



Radiosensitization

Potentiation of radiotherapy by a localized antiangiogenic gene therapy

Nathalie Crockart^{a,1}, Fabienne Danhier^{a,1}, Liévin Daugimont^a, Nelio Gonçalves^a, Bénédicte F. Jordan^b, Vincent Grégoire^c, Olivier Feron^d, Céline Bouquet^e, Bernard Gallez^b, Véronique Prétat^{a,*}

^a Université Catholique de Louvain, Louvain Drug Research Institute, Pharmaceutics and Drug Delivery; ^b Université Catholique de Louvain, Louvain Drug Research Institute, Laboratory of Biomedical Magnetic Resonance; ^c Université Catholique de Louvain, Institut de Recherche Expérimentale et Clinique, Center for Molecular Imaging and Experimental Radiotherapy, IREC; ^d Université Catholique de Louvain, Institut de Recherche Expérimentale et Clinique, Pole of Pharmacology and Therapeutics, Brussels, Belgium; ^e Bioalliance SA, Paris, France

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ABSTRACT

Background and purpose: We hypothesized that electrotransfer of a plasmid encoding an antiangiogenic factor, the recombinant disintegrin domain of ADAM-15, (pRDD) could modify the tumor microenvironment and radiosensitize tumor.

Materials and methods: pRDD was injected in the TLT tumor or FSa11 fibrosarcomas before electroporation. pO₂ in tumors and oxygen consumption *in vitro* were measured by electronic paramagnetic resonance (EPR) oximetry. Tumor perfusion was assessed by laser doppler imaging and patent blue assay.

Results: pRDD electrotransfer caused a significant delay in TLT growth and an anti-angiogenic effect. It significantly increased tumor pO₂ in TLT and FSa11 for at least 4 days. pRDD electrotransfer and radiotherapy were more effective than either treatment alone. Modifications of tumor microenvironment were evaluated: tumor perfusion and interstitial fluid pressure were not modified. Oxygen consumption by the cells was decreased resulting both from a decrease in oxygen consumption rate and from a decrease in cell viability.

Conclusion: The combination of localized antiangiogenic gene therapy and radiotherapy applied in the time of maximal oxygenation could be a promising alternative for cancer treatment.

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Angiogenesis, often described as the growth of new capillary blood vessels from pre-existing vasculature, plays a role in normal growth as well as in tumor progression and metastasis [1–4]. As a consequence, many anti-angiogenic strategies to treat human cancers have emerged. Recently, it has been suggested that gene therapy, with a gene encoding an anti-angiogenic factor, could be a promising method for the treatment of cancer due to its potential advantages compared with conventional antiangiogenic drugs [5–9]. Since effective suppression of pathological angiogenesis may require chronic treatment, gene therapy strategies could be useful to achieve the selective delivery to affected tissues thus achieving localized gene expression and high regional therapeutic agent concentrations, without significant systemic levels. It also allows prolonged expression of therapeutic agents. Gene therapy also represents a method for circumventing the production problems of many recombinant proteins [5–9].

Among the non-viral methods of gene therapy, electroporation which involves the application of high-voltage electric pulses to

tissues, has been shown to be one of the most efficient methods *in vivo* [10]. Electroporation causes the permeabilization of the plasma membrane and, therefore, provides effective means of increasing the uptake of molecules such as DNA into cells [11,12]. Electroporation is also currently used to enhance the local delivery of non-permeating anti-cancer drugs and DNA into tumors [12,13].

Recently, the family of A disintegrin and metalloproteinase proteins (ADAMs) has been shown to be involved in cancer proliferation and progression [14–16]. All family members potentially possess cell-adhesion and protease activities. ADAMs contain a disintegrin domain located on the COOH-terminal side of the metalloproteinase with an integrin-binding sequence in a disintegrin loop that interacts with integrins and may mediate cell–cell interactions. Antiangiogenic and antimetastatic activities of the recombinant disintegrin domain (RDD) of ADAM-15 have been demonstrated [17]. Intra-tumoral electrotransfer of a plasmid encoding RDD (pRDD) induced significant inhibition of B16F10 tumor growth and metastasis [18]. As compared to plasmids encoding thrombospondin 1 or the soluble isoform of VEGF receptor 1 (sFlt-1), pRDD was more effective for the treatment of B16 melanoma due to its direct antitumoral activity combined with antiangiogenesis and marked inhibition of metastasis [18]. A phase I clinical trial, conducted in advanced or metastatic melanoma, has

* Corresponding author. Address: Université Catholique de Louvain, Louvain Drug Research Institute, Pharmaceutics and Drug Delivery, Avenue Mounier, 73 B1.73.12, B-1200 Brussels, Belgium.

E-mail address: veronique.preat@uclouvain.be (V. Prétat).

¹ Shared coauthorship.

shown a very good safety profile after intratumoral electrotransfer and satisfactory signals of efficacy [19]. A Phase I/II trial on intramuscular electrotransfer is currently on going, aiming at evaluating the safety and efficacy profile via systemic route in patients with metastatic melanoma.

Against the traditional idea that the use of agents that inhibit angiogenesis would further increase tumor hypoxia and thus impair tumor response to radiation, increasing evidence shows that the combination of anti-angiogenic agents with radiation can improve tumor response to radiotherapy [20–26]. Tumor oxygenation could play a role in this radiosensitization. It has been hypothesized that a transient normalization of the tumor vasculature [24–25] may occur early after starting the antiangiogenic treatment, leading transiently to a better perfusion and oxygen/drug delivery [20,26,27]. Another mechanism observed with the use of some antiangiogenic agents was a tumor reoxygenation due to an inhibition of the oxygen consumption by the tumor cells [28,29].

We hypothesized that the electrotransfer of a plasmid encoding RDD could modify the tumor microenvironment and lead to an additive or synergic effect between this antiangiogenic treatment and irradiation. Hence, tumor oxygenation was assessed using electron paramagnetic resonance (EPR) oximetry that allows continuous measurements of pO_2 from the same site in tumors for long periods [30,31]. This technique was previously used to monitor the changes in pO_2 after classical anti-angiogenic drug administration [20,27–29]. pRDD electrotransfer dramatically increased tumor oxygenation. At maximal tumor oxygenation, radiosensitization and changes in the tumor microenvironment (tumor perfusion, interstitial pressure, vessel density, oxygen consumption, cell viability and apoptosis) were investigated.

Materials and methods

Animal tumor models

Two different syngeneic tumors (10^5 tumor cells) were implanted in the gastrocnemius muscle of 8 week old male mice (Janvier, France): the transplantable liver tumor (TLT) implanted in NMRI mice and the fibrosarcoma FSaII implanted in C3H mice. All treatments were applied when the tumor reached 8.0 ± 0.5 mm. All experiments were performed in compliance with guidelines set by national regulations and were approved by the Ethics Committee for animal care of the Université catholique de Louvain (UCL/MD/2008/025).

Animals were anesthetized by inhalation of isoflurane during the assessment of tumor microenvironment parameters. For electrotransfer, animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg Ketalar; Pfizer) and xylazine (8 mg/kg; Sigma–Aldrich, Belgium).

Electrotransfer of pRDD in tumor

Plasmid

pRDD is a pVAX1-based plasmid (Invitrogen, Belgium) encoding for the recombinant disintegrin domain of human ADAM-15 under control of the secretion signal of murine urokinase and the strong cytomegalovirus (CMV) promoter. This plasmid was kindly provided by Bioalliance (Paris, France) [17,18]. pLUC, a pVAX plasmid encoding the reporter gene luciferase was used as control. Plasmids were amplified in *Escherichia coli* and purified using Endo-free Qiagen Gigaprep kit according to the manufacturer's protocol. The quality of the plasmid was assessed by the ratio of UV absorption at 260 nm and 280 nm and by agarose gel electrophoresis. The DNA concentration was determined by measurements of the absorbance at 260 nm. Plasmids were prepared in Phosphate Buffer Saline (PBS) and stored at -20°C before use.

Electrotransfer of plasmid DNA in tumor

pRDD plasmid (200 $\mu\text{g}/60 \mu\text{l}$ PBS) or pLUC (50 $\mu\text{g}/60 \mu\text{l}$ PBS) was injected in the tumor 5 min before electrotransfer. Two electric pulses (1 high voltage pulse 1250 V/cm, 100 μs ; Lag 1000 ms; 1 low voltage pulse 140 V/cm, 400 ms) were applied by the device Cliniporator (IGEA, Italy) with two stainless steel plate electrodes spaced by 8 mm. Conductive gel was used to ensure electrical contact (EKO-gel, Italy) [18].

Luciferase expression in tumor was assessed by bioluminescence imaging. Briefly, luciferine (Xenogen Corporation, USA) was injected intraperitoneally (3 mg/100 μl) and optical imaging was acquired using an IVIS50 system (Xenogen) 10 min after injection of the substrate. Photons emitted from the tumor were quantified [18].

The effect of pRDD electrotransfer on TLT growth was assessed by daily measurements of the transverse and anteroposterior diameters of the tumors with an electronic caliper. Three groups of mice (control mice, PBS electrotransfer and pRDD electrotransfer) were used to analyze the effect of pRDD electrotransfer on tumor growth. Statistical analysis was performed comparing the time to reach 14 mm.

Measurement of tumor oxygenation

EPR oximetry using charcoal as the oxygen sensitive probe was used to evaluate tumor oxygenation changes [20,27–31]. EPR spectra were recorded using a 1.2 GHz EPR spectrometer (Magnetech, Germany). Mice were injected 2 days before treatment in the center of the tumor with 50 μl suspension of charcoal (100 mg/ml, particle size less than 25 μm CX0670-1; EM Science, USA). The localized spectroscopic EPR measurements recorded the average pO_2 in a volume of approximately 10 mm^3 . Either the plasmid pRDD or PBS was injected in TLT and FSaII tumor before electrotransfer (PBS/ET and pRDD/ET). A control group was injected with PBS without electroporation (PBS). The pO_2 was recorded before and each day after electrotransfer.

Irradiation and tumor regrowth delay assay

The effect of pRDD electrotransfer combined with X-rays applied 3 days after plasmid treatment on TLT growth was assessed by daily measurements of the transverse and anteroposterior diameters of the tumors with an electronic caliper until the tumor diameters reached 16 mm. TLT were locally irradiated under ambient air condition with 6 Gy (1.2 Gy/min) of 250 kV X-rays (RT 250, Philips Medical systems, Germany). This low dose was selected to induce no or low effect on tumor growth. The tumor was centered in a circular irradiation field measuring 3 cm in diameter. Six groups of mice were treated: group 1: PBS injection group; 2: PBS injection + irradiation at day 3; group 3: PBS injection + electrotransfer; group 4: PBS injection + electrotransfer + irradiation at day 3; group 5: pRDD electrotransfer and group 6: pRDD electrotransfer + irradiation at day 3. Statistical analysis was performed comparing the time to reach 14 mm. A second set of experiments was performed to confirm the effect of tumor oxygenation by adding a group of mice whose legs were transiently ligatured using a rubber band just before and during the irradiation to abolish the oxygen effect: group 1: PBS injection + electrotransfer; group 2: PBS injection + electrotransfer + irradiation at day 3; group 3: pRDD electrotransfer + irradiation at day 3 and group 4: pRDD electrotransfer + irradiation at day 3 on ligatured leg. Statistical analysis was performed comparing the time to reach 13 mm.

Tumor microenvironment

Laser Doppler imager (Moor instruments, United Kingdom) was used to assess superficial tumor blood flow over time [32]. The

mean flux values for the tumor were calculated from the relative flux units. Perfusion was measured in TLT before and each day after PBS injection, PBS electrotransfer or pRDD electrotransfer.

Patent Blue (Sigma–Aldrich) was used to obtain a rough estimate of the TLT perfusion three days after PBS injection, PBS electrotransfer or pRDD electrotransfer [20,33]. 200 μ l of Patent Blue (1.25%) solution was injected into the tail vein of the mice. After 1 min, mice were sacrificed. The tumors were carefully excised and cut into two size-matched halves. Pictures of each tumor cross-section were taken and the stained area of the whole cross-section was determined using an in-house program running on Interactive Data Language (RSI, USA) [20]. The mean of the percentages of the two pictures was used as an indicator of tumor perfusion.

The interstitial fluid pressure (IFP) assessed on day 3 following PBS injection, PBS electrotransfer or pRDD electrotransfer was measured using a “wick in needle” apparatus (Stryker, 295-1 Pressure, Stryker Corporation, USA). The needle was inserted in the center of the tumor, 50 μ l of saline was injected and IFP was measured [34].

Blood vessels in tumors were visualized by CD31 immunohistochemistry in TLT from mice sacrificed on days 3 and 7 after electrotransfer of pRDD or vehicle. Tumor cryoslices were immunoprobed with rat monoclonal CD31 IgG2a antibodies (BD Pharmingen, USA) and revealed by a secondary antibody coupled to tetramethyl-rhodamine isothiocyanate (Jackson, Belgium). Sections were then counterstained by a 4,6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Lab Inc, USA). Tumor slices were examined with an Axioskop microscope (Zeiss, Germany) equipped for fluorescence. Mean fluorescence in arbitrary units was determined using the Image J. NIH.

To electroporate cells *in vitro*, 400 μ l of cell suspension containing 6×10^6 cells (determined by trypan blue exclusion method) in a pulsation buffer (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) [35] were mixed with 24 μ g plasmid. Electroporation was applied using cuvettes with 2 mm spaced electrodes inserted in the Cytosol Sciences apparatus PA-201 (USA). Ten 160 V electric pulses of 5 ms were applied at 1 Hz. Cells were then incubated in 6-well dishes containing 6 ml medium (high glucose DMEM, Invitrogen) for 24 h.

Oxygen consumption rate was measured on TLT cells *in vitro* by an EPR method [28,29]. Briefly, 10^7 viable cells/ml were sealed in glass capillary tubes in the presence of 0.08 mM of the O_2 sensing probe ^{15}N 4-oxo-2,2,6,6-tetramethylpiperidine- d_{16} - ^{15}N -1-oxyl (CDN Isotopes, Canada). Measurements on these sealed tubes were performed at 37 °C on a Bruker EMX EPR spectrometer operating at 9 GHz.

TLT cells' viability *in vitro* (control, electroporated or electroporated with pRDD) was assessed by the trypan blue exclusion technique. Cells (2×10^6) were plated in 6-well plates and were then harvested by trypsinization 24 h after pulse application. Trypan blue was added and living cells were counted using an automated cell counter (BioRad TC10, USA).

Apoptosis was identified morphologically by DAPI staining. After electroporation (pRDD or PBS), 2×10^6 TLT cells were seeded in 6-well plates containing a coverslip and cultured for 24 h. Samples were then fixed with 4% paraformaldehyde and stained with 0.2 μ g/ml. Coverslips mounted onto glass slides were then examined using a microscope with a 340/380 nm excitation filter and LP 430 nm barrier filter.

Statistical analyses

The differences in tumor growth, pO_2 , LDI perfusion and mean fluorescence of CD31 were compared by a two-way ANOVA followed by a Bonferroni post-test. The oxygen consumption slopes

were compared using a Wilcoxon test. The cell viability results were compared with a *t*-test. The patent blue and IFP results were compared using a one-way ANOVA (Kruskal–Wallis test). Results are expressed as mean $\pm$ SEM.

Results

pRDD electrotransfer delays TLT growth

The efficacy of plasmid electrotransfer in TLT tumor was assessed using luciferase as a reporter gene. As compared to injection alone of pLUC, electrotransfer increased luciferase expression from $8.25 \times 10^5 \pm 4.55 \times 10^5$ to $5.53 \times 10^7 \pm 2.65 \times 10^7$ Relative Light Units (RLU) 2 days after electrotransfer. Hence, due to this 70-fold increase in gene expression induced by electrotransfer, most of the following studies were performed using electrotransfer after PBS injection as control group.

To determine if pRDD affects the growth of TLT, TLT were injected with pRDD followed by electrotransfer (pRDD/ET). A significant regrowth delay was observed compared to the control group electroporation of vehicle (PBS/ET) ($p < 0.01$) (Fig. 1). When pRDD was injected without electrotransfer, no significant regrowth delay was observed compared to control group (data not shown).

pRDD electrotransfer enhances tumor oxygenation

To check if the pRDD electrotransfer modifies the tumor micro-environment, we first assessed the tumor pO_2 in two tumor models, TLT and FSall. In the TLT model, a significant increase in pO_2 was observed after 2 days for the group treated by the pRDD electrotransfer (Fig. 2a). No such pO_2 increase was observed for the control groups (PBS or PBS/ET). The maximal pO_2 was reached 3 days after the pRDD electrotransfer in TLT: it significantly increased from 3.2 to $0.5\text{--}19.3 \pm 3.7$ mmHg as compared to 2.6 to $0.2\text{--}2.4 \pm 0.2$ mmHg in control group (PBS, data not shown) and 3.3 to $0.6\text{--}3.7 \pm 0.5$ mmHg in the group treated by electrotransfer only (PBS/ET). A slightly higher but more heterogeneous increase in pO_2 was observed 4 days after pRDD/ET (22.2 ± 8 mmHg). In conclusion, the time of maximal reoxygenation of TLT was found to be on day 3 and 4. Nevertheless, because of the variability of the response on day 4, all further experiments for tumor characterization were conducted mainly on day 3.

The same effect of pRDD electrotransfer on the tumor oxygenation of FSall tumors was observed at day 3 and 4 (Fig. 2b). The pO_2 significantly increased from 3.5 to $0.8\text{--}10.1 \pm 2.0$ mmHg in the group treated by pRDD electrotransfer compared to 3.6 to $0.6\text{--}4.5 \pm 0.7$ mmHg in the group receiving electrotransfer without

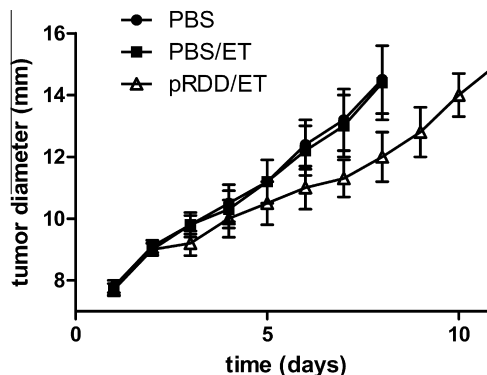


Fig. 1. Anti-tumor effect of pRDD electrotransfer. Tumor diameter (mm) in function of time (days) for TLT treated with: PBS ($n = 4$), PBS + electrotransfer (PBS/ET, $n = 5$) or pRDD plasmid electrotransfer (pRDD/ET, $n = 5$). Each point represents the mean $\pm$ SEM. (***) $p < 0.001$.

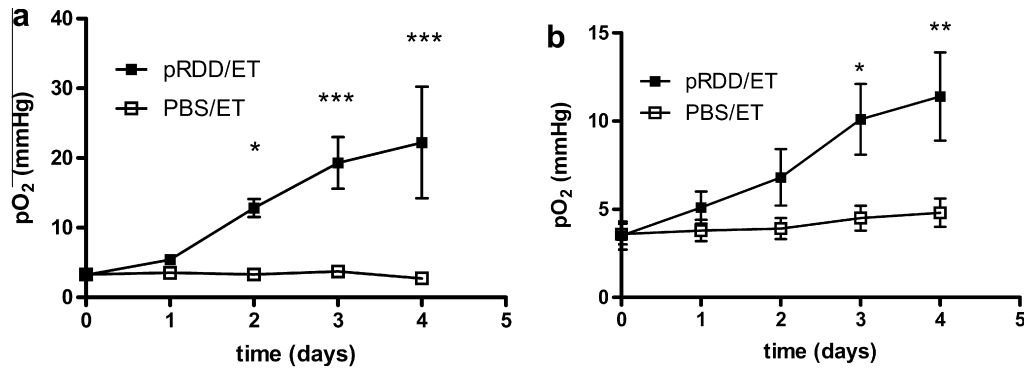


Fig. 2. Effect of the pRDD electrotransfer on tumor oxygenation. Tumor pO₂ (mmHg) measured by EPR oximetry as a function of time in (a) TLt after electrotransfer of PBS (□, n = 5) or pRDD (■, n = 4). (b) FSaII tumors after electrotransfer of PBS (□, n = 4) or pRDD (■, n = 4). Each point represents the mean ± SEM. (NS, non-significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001).

plasmid. To be consistent with TLt tumor results, further experiments on FSaII tumors were also conducted on day 3.

pRDD electrotransfer radiosensitizes tumor by an oxygen effect

We investigated whether the changes caused in the tumor microenvironment by pRDD electrotransfer, in particular the increase in tumor oxygenation, could induce a radiosensitization by the oxygen effect. Therefore, the effect of irradiation (single dose 6 Gy) at the time of maximum oxygenation i.e. 3 days after pRDD electrotransfer was assessed. According to Fig 3a, there is a significant increase from PBS/ET to PBS/ET + RX and from pRDD/ET to pRDD/ET + RX, but not from PBS to PBS + RX or from PBS to PBS/ET. To be able to detect an additivity or synergy between pRDD/ET and radiation, we had to use a low dose of RX. In a preliminary experiment (data not shown), we tested higher dose (15 Gy) and observed a significant growth delay. This low dose (6 Gy) was selected to induce no or low effect on tumor growth. Radiation combined with electroporation had an effect on tumor growth, whereas radiation and electroporation alone does not. RX added to PBS (PBS + RX) did not induce similar growth delay

as RX added to PBS/ET (PBS/ET + RX) or RX added to pRDD/ET (pRDD/ET + RX). Finally, the combination of pRDD electrotransfer and irradiation was significantly more efficient than each treatment alone (*p* < 0.001). (Fig. 3a, Supplementary Fig. 1)

Mice were also treated with the combination of treatments with a transient ligation of their leg to abolish the oxygen effect caused by pRDD. Interestingly, with the ligation, the time to reach 16 mm was decreased similarly to mice treated with PBS/ET + irradiation (*p* < 0.001). TLt not treated with pRDD electrotransfer are hypoxic (pO₂ < 5 mmHg). Consequently, the ligation of the legs did not significantly decrease the oxygenation of hypoxic tumors. Together these results indicate that the ligation of pRDD treated tumors which induced anoxia counteracted the radiosensitizing effect of pRDD electrotransfer and that the improvement in tumor oxygenation contributes to the radiosensitizing properties of pRDD electrotransfer (Fig. 3b).

pRDD electrotransfer modifies the tumor microenvironment

The tumor perfusion was assessed in the TLt model daily by laser Doppler imaging perfusion and at day 3 by the Patent Blue

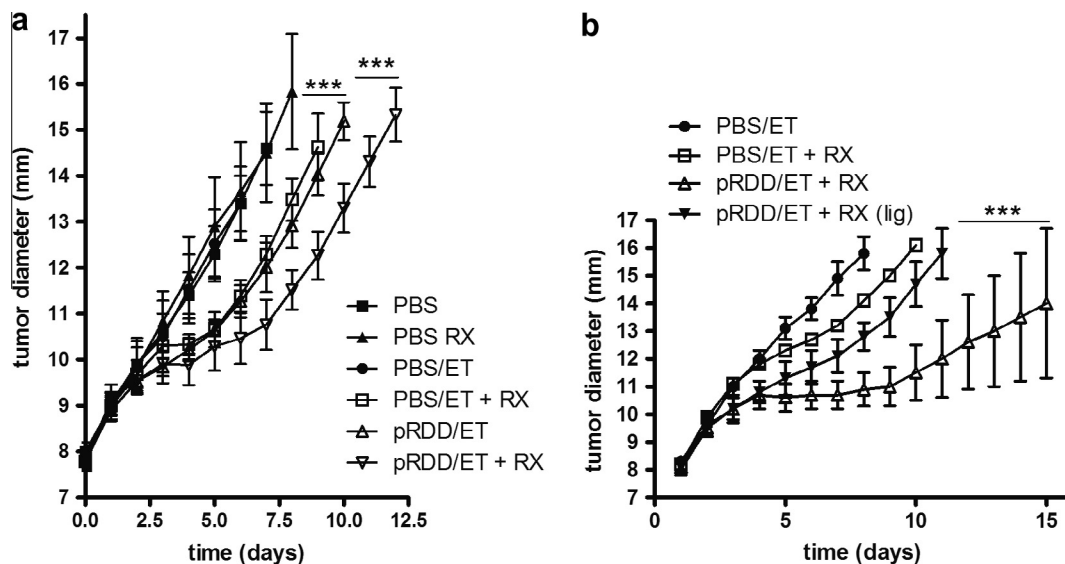


Fig. 3. Anti-tumor effect on TLt-bearing mice treated with pRDD electrotransfer in combination with irradiation. (a) Anti-tumor effect on TLt-bearing mice after PBS injection without (n = 4) or with 6 Gy irradiation 3 days after injection (n = 4); PBS injection + electrotransfer without (n = 5) or with 6 Gy irradiation 3 days after injection (n = 4) and pRDD + electrotransfer without (n = 5) or with 6 Gy irradiation 3 days after injection (n = 5). (b) Anti-tumor effect on TLt-bearing mice after PBS injection + electrotransfer without (n = 4) or with 6 Gy irradiation 3 days after injection (n = 4), pRDD + electrotransfer with 6 Gy irradiation 3 days after injection (n = 4) and pRDD + electrotransfer with 6 Gy irradiation 3 days after injection (n = 4), the irradiated legs of mice were clamped (lig).

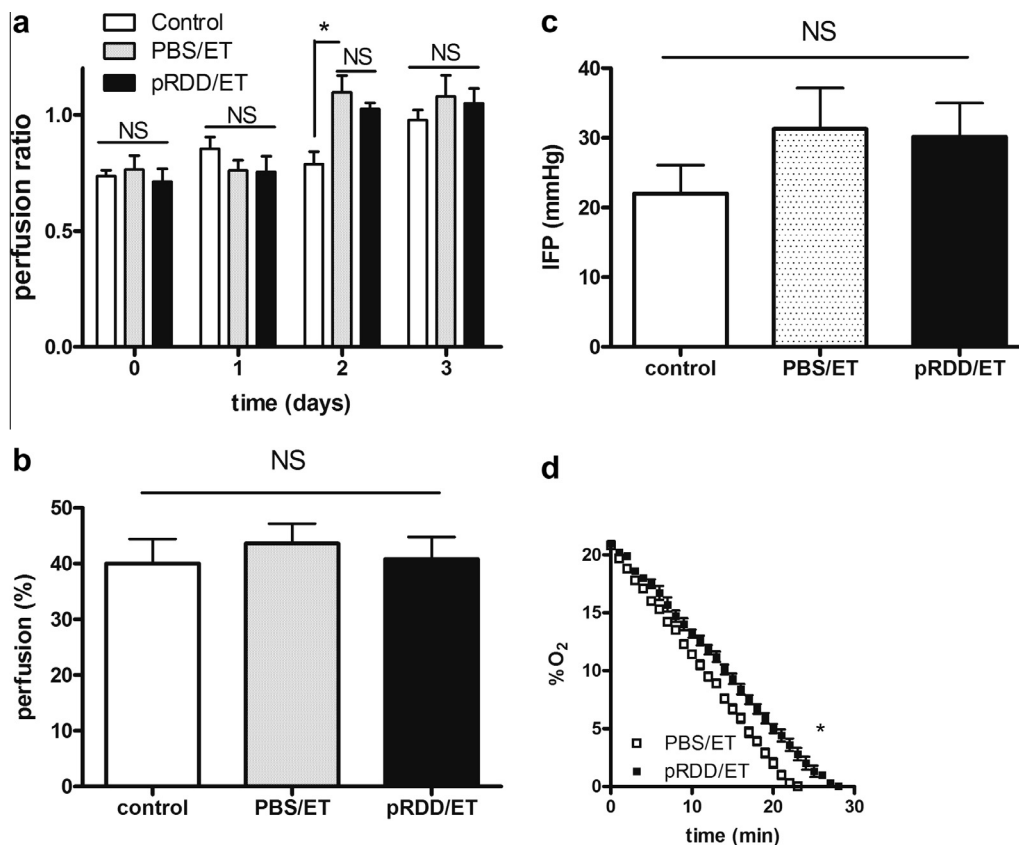


Fig. 4. Perfusion, interstitial fluid pressure and oxygen consumption rate in TLT. (a) Perfusion measured by Laser Doppler imaging before (day 0) and 3 days after treatment of PBS ($n = 10$), PBS + electrotransfer (PBS/ET) ($n = 7$) and pRDD electrotransfer (pRDD/ET) ($n = 7$). (b) Mean percentage of perfused areas with patent blue for TLT treated by PBS ($n = 5$), PBS + electrotransfer ($n = 7$) and pRDD + electrotransfer ($n = 6$) measured 3 days after treatment. (NS = non significant, $*p < 0.05$). (c) Interstitial fluid pressure measurement in TLT 3 days after treatment of PBS ($n = 7$), PBS + electrotransfer ($n = 7$) and pRDD + electrotransfer ($n = 7$). Each point represents the mean $\pm$ SEM. (NS, non significant). (d) Tumor oxygen consumption rate measured by high frequency EPR in TLT cells electrotransferred with PBS ($\square$, $n = 4$) or pRDD ($\blacksquare$, $n = 4$). Each point represents the mean $\pm$ SEM. ($*p < 0.05$).

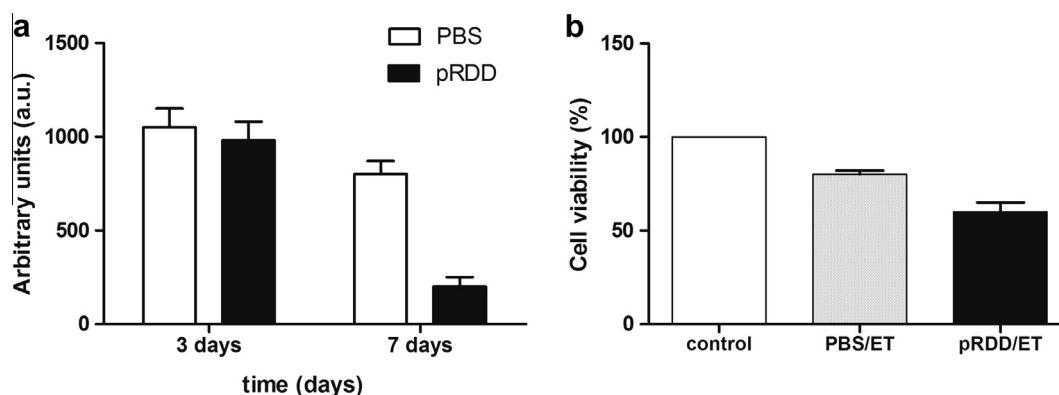


Fig. 5. CD31 immunostaining, cell viability and apoptosis of TLT tumors. (a) Fluorescence (arbitrary units) in TLT tumors 3 and 7 days after pPRD or PBS electrotransfer ($n = 4$). Each point represents the mean $\pm$ SEM. ($*p < 0.05$) (b) TLT cells' viability was assessed by the trypan blue exclusion technique, 24 h after pulse application *in vitro*. Each point represents the mean $\pm$ SEM.

assay, pRDD electrotransfer had no influence on tumor perfusion as compared to electrotransfer alone or to PBS injection at day 3 (Fig. 4a). The percentage of the tumor surface stained by patent blue was unchanged (Fig. 4b), confirming that pRDD electrotransfer did not affect tumor perfusion. Also, interstitial fluid pressure was not modified 3 days after pRDD electrotransfer (Fig. 4c).

To check if pRDD electrotransfer induces an antiangiogenic effect, immunostaining with an antibody directed against CD31

was performed. The density of endothelial cells in TLT tumors was not affected 3 days after pRDD electrotransfer but was significantly decreased 7 days after pRDD electrotransfer (Fig. 5a, Supplementary Fig. 2). Moreover, an alteration in the nuclear integrity of TLT cells was also observed.

To check if the increase in tumor pO₂ induced by electrotransfer of pRDD is due to a decrease in tumor oxygen consumption by the tumor cells, the oxygen consumption of TLT cells was investigated

in vitro 24 h after electrotransfer (time of maximal luciferase expression *in vitro*, data not shown). TLT cells consumed oxygen more slowly after pRDD electrotransfer than control cells submitted only to pulse application (Fig. 4d). The mean slopes were significantly different ($p < 0.05$) (-0.91 ± 0.06 and -0.81 ± 0.18 $\mu\text{M}/\text{min}$ for the cells treated by electrotransfer with or without pRDD, respectively).

TLT cells viability was assessed by the trypan blue exclusion technique, 24 h after pulse application *in vitro*. Compared to the control group (100%), the viability was significantly decreased to 80 ± 2 and $60 \pm 5\%$ after electrotransfer of PBS and pRDD, respectively ($p < 0.001$) (Fig. 5b). This decrease in cell viability was confirmed by DAPI staining of the nuclei: we observed many nuclear breaks, indicating apoptosis, after pRDD electrotransfer (Supplementary Fig. 3).

Discussion

Electrotransfer of pRDD significantly (i) decreased the growth of solid tumors, (ii) increased tumor oxygenation by decreasing local oxygen consumption and (iii) improved the effects of radiotherapy when irradiation was applied at maximal oxygenation time, consistent with the effects observed in the tumor microenvironment.

Antitumor and antiangiogenic effect of pRDD electrotransfer

As RDD has an antimetastatic and antiangiogenic effect on melanoma [17,18], we assessed the effect of electrotransfer of pRDD on TLT solid tumor and confirmed that pRDD electrotransfer delayed the growth of TLT compared to control groups. The antiangiogenic effect of pRDD electrotransfer assessed by immunohistostaining of CD31 was also demonstrated. This effect appeared later than the effect on tumor oxygenation. Moreover, pRDD decreased the viability of TLT cells. These data suggested that the local and sustained release of the antiangiogenic factor RDD was effective in decreasing the size of tumors and might reduce systemic side effects associated with conventional antiangiogenic drugs.

Effect of pRDD on tumor microenvironment

Even though antiangiogenic factors were first believed to lead to a decrease in tumor perfusion, recent data suggested that antiangiogenic agents could induce a transient increase in blood flow and a transient normalization of the tumor vasculature [24,25]. For the first time, we demonstrate that tumor oxygenation could be dramatically enhanced after antiangiogenic gene therapy. Such a transient increase in oxygen levels after classical antiangiogenic treatments has previously been reported [20,25,28,29]. The kinetics of this effect was assessed, showing that this increase in oxygenation was already significant after 2 days with a maximal $p\text{O}_2$ value reached 3–4 days after treatment. In the two tumor models tested, the enhancement of tumor oxygenation observed after a single pRDD electrotransfer was at least as high as the one observed after daily administration of thalidomide ($p\text{O}_2$ from 1.7 ± 0.3 mmHg to 15.9 ± 3.1 mmHg after 2 days, in TLT model) or SU5416 ($p\text{O}_2$ from 2.7 ± 0.4 mmHg to 6.8 ± 1.1 mmHg after 2 days, in TLT model) [27,28]. It was also more prolonged in time. The oxygenation data do not indicate a therapeutic window but rather a better tumor oxygenation.

To understand how pRDD electrotransfer induces this increase in oxygenation for at least 4 days, we investigated the mechanisms responsible for this effect. As tumor oxygenation results from a balance between blood supply and oxygen consumption, we assessed both variables. We demonstrated that the effect on tumor oxygenation was due to a decreased oxygen consumption of the cells. This decrease was a consequence of a decrease in both the

viability of cells and the oxygen consumption rate of the remaining viable cells. Decreased viability was also reported *in vivo* [18]. At time of maximal $p\text{O}_2$, the tumor perfusion was unchanged indicating that the oxygen effect was not caused by an increase in tumor perfusion. In most tumor models, it has been demonstrated that high voltage pulse application causes a rapid but transient blockage of the tumor blood flow reduction that recovers in 24 h and a transient reduced tumor oxygenation [37]. The absence of change in TLT perfusion 24–72 h after pRDD electrotransfer was consistent with these data. SU 5416 and ZD6474 have also been reported to increase tumor oxygenation by a decrease in oxygen consumption rate, despite an absence of effect on tumor perfusion [28,29].

Combination of antiangiogenic gene therapy and radiotherapy

As oxygenation is a key factor in the effectiveness of radiotherapy, we hypothesized that the increase in oxygenation observed 3 days after pRDD electrotransfer could radiosensitize. Hence, we assessed tumor growth after the combination of radiotherapy and pRDD electrotransfer when radiation was applied at the time of maximal oxygenation (day 3). We demonstrated the potentiation of radiotherapy by the localized antiangiogenic gene therapy. These results are consistent with previous studies with antiangiogenic agents which showed that the transient increase in tumor oxygenation enhanced the efficacy of radiotherapy [27,28,36]. Very recently, gene therapy with different encoded proteins and in various cancers has been reported to enhance radiosensitivity [38–41]. However, the mechanisms underlying the radiosensitization have not always been described.

Conclusion

The combination of antiangiogenic gene therapy and radiotherapy applied in the time of maximal oxygenation could be a promising alternative for cancer treatment. The advantage of gene therapy by electrotransfer over conventional antiangiogenic agents would be (i) the localized effect the encoded antiangiogenic agent in the tumor treated by radiotherapy without significant systemic effects and (ii) the sustained release of this encoded protein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.radonc.2013.03.018>.

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