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Research paper

Antitumoral and antimetastatic effect of antiangiogenic plasmids in B16 melanoma: Higher efficiency of the recombinant disintegrin domain of ADAM 15

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ABSTRACT

Background: Despite the discovery of novel inhibitors of tumor angiogenesis, protein-based antiangiogenic cancer therapy suffers some limitations that antiangiogenic gene therapy could overcome. We investigated whether intra-tumoral electrotransfer of three angiogenic plasmids could inhibit tumor growth and metastasis.

Methods: Plasmids encoding recombinant disintegrin domain of ADAM-15 (RDD), thrombospondin 1 (TSP-1), and the soluble isoform of the VEGF receptor 1 (sFlt-1) were injected into B16F10 melanoma-bearing C57BL/6 mice followed by electroporation. Tumor volume was measured daily using a digital caliper. Metastasis was monitored by *in vivo* bioluminescence after surgical removal of the primary luciferase-encoding B16F10 tumor 5 days after intra-tumoral electrotransfer. Markers of vascularization and cell proliferation were quantified by immunohistochemistry.

Results: Intra-tumoral electrotransfer of the antiangiogenic plasmids induced a significant inhibition of tumor growth, doubling of mean survival time and long-term survivors (~40% vs 0% in control). When the tumor was removed by surgery after intra-tumoral plasmid electrotransfer, a significant decrease in tumor metastasis was observed leading to long-term tumor-free survival especially after treatment with pRDD plasmid (84% vs 0% in control). Unlike pTSP-1 and psFlt-1, pRDD significantly decreased cell proliferation in B16F10 primary tumors which express $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins. No effect of antiangiogenic plasmid electrotransfer on normal skin blood flow was detected.

Conclusion: The intra-tumoral electrotransfer of the three antiangiogenic plasmids is a promising method for the treatment of melanoma. The plasmid encoding RDD seems to be particularly effective due to its direct antitumoral activity combined with angiogenesis suppression, and its marked inhibition of metastasis.

1. Introduction

Since Folkman [1] suggested, more than 30 years ago, inhibiting tumor growth and metastasis by interfering with tumor angiogenesis, numerous inhibitors of tumor angiogenesis have been discovered. These molecules are mainly proteins and peptides. Despite the fact that these drugs became more easily available over the last years, an antiangiogenic protein-based therapy presents several drawbacks: (i) expensive processes for protein production and purification, (ii) a short biological half-life requiring frequent dosing to maintain plasma level, (iii) invasiveness of injection, (iv) systemic toxicity. This toxicity results mainly from hypertensive and prothrombotic activities due to non-targeted delivery [2,3]. A strategy to overcome these issues may be the use of gene therapy for a sustained and localized expression of the antiangiogenic protein [4]. Over the last decades, various techniques have been developed to deliver DNA to a variety of target tissues, including viral and non-viral methods. The safety concerns arising from the use of viral vectors encouraged the use of non-viral techniques. Among these, electroporation is one of the most efficient *in vivo* [5]. Electroporation has been used as a means of introducing macromolecules, including naked plasmid DNA, in cells and is widely used for gene therapy and DNA vaccines in preclinical and clinical studies [5–7]. Intra-tumoral electrochemotherapy with bleomycin and gene therapy with IL-12-encoding plasmid have been demonstrated to be

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effective and well tolerated in preclinical studies [8] and clinical trials [9–11].

The ADAM (a disintegrin and metalloproteinase protein) family is involved in diverse biological functions including cell adhesion, migration and proteolysis, and is also involved in cancer. Many ADAMs are expressed in malignant tumors and are involved in the regulation of growth factor activities and integrin function [12]. The ADAM proteins have a particular sequence of domains including a disintegrin domain. The primary role of the disintegrin domain is to mediate cell–cell interactions especially in binding ADAMs to integrins. The disintegrin domain of ADAM-15 binds to αvβ3 and α5β1 integrins through its integrin-binding RGD motif [13]. It has been shown that the recombinant disintegrin domain (RDD) of ADAM-15 inhibits endothelial cell proliferation, adhesion and invasion *in vitro*. Moreover, *in vivo* electrotransfer of a plasmid encoding RDD inhibits angiogenesis, tumor growth, and tumor metastasis [14].

Thrombospondin-1 (TSP1) is one of the first described endogenous extracellular matrix-associated proteins that inhibit angiogenesis [15]. TSP-1 expression is frequently lost in cancer progression and its overexpression suppresses tumor growth [2]. TSP-1 has been shown to suppress tumor growth by inhibiting angiogenesis through activation of transforming growth factor beta 1 (TGFβ1). It directly inhibits endothelial cell migration induced by VEGF and could affect tumor cell through interaction with cell surface receptors and extracellular proteases [16]. Several studies have reported that the antiangiogenic activity of TSP-1 is mediated by the membrane protein CD36 [17]. The amino acids responsible for the binding of TSP-1 to CD36 have been located in the type-1 repeats of TSP-1 (TSRs). TSRs were also shown to bind (TGFβ1) which is a known endogenous regulator of angiogenesis and anti-oncogenic factor [18]. Miao et al. reported that TSRs inhibit the growth of B16F10 tumors through inhibition of angiogenesis and activation of TGFβ [19].

The vascular endothelial growth factor (VEGF) family consists of five glycoproteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). VEGF is the key mediator of angiogenesis in cancer as the production of VEGF by tumors larger than 1–2 mm results in an angiogenic switch to form new tumor vasculature [20]. The vascular system resulting from tumor angiogenesis is structurally and functionally disorganized. The multiple effects of the VEGF family are mediated through tyrosine kinase receptor VEGFR-1 (Flt-1), VEGFR-2 (Flk-1) and VEGFR-3 (Flt-4). The weak tyrosine kinase activity of Flt-1 and its high affinity for VEGF-A, the key modulator of angiogenesis, suggest that Flt-1 acts as a decoy receptor and modulates angiogenesis by sequestering excess VEGF-A and preventing signaling driven by Flk-1 [21,22]. Through alternative splicing, the Flt-1 gene encodes a soluble variant of the receptor, sFlt-1. VEGF-A binds to sFlt-1 with an affinity that is much higher than for Flk-1. Since VEGF-A binding to sFlt-1 does not result in signal transmission, sFlt-1 could be a promising tool to inhibit VEGF-A-driven angiogenesis [23].

The aims of the present study were (i) to investigate whether intra-tumoral electrotransfer of plasmids could be used for localized antiangiogenic gene therapy, (ii) to compare the effect of different plasmids encoding antiangiogenic proteins on tumor growth, (iii) to compare the effect of different plasmids encoding antiangiogenic proteins on tumor metastasis, and (iv) to understand the mechanisms of action of these plasmids, in particular to determine whether their mechanisms of action are only due to their antiangiogenic effect. Hence, mice bearing B16F10 melanoma were treated by intra-tumoral electrotransfer of plasmids encoding: (i) the disintegrin domain of ADAM-15 (pRDD), (ii) thrombospondin 1 (pTSP-1), (iii) the soluble isoform of the VEGF receptor 1 (psFlt-1). Their effect on tumor growth and metastasis as well as tumor vasculature and proliferation were investigated.

2. Materials and methods

2.1. Plasmids

All plasmids had a ubiquitous CMV promoter but had different backbones. Because plasmid backbones differ, the quantity of DNA was normalized and expressed in pmole.

Plasmids encoding luciferase pVAX LUC (pLUC) or green fluorescent protein (GFP) pGL3 GFP Reporter Vector (pGFP) (Promega Benelux, Leiden, Netherlands) were used as control.

The RDD gene with the secretion signal of murine urokinase was excised by XbaI digestion from pBi-RDD plasmid [14] and inserted into XbaI restriction site of the MCS of pVAX1 (Invitrogen, Carlsbad, CA), i.e. between the CMV promoter and the BGH poly A to generate the pVAX-RDD plasmid (pRDD).

The plasmid encoding TSP-1 (pTSP-1) was kindly provided by Prof. S.S. Yoon (Division of Surgical Oncology, Massachusetts General Hospital, Boston, MA). This plasmid is built on a pSec/Tag2 backbone with CMV promoter [24].

The expression vector encoding soluble Flt1 was prepared as follows. The insert of pBLAST45-mFlt1 vector (Invivogen, San Diego, CA) was cut out with NcoI and NheI and amplified using the following sets of sense and antisense primers: 5′ primer, 5′-ACCATGG TCAGCTGCTGGGA-3′; 3′ primer, 5′-CTACACGGCCCCCTTCTG-3′ (Invitrogen, Carlsbad, CA, USA). The product was cloned into the expression plasmid pcDNA3.1/V5-His-TOPO® (Invitrogen), which contains the cytomegalovirus (CMV) immediate early promoter/enhancer (pFlt-1). The identity and orientation of the resulting construct was further confirmed by DNA sequencing.

Plasmids were prepared using an Endo-Free Qiagen Gigaprep kit, according to the manufacturer's protocol (Qiagen, Hilden, Germany). Their quality was assessed by the ratio of light absorption (260 nm/280 nm) and by 1% agarose gel electrophoresis before and after digestion with corresponding restriction enzymes. All plasmids were stored at −20 °C until use.

2.2. Intra-tumoral electrotransfer of antiangiogenic plasmids

Eight-week-old C57BL/6 male mice were bought from Elevage Janvier (Le Genest-St-Isle, France). Mice were anesthetized with a ketamine/xylazine mixture or with isoflurane (Isoba, Schering-Plough, Belgium). Murine B16F10 melanoma cells (ATCC, Manassas, VA) and luciferase-encoding B16F10 melanoma cells (B16-Luc) (Xenogen, Caliper Life Sciences, Hopkinton, MA) were maintained in DMEM supplemented with 10% decomplemented FBS and penicillin/streptomycin (Invitrogen).

One million B16F10 or B16-luc melanoma cells suspended in 50 μL PBS buffer were injected intradermally in the left flank of the mice. The size of the tumors was measured daily with a digital caliper (a = length, b = width). The volume of the tumor was calculated as the volume of a prolate spheroid: $4/3\pi \times b^2 \times a$ [25]. Antiangiogenic plasmids were delivered by intra-tumor electrotransfer when the tumor reached a volume between 15 and 30 mm^3. Plasmid electrotransfer was repeated 2 days later. For each delivery, 20 pmol of plasmid diluted in 50 μl PBS was injected into the tumor. The tumor was then placed between two stainless steel plate electrodes, 4 mm spaced. Conductive gel was used to ensure electrical contact with the tumor (EKO-GEL, ultrasound transmission gel, Egna, Italy). A high-voltage pulse (HV, 1250 V/cm, 100 μs) followed 1 s later by a low-voltage pulse (LV, 140 V/cm, 400 ms) were applied approximately one minute after plasmid injection with the Cliniporator device (IGEA, Carpi, Italy) [26]. The ability of these parameters to efficiently transfect the B16F10 tumors was evaluated using the luciferase-encoding plasmid pLUC. Fifty micrograms of pLUC were injected into the tumor, and luciferase activity was

measured *in vivo* 3 days after the electrotransfer using a Bioluminescence Xenogen IVIS50 system (Caliper Life Sciences) 10 min after intraperitoneal injection of 150 mg/kg luciferin (Promega). Results are expressed in photons/s.

The size of the tumors was measured daily. The mice were killed when the ratio V/V_0 reached a value of 20 (V_0 = volume of the tumor on the day of first treatment, V = volume of the tumor on the day measured). Kaplan–Meier analysis of survival rates was used to discriminate between the different treatments.

2.3. Effect of antiangiogenic plasmids on the metastasis development

To study the antimetastatic activity of the plasmids, B16-Luc melanoma cells expressing luciferase were injected and electrotransfered as described earlier. Three days after the second electrotransfer, the tumors were surgically removed. Tumor recurrence as well as metastasis development was evaluated on a weekly basis using a Bioluminescence Xenogen IVIS50 system (Caliper Life Sciences) 10 min after intraperitoneal injection of 150 mg/kg luciferin. Deaths by lung metastasis were recorded. Mice were euthanized when metastases were detected.

2.4. Immunohistochemistry

Tumors that were surgically removed 3 days after the second electrotransfer were formalin fixed and embedded in paraffin for histology. Sections of five microns were cut and underwent immunostaining for Ki67 (proliferation) and CD105 (vascularization). The primary goat anti-CD105 antibody was purchased from R&D Systems (R&D Systems Europe, Abingdon, United Kingdom). The staining was revealed with the anti-goat HRP-DAB system from R&D systems. The primary anti-Ki67 antibody was rabbit clone SP6 (Neomarkers, MM, Francheville, France) and was revealed with Power Vision Poly HRP anti rabbit IgG (MM, Francheville, France). The slides were scanned using a Mirax system (Carl Zeiss, Göttingen, Germany) and captures were analyzed with ImageJ (NIH, Bethesda, MD). Results were expressed as the percentage of the sample area that was stained for Ki67 or CD105.

2.5. Flow cytometry

Here, 5×10^5 B16F10 cells were stained with conjugated mAbs directed against specific integrins: PE anti-mouse CD51 (anti-αv; eBioscience, Paris, France), PE anti-mouse CD61 (anti-β3; BD Pharmingen, Le Pont de Claix, France), anti-mouse integrin α5β1 (Chemicon, Molsheim, France) revealed by incubation with a secondary FITC conjugated Ab (Jackson Immunoresearch, Newmarket, England). Normal monoclonal immunoglobulin isotype controls were used as a negative control antibody in parallel to each integrin antibody. Stained cells were analyzed by flow cytometry (FACScan, Becton Dickinson, Le Pont de Claix, France).

2.6. Intradermal electrotransfer of the antiangiogenic plasmids

Ten pmoles of plasmid were administered by two intradermal injections (two times 15 μl) on the abdomen. Two electrotransfers of 20 pmol pRDD were also investigated. Electrotransfer was performed by application of two electric pulses (700 V/cm, 100 μs; 140 V/cm, 400 ms) using a Cliniporator (IGEA) and plate electrodes, 2 mm spaced. Conductive gel was used to ensure electrical contact with the skin (EKO-GEL) [26,27]. Two days after electrotransfer, the mean cutaneous blood flow (MCBF) was determined by Laser Doppler Imaging using a MoorLDI (Moor Instruments, Axminster, England). Scanning of the treated and contralateral regions (regions of interest, ROI) was performed and data analyzed with the MoorLDI processing software. The results are expressed as the ratio between treated ROIs MCBF and untreated ROIs MCBF to normalize the data and lower interindividual variability. An antiangiogenic effect would be translated in the lowering of this ratio (<1).

2.7. Animal ethics

All experimental protocols on mice were approved by the Ethical Committee for Animal Care and Use of the faculty of Medicine of the Université Catholique de Louvain (MD/2007/042).

2.8. Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). *T*-test or one-way ANOVA and Tukey's post test, and Mantel–Cox survival analysis were performed to demonstrate statistical differences ($p < 0.05$), using the software GraphPad Prism 5 for Windows.

3. Results and discussion

3.1. Effect of intra-tumor electrotransfer of antiangiogenic plasmids on tumor growth

In this study, we investigated the intra-tumoral electrotransfers of antiangiogenic plasmids and their effect on tumor growth and metastasis. Three antiangiogenic plasmids, acting on different targets, were selected: pRDD, encoding the recombinant disintegrin domain of ADAM-15, pTSP-1 encoding the antiangiogenic factor thrombospondin 1, and psFlt-1 encoding the decoy soluble VEGF receptor. Electrotransfer was used because it is one of the most effective non-viral techniques for gene delivery in tumors [5,26]. Intra-tumoral injection was preferred to systemic administration of the plasmid because a local action was desired. Moreover, it ensures a high local concentration of the factor expressed by the plasmid in the target tissue and avoids dilution in the whole body or systemic side effects.

The plasmids were injected in B16F10 melanoma before the application of a high-voltage and low-voltage pulse combination [26]. An *in vivo* bioluminescence assay of intra-tumoral pLUC expression demonstrated the efficiency of the electric parameters in transfecting B16F10 tumors. Luciferase activity, expressed in photons/s, significantly increased from $1.72 \times 10^4 \pm 3.04 \times 10^3$ to $1.02 \times 10^6 \pm 3.03 \times 10^5$ after intra-tumoral injection of the plasmid without and with electrotransfer, respectively ($n = 8$, $p < 0.01$).

Preliminary studies conducted with intra-tumoral electrotransfer of 10 pmol plasmid into subcutaneous 30–50 mm^3 tumors showed that this treatment was not sufficient to significantly delay tumor growth (data not shown). To obtain an effect on tumor growth, we chose to: (i) deliver a larger dose of plasmid (20 pmol per delivery); (ii) increase the number of administrations of the plasmids from one single administration to two, at 3 days of interval, in order to prolong the duration of action of the plasmids, (iii) begin the treatment earlier, i.e. when tumor volumes reached 15–30 mm^3. A control group receiving plasmid encoding luciferase (pLUC) or GFP (pGFP) under the same experimental procedure was included throughout this study. The delivery of a luciferase-encoding plasmid had no significant effect on the tumor growth, compared with untreated animals (data not shown).

Two intra-tumoral electrotransfers at 3-day interval of pRDD, pTSP-1 or psFlt-1 plasmids induced a significant decrease in tumor growth. All mice treated with the control plasmid pLUC developed fast growing tumors (Fig. 1). In contrast, in antiangiogenic plasmid-treated groups, the growth of tumors was delayed and complete tumor regressions were observed leading to long-term mouse survival. However, a few mice died from lung metastasis. The median

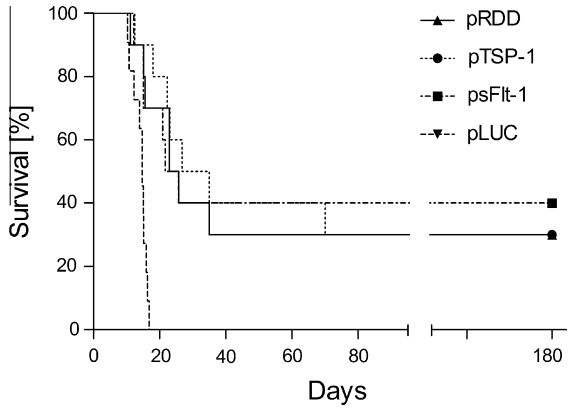

Fig. 1. B16F10 tumor growth inhibition after intra-tumoral electrotransfers of antiangiogenic plasmids. Equimolar amounts (20 pmol) of antiangiogenic plasmids were injected into subcutaneous 15–30 mm^3 B16F10 tumors (day 0) and subjected to electrotransfer. A second electrotransfer treatment was applied at day 3. Mice survival. (Mantel–Cox $p < 0.0001$; $n = 10$; treatments vs pLUC.)

survival time increased from 14.7 days after pLUC electrotransfer (control group) to 24.4, 30.9 and 23.7 days after electrotransfer of pRDD, pTSP-1 and psFlt-1 plasmids respectively (Mantel–Cox $p < 0.0001$). The intra-tumoral electrotransfers of the antiangiogenic plasmids resulted in decreased tumor growth with approximately doubling of the median survival time when compared to untreated controls and complete regression of the tumors leading to long-term survival of approximately 40% cured mice. The difference between each antiangiogenic treatment did not reach statistical significance (Mantel–Cox $p > 0.05$).

The tumor growth curves showed that after a short time of stabilization or decrease in the tumor volume, tumors entered into logarithmic growth. For TSP-1, this escape could be due to an up-regulation of various proangiogenic factors in response to TSP-1 antiangiogenic effect as reported for CT26 colon carcinoma [24]. A similar mechanism could be implicated for psFlt-1 and pRDD as one of the most important mechanisms to escape angiogenesis suppression is the up-regulation of VEGF [24]. The mechanism underlying tumor escape to angiogenesis inhibition has not been studied in the B16F10 melanoma model. A combination of TSP-1 and RDD with sFlt-1 or other anti-VEGF agents (such as the monoclonal antibody bevacizumab) could result in a synergistic effect and provide information on how melanoma cells escape angiogenesis inhibition.

The local antiangiogenic gene therapy by intra-tumoral electrotransfer allowed for long-term antitumoral activity without repeated injections of proteins [4].

3.2. Effect of intra-tumor electrotransfer of antiangiogenic plasmids on metastasis development

To assess the ability of the electrotransfer of antiangiogenic plasmids to inhibit metastasis of B16F10 melanoma cells expressing luciferase (B16-Luc), tumors were treated twice at 3-day interval by electrotransfer of the plasmids before being surgically removed 5 days later. The development of metastases was assessed weekly by IVIS imaging of the luciferase activity (Fig. 2a).

There was no local tumor recurrence after surgery. Metastases developed mainly in the lungs and randomly in brachial or inguinal lymph nodes. Mice treated with antiangiogenic plasmids developed metastasis later than those that received the control GFP-encoding plasmid. The median metastasis detection increased from 26 days for control animals to 49 and 40 days for mice treated with pTSP-1 and psFlt-1, respectively (Mantel–Cox $p < 0.001$; treatments vs pGFP). pTSP-1 and psFlt-1 did not differ significantly

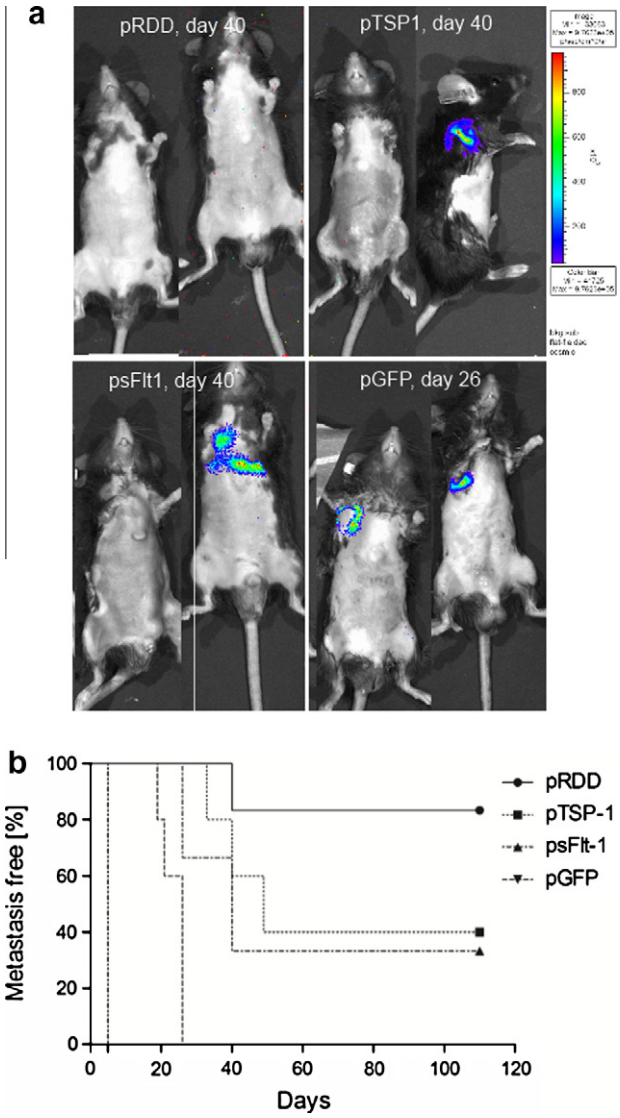

Fig. 2. Inhibition of metastasis development. Antiangiogenic plasmids (20 pmol) were injected into subcutaneous 15–30 mm^3 B16-Luc tumors (day 0) and subjected to electrotransfer. A second electrotransfer treatment was applied at day 3. At day 5, primary tumors were resected and metastasis development was followed using luciferase bioluminescence imaging. (a) Bioluminescence imaging of metastasis y in treated and pGFP control mice 40 and 26 days respectively after surgery. pGFP mice could be followed only up to 26 days due to mortality. (b) Evolution of the percentage of metastasis-free mice. (Mantel–Cox $p < 0.001$; treatments vs pGFP; $n = 5$.)

(Mantel–Cox $p > 0.05$). In this model mimicking clinical setting, i.e. treatment before surgical removal of the melanoma tumors, we demonstrated that the three plasmids significantly inhibited the development of B16F10 metastasis when compared to control. More importantly, the development of metastasis was drastically inhibited by treatment with the pRDD plasmid as a long-term metastasis-free delay (more than three months) was observed for 84% of the mice (compared to approximately 40% for pTSP-1 and psFlt-1) (Fig. 2b).

3.3. Effect of antiangiogenic plasmids on cell proliferation and vascularization of melanoma

The three plasmids encode proteins that are known to induce antiangiogenic effect in tumor [2,14,16,23,24]. To test whether the antitumoral and antimetastatic activity of the plasmids encoding RDD, TSP-1 or sFlt-1 were due to their antiangiogenic activity,

immunohistochemistry was performed on the tumors that were resected 5 days after the first electrotransfer. Both Ki67 (cell proliferation) and CD105 (angiogenic endothelial cells) biomarkers were analyzed (Fig. 3a).

As shown in Fig. 3, only pRDD electrotransfer treatment inhibited both cell proliferation and the number of blood vessels in the tumor. A quantitative analysis of the immunohistochemical staining indicated that pRDD significantly inhibited cell proliferation compared to the control plasmid, whereas pTSP1 and psFlt-1 had no effect on Ki67 staining (Fig. 3b). In contrast, the three plasmids tended to decrease the CD105 staining with a better inhibitory effect of pRDD plasmid. However, this effect was not statistically significant (Fig. 3c). Based on their known antiangiogenic activity, significant reduction in CD105 staining would be expected for the three tested plasmids. Angiogenesis and vascular regression respond to a variety of signals, including the metabolic needs of the surrounding tumor cells. It is possible that at the time the tumors were resected, the vascular network was not yet extensively modified in response to the treatment. Pharmacodynamic studies with these plasmids could determine whether the regression of the tumoral vasculature occurs concomitantly with tumor regression, or whether tumor regression precedes vascular dismantling.

The difference in metastasis inhibition could be explained by a dual mechanism of action of pRDD. Indeed, RDD has, unlike TSP-1 and sFlt-1, a direct antitumoral activity. *In vitro*, RDD has been demonstrated to inhibit proliferation and to induce apoptosis of endothelial cells [14]. RDD also inhibits melanoma cell migration and invasion [14]. This direct antitumoral activity could be attributed to the overexpression of αvβ3 and α5β1 integrins by B16F10 melanoma cells. Murine B16F10 melanoma cell line was thus characterized for its integrin expression profile. Flow cytometry analysis showed that B16F10 cells expressed αv integrin (99.9%), β3 integrin (96%) and α5β1 integrin (72%) which are targets of the RDD disintegrin domain of ADAM-15.

These findings indicate that the inhibition of the single VEGF pathway might not be sufficient to obtain an extended inhibition of metastasis: sFlt-1 could impede malignant cells migration by decreasing the vascular density but has no direct effect on tumor cells. There is also no evidence that TSP-1 could inhibit cell migration or invasiveness [15]. These data suggest that the effects of pTSP-1 and pFlt-1 on tumor growth and metastasis could mainly result from their antiangiogenic activity. Direct antiproliferative activity, as observed with pRDD, seems necessary for the control of metastasis development. Integrins are molecules of major importance for the control of cell proliferation [28]. Therefore, the decrease in cell proliferation by pRDD could be explained by the ability of RDD to bind αvβ3 and α5β1 integrins via its RGD integrin-binding motif [29,30].

3.4. Effect of antiangiogenic plasmid electrotransfer on normal skin

RDD, TSP-1, and sFlt-1 display known antiangiogenic activities in tumors, including melanomas, as reported in Fig. 3 and in the literature [2,13,15,23]. Because skin is the main tissue surrounding the tumor in the B16F10 melanoma model, we found it important to verify that electroporation and antiangiogenic gene therapy were innocuous for healthy tissues. To assess their effect on vascularization of normal skin, these plasmids (10 or 20 pmol) were administered by intradermal injections (two times 15 μl) on the abdomen skin. Two days after electrotransfer, the mean cutaneous blood flow was determined by Laser Doppler Imaging. As shown in Fig. 4, electrotransfer of antiangiogenic plasmids had no consequence on vascularization at normal skin level. The blood flow of normal skin was neither decreased nor increased by the electrotransfer of antiangiogenic plasmids, including pRDD, when compared to untreated normal skin, suggesting that their electrotransfer is safe for the vasculature of normal skin. We have shown that application of high-voltage pulses induced a mild reversible

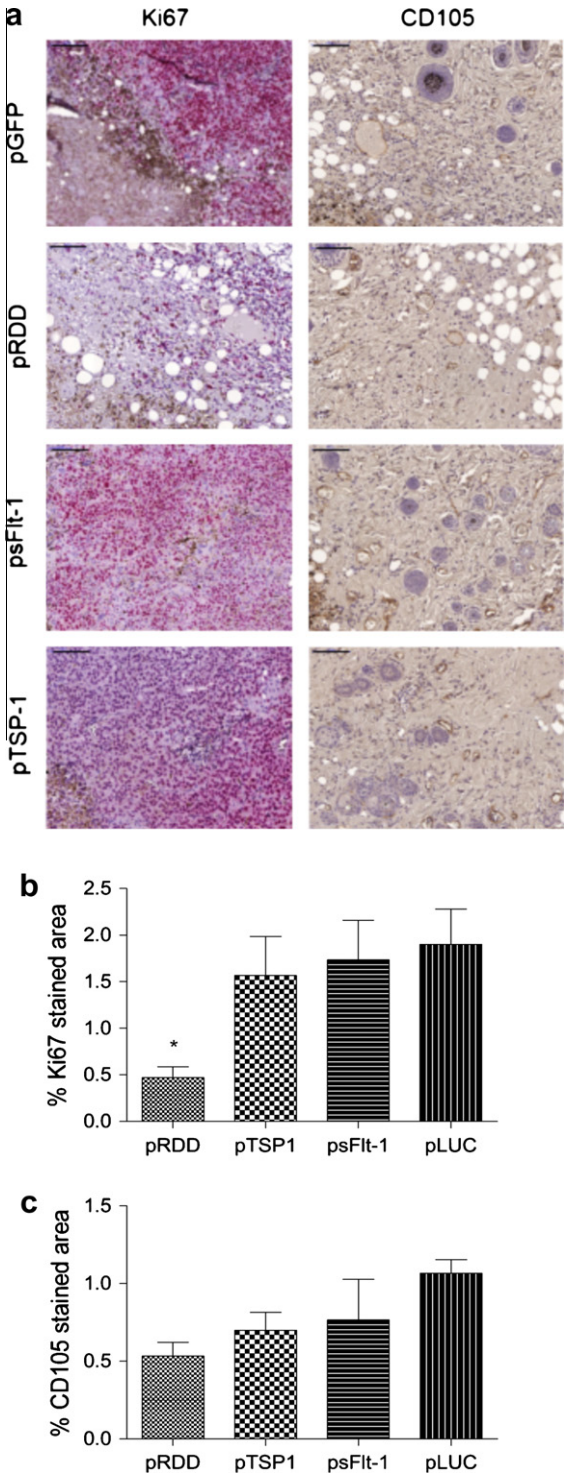

Fig. 3. Effect of antiangiogenic plasmids on cell proliferation and vascularization of melanoma. Primary subcutaneous B1610 melanoma tumors were treated at days 0 and 3 with intra-tumoral electrotransfer of pLUC (control), pRDD, psFlt-1 or pTSP1 plasmids. Tumors were collected at day 5. Paraffin-embedded tumor sections were immunolabeled with either an anti-Ki67 antibody (for cell proliferation) or an anti-CD105 antibody (for tumor blood vessels). (a) Photographs of immunohistochemical Ki67 and CD105 stainings, scale bars = 50 μm. (b) Quantitative analysis of proliferation (Ki67) (mean ± SEM; $n = 5$, *ANOVA $p < 0.05$) and (c) quantitative analysis of tumoral vascularization (CD105) (mean ± SEM; $n = 5$, ANOVA $p > 0.05$ vs pLUC).

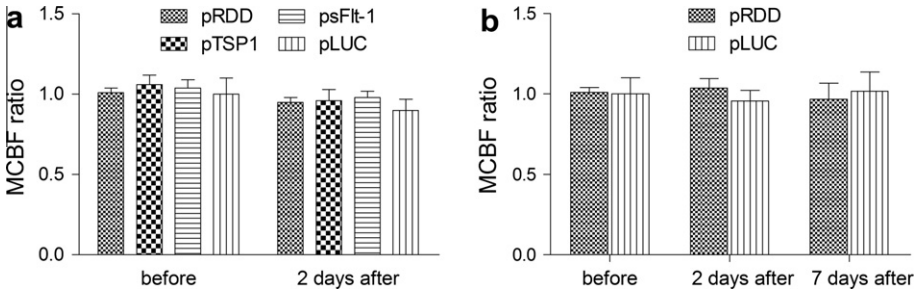

Fig. 4. Effect of antiangiogenic plasmid electrotransfer on skin blood perfusion. (a) Blood flow after electrotransfer of 10 pmol of plasmid in normal skin, expressed as ratio of mean cutaneous blood flow of treated and untreated skin measured by Laser Doppler Imaging (mean ± SEM, $n = 6$). (b) Blood flow after two electrotransfers of 20 pmol of pRDD and pLuc plasmid in normal skin (mean ± SEM, $n = 6$).

impairment of the skin barrier function and a dramatic decrease in skin resistance and transient decrease (<5 min) in blood flow was observed. Neither inflammation nor necroses were observed [31,32]. Moreover, the application of high-voltage pulses in clinical trials for the treatment of melanomas by electrochemotherapy or gene therapy is well tolerated [9,10].

4. Conclusion

In conclusion, this study demonstrated that intra-tumoral electrotransfer for gene therapy is an efficient method for the local delivery of antiangiogenic plasmids. All tested plasmids induced a significant inhibition of tumor growth and metastasis. Moreover, pRDD, due to its direct antitumoral activity combined with angiogenesis suppression, was shown to strongly delay and even suppress melanoma metastasis. More insight is needed to understand how tumors escape the treatments and how the different plasmids could be associated to obtain a better control of tumor progression and metastasis.

Conflict of interest

The authors have no financial conflict of interest.

Acknowledgments

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