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Is Troponin I Gene Therapy Effective for Osteosarcoma Treatment? Study on a Human-like Orthotopic Rat Model

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Abstract. *Background: An anti-angiogenesis strategy has been widely recognized as a viable approach to fight cancer and more and more anti-angiogenic factors are continually being identified. Among them, the muscular isoform of Troponin I (TnI) has been described as being a powerful anti-angiogenic agent in vitro as well as in vivo. We investigated the therapeutic efficacy of TnI gene therapy in a human-like orthotopic rat osteosarcoma model. Materials and Methods: In this tumor model, we evaluated whether the administration of the secreted TnI coding sequence complexed to cationic liposomes (named TnITag cDNA/lCLP) could induce a delay in tumor growth and reduce tumor vasculature. Results: Although TnI specifically inhibited endothelial cell growth in vitro, we were not able to demonstrate any therapeutic efficacy of TnI in the transplantable osteosarcoma model. Conclusion: This lack of efficacy probably resulted from the rapid degradation of recombinant TnI by matrix metalloproteinases, especially MMP2, which are present in large amounts in tumors.*

Osteosarcoma is one of the most frequent primary bone tumors. It predominantly affects teenagers and young adults and is commonly localized in the lower extremities (1,2). Surgical resection of the primary tumor remains an essential element of the treatment, whereas the ensuing chemotherapy plays an essential role in the control of sub-clinical metastatic disease (3). However, tumors are often found to be resistant to these cytostatic agents. These tumors in particular, and tumors that relapse after treatment, may benefit from novel agents such as anti-angiogenic factors. It is now well accepted that angiogenesis is an essential component of tumor growth and metastatic spread and that tumor vascular density can be an indicator of tumor malignancy (4, 5). Therefore, angiogenesis has become a target for cancer treatment (6). One of the many anti-angiogenic substances identified is the muscular isoform of Troponin I (TnI). Moses was the first to demonstrate that this contractile protein is an inhibitor of angiogenesis and metastasis *in vivo* in a murine melanoma model. (7). However, we chose to evaluate the therapeutic potential of TnI gene therapy in a more clinically relevant situation than that employed by Moses. In this study, we tested whether or not the secreted TnI coding sequence complexed to cationic liposomes could induce a delay in tumor growth and metastases and reduce tumor vasculature in a human-like orthotopic osteosarcoma model grafted in immunocompetent rats.

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Materials and Methods

TnI expression plasmid construction and lipoplex formulation. The following modified primers were used to amplify human muscular Troponin I cDNA: TnI sense 5'-GCTATCGGAGATGA GGA GAAGCG-3'; TnI antisense: 5'- GCTACCGGACTCGGA CTC AAACAT-3'. The expression plasmid for TnI (named pSecTnITag) was constructed by cloning TnI cDNA (552 bp) into the plasmid pSecTag2 (Invitrogen, Groningen, Netherlands). TnI cDNA was cloned in frame with the IgK leader sequence located upstream from the cloning site and the 6His-cMyc epitope located downstream from the cloning site. The construct was verified by sequencing (Genomexpress, Grenoble, France). Vectors were amplified in *E coli* JM 109 (Promega, Madison, USA) and purified with Qiagen Endofree maxi and giga preparations (Qiagen, Courtaboeuf, France). These plasmids were complexed to DOTAP/Chol cationic liposomes formulated by Munich BiotechAG (8) (Munich, Germany) at a final DNA concentration of 0.2 mg/ml. We called the resulting cDNA/cationic liposome complexes TnITag cDNA/CLP and cDNA/CLP, respectively, for pSecTnITag/cationic liposome complexes and pSecTag/cationic liposome complexes.

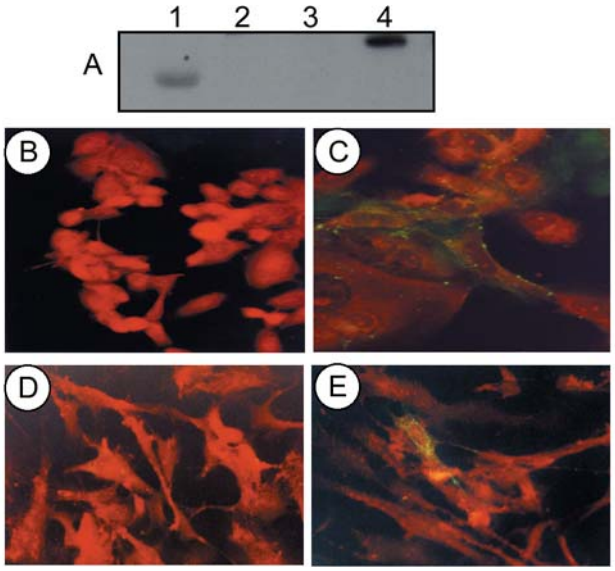

Figure 1. *Expression of recombinant TnI. A: Expression of TnI protein by 1547/pSecTnITag cells determined by Western blot. C-Myc tagged TnI is expressed as 24 kDa single band only by 1547/pSecTnITag cells (Lane 4) Lane 1: marker for molecular weight; Lane 2: untransfected 1547 cells; Lane 3: proteins from 1547 /pSecTag2 cells. B-E: Expression of TnI protein by 1547/pSecTnITag and EJG transfected cells determined by immunocytochemistry. C : 1547 Control cells; D : 1547/pSecTnITag cells (Magnification x 200) E: EJG Control cells. F: EJG/pSecTnI cells (Magnification x200).*

Cell lines. The osteosarcoma cell line 1547 (ATCC) and the capillary endothelial cell line EJG (ATCC) were cultured in MEM medium (Invitrogen), supplemented with 10% FCS (BioWhittaker, Wakersville, MD, USA), 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin (Invitrogen). EJG cells were additionally supplemented with 1% non-essential aminoacids (Invitrogen). For growth inhibition assays, EJG proliferation was stimulated by the addition of 6 ng/ml of b-FGF.

Immunodetection of recombinant TnI. The expression of TnI after cell transfection was verified by two immunodetection methods: Western Blot and *in situ* immunocytochemistry, due to the fusion of the TnI coding sequence with the c-Myc epitope.

Western blot. The 1547 cells were electroporated with pSecTnITag and pSecTag plasmids. The resulting cell sublines were referred to as 1547/pSecTnITag and 1547/pSecTag cells. Forty μg of total proteins, extracted from 5 x 10^6 1547, 1547/ pSecTnITag and 1547/pSecTag cells, were separated by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane for Western blotting with anti c-Myc antibody (dilution 1/1000; Serotec, Oxford, UK), followed by sheep horseradish peroxidase to mouse IgG (dilution 1/1000; Sigma, St Louis, Missouri, USA). The membrane was revealed by chemoluminescence with ECL (Amersham, Orsay, France) and autoradiography.

Immunocytochemistry. A c-Myc antibody (Invitrogen) was employed as primary antibody and a goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma) as secondary antibody. Forty-eight hours after transfection (Fugene 6, Roche, Meylan, France) with pSecTnITag or pSecTag plasmid, cells plated on slides were fixed with 3% paraformaldehyde and non specific antigenic sites were blocked in 10 % FCS in PBS. The cells were incubated with primary antibody (dilution 1/400) for 1 h at room temperature in a humidified atmosphere. The slides were washed twice and incubated with secondary antibody (1/400; Molecular Probes, Leiden, Netherlands) in a 1% counterstaining Evans Blue blocking solution for 1 h in the dark.

In vitro cell sensitivity to TnI. Cells were seeded in 24-well plates until they had reached 50-60 % confluence. The medium was then replaced by medium without FCS to which the different cDNA/cationic liposome complexes were added (1 μg DNA /ml – 2 μg DNA /ml)). After 1 h incubation, we replaced the transfection medium by medium with FCS. The *in vitro* sensitivity of cell lines to cDNA/cationic liposome complexes was determined at different times after transfection using the trypan blue exclusion assay. For each cell population, cells from three wells were counted four times.

Experimental in vivo model. All procedures and care given to the animals were performed according to institutional guidelines. All experiments were carried out under general anesthesia with Isoflurane (2.5 %)/oxygen (2.5%) dispensed by a Minerve anesthesia apparatus (Minerve, Esternay, France). For all experiments Sprague – Dawley rats, 21- to 28-days-old were used. The experimental model used was described by Allouche (9). Small fragments (100 mm^3) of the hyperproliferative osteogenic tumor area were grafted into the right posterior leg of rats. After 8 days the tumor had reached an average diameter of 0.6 – 0.8 cm and tumor-bearing rats were randomized into two groups: one received the TnITag cDNA/CLP (4 rats) and the other received the cDNA/CLP (4 rats). Over a period of 2 weeks, an amount of DNA/cationic liposomes complexes corresponding to 250 μg of DNA was intravenously administered three times a week to the animals. Over the treatment period, tumor evolution was evaluated by measuring the tumor volume calculated using Carlsonn's formula (10). At the end of the treatment, the animals were sacrificied. The tumors were fixed in 4% paraformaldehyde and 8-μm cryo-sections were cut from different tumor areas and submitted to histological and immunohistological analyses.

Tumor vascularisation and apoptosis detection. Tumor vessels and apoptotic areas were visualised by triple staining on frozen tumor sections blocked with 5% goat serum. Endothelial cells were detected with a mouse anti-rat RECA 1 primary antibody (1/400, Serotec); apoptotic cells were detected by a double staining TUNEL/DAPI. TUNEL staining was performed with the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) and DAPI was used at a concentration of 1 μg/mL in PBS. The secondary antibody was Cy3-goat anti-mouse IgG (1/400; Jackson Immunoresearch, West Grove, PA, USA).

Statistical analysis. All data are presented as means±SEM. Differences from the mean were tested for significance by means of the Student's *t*-test using Microsoft Excel software. The level of statistical significance was set at $p<0.05$.

Results

TnI expression by transfected cells. After electroporation of the osteosarcoma cells, a signal of 24 kD was detected by Western blot only for the 1547/psecTnITag cells. This signal correlated

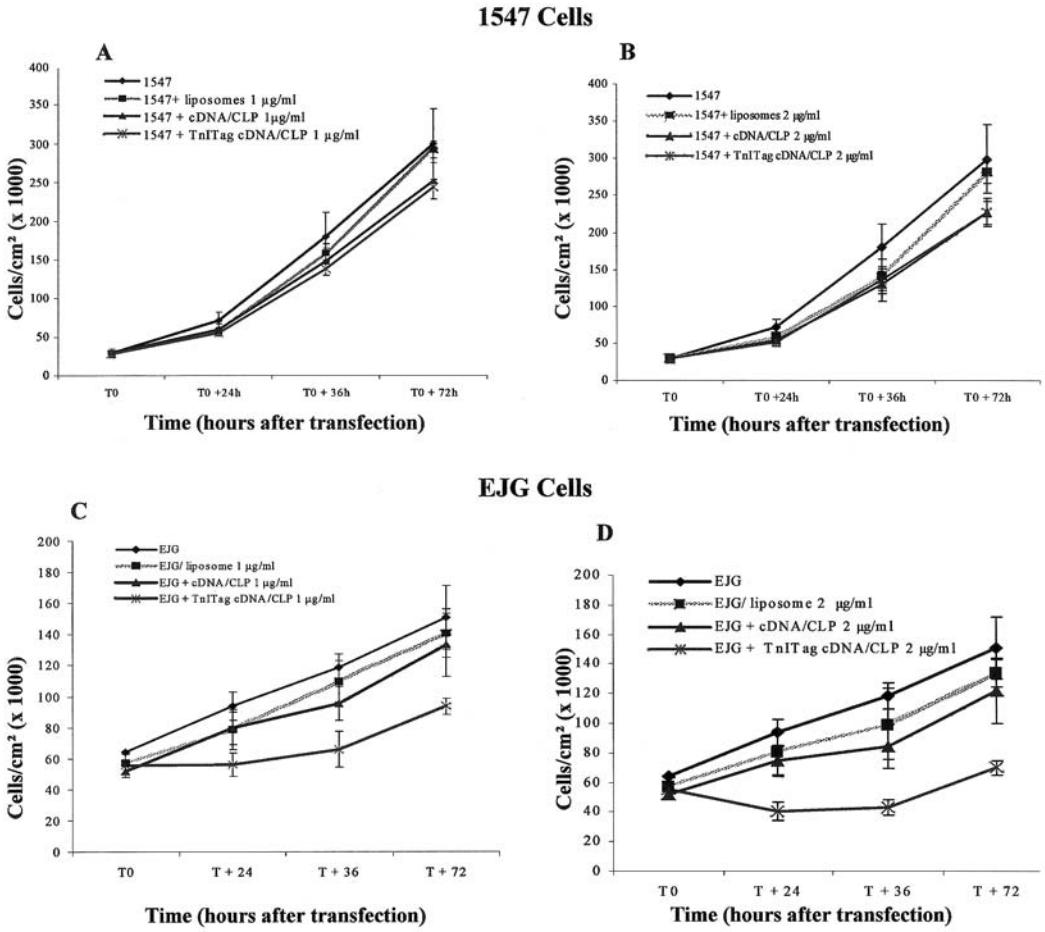

Figure 2. *Comparative effect of pSecTnITag/lipoplexes on osteosarcoma and endothelial cells growth in vitro. A-B: 1547 cell growth after transfection by lipoplexes corresponding to 1 µg DNA/ml (A) and 2 µg DNA/ml (B). No significant cell growth inhibition could be noted. C-D: EJG cell growth after transfection by lipoplexes corresponding to 1 µg DNA/ml (C) and 2 µg DNA/ml (D). A specific and significant ($p<0.05$) inhibition of EJG cell proliferation was observed after transfection by TnITag cDNA/CLP at both DNA concentrations used.*

with TnI expression by the transfected cells (Figure 1A). Specific TnI expression by pSecTnITag-transfected cells (1547 cells as well as EJG cells) was confirmed by indirect immunocytochemistry. This technique detects *in situ* protein expression in cultured cells. Goat anti-mouse antibody alone was used as control and was always found to be negative (Figure 1B and D). The results obtained are similar for the 1547 and EJG cells, whether they were stably or transiently transfected. A granular, cytoplasmic green staining was detected only in cells expressing recombinant TnI protein. Homogenous staining was observed, as expected, in 1547/pSecTnITag cells as these cells resulted from the selection of a 1547 cell clone after electroporation of 1547 cells with the pSecTnITag plasmid (Figure 1C). By contrast, the staining of the transiently transfected EJG cells was heterogenous; its intensity varied with the cells, indicating the variable expression of the fusion protein (Figure 1E). Both immunodetection methods showed that the recombinant plasmid enabled expression of the protein of interest after stable or transient cell transfection.

In vitro cell sensitivity to TnI. As shown in Figure 2A and B, no significant decrease in growth of 1547 cells was observed with DNA concentrations of 1 and 2 µg/ml, regardless of the plasmid used ($p>0.05$). Recombinant TnI coded by expression plasmid did not exert any suppressive effect on osteosarcoma cell proliferation.

The growth profiles of the EJG cells after transfection were different from those observed, under the same conditions, for 1547 cells (Figure 2C and D). Whereas cell growth was not significantly slowed after transfection with liposomes or cDNA/CLP, it was significantly suppressed for 36 h when the cells were transfected with the TnITag cDNA/CLP (Figure 2C and D) ($p<0.05$). EJG cells resumed normal growth 72 h after transfection. The transient inhibition of proliferation we

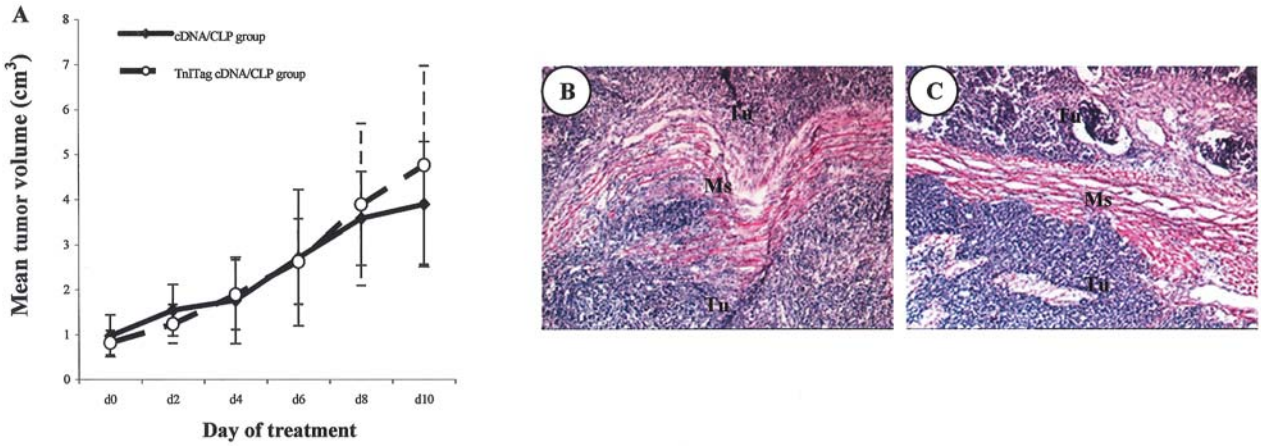

Figure 3. *Effect of TnITag cDNA/CLP on primary tumor growth. A: Evolution of mean tumor volume during the treatment period. The curves represent the mean tumor volume evolution of each group of animals. B and C: Histological analyses of tumor. B: Tumor from a cDNA/CLP animal. (Magnification x 50). C: Tumor from a TnITag cDNA/CLP-treated animal. (Magnification x 50). No differences in tumor structures could be observed after TnITag cDNA/CLP treatment. As in the cDNA/CLP group, tumors of the TnITag cDNA/CLP group invaded the surrounding muscular tissue. Ms: skeletal muscle; Tu: Tumor.*

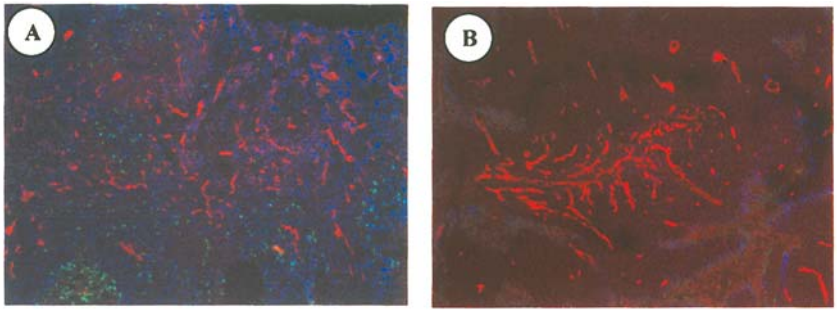

Figure 4. *Analysis of tumor vascular density and apoptosis after treatment. (A) Tumor section from cDNA/CLP rat. High vascular density with a chaotic vascular network and numerous small vessels (stained in red) was observed at the tumor periphery. No apoptosis was detected in endothelial cells and TUNEL-positive tumor cells were rare and isolated in the hyperproliferative area (intense DAPI staining). (B) Tumor section from TnITag cDNA/CLP rat. Tumor vessel density showed no differences compared to cDNA/CLP rats. Vessel distribution was as chaotic as in control tumors. No increase in apoptotic endothelial cells or tumor cells could be detected.*

observed showed that transfection with cationic liposomes resulted in an effective level of gene expression for 48 h. After this period, the expression level was too weak to produce an observable inhibitory effect and the cells resumed growing. This delay is apparently an intrinsic characteristic of the liposomes we used, as we observed it in all experiments, regardless of the cell lines used (data not shown).

In vivo effect of TnI cDNA expression on rat osteosarcoma model. During the treatment, the tumor evolution of the TnITag cDNA/CLP-treated animals was the same as that of cDNA/CLP-treated animals (Figure 3A). After six intravenous injections, the average tumor volume of the TnITag cDNA/CLP-treated group reached 4.7 cm^3 and was 15% greater than the mean tumor volume of the reference group at 3.9 cm^3 ($p>0.05$). Histological analysis performed at the end of the experiment confirmed that TnI had no effect on tumor growth and aggressiveness. No difference in tumor histological structure could be seen between the reference and the TnITag cDNA/CLP-treated groups (Figure 3B and C). All tumors infiltrated the adjacent muscular tissue and had a typical osteosarcoma histological structure, characterized by the presence of hyperproliferative peripheral regions with high cell density and many mitoses and necrotic central regions (Figure 3B and C).

The potential effect of TnI on tumor vasculature was analyzed in peripheral tumor zones, areas of the tumor that were normally the most densely vascularized. The administration of TnITag cDNA/CLP did not decrease tumor vascular density, as shown Figure 4. The tumor vascular network of both groups of animals was dense, composed of numerous irregular vessels and capillaries, sometimes regrouped in clusters forming regions of higher vascular density (Figure 4). The addition of double TUNEL and DAPI staining to RECA-1 labelled the tumor

vasculature and the possible apoptotic endothelial or tumor cells on the same slides. As for the vasculature, no increase in apoptotic tumor cells was seen in the TnITag cDNA/CLP-treated group compared to the reference group (Figure 4B).

TnI showed a poor effect *in vivo* compared to that observed *in vitro* using cell culture models. Under our experimetal conditions, TnI was unable to induce any delay in tumor growth or to decrease tumor vasculature.

Discussion

Inhibition of angiogenesis is an attractive target for anticancer therapy because the fundamental requirements for tumor growth are dependent on a blood supply (11). Moreover, unlike standard chemotherapy that targets tumor cells and other dividing cells, angiogenesis inhibitors target dividing endothelial cells that have been recruited into the tumor bed (12). Osteosarcoma is the typical example of a tumor that can benefit from anti-angiogenic gene therapy. Indeed, conventional osteosarcoma therapy is aggressive and its success relatively poor. It is therefore particularly important to develop new therapeutic strategies. On this basis, and with the objective of developing an osteosarcoma gene therapy, we studied the effects of TnI cDNA complexed to cationic liposomes on an human-like orthotopic rat osteosarcoma model.

In our study we confirmed the results of previous studies (8,13) that *in vitro* TnI is a specific inhibitor of endothelial cell proliferation. Although studies have suggested that TnI may interact with the bFGF receptor on endothelial cells and possibly that TnI competes with VEGF and bFGF on the cell surface for their receptor, the mechanism whereby TnI exerts its antiproliferative effect remains unknown (13).

More intriguing were the data obtained by applying TnI gene therapy *in vivo*. No inhibition of tumor growth and no decrease in tumor vascular density were observed after a course of six intravenous administrations of TnITag cDNA/CLP to osteosarcoma-bearing rats. The rat osteosarcoma model we used possesses a similar phenotype to osteosarcoma in humans (9, 14, 15). Like its human counterpart, this tumor model is characterized by destruction of cortical bone, invasion of the surrounding soft tissues and development of pulmonary metastasis (9). In contrast to most experimental tumor systems used in gene therapy assays (16,17), the experimental conditions of our study reproduced more successfully the clinical situation. The orthotopic osteosarcoma was grafted in immunocompetent animals and the treatment was administered to established growing tumors. Under these particular experimental conditions, TnITag cDNA/CLP seemed to have no therapeutic effect. We can suggest some hypotheses to explain why TnI gene therapy is ineffective, although the TnI protein was reported to be an angiogenesis inhibitor *in vivo* and to prevent metastasis in mice models (7, 18).

The lack of therapeutic effect could not be attributed to the absence of tumor vascular bed targeting by the DNA/CLP. We decided to use cationic liposomes as vectors for the gene therapy because of their specificity for targeting neovessels, particularly tumor neovessels (8,19). Such a gene therapy vector limits, in theory, the risks of diffusion of the anti-angiogenic agent in the body. The specific targeting of tumor neovessels by the cationic liposomes we used was demonstrated by studies of their biodistribution in our animal experimental model (data not shown): cationic liposomes significantly accumulated in osteosarcoma tissue compared to normal tissue. Thurston, and more recently Krasnici, showed that the accumulation of cationic liposomes and lipoplexes is 3.3 times greater in tumors than in the surrounding muscle tissue (8, 20). The mechanism responsible for the interaction of liposomes with endothelial cells is related to the respective charges carried by endothelial cells and cationic liposomes (19). The absence of effect observed is most probably due to the TnI coded by the expression plasmid itself, rather than being related to the liposomes:

i) TnI expressed by our plasmid construct was fused with a Myc epitope. The presence of this epitope might cause the protein to assume a three-dimensional structure *in vivo* that hides the active site (anti-angiogenic site) of TnI that was demonstrated to be located between Glu 94 and Leu 123 (18).

ii) To improve transfection efficiency, we fused the TnI coding sequence to the IgK secretion sequence of the plasmid. TnI recombinant protein is expressed in endothelial cells and then released in the environment. It is known that, in a growing tumor, proteins and enzymes that promote tumor angiogenesis are present at high levels (21). Among these proteins are the MMPs. These endopeptidases are involved in degradation of the basement membrane and remodelling of the extracellular matrix during physiological and pathological conditions such as embryonic development, wound healing and tumor angiogenesis (22). It has been reported that TnI is susceptible to proteolytic degradation by MMP-2 (23, 24). Thus, we can suppose that the secreted recombinant TnI is rapidly degraded by MMP2 before exerting its anti-angiogenic action.

As suggested by the results of Moses, and more recently by Kern, TnI might exert its anti-angiogenic activity in the early stages of angiogenesis. In the model described by Moses, TnI was administered 24 h after inoculation of tumor cells in the mice (7). Kern administered TnI peptide prior to the tumor cells (18). The fact that an anti-angiogenic action of TnI was then observed might indicate that this protein suppresses an early stage of angiogenesis. It is known that angiogenesis inhibitors do not exert their effect at the same stage of angiogenesis, some being effective against the early steps of this process whereas others, like endostatin, possess a wider spectrum of action (25, 26). This leads us to suggest that we administered TnI at an advanced stage of angiogenesis, when tumor vascular supply was already established. This highlights

the importance of appling the anti-angiogenic therapy at the early stages of angiogenesis, when new blood vessels are functionally active but lacking a perycite coating (27). If these developing blood vessels were targeted at this time-point, termed the "plasticity window", the treatment is expected to be more effective than after pericyte coating (28).

Although the findings of this study show no therapeutic effectiveness of TnI gene therapy, it is probably due to the secreted form of the protein and this lack of effectiveness cannot be generalized to other anti-angiogenic agents. In fact, in a similar study done under the same experimental conditions with endostatin as the anti-angiogenic gene, we showed the capacity of this agent to delay tumor growth and metastatic spread (in press). There is increasing evidence that targeting angiogenesis is the correct way to gain control over osteosarcoma (29-30). Whether Troponin I can become a useful agent for osteosarcoma gene therapy requires further studies.

Acknowledgements

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