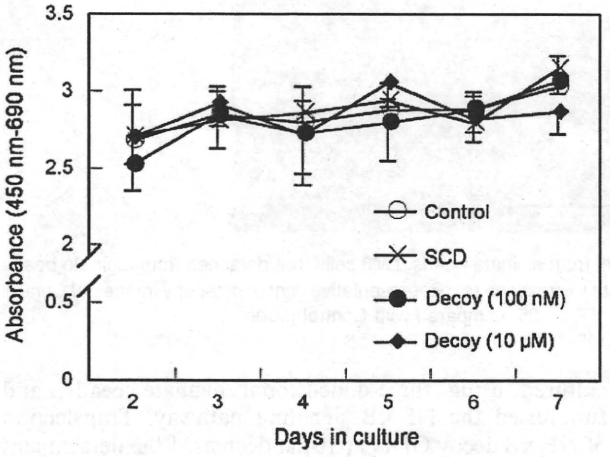

Figure 3 Effect of NF-κB decoy oligodeoxynucleotide on the cell proliferation of LM8 cells. The cell proliferation of LM8 cells cultured for 2–7days in alginate beads was analyzed by a bromodeoxyuridine (BrdU) assay. No significant differences were observed among the experimental groups.

In vivo *alginate transplantation study*

To examine the inhibitory effect of NF-κB decoy ODN on pulmonary metastasis *in vivo*, alginate beads containing LM8 cells pre-transfected with NF-κB decoy ODN were transplanted into the dorsal skin of mice. In a preliminary experiment,[23] beads containing murine OS cells, either the parental Dunn cell line or its derivative cell line LM8, were transplanted into the dorsal skin of mice to determine their metastatic potential *in vivo*. The rate of pulmonary metastasis was higher for LM8 cells than Dunn cells, suggesting that the metastatic potential of cells in alginate bead culture reflect the original *in vivo* potential.

In this study, tumor nodules were formed in all mice in both the control (10/10) and SCD (10/10) groups. However, subcutaneous tumor nodules were formed in 90% of mice by both the decoy 100 nM (9/10) and 10 μM (9/10) groups. Tumor nodules of all groups progressively increased in volume until the mice was killed on day 35 after transplantation. There were no significant

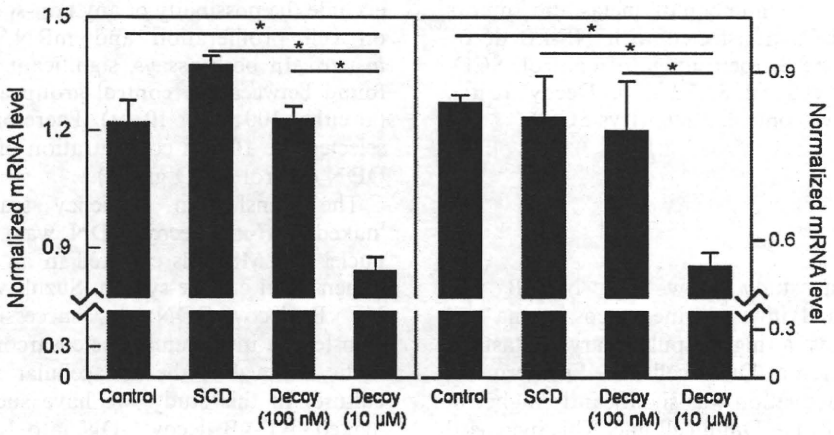

Figure 4 Effects of NF-κB decoy oligodeoxynucleotide on the mRNA expression of vascular endothelial growth factor (VEGF: **a**) and intercellular adhesion molecule-1 (ICAM-1: **b**). VEGF and ICAM-1 mRNA levels were quantified by real-time PCR and normalized by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level of each sample. *$P<0.01$, compared with the Decoy (10 μM) group. SCD, scrambled decoy ODN (100 nM).

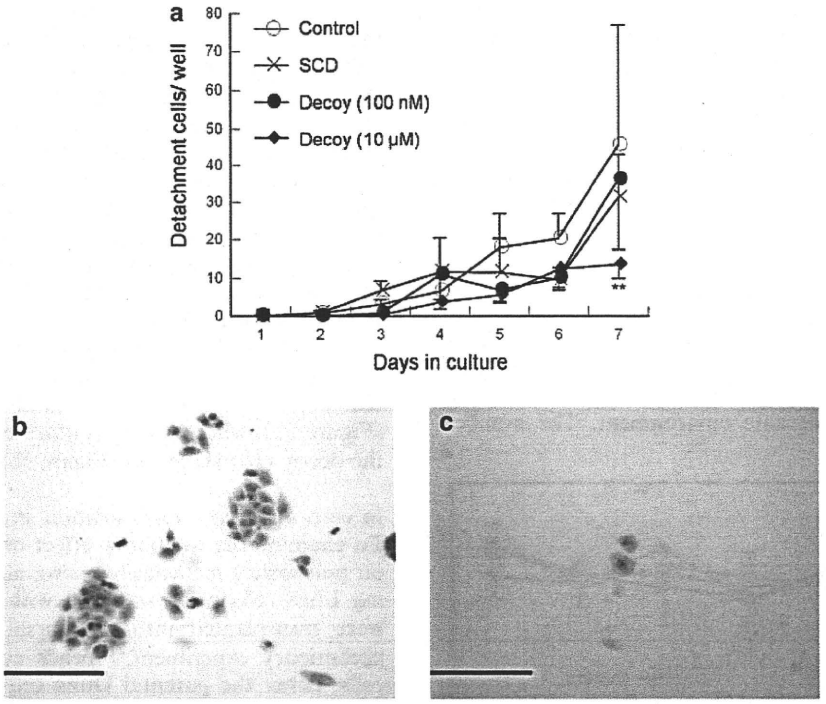

Figure 5 Effects of NF-κB decoy oligodeoxynucleotide on cell detachment from alginate beads. LM8 cells that detached from alginate beads during 24 h were stained with Hematoxylin and manually counted under a light microscope (**a**). Representative light microscopy images of Control (**b**) and Decoy 10 μM groups (**c**) on day 7 are shown. Scale bars: 200 μm. $^{**}P<0.05$, compared with Control group.

differences in tumor volume (Figure 6a) and body weight (Figure 6b) among all groups throughout the experimental period. Representative histological images of the lungs removed from each experimental group are shown in Figure 7. Microscopic analysis of the lungs found metastatic lesions in all the experimental groups. A large number of tumor nodules were found in the lungs of the control and SCD groups. Tumor nodules of the control and SCD groups were larger than those of the NF-κB decoy ODN at 100 nM and 10 μM groups. There was a marked reduction in the number of metastatic tumors produced by LM8 cells transfected with NF-κB decoy ODN at 10 μM (Figure 8) (percentage of control; SCD: 138.2 ± 35.7, Decoy 100 nM: 81.7 ± 19.6, Decoy 10 μM: 37.3 ± 15.8, $P<0.05$ vs control, $P<0.01$ vs SCD).

Discussion

The results of our study show that NF-κB was constitutively activated in a murine osteosarcoma cell line LM8, which has a higher pulmonary metastatic potential than its parental Dunn cell line. Furthermore, the level of NF-κB activation was significantly higher in the LM8 cell line than the Dunn cell line. This increased activation of NF-κB is considered to contribute to the maintenance of a highly proliferative malignant phenotype. In this study, naked NF-κB decoy ODN was successfully transfected into the nuclei of LM8 cells cultured using three-dimensional alginate beads, and suppressed the NF-κB signaling pathway. Transfection of NF-κB decoy ODN at 10 μM decreased the detachment of cells from the alginate beads and suppressed the expression of such metastasis-related genes as VEGF and ICAM-1. In the *in vivo* alginate transplantation study, the number of pulmonary metastases was markedly decreased by transfection of NF-κB decoy ODN.

In a preliminary study, we used two concentrations of scrambled decoy ODN (SCD; 100 nM and 10 μM) to exclude the possibility of any non-specific effects of ODN on cell proliferation and mRNA expression assays *in vitro*. In both assays, significant differences were not found between the control group and the SCD groups (at either 100 nM or 10 μM). Therefore, for this study, we selected the 100 nM concentration of SCD for use as the ODN control (SCD group).

The transfection efficiency study confirmed that 'naked' NF-κB decoy ODN was transfected into the nuclei of LM8 cells cultured in an alginate bead three-dimensional culture system. Suzuki *et al.*[29] reported that NF-κB decoy ODN was successfully and efficiently transfected into human osteosarcoma cells after enzymatic removal of the extracellular matrix in monolayer culture. In this study, we have successfully transfected 'naked' NF-κB decoy ODN into LM8 cells in a three-dimensional alginate environment. We speculated that NF-κB decoy ODN could be transfected into LM8 cells suspended in alginate environment before formation of an extracellular matrix and/or cell-to-cell adhesion, proving

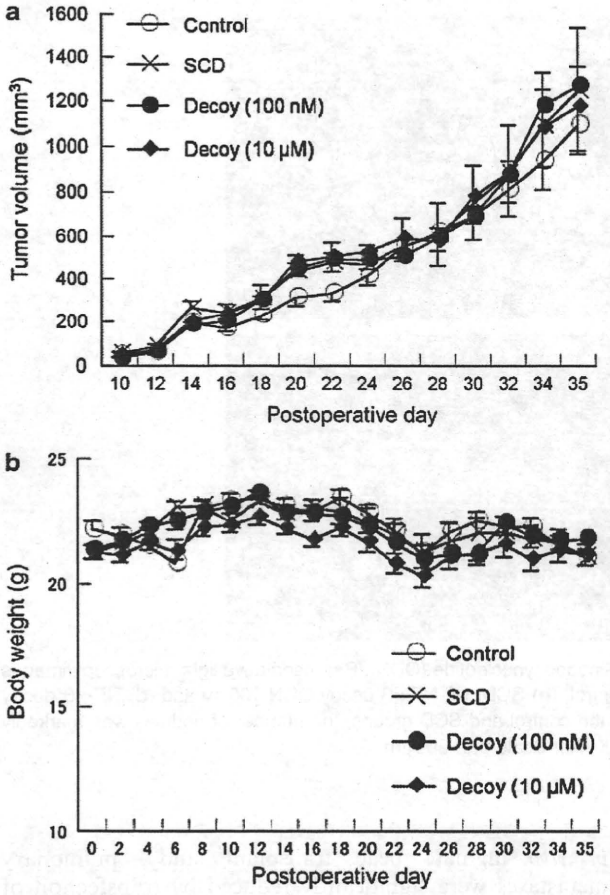

Figure 6 Effects of NF-κB decoy oligodeoxynucleotide on local tumor volume (**a**) and weight of mice (**b**) *in vivo*. Five alginate beads ($\sim 2.0 \times 10^5$ LM8 cells) were transplanted subcutaneously into the dorsal skin of 10 mice in each experimental group. Tumor volume and weight were measured every other day. There were no significant differences in tumor volume or body weight.

the advantage of using a three-dimensional culture system. The results of this study showed that activation of NF-κB signaling activity was markedly suppressed by transfection of NF-κB decoy ODN in a dose-dependent manner; suggesting that NF-κB signaling activity can be effectively suppressed by transfection of NF-κB decoy ODN.

It has been reported that activation of NF-κB causes certain types of malignant tumor cells to suppress apoptosis.[30,31] Therefore, apoptosis should be induced when NF-κB signaling is inhibited.[32] In human osteosarcoma, genes relating to apoptosis exist in the downstream of NF-κB signaling.[33] Asai *et al.*[14] showed in an *in vitro* study that transfecting valosin-containing protein (VCP; an inactivator of inhibitor-κB) into osteosarcoma cells significantly decreased the rate of apoptosis compared with non-treated osteosarcoma cells. In our study, the DNA-binding activity of NF-κB by LM8 cells was markedly inhibited by transfection of NF-κB decoy ODN. However, the BrdU assay showed that NF-κB decoy ODN did not affect the cell proliferative activity of LM8. This finding suggests that the NF-κB signaling pathway may not have been completely blocked by the transfection of NF-κB decoy ODN. Alternatively, other anti-apoptosis signals, such as Bcl-2, murine double minute 2 (MDM2), DNA-dependent protein kinase (DNA-PK) and epidermal growth factor receptor (EGFR) might be involved.[34]

The capacity for angiogenesis and adhesion in distant organs is a crucial factor enabling distant metastasis of malignant tumors.[35] VEGF has been reported to induce the proliferation of endothelial cells and increased vascular permeability,[10] whereas ICAM-1 is known to have an important role in lymphatic and vascular invasion, and subsequent adhesion to other tissues.[36] It has been reported that the expression of VEGF and/or ICAM-1 is correlated to the malignancy, or metastatic potential of tumors including osteosarcoma.[37,38] In our study, the mRNA levels of VEGF and ICAM-1 were significantly decreased by transfection of NF-κB decoy ODN, indicating that the metastatic potential of LM8 cells was decreased by the transfection of NF-κB decoy ODN.

We evaluated the number of cells detached from alginate beads because the detachment observed in this culture system may reflect the initial step of metastasis.[23,39] The number of detached cells was significantly reduced by transfection of NF-κB decoy ODN at 10 μM. This result leads us to speculate that the cellular kinetics related to migration or invasion was inhibited by administration of NF-κB decoy ODN. Next, the *in vivo* study demonstrated that although tumor volume was not reduced, the number of pulmonary metastases was significantly reduced by transfection with NF-κB decoy ODN at 10 μM. As with the *in vitro* results, transfection with NF-κB decoy ODN did not affect the activity of cell proliferation, but did alleviate the metastatic potential of LM8 cells *in vivo*. Similarly, Kawamura *et al.*[21] reported that combined treatment of NF-κB decoy ODN with an anti-cancer drug did not suppress tumor cell proliferation, but did reduce hepatic metastasis in a mouse tumor model. Taken together, these results suggest that cytokines regulated by NF-κB may have pivotal roles in the process of metastasis.

The clinical application of NF-κB decoy ODN is hampered by the instability of oligonucleotides in blood and their rapid degradation by nuclease.[40,41] The intravenous injection of 'naked' NF-κB decoy ODN in an animal model showed accumulation in the kidney (30% of the dose per g tissue), but little accumulation in other organs.[42] Therefore, alternative delivery methods such as local administration,[43,44] combination use with cationic liposome as a carrier,[40] and physical stimulation by ultrasound wave[45,46] have been reported. In other series of study, we directly injected 'naked' NF-κB decoy ODN into a LM8 tumor mass and examined the biodistribution and the inhibitory effects of the decoy in a murine spontaneous pulmonary metastasis model.[47] The results demonstrated that NF-κB decoy ODN was transfected only into cells marginal to the tumor and no inhibitory

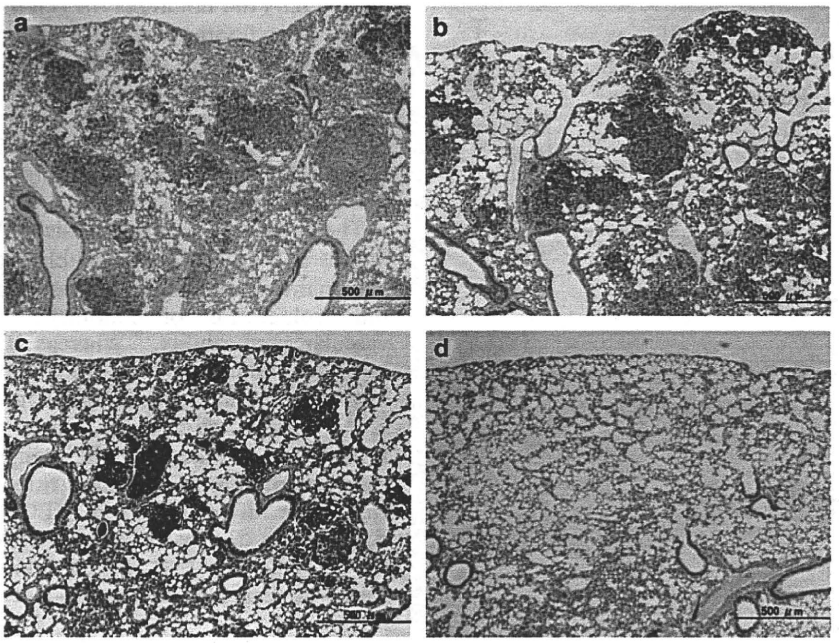

Figure 7 Histological images of lungs after transfection of NF-κB decoy oligodeoxynucleotide (ODN). Representative light microscopy images of lungs removed from each experimental group are shown, where (**a**) Control, (**b**) SCD, (**c**) NF-κB decoy ODN 100 nM and (**d**) NF-κB decoy ODN 10 μM. Larger numbers of tumor nodules were found in the lungs of the control and SCD groups, the number of nodules was markedly reduced in the decoy-transfected groups. SCD, scrambled decoy ODN (100 nM). Scale bar: 500 μm.

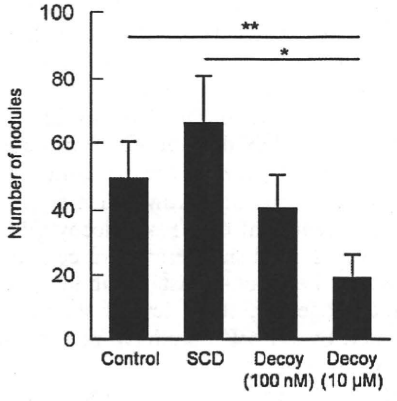

Figure 8 Effect of NF-κB decoy oligodeoxynucleotide on pulmonary metastasis of LM8 osteosarcoma in *in vivo*. The number of tumor nodules (pulmonary metastases) in the maximal area of each lung section was counted microscopically. $*P<0.01$ compared with SCD group, $**P<0.05$, compared with control group. SCD, scrambled decoy ODN (100 nM).

effects on pulmonary metastasis were observed. For clinical applications, effective transfection methods to place decoy ODN into tumor cells are needed.

In conclusion, we successfully transfected 'naked' NF-κB decoy ODN into LM8 cells cultured in an alginate-encapsulated tumor spheroid model. The DNA-binding capacity of NF-κB by LM8 cells was significantly suppressed by transfection of NF-κB decoy ODN. In the *in vivo* alginate bead transplant study, pulmonary metastases were significantly reduced by transfection of NF-κB decoy ODN. The results on cell proliferation and mRNA expression of VEGF and ICAM-1 *in vitro* provide biological evidence for the mechanism for inhibition of pulmonary metastasis by LM8 cells. Our results suggest that NF-κB has crucial and specific roles in the regulation of tumor metastasis and may be an important therapeutic target for the development of anti-metastatic treatment for osteosarcoma.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Extraskeletal subcutaneous osteosarcoma of the upper arm: A case report

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Abstract. Extraskeletal osteosarcoma (ESOS) occurs in approximately 1% of soft tissue sarcomas and 2-4% of all osteosarcomas. In particular, subcutaneous osteosarcoma is extremely rare, occurring in less than 10% of ESOS cases. This report presents a case of a subcutaneous tumor in the upper arm of a 79-year-old male. Imaging and pathological findings led to the conclusion that the soft tissue tumor should be diagnosed as subcutaneous osteosarcoma. Additionally, this case report documented the clinicopathological findings of the extraskeletal subcutaneous osteosarcoma in this case and discussed its clinical features by reviewing cases previously described in the literature.

Introduction

Extraskeletal osteosarcoma (ESOS) is a rare malignancy that accounts for approximately 1% of all soft tissue sarcomas and for 2-4% of all osteosarcomas (1-3). ESOS usually occurs in the deep soft tissue of the extremities of adults (4). It typically arises in the deep soft tissue of the thigh. Other less frequent sites include the buttock, shoulder, trunk and retroperitoneum. Approximately 24% of cases have been associated with previous radiotherapy or trauma (5). In contrast to primary osteosarcoma of the bone, this variant typically develops after the fifth decade of life, and the prognosis is uniformly poor (5,6). The present report documented the clinicopathological findings in a patient who had an ESOS arising from the subcutaneous tissue of the upper arm and reviews previous cases of subcutaneous ESOS.

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Key words: osteosarcoma, subcutaneous tissue, extraskeletal

Case report

A 79-year-old male was referred to the Mie University Hospital due to an enlarged, slightly painful mass in the left upper arm. The patient first noted the mass 3 years prior to presentation. No history of trauma or therapy had previously occurred at this site. Moreover, the patient had experienced no recent health problems and recalled no family history of cancer. A physical examination confirmed the presence of a lobulated hard mass with a diameter of 4 cm at the lateral side of the left upper arm. Radiographs of the left upper arm revealed a mass with ossification which appeared to be separated from the humerus (Fig. 1). Magnetic resonance images (MRI) showed a soft tissue mass above the fascia of the triceps with a low signal intensity on T1-weighted images and a heterogeneous signal intensity on T2-weighted images (Fig. 2). Computed tomography (CT) of the chest did not demonstrate any pulmonary masses. All routine laboratory data were normal. The long clinical course from the first awareness of the tumor and the clinical findings, suggested that the tumor was benign or a malignant calcifying epithelioma with ossification. The patient was treated with wide resection and a skin graft from an inguinal lesion. Gross sectioning of the specimen showed a 4x4x2.5 cm firm and solid mass in the deep dermis and subcutaneous tissue (Fig. 3). A microscopic examination showed the presence of numerous spindle and atypical cells often exhibiting pronounced nuclear atypia or multinucleated giant cells with bone and osteoid formation. A high mitotic activity with numerous atypical mitoses was noted (Fig. 4). These findings led to the conclusion that this soft tissue tumor was a subcutaneous ESOS. The patient had no evidence of local recurrence and distant metastasis 1 year following resection.

Discussion

Since ESOS was initially described by Wilson in 1941, approximately 300 cases have been reported thus far (5,6). ESOS is defined as a malignant mesenchymal neoplasm composed of cells producing osteoid, bone and/or chondroid material, with no attachment to bone or periosteum (7). It occurs most often in the deep soft tissues of the extremities of adults, at an average age of 50 years. Clinically, ESOS usually carries an extremely poor prognosis. The 5-year survival rate ranges

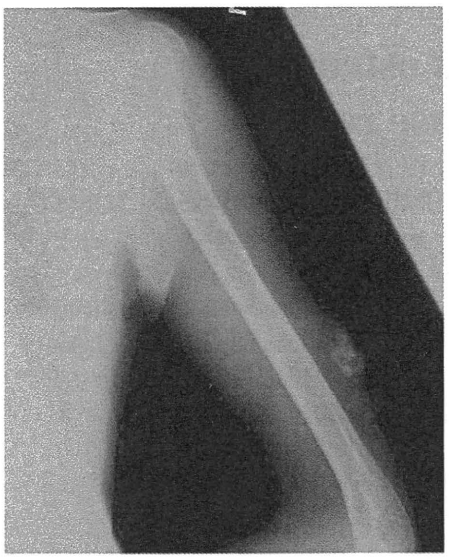
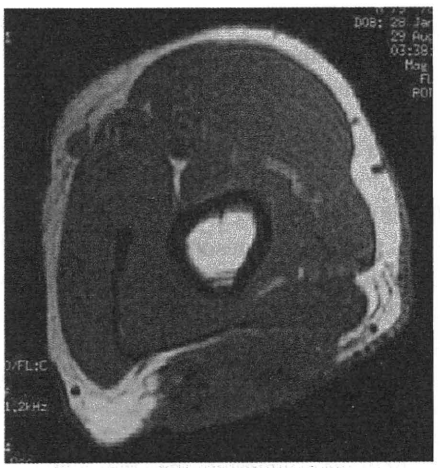


Figure 1. Radiographs revealed a subcutaneous soft tissue mass with ossification in the upper arm.

A



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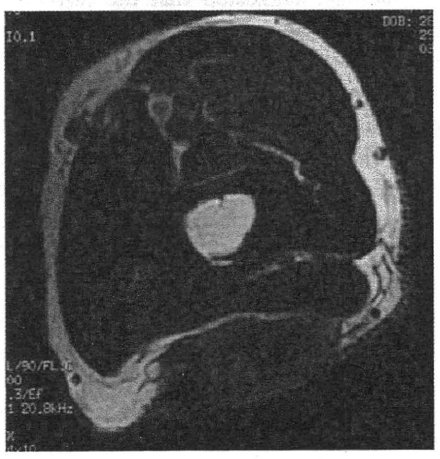
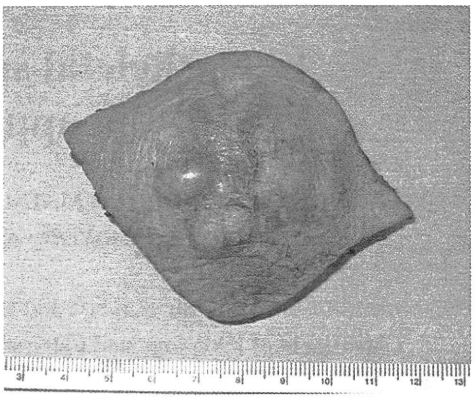


Figure 2. MRI showed a soft-tissue mass above the fascia of the triceps with (A) a low signal intensity on T1-weighted images and (B) a heterogeneous signal intensity on T2-weighted images.

from 25 to 37% for ESOS as previously reported (3,4). The tumor size appears to be the only reliable prognostic variable in that tumors greater than 5 cm have a poor prognosis (3).

A



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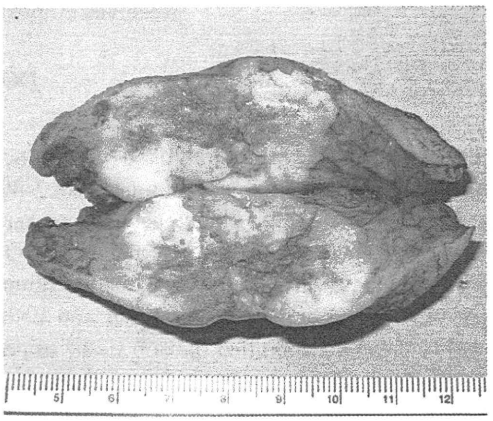
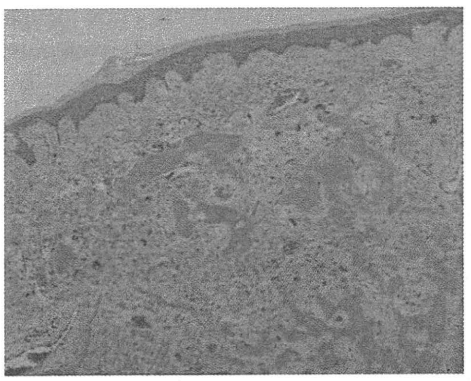


Figure 3. Gross appearance of the tumor (A, surface; B, cut surface). (A) Gross appearance of the resected tumor showed a lobulated hard mass without cutaneous ulceration. (B) The cut surface of the specimen showed a 4x4x2.5 cm firm and solid mass in the deep dermis and subcutaneous tissue.

A



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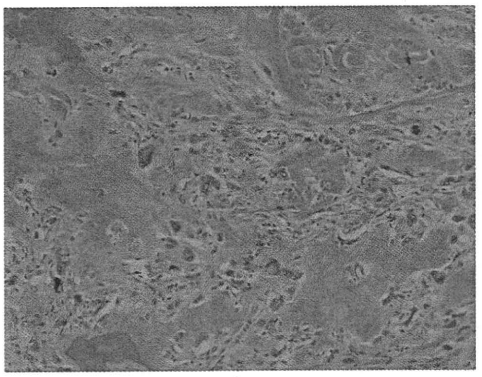

Figure 4. Microscopic findings showed numerous spindle and atypical cells with one or more nuclei, occasional multinucleated giant cells and osteoid and bone formation. H&E staining (A, magnification, x10; B, magnification, x40).

Table I. Review of the literature describing cases of primary subcutaneous extraskeletal osteosarcoma.

Author	Age/gender	Location	Size (cm)	Therapy	Outcome
Fang *et al* (8)	59/female	Abdominal wall	1.0	Surgery	CDF, 16 years
Yamakage *et al* (9)	62/male	Forehead	3.0	Surgery	DOD, 2 months (brain metastasis)
Dubec *et al* (10)	75/female	Lower leg	15.0	Surgery	CDF, 12 months
Pillay *et al* (11)	56/male	Scalp	10.0	Surgery and chemotherapy	Unknown
Oonuma *et al* (6)	55/female	Buttock	1.0	Surgery and chemotherapy	CDF, 48 months
Matsumoto *et al* (12)	68/ female	Buttock	1.0	Surgery	CDF, 16 months
Hatano *et al* (13)	25/male	Jaw	1.5	Surgery and chemotherapy	CDF, 16 months
Nakamura *et al* (Present study)	79/male	Upper arm	4.0	Surgery	CDF, 12 months

CDF, continuously disease-free; DOD, dead of disease.

Subcutaneous ESOS was rarely reported. Only eight cases, including the present one, were found in the literature (6,8-13) (Table I). Patients in those studies included 4 males and 4 females, ranging in age from 25 to 79 years. Lesions were located in the buttock in 2 cases and in the scalp, forehead, jaw, abdominal wall, lower leg and upper arm each in 1 case. In general, ESOS develops in the lower extremities, with the thigh being involved most; however, these 8 cases developed ESOS in various anatomical sites. The tumor size was less than 5 cm in all but 2 cases. A surgical resection was performed in all cases. The consequent surgical margin was wide in 7 patients, including an additional wide resection in 2 cases and an intralesional margin in 1 case. A total of three patients received chemotherapy. A wide surgical resection was performed in the present case. No chemotherapy was administered as a result of the advanced age of the patient. A wide margin is generally recommended for ESOS, as for other high-grade sarcomas (14). Lee *et al* reported that recurrence is common in ESOS and usually occurs in more than half of the patients (3). However, a wide (or radical) resection should decrease the recurrence of ESOS.

The role of adjuvant chemotherapy in ESOS is unclear. A recent series (14,15) found that the 5-year survival rate of patients with ESOS receiving chemotherapy showed an obvious improvement in comparison to what was described in previous reports (3,4). The two most recent reports found that the 5-year survival rate of patients with chemotherapy was approximately 70% (14,15). Although adjuvant therapy for ESOS remains controversial, chemotherapy may be useful in an aggressive multimodality approach to this tumor.

The 5-year survival rates associated with ESOS are relatively poor. However, 7 of the 8 cases of subcutaneous ESOS were continuously disease-free. The prognostic significance of the tumor location with respect to its relationship to the superficial fascia of the extremity or trunk was incorporated into the staging system of soft tissue sarcoma in 1998 (16). Although only 9 patients with primary subcutaneous ESOS were previously reported in the literature, these reports may indicate that subcutaneous ESOS has a more favorable prognosis than their more deeply situated counterparts.

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SHORT COMMUNICATION

A tumor endoprosthesis is useful in elderly rheumatoid arthritis patient with acute intercondylar fracture of the distal femur

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Abstract The purpose of this paper is to report the use of total knee arthroplasty using a tumor prosthesis in the treatment of elderly patients with an intercondylar fracture of the distal femur. Supracondylar fractures of the femur in patients with rheumatoid arthritis are difficult to treat due to joint deformity. We present outcomes for treating intercondylar fractures of the distal femur in rheumatoid arthritis patient using a tumor endoprosthesis. This technique allows early mobilization of the patient, with restoration of a good range of knee motion. A tumor prosthesis appears to be a viable treatment option for intercondylar femoral fractures in elderly patients. It is well tolerated and permits early ambulation and return to activities of daily living.

Keywords Tumor endoprosthesis · Rheumatoid arthritis · Supracondylar fracture · Distal femur

Introduction

The treatment of fractures around rheumatoid arthritis (RA) joints is difficult because of coexisting RA disease, severe osteopenia, and significant arthritic change. We present outcomes for the treatment of intercondylar fractures of the distal femur in RA patient that allows restoration of a good range of knee motion. It is useful for RA patient with intercondylar fractures, especially in low-demand elderly patient. This report is the second case report that treated acute supracondylar fracture using a tumor endoprosthesis.

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Case report

A 77-year-old woman was referred to our institute (Institute of Orthopeadic Research) due to progressive pain in both knees and wrists. She was able to walk with a cane. At the age of 70 years, she had been diagnosed with RA and was managed with oral corticosteroid (prednisolone, 5 mg once daily) and an anti-inflammatory drug (meloxicam, 10 mg once daily). She had been treated with DMARD therapy (tacrolimus, 1.5 mg once daily) and had controlled active inflammation. The results of the laboratory investigations were as follows: erythrocyte sedimentation rate, 44 mm; C-reactive protein, 0.36 mg/dl; and matrix metalloprotease, 299 ng/ml.

According to the Steinbrocker classification, she had Stage III and Class III RA. The X-rays of both knees showed narrowing of the joint space and were classified as Larsen grade III. The patient refused total knee arthroplasty (TKA).

She fell at home, sustaining a supracondylar fracture of the left femur. X-ray of the left knee showed intra-articular fractures in 4 parts, classified as Orthopaedic Trauma Association type 33 C2 [1] (Fig. 1a, b).

Surgery was performed in February 2008. The fracture site was identified, and the distal fracture parts were removed via a medial parapatellar approach incision. The length of the femur to be removed was measured using calipers and matched with the available implants. A standard proximal tibial cut was made perpendicular to the mechanical axis using an extramedullary alignment guide. The tibia was prepared using a series of broaches to allow

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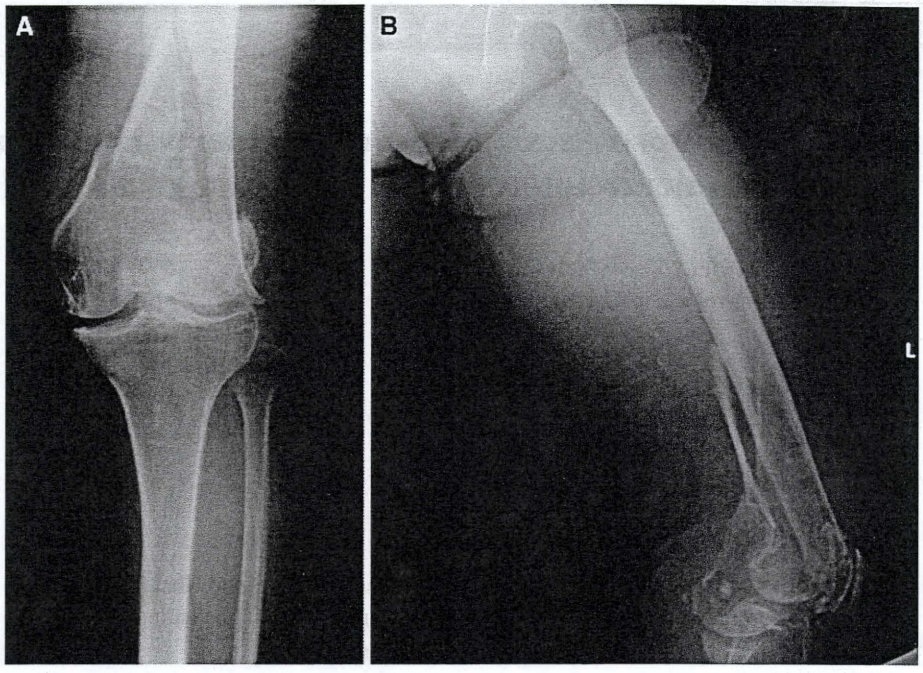

Fig. 1 **a** Anteroposterior and **b** lateral radiographs of the *left* knee before surgery

for acceptance of the stem and the fin. Appropriately sized trial prostheses were implanted, and the limb lengths and patellar tracking were checked. The knee joint and the segmental bone defect were reconstructed using the Kyocera limb salvage (KLS) tumor endoprosthesis.

The left knee was maintained in a no cast brace and mobilized starting 1 day after surgery. Follow-up at 24 months revealed an excellent, pain-free level of function. The left knee had a range of motion of 0–135°, and the patient was able to walk with a cane. The American Knee Society Scores were recorded as 95/100 for the knee score and 65/100 for the functional score. Radiographs demonstrated no evidence of prosthesis loosening or migration and no erosion of the femur or tibia (Fig. 2a, b).

Discussion

Most fractures at the distal end of the femur occur following a blow to the flexed knee in elderly individuals who fall directly onto the knee. The aim of treatment of supracondylar/condylar femoral fractures is to restore function to pre-injury levels. In such cases, the fracture usually involves the joint and is frequently comminuted, often with some bone loss, thus making open reduction and internal fixation difficult.

Rigid fixation is best for early mobilization of the knee. However, in elderly osteopenia patients, rigid fixation often cannot be achieved, and some form of external splinting is also required. Even if rigid fixation can be achieved and early mobilization can be started, full weight bearing should not be allowed for a number of weeks. In cases, where rheumatic osteopenia is so severe that acceptable fixation cannot be achieved and the surface of the joint is so shattered that reconstruction seems foolhardy, it is impossible to secure the fracture adequately to allow knee movement, so adhesions are likely to develop, causing further knee stiffness.

For these fractures, there is a 2-stage procedure. The first surgery requires open reduction and internal fixation. After removal of the fixation devices, a total knee arthroplasty (TKA) can be performed. However, the 2-stage procedure has disadvantages. These patients are at increased risk of restricted motion and perioperative complications following TKA such as skin necrosis and infection. And fracture fixation at the first surgery may make the subsequent TKA difficult because of arthrofibrosis, patella infra, and reduced range of motion [2, 3]. Two-stage procedures would confine patients to bed for a longer time and prolong the duration of treatment. Thus, a primary knee replacement can be used to overcome a problem that is otherwise insurmountable for elderly patients [4–8].

If the fracture site is more proximal and the patient has a reasonable chance of regaining good knee function, the fracture needs to be fixed and the knee replaced. In such cases, a primary knee replacement with a tumor endoprosthesis may be appropriate. In previous reports, a tumor endoprosthesis was used for periprosthetic supracondylar TKA fractures [9, 10], complex TKA revision [11], and failed internal fixation or nonunion of a distal femur

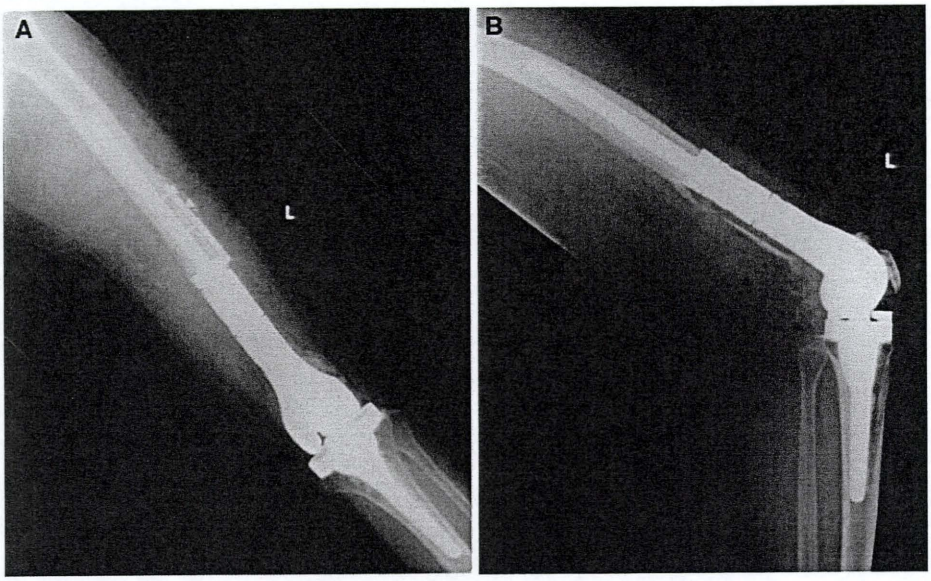

Fig. 2 **a** Anteroposterior and **b** lateral radiographs of the *left* knee 24 months after surgery

fracture [10, 12–14]. There has been only one patient with an acute supracondylar fracture, reported by Freedman et al. [12], which was reconstructed using a tumor endoprosthesis.

We performed replacement using a tumor endoprosthesis because of type C supracondylar fractures with coexisting RA disease, severe osteopenia, and significant arthritic change within the knee joint.

One year after surgery, the present patient underwent TKA surgery for her right knee because she was satisfied with the treatment for the left knee. Despite the operative challenges for an acute fracture, a tumor endoprosthesis provided significant pain relief and functional improvement for an intercondylar fracture of the distal femur.

In RA patient with a fracture at the end of the distal femur, replacement with a tumor endoprosthesis is a new surgical option. We recommend this method in type C supracondylar fractures in which there is coexisting disease or significant arthritic change within the joint.

Conflict of interest The authors have declared no conflict of interest.

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TNF inhibitor suppresses bone metastasis in a breast cancer cell line

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ABSTRACT

In the evolution of cancer, tumor necrosis factor-alpha (TNF-α) plays a paradoxical role. High doses induce significant anticancer effects, but conversely, physiologic and pathologic levels of TNF-α may be involved in cancer promotion, tumor growth, and metastasis.

Infliximab is a chimeric murine monoclonal antibody that binds with high affinity to soluble and membrane TNF-α and inhibits binding of TNF-α to its receptors. In the present study, we investigated the effect of infliximab, a TNF-α antagonist, on breast cancer aggressiveness and bone metastases.

Infliximab greatly reduced cell motility and bone metastases in a metastatic breast cancer cell line, MDA-MB-231. The mechanism of bone metastasis inhibition involved decreased expression of CXC chemokine receptor 4 (CXCR4) and increased expression of decorin, which is the prototype of an expanding family of small leucine-rich proteoglycans. These results suggest a novel role for TNF-α inhibition in the reduction or prevention of bone metastases in this breast cancer model. Our study suggests that inhibition of TNF-α using infliximab may become a preventive therapeutic option for breast cancer.

1. Introduction

Breast cancer metastasizes to bone in more than 80% of patients with advanced disease [1]. A recent study showed that the presence of isolated tumor cells in bone marrow at the time of diagnosis of breast cancer is associated with a poor prognosis [2]. Tumor growth at the bone site can be extremely painful due to the presence of tumor mass in the bone-marrow cavity and to nerve compression. Metastasized tumor growth at the bone site can lead to debilitating fractures of, particularly, the hip and spine [3].

Tumor necrosis factor-alpha (TNF-α) plays a paradoxical role in the evolution of cancer. It can act as a tumor necrosis factor and also as a tumor-promoting factor [4]. Local administration of high-dose TNF-α is antiangiogenic and has a powerful antitumor effect [5]. On the other hand, endogenous TNF-α chronically produced in the tumor microenvironment enhances tumor development and spread.

TNF-α, a major inducer of chemokines, is a key player in the tumor microenvironment and is involved in the pathogenesis of breast cancer [6]. TNF-α also stimulates the production of interleukin-6 (IL-6), and it is a potent growth factor and directly stimulates angiogenesis [7]. It is also an important factor for breast cancer progression [8]. High serum TNF-α concentration in breast cancer patients correlates with aggressive tumor biology [9].

Infliximab is a chimeric human-mouse monoclonal antibody consisting of human immunoglobulin G1 (IgG1) Fc regions fused to the variable Fv region of a high-affinity neutralizing murine antihuman TNF antibody [10]. It prevents the binding of TNF-α to its receptors, TNF-R1 (p55 receptor) and TNF-R2 (p75 receptor), and causes cell death via complement-mediated lysis through interaction with membrane-bound TNF-α [11]. Infliximab is well tolerated, is licensed for use in Crohn's disease and rheumatoid arthritis at doses of 3–10 mg/kg, and has been used in more than 750,000 patients worldwide.

Neutralization of TNF-α results in reduction of TNF-α-regulated cytokines, proteases, and other growth factors at the inflammatory site, minimizing clinical symptoms and thus reversing the clinical disease [12,13].

In the present study, the effect of infliximab on bone metastases of a breast cancer cell line (MDA-MB-231) was investigated in vitro and in vivo.

2. Materials and methods

2.1. Reagents

Infliximab was purchased from Mitsubishi Tanabe K.K. (Tokyo, Japan). Rabbit polyclonal antibodies to phospho-NF-κB, phospho-SPAK/JNK and CXC chemokine receptor 4 (CXCR4) were from Cell Signaling Technology (Danvers, MA). Goat polyclonal antibody to decorin was from R&D Systems (Minneapolis, MN). Goat polyclonal

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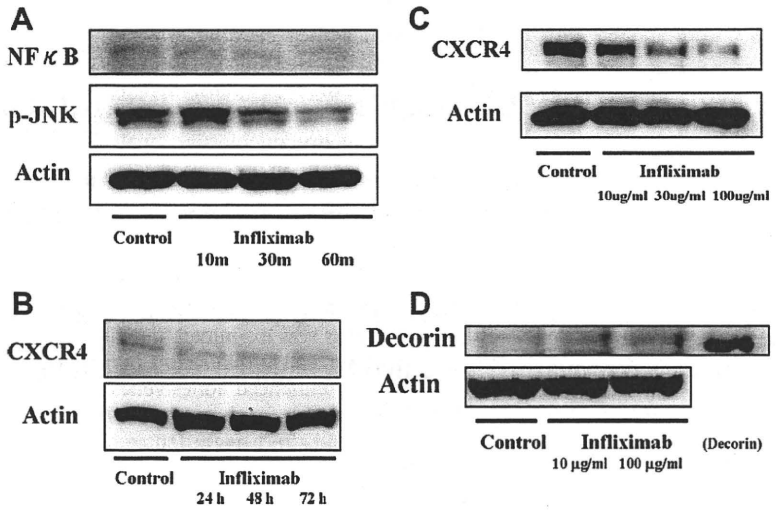

Fig. 1. Effect of anti-TNF α antibody on TNF-α signaling (A), CXCR4 expression (B, C) and decorin expression (D) in MDA-231 cells. Phospho-JNK is a MAPK activated by a variety of environmental stresses, including TNF-α. Phospho-JNK as well as NFκB in MDA-231 cells is suppressed with infliximab, likely the result of infliximab suppressing TNF-α signaling (A). CXCR4 expression was investigated by immunoblotting analysis in MDA-231 cells. Treatment with 100 μg/ml infliximab inhibits CXCR4 protein expression in a time-dependent manner (B). And infliximab inhibits CXCR4 protein expression in a dose-dependent manner (C). Infliximab, at doses of 10 and 100 μg/ml, increases decorin expression in MDA-231 cells in a dose-dependent manner (D). "(Decorin)" as positive control is extracts of MDA-DCN, which stably expresses human decorin.

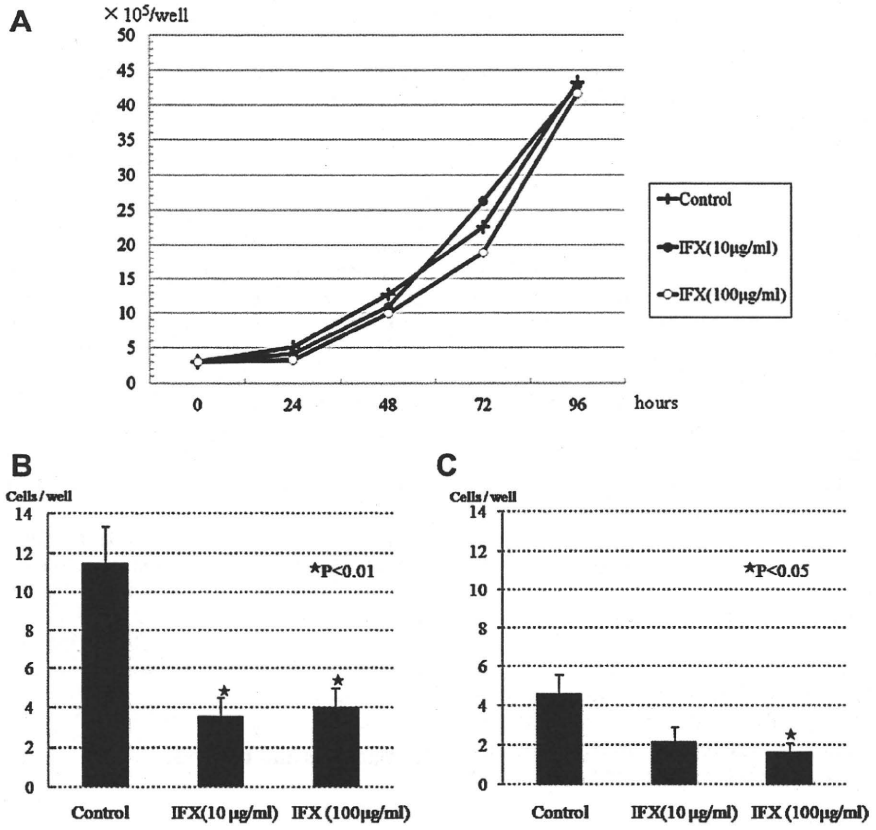

Fig. 2. Cell proliferation assay (A), migration assay (B) and invasion assay (C) in vitro. Infliximab (IFX; ⩽100 μg/ml) has no effect on tumor cell proliferation in MDA-231 cells. Migration assay and invasion assay were counted the number of cells that moved through a membrane. Both 10 and 100 μg/ml infliximab suppress cell migration ($p < 0.01$), and 100 μg/ml infliximab suppresses cell invasion ($p < 0.05$). Columns, mean; bars, SE.

5 years, whereas those with minor metastases in the bone can survive up to 10 years or more. Bone metastasis is clinically significant because of decreased patient mobility and concomitant deterioration of the patient's quality of life.

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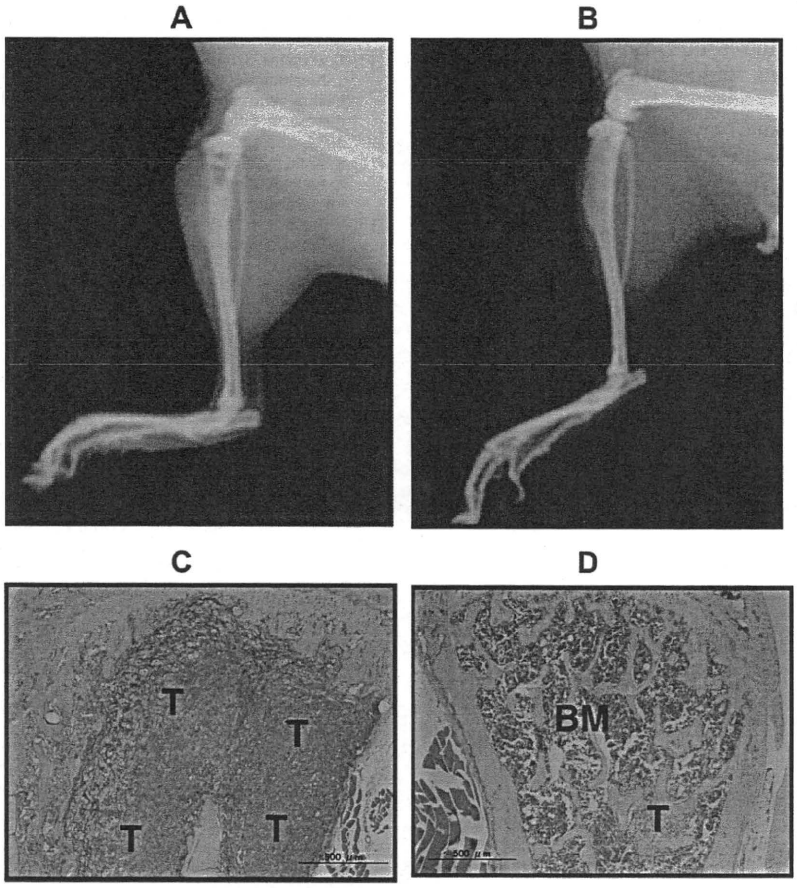

Fig. 3. Effects of infliximab on bone metastases of MDA-MB-231 human breast cancer cells. Representative radiographs of bone metastases treated without (A) or with infliximab (10 mg/kg/week) (B). Representative histological view of bone metastases treated without (C) or with infliximab (10 mg/kg/week) (D) is shown hematoxylin-eosin staining (T: tumor, BM: bone marrow, scale bar = 500 μm).

Table 1
Effects of infliximab on bone metastases of MDA-MB-231 human breast cancer cells. Representative histological view of bone metastases treated without or with infliximab were examined. Metastatic tumor burden in bone was assessed by histomorphometry as described in Section 2. Data are shown as rate and area of metastases/hindlimb.

	Rate (%)	Area (%)
Control	47.5 ± 6.9	34.3 ± 6.8
Infliximab	19.4 ± 8.1*	13.3 ± 5.4*

* Significantly different from control ($p < 0.05$).

Inflammatory processes can have quite diverse effects on cancer development. In many inflammatory scenarios, the cytokine TNF-α plays a central role. TNF-α actually has a bimodal role in cancer [4]. Local administration of high-dose TNF-α is antiangiogenic and has a powerful antitumor effect [5]. On the other hand, endogenous TNF-α chronically produced in the tumor microenvironment enhances tumor growth and invasion by inducing other cytokines/chemokines involved in cancer progression. Tumor cell-derived TNF-α plays a profound role in malignant tumors [23–26].

Animal models of bone metastasis using cancer cell lines derived from human carcinomas have been studied in order to understand the intrinsic pathological event. The most common tumor to cause osteolytic lesions is breast carcinoma; MDA-231 cells of human breast carcinoma selectively colonize the tibias and femurs of nude mice, including osteolytic metastases [14]. In this report, it was confirmed that the breast cancer cell line MDA-231 secreted TNF-α and expressed its receptor, TNF-R1.

Infliximab, an anti-TNF-α antibody, has been shown to bind and inhibit exclusively human and chimpanzee but not rodent TNF-α [10]. In this model, widespread skeletal metastases to femur and tibia were observed, while treatment with infliximab significantly reduced both the incidence and extent of bone metastases in vivo. This is the first report showing that anti-TNF therapy suppressed bone metastases. Inhibition of TNF-α with infliximab showed effects on cell migration and invasion in vitro, but not on cell proliferation. Supporting our results and in line with some reports [23,24], inhibition of TNF-α had no influence on tumor cell growth in vitro. The antitumor mechanism of action may be through modulation of the cytokine-dependent communication between cells in the tumor microenvironment rather than direct antibody-mediated cytotoxicity.

Tumor cell migration and invasion are chemokine-dependent. CXCR4 plays a critical role in the homing of cancer cells to specific metastatic sites [16]. The results of the present study show that treatment with infliximab suppressed CXCR4 expression in MDA231, suggesting that infliximab may suppress bone metastases by down-regulating the expression of CXCR4 in MDA231 cells.

Decorin expression in MDA-231 cells was examined as another possible mechanism. It is known that low levels of decorin in invasive breast carcinomas have been associated with larger tumor size, shortened time to progression, and worse outcome [27]. We previously reported that decorin suppressed lung metastases and bone metastases in an animal model [14,28]. In previous reports,

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TNF-α inhibited decorin gene expression. Interestingly, infliximab increased decorin expression in MDA-231 cells. Our data suggest that the increased expression of decorin may inhibit bone metastases.

In this report, it was demonstrated that anti-TNF-α therapy using infliximab suppresses bone metastases of breast cancer. The results also suggest that TNF-α is a target in breast cancer. Clinical trials with anti-TNF-α therapy are currently under way in patients with solid cancers [29–32]. Indeed, preliminary results from phase II clinical trials in patients with breast cancer provide some evidence for this [29]. We believe that the information provided in this article may aid in the design of future clinical trials of TNF-α antagonists, suggest suitable combinations with other targeted therapies, and identify biomarkers for patient selection and monitoring response to treatment.

5. Conflict of interest

The authors have declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.051.

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