European Journal of Pharmaceutics and Biopharmaceutics 78 (2011) 314–319

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

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

Liévin Daugimont [a], Gaelle Vandermeulen [a], Florence Defresne [b], Caroline Bouzin [b], Lluis M. Mir [c], Céline Bouquet [d], Olivier Feron [b], Véronique Préat [a,*]

[a] Université Catholique de Louvain, Louvain Drug Research Institute, Unité de Pharmacie galénique, Brussels, Belgium
[b] Université Catholique de Louvain, Institut de recherche expérimentale et clinique, Pôle de Pharmacologie, Brussels, Belgium
[c] CNRS, UMR 8203, Institute Gustave-Roussy, Villejuif, France
[d] BioAlliance Pharma, Paris, France

ARTICLE INFO

Article history:
Received 14 October 2010
Accepted in revised form 4 February 2011
Available online 18 February 2011

Keywords:
Antiangiogenic plasmid
Melanoma
Electroporation
Disintegrin domain of ADAM 15

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

* Corresponding author. Université Catholique de Louvain, Louvain Drug Research Institute, Unité de Pharmacie galénique, Avenue E. Mounier 73-20, 1200 Brussels, Belgium. Tel.: +32 2 764 73 09; fax: +32 2 764 73 98.
E-mail address: veronique.preat@uclouvain.be (V. Préat).

0939-6411/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2011.02.001

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 $\alpha v\beta 3$ and $\alpha 5\beta 1$ 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