

Preclinical Evidence that Use of TRAIL in Ewing's Sarcoma and Osteosarcoma Therapy Inhibits Tumor Growth, Prevents Osteolysis, and Increases Animal Survival

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Abstract

Purpose: Osteosarcoma and Ewing's sarcoma are high-grade neoplasms typically arising in the bones of children and adolescents. Despite improvement in therapy, the five-year survival rate is only 20% for patients not responding to treatment or presenting with metastases. Among new therapeutic strategies, the efficacy of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily with strong antitumoral activity and minimal toxicity to most normal cells and tissues, was investigated by complementary approaches both *in vitro* and in preclinical models.

Experimental design: The sensitivity of osteosarcoma and Ewing's sarcoma cell lines to TRAIL was investigated *in vitro* by determining TRAIL receptor expression together with TRAIL effects on cell viability and apoptosis. Complementary preclinical studies were carried out in respective tumor models by inoculation of osteosarcoma or Ewing's sarcoma tumor cells in paraosseous location. In addition, a model of lung nodule dissemination was developed by i.v. injection of osteosarcoma cells.

Results: *In vitro*, both osteosarcoma and Ewing's sarcoma cells that express the TRAIL death receptors were highly sensitive to TRAIL-induced caspase-8-mediated apoptosis. TRAIL administered *in vivo* by non-viral gene therapy inhibited primary bone tumor incidence and growth by 87% and prevented tumor-induced osteolysis, leading to a significant 2-fold increase in animal survival 40 days after tumor induction. Furthermore, TRAIL inhibited tumor nodule dissemination in lungs and increased survival in an osteosarcoma model.

Conclusion: These findings suggest that TRAIL is a promising candidate for the development of new therapeutic strategies in the most frequent malignant primary bone tumors. *Clin Cancer Res*; 16(8); 2363-74. ©2010 AACR.

Bone sarcomas are rare malignancies diagnosed in fewer than 700 individuals per year in the United States. Osteosarcoma, the most common primary malignant bone tumor in both children and adults, is characterized by the development of bone or osteoid substance by the tumor cells (1). The disease develops mainly in young patients between ages 10 and 25 years with a peak of incidence

at 18 years (2). Current therapeutic protocols consist in neoadjuvant and adjuvant poly-chemotherapy associated to definitive surgery with limb salvage. The rate of long-term survival is 50% to 70% at 5 years for patients with localized tumor but only 15% to 20% when pulmonary metastases are detected at diagnosis or not responding to therapy, or with disease relapse. Ewing's sarcoma is a high-grade neoplasm that accounts for approximately half of osteosarcoma cases in children, representing 2% of childhood cancers with a peak incidence at age 15 years (3-5). It is characterized by a rapid tumor growth and extensive bone destruction that can result in bone pain and pathologic fracture (6). Ewing's tumors show a typical chromosomal translocation in >90% of cases linking the *EWS* gene on chromosome 22q12 to a member of the ETS transcription gene family, most commonly to *Fli-1* on 11q24 (7, 8), leading to an aberrant transcription factor that promotes tumorigenicity (9-11). Current treatment consists of several rounds of multidrug cytostatic therapy combined with surgery and radiotherapy for local control. With the use of alkylating agents, long-term survival can

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doi: 10.1158/1078-0432.CCR-09-1779

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Translational Relevance

Despite improvement in therapy, the five-year survival rate for the two more frequent primary bone tumors in children (osteosarcoma and Ewing's sarcoma) is only 20% for patients not responding to treatment or presenting with metastases. The results of preclinical studies presented in this article show the proof of concept of the beneficial use of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) in osteosarcoma and Ewing's sarcoma therapy. These data could be easily translated to cancer medicine given that agonist antibodies to TRAIL death receptors are already tested in clinical trial with encouraging results in solid tumors other than primary bone tumors. Furthermore, we showed that the expression of death receptor is a prerequisite for TRAIL sensitivity in bone tumor cell lines. Given that 70% of osteosarcoma and Ewing's sarcoma biopsies express DR4 or DR5, it can be suggested that these patients may benefit from such TRAIL therapy.

be achieved in >50% of patients with localized disease, whereas patients with clinically detectable metastases at diagnosis or who are not responding to therapy have a significantly poorer prognosis, with long-term survival decreasing to 15% to 25%, especially for patients with bone metastases (12).

As the survival rates for metastatic and relapsed osteosarcoma or Ewing's sarcoma patients have not changed in the past 30 years despite the use of intensive chemotherapy (13), new therapeutic approaches are therefore needed.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL or APO-2L) is a member of the TNF superfamily that has a strong antitumor activity in a wide range of cancer cell lines while being minimally cytotoxic to most normal cells and tissues (14). TRAIL is able to bind five cognate receptors: two death receptors acting as activating receptors [DR4 (TRAIL-R1) and DR5 (TRAIL-R2)] and three decoy receptors [DcR1 (TRAIL-R4), DcR2 (TRAIL-R5), and secreted osteoprotegerin (OPG); ref. 15]. The TRAIL decoy receptors are unable to trigger a death signal because they lack a functional death domain (16). TRAIL activates the extrinsic apoptotic pathway by inducing Fas-associated death domain protein recruitment to the death domain, leading to caspase-8 activation and caspase-3 cleavage (17). In addition, it has been shown that TRAIL can induce the mitochondrial apoptotic pathway through caspase-8 activation followed by NF κ B and Bid recruitment (18). Clinical trials are currently in progress testing fully humanized activating monoclonal antibodies directed against DR4 or DR5. Two phase 1 studies targeting DR4 (Mapatumab, HGS-ETR1, TRM-1;

ref. 19) or DR5 (Lexatumumab; ref. 20) are currently carried out in patients with solid malignancies and a phase 2 study with lexatumumab is in progress in patients with solid tumors (21). These studies show promising results on tumor progression, but over a limited time span. Except hepatic toxicity, the major limitation of the therapeutic use of TRAIL is the resistance that occurs in cancer cell lines, which can be attributed to dysfunctions in the TRAIL signaling pathway (16, 22, 23).

In the present study, complementary experimental approaches were used to study TRAIL mechanism of action on osteosarcoma and Ewing's sarcoma cell lines *in vitro*. Then, its potential therapeutic interest was investigated by gene transfer *in vivo* in respective tumor models induced by osteosarcoma POS-1 and Ewing's sarcoma A673 cell inoculation in mice. The results showed antitumor effects of TRAIL in both models, with significant prevention of bone lesions, inhibition of bone tumor development, and increased survival rates. TRAIL also diminished lung nodule dissemination induced by osteosarcoma cells.

Materials and Methods

Cell lines

Ewing's sarcoma. Eight human Ewing's sarcoma cell lines were used. A673, TC32, SKES1, SKNMC, and RDES cell lines were kindly provided by Dr. S. Burchill (Children's Hospital, Leeds, United Kingdom) and the EW24, TC71, and EW7 by Dr. O. Delattre (Institut National de la Sante et de la Recherche Medicale U830, Paris, France). The cell lines A673, TC32, SKES1, and RDES were cultured in DMEM (BioWhittaker) with 10% fetal bovine serum (FBS; Hyclone) and SKNMC, EW24, TC71, and EW7 cells were cultured in RPMI (BioWhittaker) with 10% FBS. In addition, EW7 cells require type I collagen to grow.

Osteosarcoma. Seven osteosarcoma cell lines were studied as follows: human HOS, U2OS, MG63, rat ROS and OSRGa, and mouse POS-1, MOS-J. The mouse POS-1 osteosarcoma cell line was kindly provided by Dr. A. Kamijo (Yokohama City University School of Medicine, Yokohama, Japan; ref. 24). Rat osteosarcoma OSRGa cell line was established from an initially radioinduced osteosarcoma (25). Murine MOS-J osteosarcoma cell line was kindly provided by Dr. L. Shultz (Jackson Laboratory, Bar Harbor, Maine; ref. 26); Human MG63, U2OS, HOS, and rat ROS cell lines were purchased from the American Type Culture Collection (Promochem). All cell lines were cultured in DMEM (Lonza) supplemented with 5% FCS (Hyclone) and 2 mmol/L L-glutamine (Lonza) except the POS-1 and MOS-J cell lines, which were cultured in RPMI (Lonza) supplemented with 10% FCS and 2 mmol/L L-glutamine.

Other. Cells (2.93) derived from human fetal kidney used in *in vitro* cell transfection assays (27) were cultured in DMEM supplemented with 10% FBS and 2 mmol/L L-glutamine.

In vitro experiments

Plasmid constructs. The pBF42-hTRAIL construct (kindly provided by Dr. B. Fang, The University of Texas MD Anderson Cancer Center, Houston, Texas), contains the DNA sequence encoding human TRAIL inserted into the pBF42 plasmid between the *Xho1* and *BamH1* sites. For both *in vitro* and *in vivo* studies, the empty pBF42 plasmid was used as control.

Cell transfection. To assess the cellular expression of hTRAIL, 80% confluent 2.93 cells in six-well plates were transfected with 8 μg of pBF42 or pBF42-hTRAIL using a synthetic transfection reagent, the lipophosphoramidate KLN143. Transfections were done in serum-free optiMEM, and after 2 h and 30 min at 37°C, the transfection medium was replaced by 2 mL of DMEM containing 10% FBS and 1% penicillin/streptomycin (complete medium). The transfection efficiency was quantified *in vitro* by using the pEGFP-N3 construct encoding the green fluorescent protein. Expression of the hTRAIL transgene was quantified at the protein level 48 h after transfection. In addition, to validate the biological activity of pBF42-hTRAIL *in vitro*, the proliferation of pBF42-TRAIL-transfected A673 cells was compared with the proliferation of pBF42-transfected cells during 72 h, by using the proliferation assay described below.

Cell proliferation and viability. Subconfluent cultures of osteosarcoma or Ewing's sarcoma cells in 96-well plates were treated for 72 h with 50 ng/mL^{-1} hTRAIL (R&D systems). Cell viability was determined using the sodium 3'[(1-(phenylaminocarbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) cell proliferation reagent assay kit (Roche). Cell viability was also assessed by trypan blue exclusion. The percentage of cells exhibiting intracytoplasmic trypan blue staining was determined using a Malassez counting chamber.

Apoptosis. Subconfluent tumor cells cultured in 24-well plates were treated with 50 ng/mL TRAIL for 1 to 6 h, washed, and lysed in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 1 mmol/L Na_3VO_4 , 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF, 10 $\mu\text{g}/\text{mL}$ leupeptin, and 10 $\mu\text{g}/\text{mL}$ aprotinin] for 30 min. The cells were then scraped off and the lysates were cleared of debris by centrifugation at 12,000 g for 15 min, after which total protein was quantified using the bicinchoninic acid + copper II sulfate assay (Pierce Chemical). Caspase-3 activity was assessed in 10 μL of cell lysate with the CaspACE assay kit (Promega) following the manufacturer's recommendations. Cells treated with 1 $\mu\text{mol}/\text{L}$ staurosporin 4 h before harvesting were used as positive control for caspase activity. Caspase-8 activity was assessed by Western blot analysis as described below.

Gene expression analysis. Expression of hTRAIL death receptors DR4 and DR5 was assessed in subconfluent tumor cells cultured in six-well plates. TRAIL receptor mRNA expression was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) after total RNA extraction using Trizol reagent (Invitrogen).

Briefly, 10 ng of cDNA were amplified using the IQ SYBR Green Supermix (Bio-Rad) for the DR4 and DR5 gene. Sense and antisense primers used are as follow (DR4: sense, ggaggcacagtgtctgctg; antisense, cagcaccatttctggaac; DR5: sense, agagccaacaggtgtcaacat; antisense, gcctcctctctgagacctt). Each sample was analyzed twice and quantified with the analysis software for iCycler iQ Real-time PCR Detection System (Bio-Rad).

hTRAIL ELISA. Ninety-six-well plates were coated with polyclonal anti-human TRAIL antibody (0.2 $\mu\text{g}/\text{well}$, R&D Systems) overnight at room temperature and then washed and incubated for 1 h with a blocking solution (1% bovine serum albumin/5% sucrose/PBS). After washing, the wells were incubated at room temperature with dilute serum or with media from transfected 2.93 cells for 2 h, followed by a 2-h incubation with Biotin-SP-conjugated Donkey Anti-Human IgG (1:20,000; R&D Systems). Detection was done by incubating with streptavidin-horseradish peroxidase (1:200; R&D systems) for 30 min. The reaction was stopped by the addition of 50 μL of 1 mol/L H_2SO_4 and then absorbance at 410 nm was measured using a VICTOR² Multilabel counter (Perkin-Elmer). The ELISA detection limit was 0.02 ng/mL . TRAIL production was also assessed in the muscle and tumor after tissue lysing and crushing as described below (*in vivo* analysis of transgene expression).

Western blot analysis. Fifty micrograms of total protein were separated by SDS-PAGE and were electrophoretically transferred to an Immobilon-P membrane (Millipore). The membrane was incubated with antibodies [caspase-8 and DR5 (Cell Signaling Technology) and DR4 (R & D systems)] in PBS containing 0.05% Tween 20 and 3% bovine serum albumin. The membrane was washed and probed with secondary antibody coupled to horseradish peroxidase. Antibody binding was visualized with an enhanced chemiluminescence system (ECL kit, Roche). For quantification, luminescence was detected with a Charge Couple Device (CCD) camera (Syngene) and was analyzed using the GeneTools program (Syngene).

Fluorescence-activated cell sorting analysis. Cells (2×10^5) were washed twice and were resuspended in 100 μL of PBS and 4% FBS. Two microliters of phycoerythrin-conjugated anti-TRAIL-R1 (DR4), anti-TRAIL-R2 (DR5), anti-TRAIL-R3 (DcR1), or anti-TRAIL-R4 (DcR2) antibodies (R&D systems) were added in 50 μL PBS 0.1% FBS. The cells were incubated on ice for 30 min, washed twice with PBS 2% FBS, and then analyzed using the Cytomics FC500 flow cytometer (Beckman-Coulter).

In vivo experiments

Osteosarcoma model. Four-week-old male C3H/He mice (Elevages Janvier) were maintained under pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine, Nantes, France) in accordance with the institutional guidelines of the French Ethical Committee (CEEA Pays de la Loire n°06) and under the supervision of authorized investigators. Animals were housed 1 wk before the start of experiments. The mice were anesthetized by

inhalation of an isoflurane/air mixture (1.5%, 1 L/min) combined with 0.05 mg/kg buprenorphine (Temgesic, Schering-Plough) before receiving an injection of 2×10^6 POS-1 cells in close proximity to the metatarsus. Tumors appeared at the injection site ~8 d later. The tumor volume (V) was calculated from the measurement of two perpendicular diameters using a calliper, according to the following formula: $V = 0.5 \times L \times (S)^2$, in which L and S are, respectively, the largest and smallest perpendicular tumor diameters. Mice were sacrificed when tumor volume reached 1,000 mm³ for ethical reason.

Ewing's sarcoma model. Four-week-old Rj:NMRI-nude mice (Elevages Janvier) were housed at the experimental Therapy Unit as described above. They were anesthetized as described before receiving an intramuscular injection of 2×10^6 A673 cells.

Models of pulmonary nodule dissemination. Four-week-old male C3H/He mice were anesthetized as described before i.v. injection of 1.5×10^5 murine osteosarcoma POS-1 cells. Under these conditions, pulmonary nodules develop rapidly, leading to the death of the animals in 3 to 4 wk after POS-1 cell injection (28).

Formulation preparations. Experiments were done with the third-generation phosphonolipid transfection reagent KLN143, containing an ammonium polar head. It was used and formulated as previously described for the second generation of cationic phosphonolipids (29, 30). Briefly, cationic lipids were dissolved in chloroform in a glass vial. Chloroform was evaporated under vacuum to produce a dry lipid film. Sterile saline (NaCl 0.9% w/v) was then added to the lipid film, and after storage at 4°C overnight, the resulting solution was sonicated for 10 min before use. Cationic lipids were mixed with plasmid DNA and diluted in sodium chloride solution (0.9%) as required. The mixture was incubated at room temperature for 30 min before administration.

Experimental protocols. To determine the effect of hTRAIL delivered by synthetic vectors on primary tumor growth or pulmonary nodule formation, mice were injected with tumor cells as described above. Three groups of eight mice were then treated as follows: controls (vehicle treated), pBF42 (empty plasmid treated controls: KLN143/empty pBF42), and hTRAIL (KLN143/pBF42-hTRAIL). The KLN143 reagent alone did not affect tumor development (data not shown). TRAIL treatment in both models consisted of weekly i.v. injections of the lipophosphoramidate/DNA formulations in the retro-orbital vein, beginning at the time of tumor cell inoculation until 28 to 42 d posttumor cell injection, depending on the series. Treatment was continued until the volume reached 5,000 mm³ for Ewing's sarcoma or 1,000 mm³ for osteosarcoma at which point mice were sacrificed by CO₂ inhalation, in accordance with ethical regulations. In the model of lung dissemination following i.v. injection of POS-1 cells, the animals were sacrificed when respiratory distress was observed and the number of nodules was assessed at necropsy.

In vivo analysis of transgene expression. Blood was drawn intermittently from the retro-orbital vein to moni-

tor circulating hTRAIL protein levels throughout the experiment. At necropsy, the tumor and muscle tissues were lysed in reporter lysis buffer (Promega) supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals) and crushed for 30 s using Ultraturax. After which, the lysates were centrifuged at 10,000 rpm for 5 min at 4°C and processed for hTRAIL detection using the ELISA described above.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining for detection of apoptosis in vivo. The proportion of apoptotic cells in primary bone tumor was determined using the *in situ* cell death detection kit (Roche Diagnostics), based on the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. TUNEL-positive cells were counted by microscopic examination with a $\times 40$ objective lens and indices were determined as the mean percentages of positive cells among total cells.

Radiographic analysis. Radiographs on anesthetized animals [xylazine (Rompun)-ketamine (Imalgène 500) 8% and 13%, respectively, in PBS; 100 μ L/10 g] were taken every week and at necropsy with a PLANMED Sophie mammography apparatus (SN RAH 40710).

Statistics

For analysis of TRAIL concentrations, results from treated groups were compared with the control using Dunnett's multiple comparison test. *In vivo*, Gaussian distributions were tested for the analysis of tumor volumes in the three groups by the Kolmogorov and Smirnov test. A two-tailed unpaired *t* test with Welch correction was used to compare mean tumor volumes. To compare metastatic incidence, a Fisher exact test was performed. Mouse survival was analyzed using the Kaplan Meier survival curves with log-rank analysis.

Results

Eight human Ewing's sarcoma and seven mouse, rat, or human osteosarcoma cell lines available in our laboratory were screened for their sensitivity to TRAIL *in vitro*: four Ewing's sarcoma (A673, TC71, RDES, and EW7) and four osteosarcoma (HOS, MG63, U2OS, and POS-1) cell lines were found sensitive to TRAIL. The further studies on TRAIL mechanisms of action were done with all sensitive cell lines, but to simplify the article, the results obtained with one representative sensitive cell line of each tumor type are shown.

Osteosarcoma and Ewing's sarcoma cells sensitive to TRAIL express death receptors DR4 and DR5

The expression of TRAIL death receptors was determined in all sensitive osteosarcoma and Ewing's sarcoma cell lines at the mRNA and protein levels, respectively, by RT-qPCR and fluorescence-activated cell sorting (FACS) analyses. Figure 1A shows an example of the results obtained with the A673 (Ewing's sarcoma) and HOS (osteosarcoma) cells at the mRNA level, showing the expression

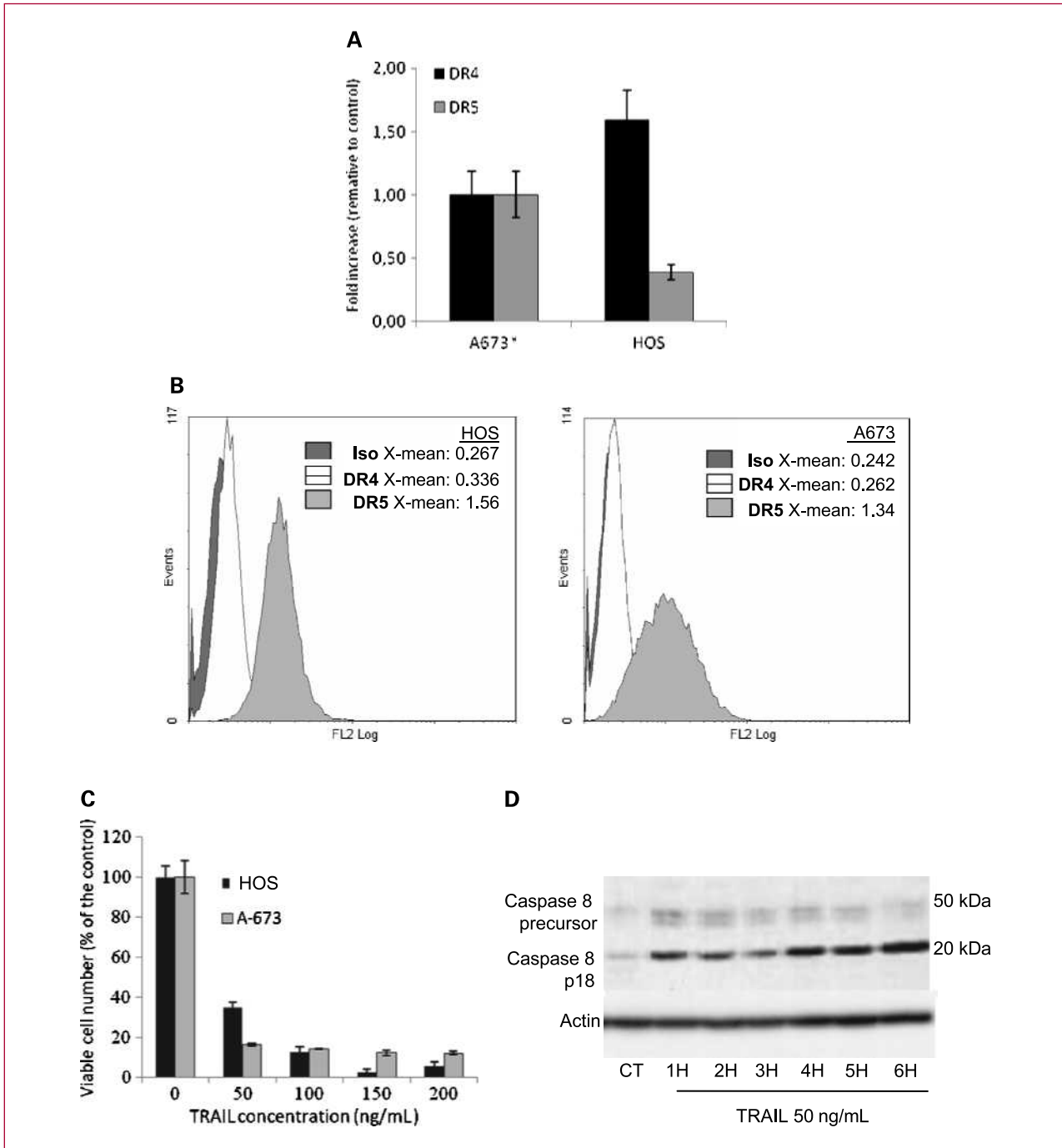


Fig. 1. Human A673 Ewing's sarcoma and HOS osteosarcoma cells express TRAIL death receptors and are sensitive to TRAIL-induced apoptosis. TRAIL DR4 and DR5 death receptor expression was assessed at the transcript level by RT-qPCR (A) and at the protein level by FACS analysis (B) in Ewing's sarcoma A673 and osteosarcoma HOS cells. TRAIL sensitivity of primary bone cell lines was determined by XTT assay after treatment with 0 to 200 ng/mL TRAIL for 72 h (A673 and HOS cells as an example (C)). Caspase-8 activation was analyzed in response to TRAIL in the Ewing's sarcoma A673 cell line by the Western blot of caspase-8 cleavage (caspase-8 p18) after treatment of A673 cells with 50 ng/mL TRAIL for 1 to 6 h (D).

of the death receptors DR4 and DR5 by both cell lines, DR4 being more expressed in the HOS cell line than DR5 (Fig. 1A). However, at the protein level, FACS analysis reveals a higher expression of DR5 at the surface of both A673 and HOS cells than DR4 (Fig. 1B).

TRAIL induces apoptosis in osteosarcoma and Ewing's sarcoma cells *in vitro*

The effect of recombinant human TRAIL was investigated on the viability of osteosarcoma and Ewing's sarcoma cell lines using an XTT assay. Treatment with increasing

concentrations (0-200 ng/mL) of TRAIL for 72 h decreased cell viability in both cell types, with maximum inhibition achieved at 150 ng/mL (IC_{50} , 38.3 ng/mL) for HOS and 50 ng/mL for A673 cells (IC_{50} , 29.9 ng/mL) for example (Fig. 1C). Treatment of cells with 50 ng/mL TRAIL for 24 to 72 hours induced a time-dependent decrease in the number of viable cells, compared with the control, with a maximum decrease observed after 72 hours of treatment (data not shown). To determine whether the decrease in viable cell number was due to induction of cell death, cell viability was analyzed by trypan blue dye exclusion, revealing a 27% increase in cell death in response to treatment with 50 ng/mL TRAIL for 48 hours in both cell lines (data not shown). Activation of caspase-8 was investigated in osteosarcoma and Ewing's sarcoma cells incubated with 50 ng/mL TRAIL. Western blot analyses of caspase-8 activation are given for the Ewing's sarcoma A673 cell line (Fig. 1D) and show that caspase-8 is activated in a time-dependent manner in response to TRAIL, confirming the induction of death receptor-mediated apoptotic cell death.

***In vitro* validation of TRAIL transgene expression and biological activity**

To show the transfection efficiency achieved using the synthetic transfection reagent KLN143, hTRAIL transgene expression was analyzed by ELISA in the supernatant of 2.93 cells 48 hours after transfection with empty plasmid pBF42 or pBF42-hTRAIL using the KLN143 reagent. Results presented in Fig. 2A confirmed that pBF42-hTRAIL-transfected cells expressed a high level of hTRAIL (3.78 ng/mL), whereas no TRAIL was detected in the supernatant of pBF42 empty plasmid-transfected cells, as expected. The biological activity of hTRAIL produced by pBF42-hTRAIL-transfected cells was confirmed *in vitro* by a ~40% inhibition of pBF42-TRAIL-transfected A673 cell proliferation after 72 hours, compared with cells transfected by the empty plasmid pBF42 (Fig. 2B).

***In vivo* analysis of TRAIL transgene expression**

In vivo, TRAIL transgene expression was analyzed at systemic and local levels: TRAIL overexpression was detected

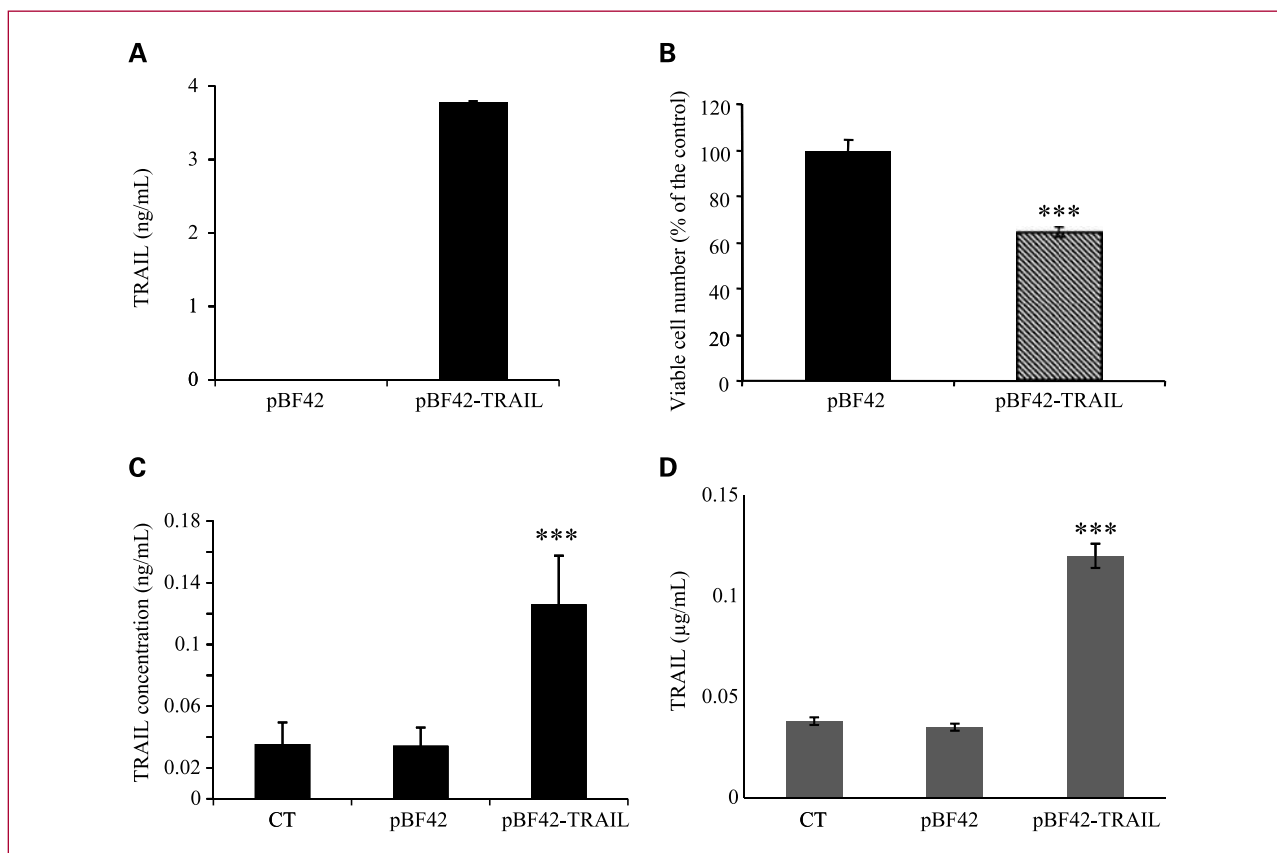


Fig. 2. *In vitro* and *in vivo* validation of TRAIL transgene overexpression and biological activity. TRAIL transgene expression was assessed at the protein level by ELISA in the culture medium of 2.93 cells 48 h after transfection with the pBF42-hTRAIL plasmid using the KLN143 synthetic vector (A). The biological activity of TRAIL transgene protein was assessed by its ability to induce apoptosis in A673 Ewing's sarcoma cells: cells transfected with KLN143/pBF42 or KLN143/pBF42-TRAIL were cultured for 72 h and the viable cell number was determined using an XTT assay (B). *In vivo*, KLN143/DNA formulations were administered weekly through the retro-orbital sinus of the mice bearing Ewing's sarcoma and TRAIL expression was analyzed 7 d postinjection by ELISA in the (C) serum or in the (D) tumor tissue (***, $P < 0.0001$ compared with respective control group); CT, control.

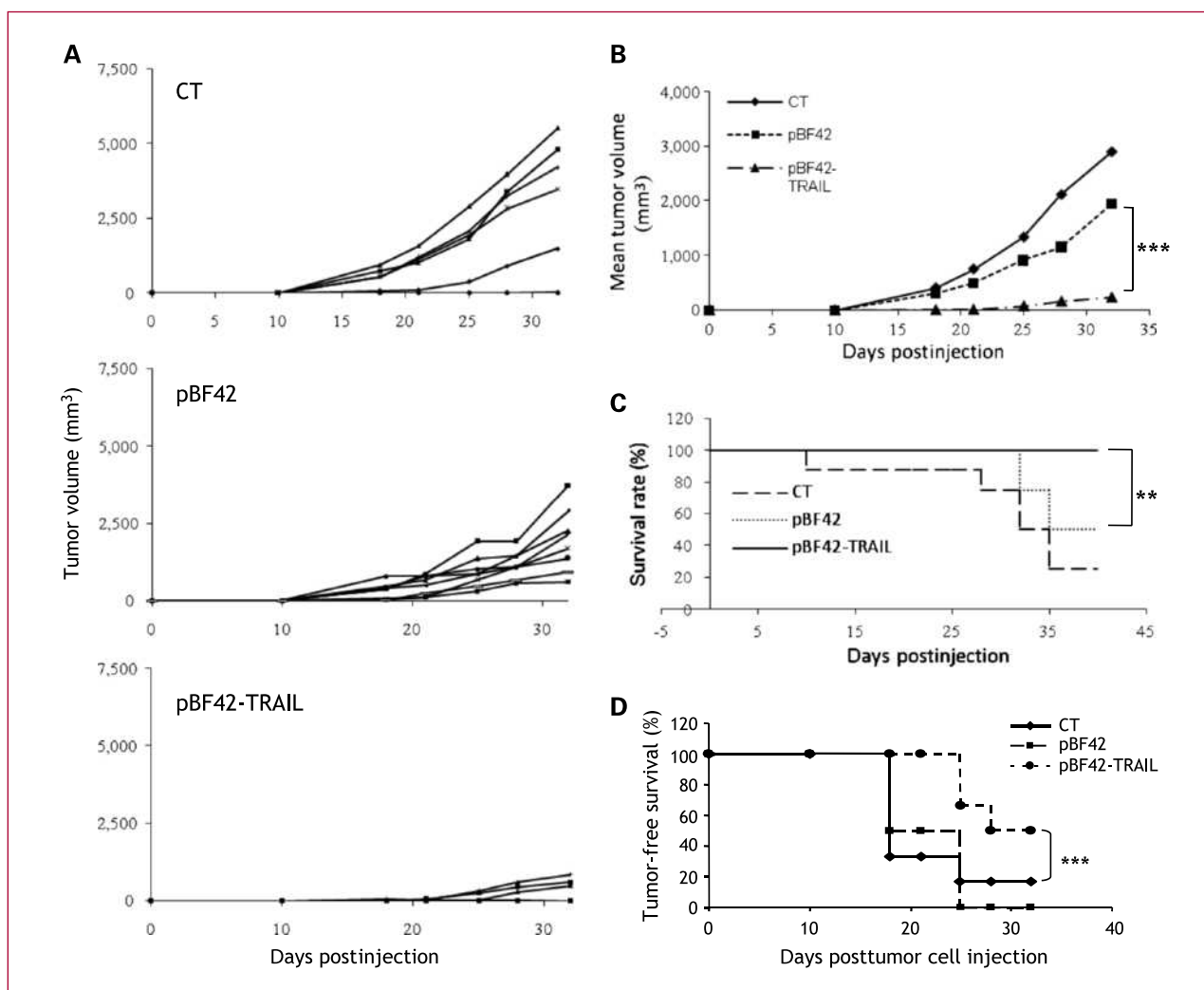


Fig. 3. TRAIL prevents Ewing's sarcoma development and increases mouse survival. Mice were injected with 2 million A673 cells as described in Materials and Methods. Groups of six to eight mice were assigned to controls (CT: vehicle treated), pBF42 (empty pBF42 plasmid/KLN143 reagent), and pBF42-TRAIL (KLN143 reagent/pBF42-hTRAIL). The KLN143/DNA formulations were administered through injection into the retro-orbital vein weekly, beginning at the time of tumor cell inoculation and continued for up to 28 d. A, tumor volumes of individual mice. B, mean tumor volume per group (***, $P = 0.0002$). C, overall survival rates for the three groups over a 40-d period (**, $P = 0.009$). D, tumor-free survival over a 32-d period (***, $P < 0.001$).

in the serum 2 days after i.v injection of plasmids pBF42-hTRAIL formulated with KLN143 compared with pBF42 empty plasmid or controls (respectively, 0.12 ng/mL versus 0.03 ng/mL, $P < 0.0001$; Fig. 2C). Local transgene TRAIL overexpression was also investigated at the site of injection (muscle) and at the tumor level by ELISA test. As expected, higher expression of TRAIL was detected in the muscle injected with KLN143/pBF42-TRAIL formulation compared with empty plasmid pBF42 (data not shown). This overexpression was also observable at the tumor site: the group treated by pBF42-hTRAIL expresses significantly higher amount of TRAIL (0.12 $\mu\text{g/mL}$) compared with both pBF42 empty plasmid and control groups (0.035 and 0.038 $\mu\text{g/mL}$, respectively; $P < 0.0001$; Fig. 2D).

hTRAIL transgene expression decreases osteosarcoma and Ewing's Sarcoma incidence and progression, and increases animal survival

Models of primary Ewing's Sarcoma and osteosarcoma were induced by para-osseous injection of 2×10^6 human A673 or mouse POS-1 tumor cells into nude mice or C3H/HeN mice, respectively. Mice received the first i.v. injection of KLN143 alone or KLN143/DNA formulations at the time of tumor cell inoculation, and then weekly thereafter for 4 weeks. Tumor volume was measured and calculated twice weekly.

A673 Ewing's sarcoma model. A significant decrease in tumor incidence was observed in the TRAIL-treated group with only 50% of mice developing a tumor compared with 100% in the pBF42 empty vector-treated and 83% in the vehicle-treated control groups at day 32 (Fig. 3A).

Moreover, tumor volumes for the mice that develop tumors were significantly lower in the TRAIL-treated group compared with the pBF42 empty plasmid or the vehicle-treated control groups (Fig. 3A). For example, 32 days after tumor cell inoculation, mean tumor volume in the TRAIL-treated group was 241.6 mm³ compared with 1,943.6 and 2,894.8 mm³, respectively, for the pBF42 empty plasmid and the vehicle-treated control groups ($P = 0.0002$; Fig. 3B). Animals had to be sacrificed due to progressive tumor development. Overall and tumor-free survivals were analyzed by comparing Kaplan-Meier curves with a log-rank test. No significant difference was found between the survival curves of the vehicle-treated and empty plasmid-treated groups (Fig. 3C and D); however, a significant increase in overall survival was observed at day 40 with 100% of survival in the TRAIL-treated group versus 50% in the empty plasmid-treated group and 25% in the vehicle-treated group ($P = 0.009$; Fig. 3C). Tumor-free survival curves also showed significant increase in the TRAIL-treated group compared with controls or empty plasmid groups ($P < 0.001$; Fig. 3D).

Mouse POS-1 osteosarcoma model. A model of primary osteosarcoma was developed by injecting tumor cells in the foot pad of syngenic mice by a para-osseous approach in close contact to the metatarsus as previously described (31). Mice treated with empty plasmid show a rapid development of tumors at the site of tumor cell injection in 83% of mice, whereas TRAIL gene transfer induced a tumor progression in only 33% of mice ($P < 0.001$; Fig. 4A).

TRAIL increases mouse survival by preventing pulmonary nodule development

TRAIL efficacy was also investigated in a model of tumor nodules developed secondary to Ewing's sarcoma. A673 or TC71 human Ewing's sarcoma cells (2.5×10^6) were injected i.v. in nude mice similar to models previously described (32). However, this approach led to the development of only few lung nodules in a small number of animals (<25%), impossible to use for preclinical studies in which high number of animals are needed per group. Therefore, because we previously showed that i.v. injection of 1.5×10^5 murine osteosarcoma POS-1 cells

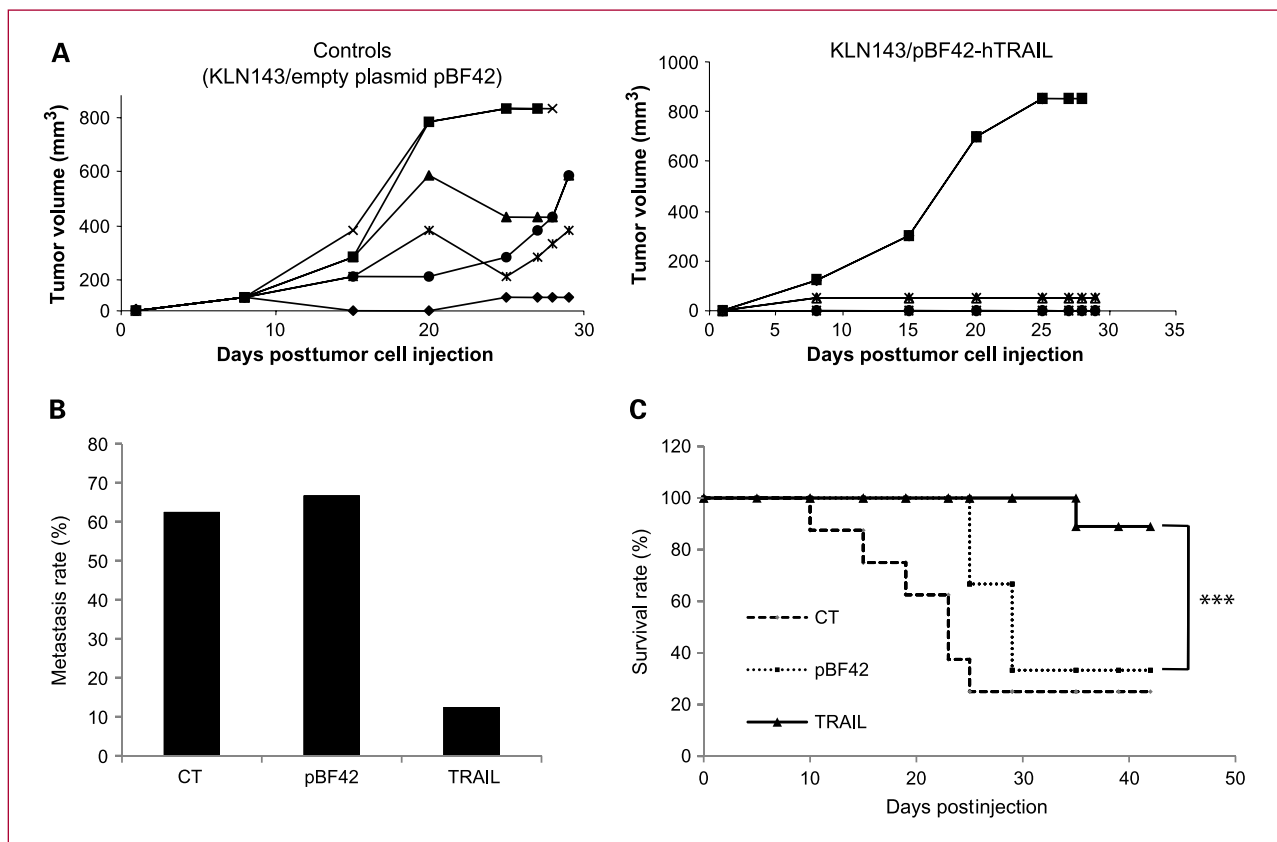


Fig. 4. TRAIL gene transfer inhibits osteosarcoma incidence and prolongs mouse survival by inhibiting pulmonary tumor nodule dissemination. Four-week-old male C3H/HeN mice were injected with 2×10^6 murine POS-1 cells near the metatarsus. Primary osteosarcoma develops at the site of injection. Mice were treated i.v. with PBS (CT), pBF42, or pBF42-TRAIL (TRAIL) weekly, starting at time of tumor cell inoculation and continued until day 28. A, individual tumor volume of empty pBF42 plasmid- and pBF42-TRAIL-treated groups are shown. For the lung nodule model, mice were i.v. injected with 150,000 POS-1 cells and treated with PBS (CT), pBF42, or pBF42-TRAIL (TRAIL) weekly until day 42 as described above. B, metastasis rate was calculated at necropsy by the proportion of mice bearing pulmonary tumor nodules. C, survival rate at day 42 is shown by Kaplan-Meier curve analysis ***, $P = 0.0025$.

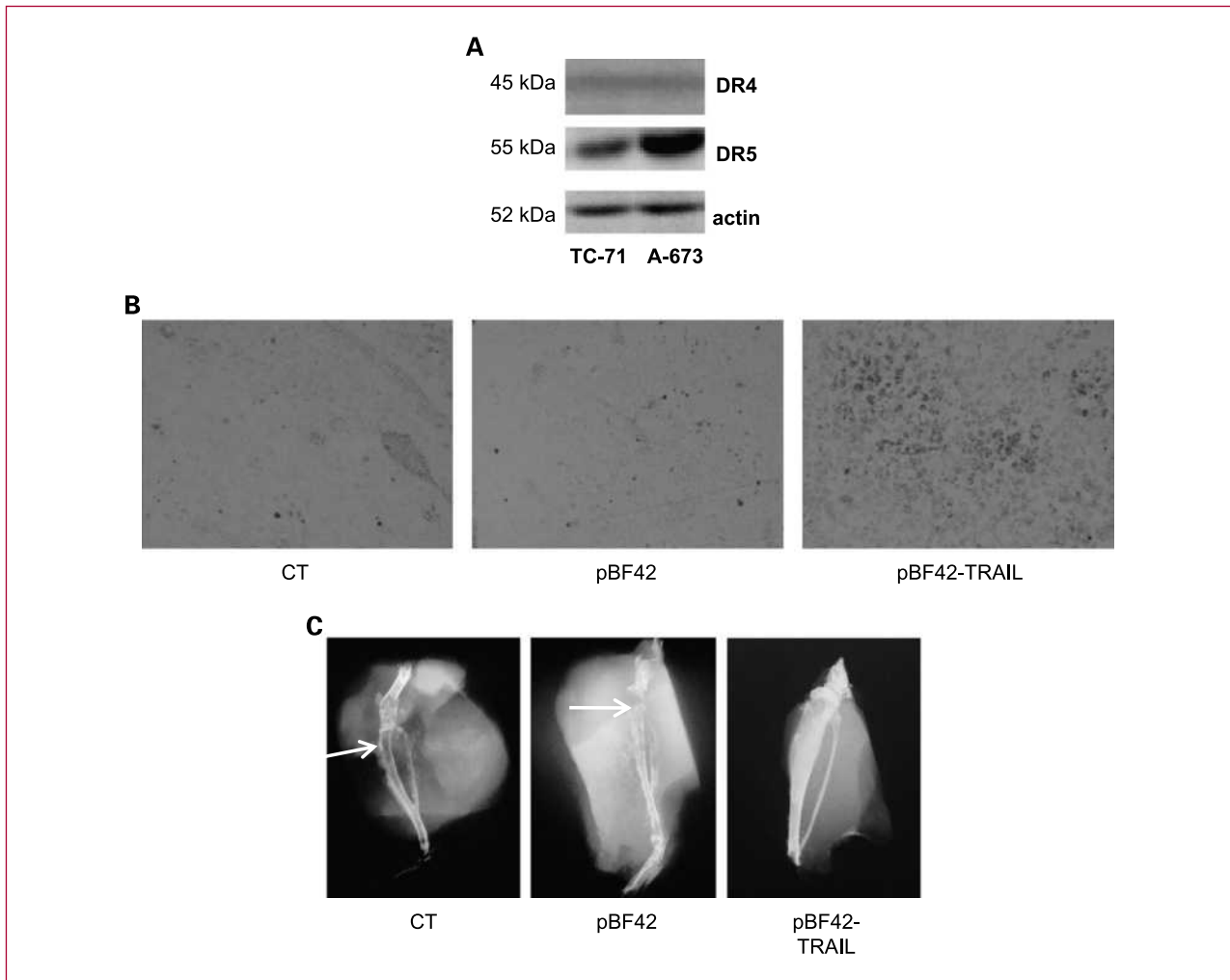


Fig. 5. TRAIL mechanisms of action *in vivo*: expression of death receptors, induction of apoptosis, and prevention of tumor-associated osteolysis. A, death receptors DR4 and DR5 expression was measured by Western blot in Ewing's sarcoma A673 tumor tissue after lysing and crushing the tissue as described in Material and Methods. B, TUNEL staining was done on 6- μ m sections of A673 Ewing's sarcoma tumors from empty pBF42- and pBF42-TRAIL-treated mice (magnification, $\times 200$). C, tumor-associated osteolysis was analyzed by radiography at day 40 on control mice (CT), mice treated with empty pBF42 plasmid (pBF42), or mice treated with pBF42-TRAIL formulation (pBF42-TRAIL). Arrows, osteolytic lesions.

leads to the development of lung nodules in 80% to 90% of mice (28); this model was further used to test TRAIL efficacy against lung nodule dissemination. The results presented in Fig. 4B show a significant decrease in the development of lung nodules counted at autopsy in the TRAIL-treated group, with only 12.5% of mice developing lung nodules versus 66.7% and 62.5% in the pBF42 empty plasmid- and the vehicle-treated control groups, respectively. Consequently, a significant increase in animal survival was observed in the TRAIL-treated group compared with the empty plasmid and vehicle-treated control groups, with, respectively, 89% of survival 42 days after tumor cell injection in the TRAIL group versus 33.3% and 25% for the control groups ($P = 0.0025$; Fig. 4C). These results show that TRAIL does not only exert an inhibitory effect on primary bone tumor growth but also on lung nodule dissemination.

Mechanisms of TRAIL action *in situ*

To better understand the mechanisms of action of TRAIL *in vivo*, several parameters have been analyzed as follows: the expression of death receptors by the tumor cells *in situ*, the induced apoptosis, and bone lesions. DR4 and DR5 expression analyzed in A673 and TC71 human Ewing's sarcoma tumors by Western blot shows a higher expression of DR5 compared with DR4 in tumors induced by both cell lines (Fig. 5A). This result confirms the difference already observed *in vitro* by FACS analysis on cultured A673 cells (Fig. 1B). Similar higher expression of DR5 compared with DR4 was observed in the osteosarcoma POS-1 tumor (data not shown). To analyze the antitumor effect of TRAIL *in vivo*, TUNEL staining was done on histologic sections from A673 tumors of TRAIL-treated mice versus controls and empty plasmid-treated mice. Results showed a higher proportion of TUNEL-stained cells in

tumors from the TRAIL-treated group compared with both control groups (90 positively stained cells in the TRAIL group versus 9 and 14 in the vehicle-treated and pBF42 empty plasmid-treated groups, respectively; Fig. 5B). As a consequence of tumor inhibition, TRAIL also diminished the osteolytic lesions associated with Ewing's sarcoma development (Fig. 5C). Radiographic analysis of tibia close to Ewing's sarcoma development show an intensive cortical bone degradation in the Ewing's sarcoma-bearing mice or in pBF42 empty plasmid-treated animals, which was totally prevented in pBF42-hTRAIL-treated animals (Fig. 5C).

Taken together, these results show that TRAIL induces a significant slowdown of tumor progression and an increase in animal survival in both Ewing's Sarcoma and osteosarcoma models by exerting a direct effect on tumor cells, as shown both *in vitro* (Fig. 1C and D) and *in vivo* (Fig. 5B).

Discussion

Osteosarcoma and Ewing's sarcoma are rare primary bone tumors with a poor prognosis for patients with disease relapse or with metastases at diagnosis. There is an urgent need to develop new adjuvant therapeutic approaches to improve patient survival and to diminish chemotherapy dosing. TRAIL, a member of the TNF superfamily, represents a promising potential therapeutic option owing to its ability to kill transformed cells. Several studies have reported TRAIL efficacy in Ewing's sarcoma and osteosarcoma cell lines *in vitro* (33, 34), but very few preclinical studies are available (35).

TRAIL can bind five cognate receptors and several studies done with tumor cell lines showed that TRAIL sensitivity is linked to the presence of activating DR4 and/or DR5 death receptors expressed on the surface of tumor cells (36). We confirm that osteosarcoma and Ewing's sarcoma cell lines sensitive to TRAIL express the death receptors DR4 and DR5 at the mRNA level and mostly DR5 at the cell membrane as evidenced by cytometry analysis in cell culture or by Western blot *in situ*. This finding may be related to publications that describe that DR5 acts as the primary transducer of the TRAIL signal (37). Similarly, it has been reported that normal cells are sensitized to TRAIL when DR5 is upregulated by overexpression of *c-myc* or oncogenic ras mutants (38).

The actions of TRAIL are generally thought to be mediated through the activation of the extrinsic apoptotic pathway, but the intrinsic apoptotic pathway may also be involved through the cleavage of Bid. Depending on the cell line, the intrinsic pathway may be necessary as a primary mechanism of apoptosis or serve to amplify the signal (39). In the sarcomas and osteosarcoma cell lines, we confirmed that TRAIL-mediated apoptosis is caspase dependent, as shown by caspase-3 and caspase-8 activation within 2 hours of TRAIL treatment. The intrinsic pathway does not seem to be involved, as no Bid cleavage could be detected (data not shown).

To test the effectiveness of TRAIL therapy *in vivo*, preclinical models of Ewing's sarcoma and osteosarcoma induced, respectively, by injection of human A673 or murine POS-1 cells were used. These models closely reproduce the clinical development of bone tumors, as the tumor normally develops in the vicinity of the bone, stimulates degradation of the cortical bone through osteoclasts, and then invades the medullar cavity, with development of extensive osteolysis. TRAIL delivery as a recombinant protein has been effective in limiting tumor development in several preclinical models of solid tumors, including lung and breast carcinoma (40–42), as well as in a xenograft model of Ewing's sarcoma (35). A novel approach was used in the present study by the use of the cationic lipid vector KLN143. Previous studies from our laboratory using other synthetic vectors, such as block copolymers, have shown the feasibility of providing efficient and long-lasting protein expression using nonviral gene transfer approaches in osteosarcoma (43). Synthetic vectors present real interest for subsequent clinical application, as a nontoxic alternative to gene delivery through retroviral vectors, which is associated with toxicity due to innate and adaptive immune responses. TRAIL overexpression through gene delivery using cationic vectors was validated *in vivo* both at the local (muscle) and systemic (serum) level. It was also validated at the tumor level (even if the tumors were small) by ELISA on the tumor tissue. The biological activity of TRAIL was evidenced by inhibition of tumor progression and tumor incidence as a result of direct tumor cell apoptosis, which was confirmed by TUNEL staining on histologic sections.

TRAIL-induced inhibition of tumor growth was accompanied by a prevention of associated bone osteolysis. Bone osteolysis represents an important feature of primary bone tumor development and can significantly diminish the patient's quality of life. This is the first study to show that TRAIL inhibits not only the tumor development but also the tumor-associated osteolysis observed in primary bone tumors. This effect could be explained by the blockade of the vicious cycle between tumor cells and osteoclasts that occurs during tumor development in bone more than a direct effect of TRAIL on osteoclasts. This theory was first hypothesized by Pajet in the late 19th century as the "seed and soil" theory (44). Subsequent studies showed that tumor cells produce osteoclast-activating factors such as receptor activator of NF- κ B ligand or TNF, which induce the differentiation and activation of osteoclast precursors (45, 46). When activated, osteoclasts degrade bone, allowing the release of growth factors stored in the bone matrix, including insulin-like growth factor, transforming growth factor, and fibroblast growth factors, which in turn activate tumor cell proliferation. We showed in this study that targeting tumor cells with TRAIL blocks this vicious cycle, leading to indirect inhibition of the severe bone lesions observed in Ewing's sarcoma for instance.

Our study also revealed that TRAIL not only decreased tumor growth and indirectly osteolysis but also significantly increased animal survival. Because osteosarcoma

and Ewing's sarcoma patient's death is mainly caused by metastasis dissemination in lungs, complementary studies were undertaken in a model of lung nodule dissemination. However, models of lung nodule dissemination derived from Ewing's sarcoma cell injection were difficult to set up with a poor incidence and variable number of lung nodules following 2 to 5×10^6 cells injected i.v. (32). It was therefore not possible to conduct preclinical studies with significant number of animals with lung nodules per group with homogenous nodule incidence. This is the reason why we have continued our studies with a model of lung nodules induced with POS-1 osteosarcoma cells. This model has already been published (29) and was induced by injection of tumor cells isolated from osteosarcoma into the retro-orbital sinus of the mice. In this model, TRAIL significantly prolonged animal survival associated with a strong inhibition of lung nodule formation. These results are consistent with previous studies in other tumor models, such as skin carcinoma (47), which have shown the ability of TRAIL to limit metastasis development.

Taken together, the results presented here are encouraging and warrant the evaluation of TRAIL therapy in clinical trials for Ewing's sarcoma and osteosarcoma. However, the major limitation of the therapeutic use of TRAIL is the occurrence of cellular resistance, which is attributed to several dysfunctions arising at various levels in the TRAIL signaling pathway, such as an imbalance in death/decoy receptors or upregulation of c-FLIP expression (16). Therefore, before TRAIL can be successfully used for the treatment of patients, it is crucial to study the mechanisms of resistance in Ewing's sarcoma. Nevertheless, it has been shown that ~70% of Ewing's sarcoma patients express one of the two death receptors DR4 or DR5 (48), suggest-

ing that TRAIL treatment may represent a good therapeutic option for these patients. Moreover, three clinical trials currently in progress using fully humanized agonist antibodies targeting DR4 or DR5 in solid tumors, including osteosarcoma, show encouraging results (19–21). TRAIL toxicity previously reported in several studies has been recently shown to be due to the exogenous poly-histidine tag of the recombinant TRAIL used in these clinical studies (49), which induces apoptosis of hepatocytes. A phase I/II clinical trial is currently in progress using a more stable version of TRAIL comprising the extracellular domain, showing promising results without dose-limiting toxicity or hepatotoxicity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank C. Bailly, C. Le Corre, A. Lefebvre, and M-A. Muller from the Experimental Therapy Unit Platform (IFR26, Nantes, France) for their technical assistance and G. Odri for his help with statistical analyses.

Grant Support

Association pour la Recherche sur le Cancer fellowship (F. Lamoureux), Ligue Contre le Cancer (comité régional des Pays de la Loire), Fondation de l'avenir, Institut National du Cancer n°R07022NN fellowship (G. Picarda), and Société Française des Cancers de l'Enfant.

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Received 07/08/2009; revised 02/02/2010; accepted 02/20/2010; published OnlineFirst 04/06/2010.

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