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A new anti-tumor strategy based on *in vivo* tumstatin overexpression after plasmid electrotransfer in muscle

Jessica Thevenard [a], Laurent Ramont [a,d], Lluis M. Mir [b,c], Aurélie Dupont-Deshorgue [a], François-Xavier Maquart [a,d], Jean-Claude Monboisse [a,d], Sylvie Brassart-Pasco [a,*]

[a] FRE CNRS/URCA 3481, University of Reims Champagne-Ardenne, 51 rue Cognacq Jay, F-51095 Reims, France
[b] CNRS, UMR 8203, Institut Gustave Roussy, 114, Rue Edouard Vaillant, F-94805 Villejuif Cedex, France
[c] Université Paris-Sud, UMR 8203, Institut Gustave Roussy, 114, Rue Edouard Vaillant, F-94405 Orsay Cedex, France
[d] CHU de Reims, Avenue du Général Koenig, F-51092 Reims, France

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ABSTRACT

The NC1 domains from the different α(IV) collagen chains were found to exert anti-tumorigenic and/or anti-angiogenic activities. A limitation to the therapeutic use of these matrikines is the large amount of purified recombinant proteins, in the milligram range in mice that should be administered daily throughout the experimental procedures. In the current study, we developed a new therapeutic approach based on tumstatin (NC1α3(IV)) overexpression *in vivo* in a mouse melanoma model. Gene electrotransfer of naked plasmid DNA (pDNA) is particularly attractive because of its simplicity, its lack of immune responsiveness and its safety. The pDNA electrotransfer in muscle mediates a substantial gene expression that lasts several months. A pVAX1© vector containing the tumstatin cDNA was injected into the legs of C57BL/6 mice and submitted to electrotranfer. Sera were collected at different times and tumstatin was quantified by ELISA. Tumstatin secretion reached a plateau at day 21 with an expression level of 12 μg/mL. For testing the effects of tumstatin expression on tumor growth *in vivo*, B16F1 melanoma cells were subcutaneously injected in mice 7 days after empty pVAX1© (Mock) or pVAX1©–tumstatin electrotransfer. Tumstatin expression triggered a large decrease in tumor growth and an increase in mouse survival. This new therapeutic approach seems promising to inhibit tumor progression *in vivo*.

1. Introduction

The extracellular matrix plays a pivotal role in cellular migration, proliferation, and gene regulation during tumor progression. A new class of ligands, the matrikines [1], have been characterized as subdomains of various ECM proteins capable to bind cell surface receptors and to trigger intracellular pathways leading to a modified cell behavior. Among them, the NC1 domains of the different α(IV) chains were shown to exert anti-angiogenic and/or anti-tumor properties [2], and mainly tumstatin, the 28 kDa NC1 domain of the α3 chain of type IV collagen (α3(IV)NC1). α3(IV) collagen chain knock-out mice (COL4A3$^{-/-}$) are consequently deficient in tumstatin and an increased pathological angiogenesis and accelerated tumor growth was observed. This effect could be reversed if exogenous tumstatin was administered to the mice at physiological circulating concentration [3]. *In vivo* overexpression of tumstatin domains by tumor cells was also shown to inhibit the invasive properties of these cells in a mouse melanoma model [4]. A fragment of tumstatin containing amino acids 54–132 inhibited endothelial cell proliferation through a RGD-independent alpha(v)beta(3) integrin binding site [5,6]. The anti-angiogenic site was further localized to a 25 amino acid peptide (T7-peptide) consisting of the residues 74–98 [7]. Another tumstatin derived-fragment encompassing residues 185–203 also inhibited the proliferation and migration of melanoma cells through a RGD-independent alpha(v)beta(3) integrin binding site [8–10]. Matrikine overexpression has been used in different cancer mouse experimental models by using viral vectors or matrikine-encoding cDNA alone or in combination with conventional chemotherapies or ionizing radiation [11]. In the different models, the overexpression of a matrikine induced a decrease in tumor growth or an enhanced response to chemotherapy or ionizing radiation.

Gene electrotransfer of naked plasmid DNA (pDNA) is particularly attractive because of its simplicity, its lack of immune responsiveness and its safety. The pDNA electrotransfer in muscle mediates a substantial gene expression that lasts for several months, as demonstrated in mouse [12–14]. In the current study, we developed a

* Corresponding author. Address: Laboratoire de Biochimie, UFR Medecine, F-51095 Reims, France. Fax: +33 326918055.
E-mail addresses: jessica.thevenard@univ-reims.fr (J. Thevenard), lramont@chu-reims.fr (L. Ramont), luis.mir@igr.fr (L.M. Mir), aurelie.dupont@univ-reims.fr (A. Dupont-Deshorgue), fmaquart@chu-reims.fr (F.-X. Maquart), jc.monboisse@univ-reims.fr (J.-C. Monboisse), sylvie.brassart-pasco@univ-reims.fr (S. Brassart-Pasco).

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new experimental therapeutic approach based on *in vivo* tumstatin overexpression, induced by cDNA electrotransfer, in a mouse model. We showed that the tumstatin overexpression induced a strong decrease in tumor growth and enhanced mouse survival.

2. Materials and methods

2.1. Reagents

Culture reagents, molecular biology products, G418, LIPOFECTAMINE 2000 Reagent and pVAX1© vector were from Gibco BRL (distributed by Fisher Sc, Illkirch, France). The anti-NC1[α3(IV)] polyclonal antibody was developed by Covalab (Villeurbanne, France). It was raised in rabbit against the CNYYSNSYSFWLASLNPER peptide of murine NC1[α3(IV)] domain [4].

2.2. Cell culture

B16F1 cells, a lung metastatic subline of murine B16 melanoma, were a generous gift from Dr. M. Grégoire (INSERM UMRS 419, Nantes, France). Cells were grown in RPMI 1640 medium supplemented with 5% Fetal Bovine Serum (FBS) in 25 cm^2 flasks (Nunclon, VWR International, Strasbourg, France) at 37 °C in a humid atmosphere (5% CO_2, 95% air).

2.3. Cloning of the tumstatin domain

The sequence encoding the murine NC1[α3(IV)1–232] (tumstatin) region was amplified by reverse transcription-polymerase chain reaction from kidney mRNA using the following sets of primers: 5′-TTAAAGGGAAATCCTGGTGAC-3′ and 5′-GTTCTGGTTTCTT TGATTTCG-3′. The resulting cDNA fragment was cloned into a pVAX1© vector. The orientation and the complete sequence of the insert were verified by sequencing. The pVAX1©expression vector is a 3.0 kb plasmid vector designed for use in the development of DNA vaccines. It contains a human cytomegalovirus (CMV) promoter that drives a high level expression while the hGH polyadenylation signal enhances mRNA longevity. The orientation and the complete sequence of the insert were verified by sequencing.

2.4. Plasmid electrotransfer into skeletal muscle

Female C57BL/6 mice were purchased from Harlan (Gannat, France). All the animals were maintained with food and water ad libitum, with a 12-h light/dark cycle. All animal experiments were performed in level 2 animal facilities at the Faculty of Medicine and Pharmacy of Reims, in accordance with the CNRS institutional guidelines (http://ethique.ipbs.fr/) and in conformity with the French Ministry of Research and Agriculture Charter on Animal Experimentation Ethics. Procedure of animal study was approved by the Ethics Committee of the Research Federative Structure (SFR CAP-Santé) of the Reims Champagne-Ardenne University. Before the pDNA electrotransfer procedure, mice were anesthetized with ketamine (100 mg/kg body weight; Ketalar, Panpharma, France) and xylazine (40 mg/kg; Rompun, Bayer, France) injected intraperitoneally. In all experiments, both posterior legs of each mouse underwent electrotransfer. The experimental conditions for electrotransfer have already been described [12]. Briefly, thirty micrograms of plasmids in 30 μL of sterile 0.9% saline were injected into each *tibialis cranialis* of 8-week-old C57Bl6 mice using a Hamilton syringe. One pulse of 700 V/cm and 100 μs of duration followed 1 s later by a pulse of 100 V/cm and 400 ms of duration [15] were applied by two stainless steel plate electrodes, approximately 5.7 mm apart, placed at each side of the leg, at a repetition frequency of 1 Hz using a Cliniporator™ device (IGEA, Carpi, Italy). The control group received pVAX1© empty plasmid injections (Mock), and the tumstatin group was injected with the pVAX1©–tumstatin plasmid.

2.5. Tumstatin immunodetection

To detect tumstatin by Western blot, muscles were extracted in 0.5 mM Tris–HCl buffer, pH 7.6, 0.5 M NaCl, 0.1% SDS, 1 mM PMSF, 5 mM EDTA, 1 mM iodoacetamide, homogenized on ice using an ultra-turrax (13,500 rpm). Lysates were centrifuged at 10,000 g and the supernatants were kept frozen. 50 μg of proteins were separated by SDS–PAGE and transferred to an Immobilon-PTM membrane. After blocking with 5% non-fat dry milk in TBS buffer (50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 8.0) containing 0.1% Tween 20 (TBS-T), the membrane was incubated with an anti-NC1[α3(IV)] antibody (1/1000 in TBS-T containing 1% non fat dry milk) overnight at 4 °C. Then, the membrane was washed in TBS-T buffer and incubated with a peroxidase-conjugated goat anti-rabbit IgG (1/10,000) for 1 h 30 min. Signals were detected using an ECL plus Western Blotting Detection System(GE Healthcare, Orsay, France), according to the manufacturer's instructions.

2.6. Tumstatin enzyme-linked immunosorbent assay

96 well-plates were coated overnight at room temperature with recombinant tumstatin (calibrator) or the murine serum in 0.05 M sodium carbonate/sodium bicarbonate buffer. The plates were washed 3 times with 0.1% Tween 20, 50 mM Tris–HCl, buffer, pH 7.5 150 mM NaCl (TBS-T), saturated with 1% BSA in TBS-T for 1 h at 37 °C, washed 3 times with TBS-T, incubated with the anti-tumstatin antibody (dilution: 1/500) for 2 h at room temperature, washed 3 times with TBS-T, incubated with the secondary antibody (dilution: 1/10,000) for 1 h at room temperature, washed 3 times with TBS-T and once with TBS and incubated with 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate (Sigma–Aldrich, Saint Quentin Fallavier, France) for 30 min in the dark. The reaction was stopped by addition of 0.5 M H_2SO_4. The absorbance was read at 450 nm.

2.7. In vivo tumor growth and survival measurement

Seven days after electrotransfer, 2.5×10^5 B16F1 murine melanoma cells were subcutaneously injected into the left side of mice. Each group contains at least 7 mice. Tumor volumes were determined according to $v = ½ A \times B^2$, where A denotes the largest diameter of the tumor and B represents the largest diameter perpendicular to the first one [16]. For ethical reasons, mice were sacrificed when the tumor reached 1 cm^3.

2.8. Statistical analyses

For *in vivo* experiments, volumes of primary tumors were statistically analyzed using the non parametric *u*-test of Mann and Whitney. Results were expressed as mean ± SE.

3. Results

3.1. pVAX1©–tumstatin electrotranfer in C57BL/6 mouse leg induces tumstatin secretion in vivo

To induce tumstatin overexpression in mice, the pVAX1© containing the cDNA encoding the tumstatin domain was injected in each *tibialis cranialis* muscle of 8-week-old C57BL/6 mice and electrotransfer was realized using a Cliniporator™ as described in Section 2. Sera and muscles were collected from mice at days 0,

1, 4, 7, 14, 21 and 28. The expression of tumstatin in muscles was checked by Western blot. The expression was detected at day 1 and reached a plateau at day 21 (Fig. 1). The sera were submitted to an enzyme-linked immunosorbent assay as described in Section 2. At day 0, an average of 0.2 μg/mL of tumstatin was detected in the sera before the electrotransfer. The concentration increased to 4 μg/mL as soon as day 1 and reached a plateau at about 12 μg/mL at day 21 (Fig. 2). Tumstatin-overexpressing mice were kept alive for 10 months without detectable side effects.

3.2. Tumstatin overexpression inhibits in vivo tumor growth

Seven days after the electrotransfer, 2.5×10^5 B16F1 murine melanoma cells were subcutaneously injected into the left side of mice. Tumors appeared at day 10 after the cells injection. Tumor volume was decreased by 66% at day 14 after tumor induction in tumstatin overexpressing mice versus control mice (Mock) (Fig. 3).

3.3. Tumstatin overexpression increases mice survival

Tumstatin overexpressing mice presented an increased survival (more than 30%) compared to control Mock-transfected mice. As shown in Fig. 4, survival curves differed significantly as soon as day 14 and the difference in the number of living mice was observed until the last day of the experimental period.

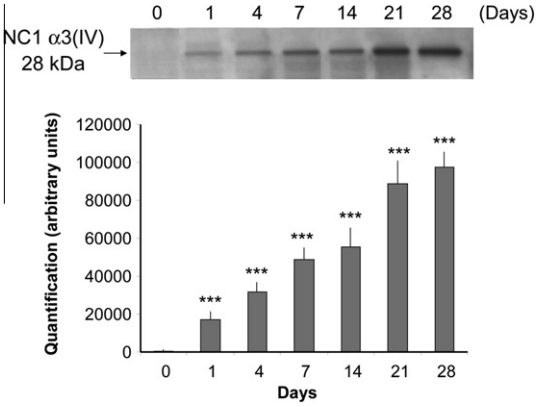

Fig. 1. pVAX1©–tumstatin electrotranfer in C57BL/6 mice leg induces tumstatin expression in muscle Thirty micrograms of pVAX1©–tumstatin were injected into each *tibialis cranialis* of 8-week-old C57BL/6 mice and electrotransferred. Muscles were collected from mice at days 0, 1, 4, 7, 14, 21, 28 and tumstatin expression was analyzed by Western blot. Quantifications were performed by densitometry using the Bio-1D software. Results are expressed as arbitrary units (AU). NS: Non significant. $^{***}p < 0.001$.

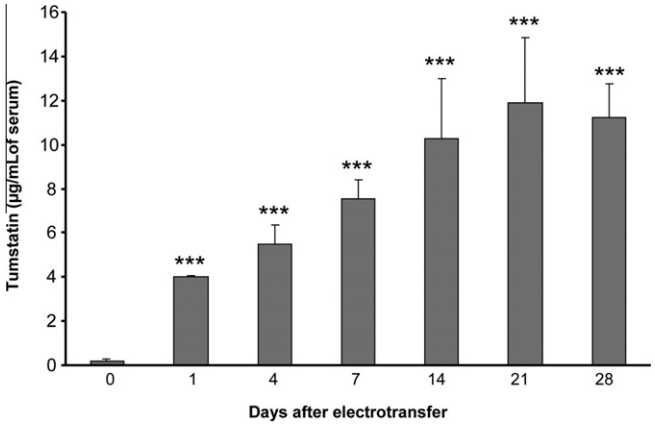

Fig. 2. pVAX1©–tumstatin electrotranfer in C57BL/6 mice leg induces tumstatin secretion *in vivo* Thirty micrograms of pVAX1©–tumstatin were injected into each *tibialis cranialis* of 8-week-old C57BL/6 mice and electrotransferred. Sera were collected from mice at days 0, 1, 4, 7, 14, 21, 28 and tumstatin content was measured by ELISA. Results are expressed as the mean ($n = 3$ mice) ±SD; $^{***}p < 0.001$.

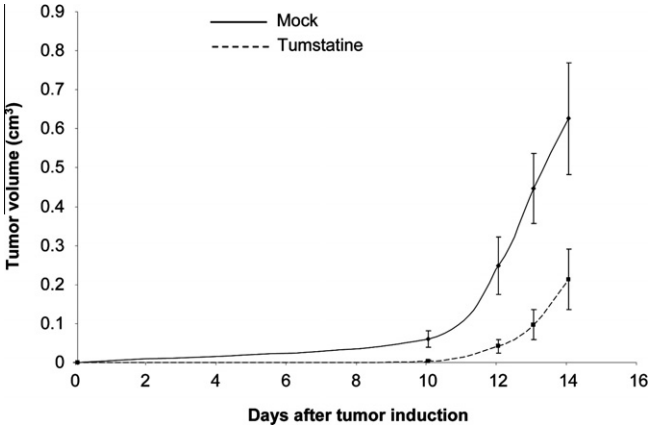

Fig. 3. Tumstatin overexpression inhibits *in vivo* tumor growth. The control group received pVAX1© empty plasmid injections (Mock), and the tumstatin group was injected with pVAX1©–tumstatin plasmid. Seven days after the electrotransfer, 2.5×10^5 B16F1 murine melanoma cells were subcutaneously injected into the left side of mice. Tumor volume was measured every two days after cell injection. Results are expressed as means ($n = 10$ mice) ±SD. $^{*}p < 0.05$; $^{**}p < 0.01$.

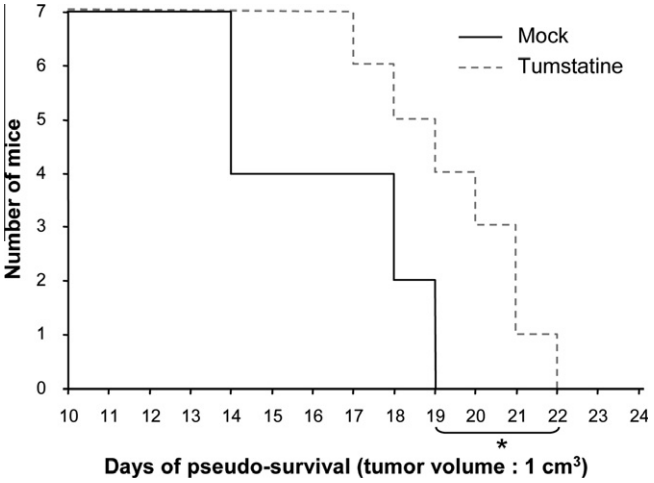

Fig. 4. Tumstatin overexpression increases mice survival. The control group received pVAX1© empty plasmid injections (Mock), and the tumstatin group was injected with pVAX1©–tumstatin plasmid. Seven days after the electrotransfer, 2.5×10^5 B16F1 murine melanoma cells were subcutaneously injected into the left side of mice. Mice were sacrificed when the tumor reached 1 cm^3. $^{*}p < 0.05$.

4. Discussion

Although the basement membrane provides a structural support for epithelial or endothelial cells, recent studies provide intriguing evidence that it also acts as a potential regulator of cell behavior. Among basement membrane components, type IV collagen plays a pivotal role in the regulation of cell proliferation, adhesion and migration either through its triple helical domain [17,18] or through the NC1 domains of its constitutive α(IV) chains [2]. Tumstatin exerts anti-angiogenic and anti-tumor properties [6,7,9] both *in vitro* and *in vivo*. A limitation to the therapeutic use of matrikines is the large amount of purified recombinant proteins that should be administered daily in mice throughout the experimental procedures. Gu et al. [19] have shown that doses of 10 mg/kg/day recombinant tumstatin (produced in *Escherichia coli*) were needed to inhibit tumor growth in BALBc mice injected with

B16 melanoma cells. More recently [20], Boosani et al., showed that doses of 1 mg/kg of recombinant tumstatin (produced in HEK 293 cells) were required to inhibit tumor growth in C57BL/6 mice injected with Lewis lung carcinoma (LLC) cells. Although bacterial expression of recombinant collagen IV derived matrikines results in high yields of protein, most of it is insoluble after dialysis, and unsuitable for peritoneal or peritumoral injections. The yield of recombinant matrikine production by eukaryotic cells is very low and requires long experimental processes [20].

Gene electrotransfer of naked plasmid DNA (pDNA) is a particularly attractive technology [12,13,21,22] because of its simplicity, its lack of immune responsiveness and its safety. The pDNA electrotransfer in muscle mediates a substantial gene expression that lasts several months as demonstrated by several studies in mouse [12,21] with circulating transgene levels reaching 10–250 ng/mL. In the present paper, we show that tumstatin cDNA electrotransfer into the mouse muscle rapidly led to tumstatin production in the muscle: the tumstatin domain was secreted by the muscle into the blood as soon as day one with a plateau at day 21. In our experiments, the basal expression of tumstatin in mouse serum was 200 ± 40 ng/mL. A tumstatin circulating physiological concentration of about 336 ± 28 ng/mL was also reported by Hamano and collaborators [3] in normal mice.

To study the effect of *in vivo* tumstatin overexpression, B16F1 melanoma cells were subcutaneously injected into the left side of C57BL/6 mice. *In vivo* tumor growth was decreased by 66%. In another experimental cancer model, doses of 1 mg/kg of recombinant tumstatin inhibited Lewis lung carcinoma tumor growth [20]. This is in accordance with our results. At day 21, 12 ± 3 μg/mL of tumstatin were detected in the serum of 30 g mice, corresponding approximately to 0.8 mg/kg. Yao et al. [23] also showed that intramuscular delivery of plasmid DNA encoding tumstatin (100 μg/100 μL), without electrotransfer, inhibited *in vivo* tumor growth in kidney or lung carcinomas. They did not detect tumstatin in the sera at day 0. This result is not in accordance with the literature [3,24]. In their model, recombinant tumstatin expression reached a plateau at about 30 ng/mL at day 20, suggesting that the induction of tumstatin expression is at least three times less efficient than that induced with DNA electrotransfer, compared with our results.

Up to now, a limitation to the therapeutic use of these matrikines was the large amount of purified recombinant proteins, in the milligram range, that should be administered daily in mice through experimental procedures. In the current study the new therapeutic approach we develop is based on *in vivo* tumstatin overexpression using gene electrotransfer. This approach only requires a unique injection, is less invasive and safer than the daily injection of important doses of recombinant protein. This therapy could also be used in combination with other anti-tumor agents, as demonstrated in several experimental murine cancer models, with gencitabine or ionizing radiation. In these models, the use of *in vivo* induced matrikine overexpression strongly increased the inhibitory potential of the conventional therapies [11].

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