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Biological Properties of Melanoma and Endothelial Cells after Plasmid AMEP Gene Electrotransfer Depend on Integrin Quantity on Cells

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Abstract The data on the biological responsiveness of melanoma and endothelial cells that are targeted by Anti-angiogenic MEtargidin Peptide (AMEP) are limited; therefore, the antiproliferative, antimetastatic and antiangiogenic effects of AMEP were investigated in murine melanoma and human endothelial cells after plasmid AMEP gene electrotransfer into the cells in vitro. Plasmid AMEP, a plasmid coding for the disintegrin domain of metargidin targeting specific integrins, had cytotoxic and antiproliferative effects on murine melanoma and human endothelial cells. Among the metastatic properties of cells, migration, invasion and adhesion were investigated. Plasmid AMEP strongly affected the migration of murine melanoma and human endothelial cell lines and also affected the invasion of highly metastatic murine melanoma B16F10 and human endothelial cell lines. There was no effect on cell adhesion on Matrigel^{TM} or fibronectin in all cell lines. The antiangiogenic effect was shown with tube formation assay, where human microvascular endothelial cell line (HMEC-1) proved to be more sensitive to plasmid AMEP gene electrotransfer than the human umbilical vein endothelial cell line (HUVEC). The study indicates that antiproliferative and antimetastatic biological responses to gene electrotransfer of plasmid AMEP in murine melanoma cells were dependent on the integrin quantity on melanoma cells and not on the expression level of AMEP. The strong antiangiogenic effect expressed in human endothelial cell lines was only partly dependent on the quantity of integrins and seemed to be plasmid AMEP dose dependent.

Keywords Antiangiogenic · Gene electrotransfer · Human endothelial cells · Integrins · Murine melanoma cells · Plasmid AMEP

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Introduction

Angiogenesis plays a critical role in the development of tumors (Hanahan and Weinberg 2011). The newly formed vessels have a specific physiology, providing an excellent target for the development of biological agents. Among these are several antibodies targeting either angiogenic factors (VEGF, PDGF) or their receptors (VEGFR-1, VGRF-2, PGDF-R) on the cells (Adamis and Shima 2005; Ohno 2006). In development are also drugs targeting other cell receptors, like integrins, which are overexpressed and involved in angiogenesis and metastasis (Van Belle et al. 1999; Kuphal et al. 2005; Avraamides et al. 2008). One of these drugs is also AMEP (Zhang et al. 1998; Nath et al. 1999; Trochon-Joseph et al. 2004).

AMEP is the recombinant disintegrin domain of metargidin ADAM-15 (adamalysin), which is overexpressed in melanoma cells and activated angiogenic endothelial cells. AMEP is able to bind integrins, specific transmembrane cell receptors, which were originally characterized as receptors responsible for the anchoring of cells to the extracellular matrix (Varner and Cheresh 1996). More recently, integrins have been shown to impact on dynamic processes in normal and tumor cells, such as intracellular signaling and gene expression, controlling the cell differentiation, proliferation, migration, and survival (Mizejewski 1999; Avraamides et al. 2008; Desgrosellier and Cheresh 2010). The integrin family is composed of different α and β subunits forming over twenty different αβ heterodimer complexes (Varner and Cheresh 1996). After binding extracellular matrix proteins, which are their main ligands, α and β integrin subunits cluster and induce a cascade reaction that serves as a signaling pathway (Mizejewski 1999). AMEP binds α5β1 and αvβ3 integrins via its Arg-Gly-Asp (RGD) integrin binding sequence and thus acts as an antagonist of integrin–extracellular matrix protein interaction, and consequently the normal signaling pathway is affected (Zhang et al. 1998; Nath et al. 1999; Desgrosellier and Cheresh 2010; Danhier et al. 2012).

Previous preclinical data have shown antitumor and antiangiogenic effectiveness of AMEP in vitro using a recombinant protein and in vivo using AMEP coding plasmid (Trochon-Joseph et al. 2004; Daugimont et al. 2011). On the basis of these results, a first-in-man clinical study was conducted using intratumoral gene electrotransfer of plasmid AMEP, a plasmid DNA devoid of any antibiotic resistance gene and coding for AMEP peptide under the control of the cytomegalovirus (CMV) promoter. The study has indicated a good safety profile and also some local efficacy of plasmid AMEP gene electrotransfer in the melanoma cutaneous nodules (Spanggaard et al. 2012).

However, there are few data on the changes of biological responsiveness of melanoma and endothelial cells, after plasmid AMEP gene electrotransfer into the cells in vitro. As a result of the initiation of several new clinical trials, information on the cytotoxic and antiangiogenic effects of AMEP, as well as on its effect on the invasiveness of melanoma cells, is required. Therefore, in the present study, we investigated, after plasmid AMEP gene electrotransfer, the expression level of AMEP, evaluated its effect on the functional properties of murine melanoma and human endothelial cells and correlated the observed effect with the presence and quantity of integrins in these cells in vitro. The study indicates that the biological response of melanoma cells is in correlation with integrin quantity on the cells and not with the expression level of AMEP after gene electrotransfer of plasmid AMEP, whereas antiangiogenic effects of AMEP seem to also involve other mechanisms.

Materials and Methods

Cell Lines

Murine melanoma cell lines; B16F1 with low metastatic potential and B16F10 with high metastatic potential (American Type Culture Collection, Manassas, VA) were cultured in advanced minimum essential medium (AMEM, Life Technologies, Grand Island, NY) supplemented with 5 % fetal bovine serum (FBS, Life Technologies), 10 mM/l L-glutamine (Life Technologies), 100 U/ml penicillin (Grünenthal, Aachen, DE) and 50 mg/ml gentamicin (Krka, Novo Mesto, Slovenia) in a 5 % CO_2 humidified incubator at 37 °C.

Human umbilical vein endothelial cell line HUVEC (the gift of Urska Batista, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia) was cultured in advanced Dulbecco modified Eagle medium (DMEM, Life Technologies) supplemented with 5 % FBS, 10 mM/l L-glutamine, 100 U/ml penicillin and 50 mg/ml gentamicin in a 5 % CO_2 humidified incubator at 37 °C.

Human microvascular endothelial cell line HMEC-1 (Centers for Disease Control and Prevention, Atlanta, GA) was cultured in MCDB 131 medium (Life Technologies) supplemented with 10 % FBS, 10 mM/l L-glutamine, 10 μg/l epidermal growth factor, 1 mg/l hydrocortisone (Sigma Aldrich, St. Louis, MO), 100 U/ml penicillin and 50 mg/ml gentamicin in a 5 % CO_2 humidified incubator at 37 °C.

Plasmids

Plasmid AMEP is a 2.5 kb plasmid consisting of an expression cassette for AMEP, the disintegrin domain of human metargidin (ADAM-15), inserted in a plasmid backbone devoid of any antibiotic resistance gene (ORT technology, Cobra Biologics, UK). The AMEP transgene is a fusion between the sequences encoding the secretion signal peptide from human urokinase and the human AMEP and is placed downstream of the human cytomegalovirus CMV-intron A enhancer/promoter and upstream to the late bovine growth hormone polyadenylation signal. It was provided as a lyophilized powder in a dose of 2 mg per vial. For reconstitution and dilution of plasmid AMEP, endotoxin-free water was used. Six different concentrations—0.1, 0.2, 0.5, 1.0, 2.5 and 5.0 mg/ml (corresponding to 1, 2, 5, 10, 25 and 50 μg of plasmid AMEP)—were first prepared to determine the cytotoxic effect of plasmid DNA. For further experiments, the 1 mg/ml concentration was used.

Plasmid DNA encoding enhanced green fluorescent protein (eGFP) under the control of the CMV promoter, CMV-EGFP-N1 (pEGFP, BD Biosciences Clontech, Palo Alto, CA), was used as a control plasmid. Plasmid pEGFP

was amplified in a competent *Escherichia coli* (TOP10; Life Technologies); isolation and purification were performed with the Qiagen Maxi-Endo-Free Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The spectrophotometric method (Epoch Microplate Spectrophotometer, Take3^{TM} Micro-Volume Plate, BioTek, Bad Friedrichshall, Germany) and agarose gel electrophoresis were used to determine the quality and quantity of isolated plasmid pEGFP. A final concentration of 1 mg/ml was prepared by dilution in endotoxin-free water.

In Vitro Gene Electrotransfer

A monolayer of 80 % confluent cell cultures was trypsinized, washed with appropriate media containing 5 % FBS and washed again in ice-cold electroporation buffer (EP buffer: 125 mM sucrose, 10 mM K_2HPO_4, 2.5 mM KH_2PO_4, 2 mM $MgCl_2 \cdot 6H_2O$). Cell suspension for the electroporation was prepared in ice-cold EP buffer (25×10^6 cells/ml) and was later divided into several aliquots of 44 µl. A total of 11 µl of different plasmids (pEGFP, plasmid AMEP) or endotoxin-free water was added. Then 50 µl of the resulting mixture (1×10^6 cells) was pipetted between two stainless steel parallel plate electrodes with a 2 mm gap in between. Eight square wave electric pulses (EP), with voltage-to-distance ratio of 600 V/cm, pulse duration of 5 ms and frequency of 1 Hz were generated by electric pulse generator GT-01 (Faculty of Electrical Engineering, University of Ljubljana, Slovenia). After the electroporation, the cells were incubated for 5 min with 100 µl of FBS and then plated in their corresponding complete medium for further assays.

Cytotoxicity Assay

After addition of plasmid AMEP at different increasing concentrations (0.1–5.0 µg/µl) alone or in combination with EP, 1×10^3 B16F1 or B16F10 cells, 1.5×10^3 HMEC-1 or 1×10^3 HUVEC cells were plated in 0.1 ml of appropriate complete media on 96-well plates (Corning Incorporated, Corning, NY, USA). Cells were incubated at 37 °C in a 5 % CO_2 humidified incubator. To determine cell viability, Presto Blue assay (Life Technologies) was used according to the manufacturer's instructions 72 h after the cells were plated. To measure the fluorescence intensity of Presto Blue, a microplate reader (Infinite 200, Tecan, Männedorf, Switzerland) was used. The survival curve for cells that were electrotransfected with plasmid AMEP was normalized to the cytotoxicity of EP alone. The survival curve for cells where only different concentrations of plasmid AMEP were added was normalized to the untreated control group.

Clonogenic Assay

After gene electrotransfer of plasmid AMEP with increasing concentrations (0.1–5.0 µg/µl), into B16F10 and B16F1 cells, the cells were plated in 3 ml of appropriate complete media (300 cells per petri dish). Seven days later, when the colonies were formed, they were fixed, stained with crystal violet (Sigma Aldrich) and counted. The survival curve for plasmid AMEP electrotransfected cells was normalized to the cytotoxicity of EP alone. The survival curve for cells where only different concentrations of plasmid AMEP were added was normalized to the untreated control group. The inhibitory concentration of plasmid AMEP that reduced cell survival to 50 % (IC_{50}) was determined.

Total mRNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

To determine AMEP expression at the mRNA level, 3 days after in vitro gene electrotransfer of cells, RNA extraction and qRT-PCR analysis were performed. It was demonstrated before that maximum expression of protein is reached 2 days after gene electrotransfer of plasmid DNA and then remains at the same level over the observation period of 6 days (Cemazar et al. 2004). Accordingly to the reference, AMEP expression was evaluated 3 days after gene electrotransfer to assure that expression level of AMEP reached maximum. Cells were first trypsinized and harvested and then centrifuged. Afterwards the total RNA was extracted from the cells with TRIzol Plus RNA Purification System (Life Technologies) according to the manufacturer's instructions. Spectrophotometric method was used to determine concentrations and purity of RNA. Transcription of extracted RNA into cDNA was then performed on 250 ng of total RNA extract using the SuperScript VILO cDNA Synthesis Kit (Life Technologies), according to the manufacturer's instructions. The 10× and 100× diluted mixtures of transcribed cDNA were used as a template for the quantitative polymerase chain reaction (qPCR) using TaqMan Gene Expression Master Mix (Life Technologies) and TaqMan Gene Expression Assay (applied biosystems), which contained primers and custom made TaqMan probe sequence (CCTGTTGTCAAAATTG) to amplify the fragment of human AMEP cDNA (AMEP). To amplify human or murine 18S ribosomal RNA, TaqMan probes (Hs00172187_m1 and Mm03928990_g1) were used as an internal control. qPCR was performed on 7300 System (Applied Biosystems). The thermal cycler protocol consisted of activation of Uracil-DNA Glycosylase (2 min at 50 °C), hot start activation of AmpliTaq Gold Enzyme (10 min at 95 °C), 45 cycles of denaturation (15 s at 95 °C), annealing and extension (1 min 60 °C). The 7300

System SDS software (Applied Biosystems) was used for the qPCR products analysis. The level of AMEP mRNA after the gene electrotransfer was normalized to values obtained for the reference genes from untreated control group extracts.

Proliferation Assay

After gene electrotransfer, the cells were plated on a 6 cm petri dish (Corning) for 16 h to recover. Only viable B16F1 (2.5×10^2), B16F10 (2.5×10^2), HMEC-1 (1×10^3) or HUVEC (3×10^2) cells were plated for proliferation assay in 0.1 ml of appropriate media in 96-well plates. Cells were incubated at 37 °C in a 5 % CO_2 humidified incubator. Presto Blue assay was performed 2, 48 and 96 h after the cells were plated, according to manufacturer's instructions. Proliferation of cells in each experimental group was normalized to day 0. To determine the numerical values of proliferation reduction at day 4, additional normalization to the untreated control group at day 4 was performed.

Migratory Potential Assay

To determine the effect of plasmid AMEP on migratory potential of melanoma and endothelial cell lines, the xCELLigence real time cell analyzer (RTCA) (Roche Diagnostics GmbH, Mannheim, Germany) and CIM-16 plates (Roche) were used. The bottoms of CIM-16 plates were coated with 0.3 μg of human fibronectin (BD Biosciences) and incubated in a laminar air flow chamber for 30 min. The upper compartments of the CIM-16 plates were then coated with 0.5 μg of human fibronectin (BD Biosciences) and incubated at 37 °C in a 5 % CO_2. After 2 h, the upper compartments were washed with 50 μl of phosphate-buffered saline (PBS). The lower compartments were filled with 180 μl of appropriate complete media (containing FBS). The top and bottom compartment of the CIM-16 plates were assembled together, and 80 μl of FBS-free medium was added to the top compartment. The assembled CIM-16 plates were allowed to equilibrate for 10 min at 37 °C, 5 % CO_2 prior to addition of cells. 80 μl of B16F1 or B16F10 (1×10^4 cells/well), HMEC-1 (2.5×10^4 cells/well) and HUVEC (1×10^4 cells/well) cell suspension was seeded into the top chambers of CIM-16 plates and placed into the xCELLigence system for data collection. Impendence data, reported as cell index, were collected with the xCELLigence software every 15 min during the following 72 h. The migration of cells was shown as a curve in a two-dimensional system (time, cell index). For the analysis of the data only the linear part of the curve was considered. In the interval where the curves were linear, the slopes of the curves were compared and the percentage of migration (%) was calculated by the ratio of the slope of migrated treated cells to the slope of migrated untreated control cells.

Invasion Assay

Two-dimensional invasion assay of melanoma and endothelial cells was performed using the xCELLigence (RTCA) similar to Migratory potential assay, described before, with minor modifications. The bottoms of CIM-16 plates were coated with 0.3 μg of human fibronectin and incubated in a laminar air flow chamber for 30 min. The upper compartments of CIM-16 plates were coated with a thick layer (20 μl per membrane) of 0.75 or 1 mg/ml Matrigel™ (BD Bioscience) prepared in appropriate FBS-free media for melanoma and endothelial cells, respectively. The optimization of concentration of Matrigel™ required for invasion assay of different cell lines was performed in our laboratory in previous experiments on murine melanoma and human endothelial cell lines (data not shown). Matrigel™ was allowed to gelatinize for 2 h at 37 °C in a 5 % CO_2. Appropriate medium was added to the top and bottom compartments as previously described. Eighty microliters of B16F1 (3×10^4 cells/well) or B16F10 (3×10^4 cells/well), HMEC-1 (4×10^4 cells/well) or HUVEC (2×10^4 cells/well) cells were added and the plate was set for data collection as described above. The percentage of invasion was calculated by the ratio of the slope of invaded treated cells to the slope of invaded untreated control cells.

Adhesion Assay

In order to determine whether plasmid AMEP affects cell attachment on basement membrane or on proteins of extracellular matrix, the cell adhesion assay was performed. Three days after gene electrotransfer, the cells were seeded into 96-well plates that were first coated with BD Matrigel™ Basement Membrane Matrix (Phenol Red Free, BD Biosciences) or in a precoated fibronectin plates (Fibronectin 96-well Microplate, BD BioCoat™). A 3×10^4 B16F1 or B16F10 cells, 1.5×10^3 HMEC-1 cells or 2×10^4 HUVEC cells were plated in 0.1 ml of appropriate FBS-free media containing antibiotics. Cells were incubated at 37 °C in a 5 % CO_2 humidified incubator for 2 h. Each well was then washed twice with PBS to remove the unattached cells. Presto Blue assay was performed to determine cell adhesion, according to manufacturer's instructions. Adhesion of cells in each experimental group was normalized to the untreated control group.

Tube Formation Assay

To determine the effect of plasmid AMEP on the capability of human endothelial cells to form capillary like structures

in vitro, the tube formation assay was performed. In the preliminary experiments we determined that the optimal time for tube formation assay is 2 days for HMEC-1 and 3 days for HUVEC, after the gene electrotransfer into the cells. On the determined days after gene electrotransfer HMEC-1 (1.3×10^4) and HUVEC (2×10^4) cells were plated on μ-Slide Angiogenesis (Ibidi, Munich, Germany) covered with BD Matrigel™ Basement Membrane Matrix (Phenol Red Free, BD Biosciences) and incubated for 4–5 h until the formation of tubular complexes. The tubular complexes were stained with Calcein AM (Sigma). Images were captured with a DP72 CCD camera (Olympus, Hamburg, Germany) connected to an IX-70 inverted microscope (Olympus). AxioVision program (Carl Zeiss, Jena, Germany) was used to convert raw images into binary masks, which were quantified with AngioQuant image analysis program (Niemisto et al. 2005). The total length of tubular complexes, the total size of tubular complexes and the total number of junctions were quantified. The determined parameters of tube formation assay of each experimental group were normalized to the determined parameters of tube formation assay of untreated control group.

Integrin Determination

Expression of integrins in tested cell lines was determined by flow cytometry assay. Cells were harvested and stained with monoclonal antibodies (10 μg/ml) directed against human αvβ3 (clone LM609; FITC conjugated, Chemicon) or against murine α5β1 (clone BMB5, Chemicon). Corresponding isotype controls were used in parallel as negative control antibodies. After centrifugation of 5×10^5 cells at 1,200 rpm for 5 min (5 °C), supernatant was drawn up, each well was washed with PBS + Mg + Ca 1× (Life Technologies) (200 μl per well, followed by centrifugation at 1,200 rpm, 5 min, 5 °C). Avoiding exposure to strong light was now needed to preserve the fluorescence of conjugated antibodies. The pellet was resuspended with 20 μl of each antibody dilution, then incubated on ice and in the dark for 1 h. Each well was washed twice as described above. For conjugated anti-integrin antibodies, cells were fixed with 20 μl of formalin fixing solution (Sigma) for 5 min in the dark; 200 μl PBS + Mg + Ca 1× per well was added for reading. For nonconjugated anti-integrin antibodies, 20 μl per well of the diluted FITC secondary antibodies (15 μg/ml; donkey anti-mouse IgG, or donkey anti-rat IgG, Jackson ImmunoResearch, Suffolk, UK) was added. After 1 h of incubation as above, wells were washed twice, fixed and collected as described above. Reading was performed using a flow cytometry device (FACScan, Becton Dickinson, or equivalent). The integrin pattern was analyzed using the software BD Cell Quest (Becton Dickinson) or equivalent.

Statistical Analysis

All data were tested for distribution normality with the Shapiro–Wilk test. The differences between the experimental groups were statistically evaluated by one-way analysis of variance (one-way ANOVA) followed by a Holm–Sidak test for multiple comparison. A P value of <0.05 was considered to be statistically significant. SigmaPlot Software (Systat Software, Chicago, IL) was used for statistical analysis and graphical representation.

Results

AMEP Expression in Melanoma and Endothelial Cells

AMEP expression in B16F1 and B16F10 murine melanoma and in HUVEC and HMEC-1 human endothelial cell lines was determined three days after gene electrotransfer by qRT-PCR (Table 1). In all cell lines, AMEP mRNA levels were statistically significant higher after gene electrotransfer of plasmid AMEP (plasmid AMEP + EP) compared to all other groups (control, plasmid AMEP, pEGFP, EP, pEGFP + EP) where there was no significant change in AMEP expression. The expression level of AMEP after gene electrotransfer of plasmid AMEP was cell type dependent (Table 1).

Cytotoxicity of AMEP

The cytotoxic effect of AMEP expression after plasmid AMEP gene electrotransfer into murine melanoma and human endothelial cell lines was determined with cell viability and clonogenic assays. Plasmid AMEP gene electrotransfer in all four cell lines statistically significantly reduced cell survival (Fig. 1a, b, e, f). The cytotoxic effect of AMEP on endothelial cell lines was comparable to the effect on melanoma B16F1 cell line, whereas the B16F10 cell line was statistically significant less sensitive to plasmid AMEP gene electrotransfer. The difference in the sensitivity of B16F1 and B16F10 cell lines differing in metastatic potential was also obvious in clonogenic assay, where the reproductive potential of cells was determined. Gene electrotransfer of plasmid AMEP reduced the

Table 1 Expression levels of AMEP 3 days after plasmid AMEP gene electrotransfer in different cell lines

Cell line	Fold increase in AMEP expression
B16F1	283 ± 26
B16F10	4,407 ± 1,171
HUVEC	40 ± 1
HMEC-1	248 ± 56

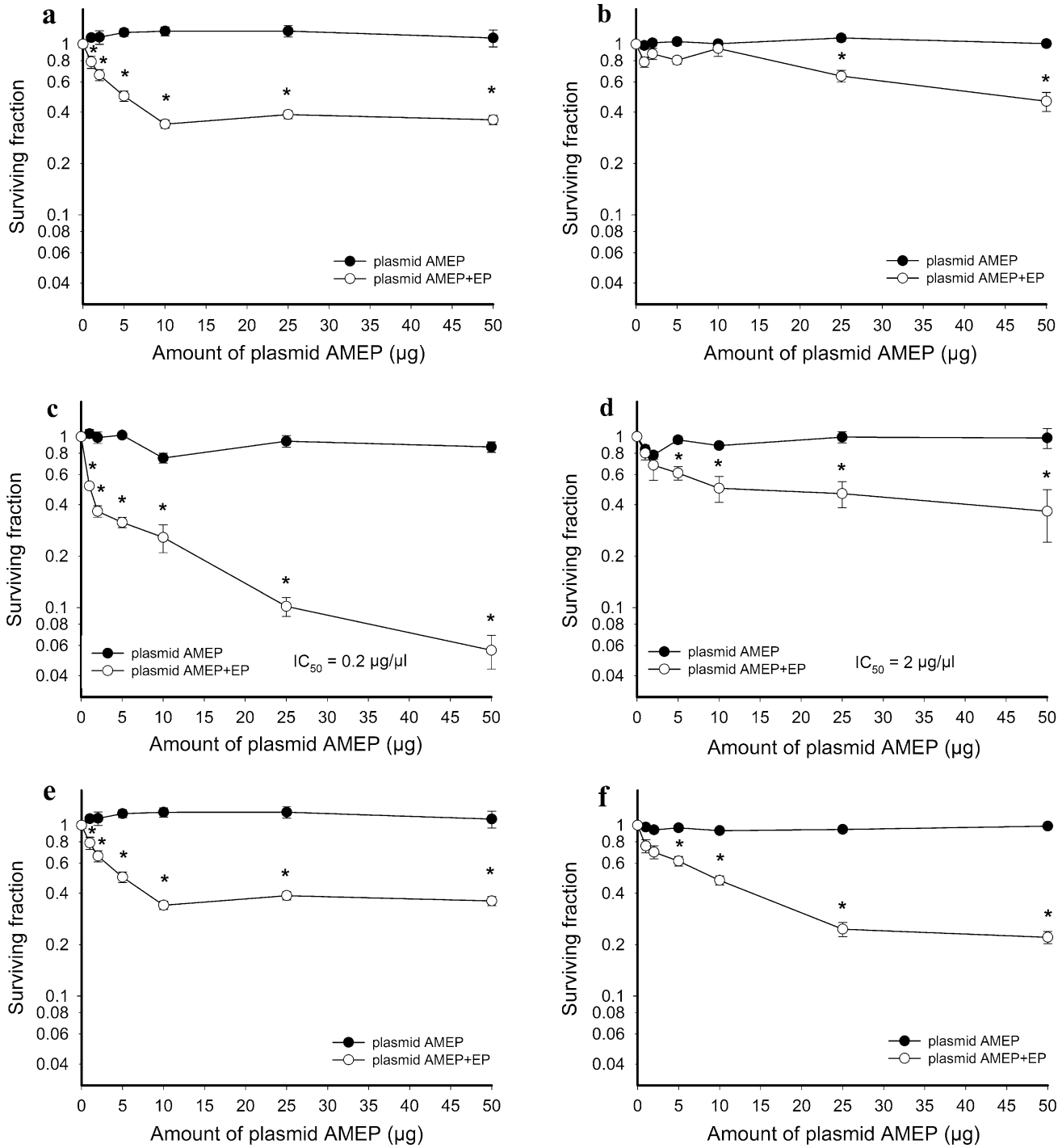

Fig. 1 Gene electrotransfer of plasmid AMEP in different concentrations resulted in cytotoxic effects. The cytotoxicity was increasing with higher amount of plasmid AMEP. Cytotoxicity of AMEP in murine melanoma B16F1 (**a**) and B16F10 (**b**) cells after the addition of plasmid AMEP in different concentrations alone (plasmid AMEP) or in combination with electric pulses (plasmid AMEP + EP) was determined 3 days after the treatment. The inhibitory concentration of plasmid AMEP that reduced cell survival to 50 % (IC_{50}) was determined 7 days after the gene electrotransfer of plasmid AMEP in different concentrations (0.1–5.0 μg/μl) in B16F1 (**c**) and B16F10 (**d**) cells according to the clonogenic assay protocol. The cytotoxicity of AMEP in human endothelial HUVEC (**e**) and HMEC-1 (**f**) cells after the addition of plasmid AMEP in different concentrations alone (plasmid AMEP) or in combination with electric pulses (plasmid AMEP + EP) was determined after 3 days. The survival curves for all cells that were electrotransfected with plasmid AMEP were normalized to the cytotoxicity of EP treatment alone. $^*P < 0.05$ versus EP treatment alone (B16F1, B16F10, HUVEC, HMEC-1)

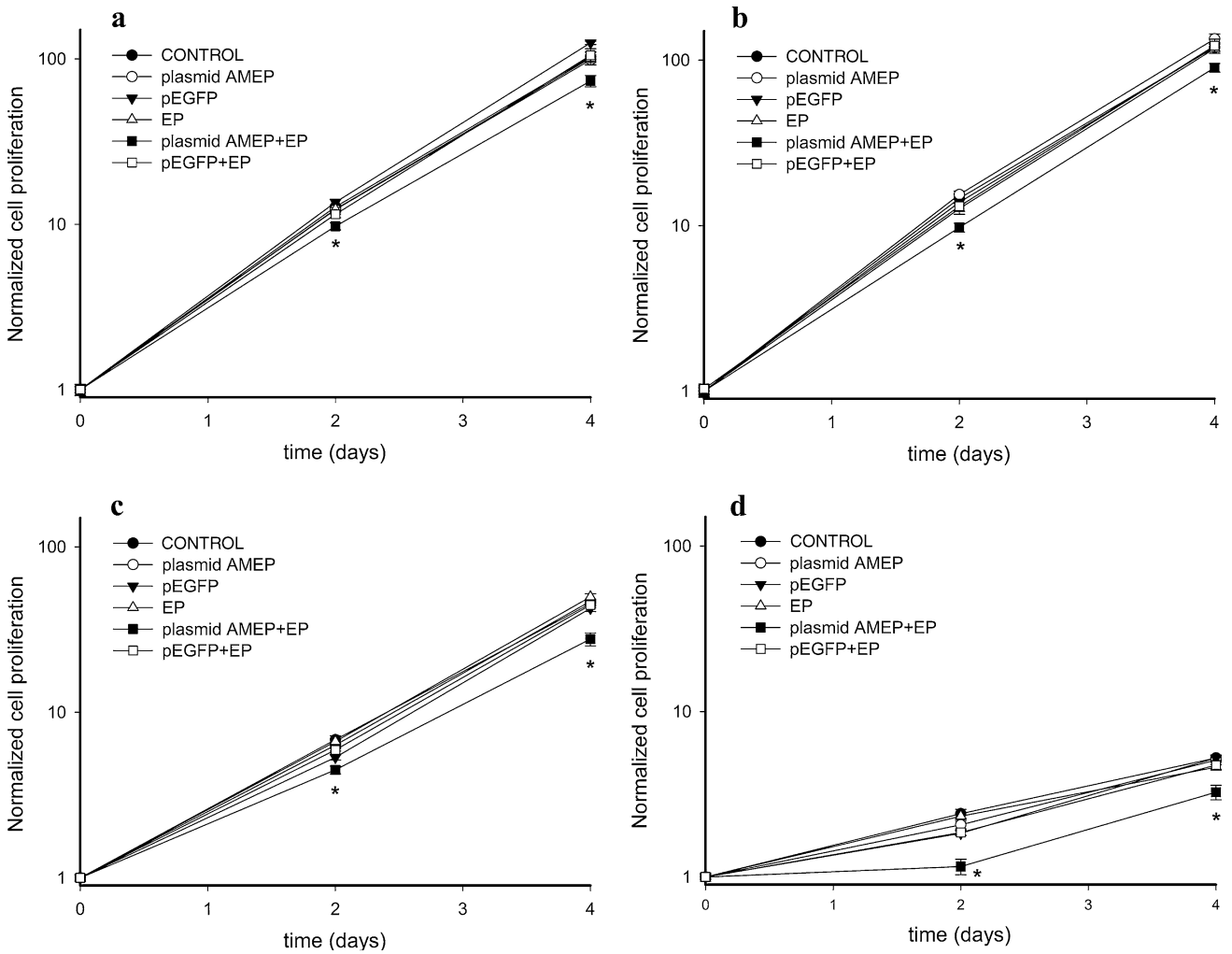

Fig. 2 Gene electrotransfer of plasmid AMEP reduced cell proliferation. Proliferation of murine melanoma B16F1 (**a**) and B16F10 (**b**) or human endothelial HUVEC (**c**) and HMEC-1 (**d**) cells in untreated cells alone (CONTROL) or in combination with electric pulses (EP) and after the addition of plasmid AMEP or pEGFP alone (plasmid AMEP, pEGFP) or in combination with electric pulses (plasmid AMEP + EP, pEGFP + EP). The proliferation of cells in each experimental group was normalized to day 0. $*P < 0.05$ versus untreated control cells (B16F1, B16F10, HUVEC, HMEC-1)

surviving fraction of B16F1 cells for 95 %, whereas in B16F10 for 60 % (Fig. 1c, d).

Effects on Cell Proliferation

The ability of plasmid AMEP to inhibit murine melanoma and human endothelial cell proliferation was determined with cell proliferation assay. Plasmid AMEP gene electrotransfer (10 μg) statistically significantly reduced the proliferation of all cell lines (Fig. 2). The proliferation of cells was monitored up to 4 days after gene electrotransfer. At day 4 the proliferation of both human endothelial cell lines was reduced to ~60 %, whereas in murine melanoma B16F1 and B16F10 cell lines proliferation was reduced to 75 and to 70 %, respectively (Fig. 3). There was no statistically significant difference in proliferation reduction between melanoma B16F1 and B16F10 cell lines. To exclude the effect of empty plasmid DNA on the proliferation of cells, gene electrotransfer of pEGFP was performed. The electrotransfer of pEGFP did not affect cell proliferation.

Effects on Migratory Potential of Cells

The effects of plasmid AMEP on migration of murine melanoma and human endothelial cells were determined with the xCELLigence RTCA. The migration of B16F1 and B16F10 murine melanoma cells was statistically significantly reduced after gene electrotransfer of plasmid AMEP (10 μg) for ~55 and ~40 %, respectively (Fig. 4). Furthermore, the migration of endothelial HUVEC and HMEC-1 cells was also statistically significantly reduced

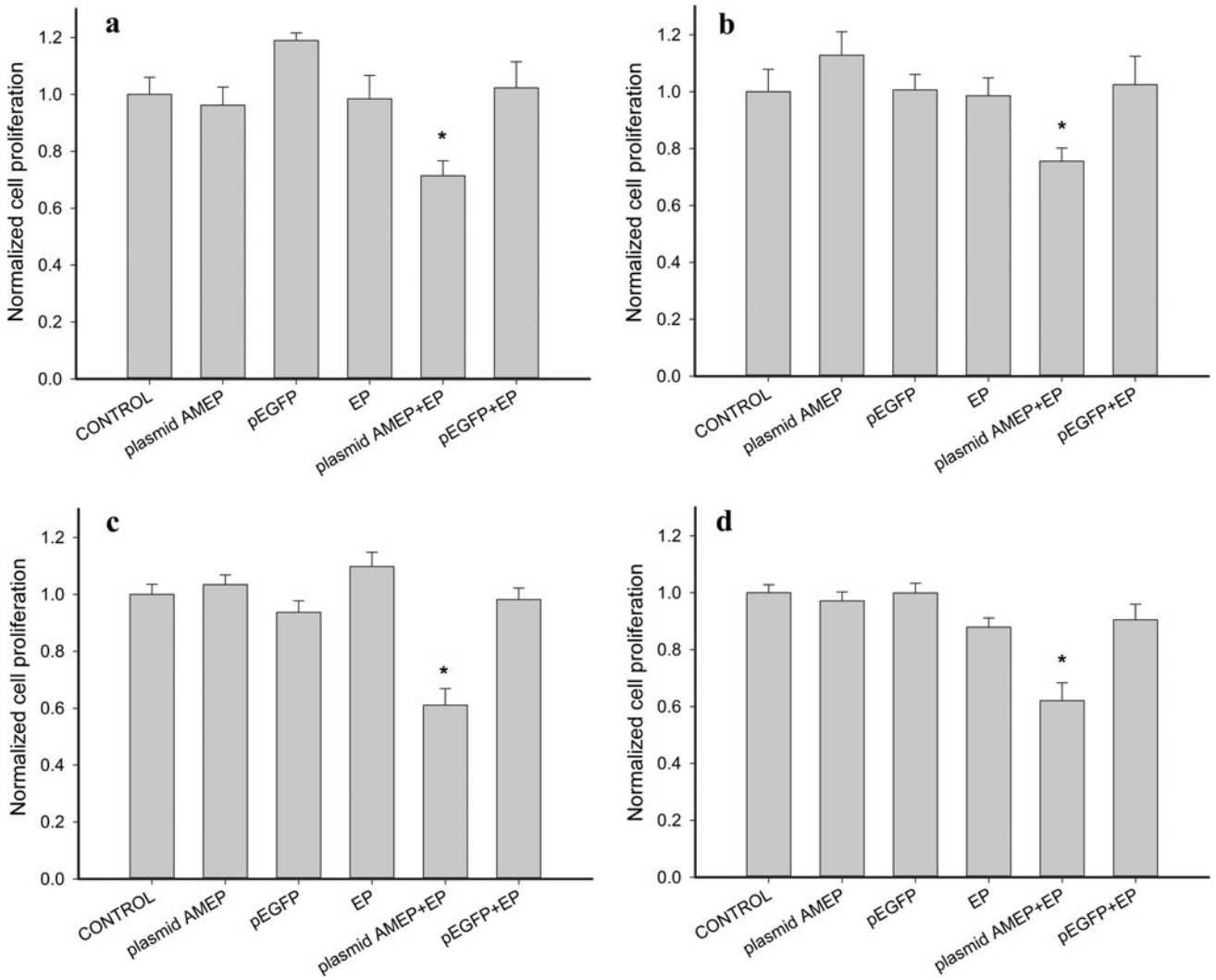

Fig. 3 Gene electrotransfer of plasmid AMEP reduced cell proliferation at day 4. Proliferation of murine melanoma B16F1 (**a**) and B16F10 (**b**) or human endothelial HUVEC (**c**) and HMEC-1 (**d**) cells in untreated cells alone (CONTROL) or in combination with electric pulses (EP) and after the addition of plasmid AMEP or pEGFP alone (plasmid AMEP, pEGFP) or in combination with electric pulses (plasmid AMEP + EP, pEGFP + EP). The proliferation of cells in each experimental group was normalized first to day 0 and then to the untreated control group at day 4. $*P < 0.05$ versus untreated control cells (B16F1, B16F10, HUVEC, HMEC-1)

after plasmid AMEP electrotransfer; 50 % for HUVEC and 30 % for HMEC-1 cells (Fig. 4). The gene electrotransfer of the control pEGFP (10 μg) also reduced cell migration in both melanoma and endothelial cells, but the difference in reduction of migration between transfection with plasmid AMEP and pEGFP was large enough to be statistically significant (Fig. 4).

Effects on Cell Invasiveness

The effects of plasmid AMEP on invasion of melanoma and endothelial cells were determined with the xCELLigence RTCA. Cell invasion through basement membranes allows cancer cells to metastasize. The B16F1 melanoma cells with low metastatic potential barely invaded through the basement membrane on the xCELLigence system, which is in line with previous statement of poor metastatic potential (Fig. 5a). Thus, the invasion assay of melanoma B16F1 cells was impossible to perform on xCELLigence system coated with 1 or 0.75 mg/ml of Matrigel™. However, in the other three cell lines (B16F10, HUVEC, HMEC-1), which invaded through the 1 mg/ml (for endothelial cell lines) or 0.75 mg/ml (for melanoma cell line) of Matrigel™, the plasmid AMEP (10 μg) gene electrotransfer statistically significantly reduced their invasion for ~35, ~50 and ~50 % respectively (Fig. 5b, c, d). There was no statistically significant change in cell invasion after gene electrotransfer of pEGFP (10 μg) (Fig. 5b, c, d).

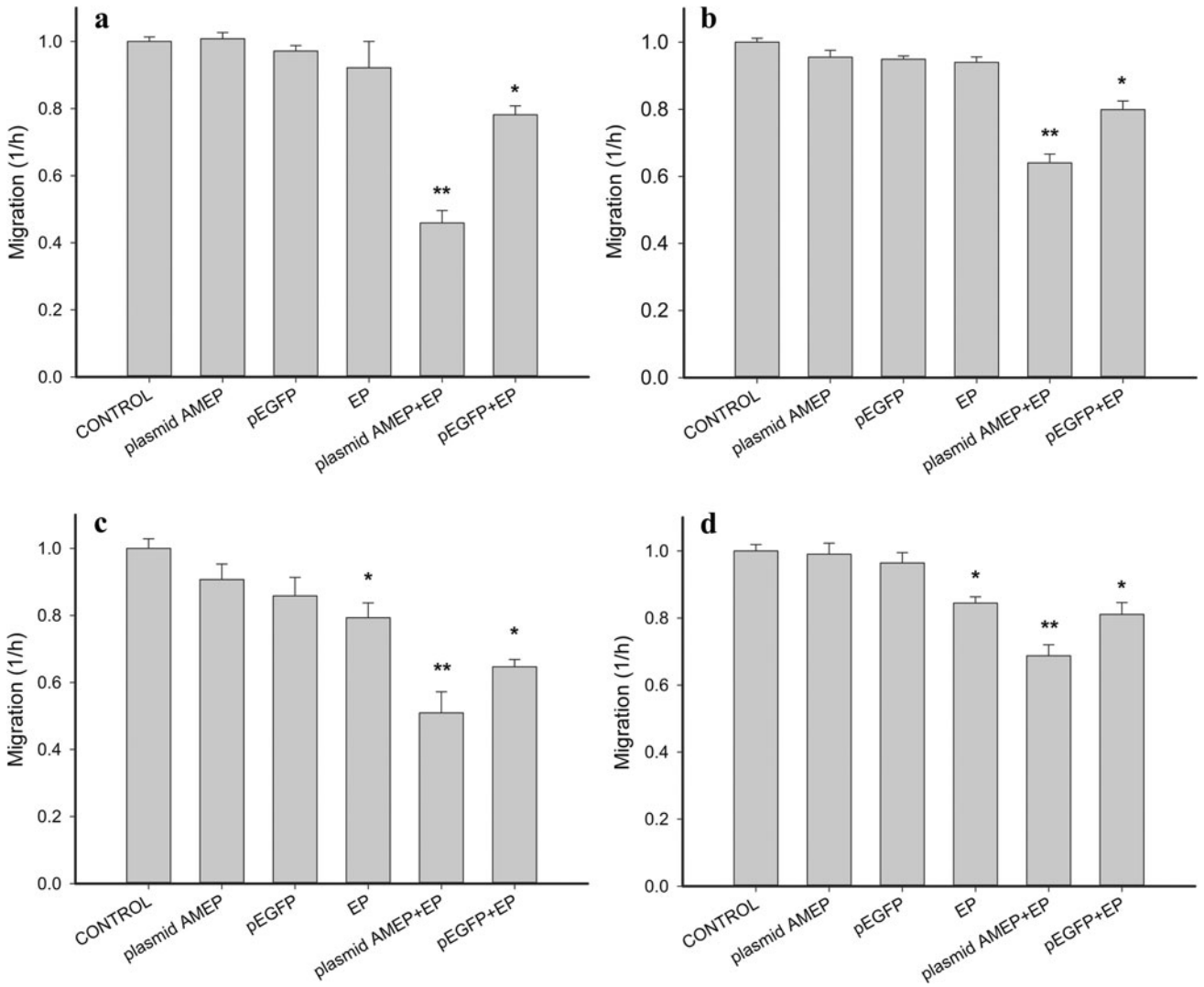

Fig. 4 Gene electrotransfer of plasmid AMEP reduced cell migration. The migration of murine melanoma B16F1 (**a**) and B16F10 (**b**) or human endothelial HUVEC (**c**) and HMEC-1 (**d**) cells in untreated cells alone (CONTROL) or in combination with electric pulses (EP) and after the addition of plasmid AMEP or pEGFP alone (plasmid AMEP, pEGFP) or in combination with electric pulses (plasmid AMEP + EP, pEGFP + EP). The migration of cells in each experimental group was normalized to the untreated control group. $*P < 0.05$ versus untreated control cells (B16F1, B16F10, HUVEC, HMEC-1). $**P < 0.05$ versus untreated control groups, electric pulses alone and in combination with pEGFP (B16F1, B16F10, HUVEC, HMEC-1)

Effects on Cell Adhesion

To determine if plasmid AMEP has any effect on cell attachment on basement membrane or on extracellular matrix proteins, cell adhesion assay was performed. There was no statistically significant change in murine melanoma cells attachment on Matrigel^{TM}, which mimics basement membrane matrix, and corresponds to an environment very similar to the one found in tumors, after gene electrotransfer of plasmid AMEP or plasmid EGFP. Furthermore, the gene electrotransfer of plasmid AMEP also did not affect the attachment of human endothelial cells (Fig. 6a, b, c, d).

Since plasmid AMEP should affect the $\alpha 5\beta 1$ integrin mediated binding to fibronectin, the murine melanoma and human endothelial cell's attachment on fibronectin coated plates was determined. Gene electrotransfer of plasmid AMEP into the cells did not affect the adhesion of cells on fibronectin, indicating that other adhesion molecules were involved (Fig. 6a, b, c, d).

Effect on Tube Formation of Endothelial Cells

The formation of capillary-like structures (tube formation) in vitro (also called in vitro angiogenesis assay) is a good indicator if the substance has an antiangiogenic effect.

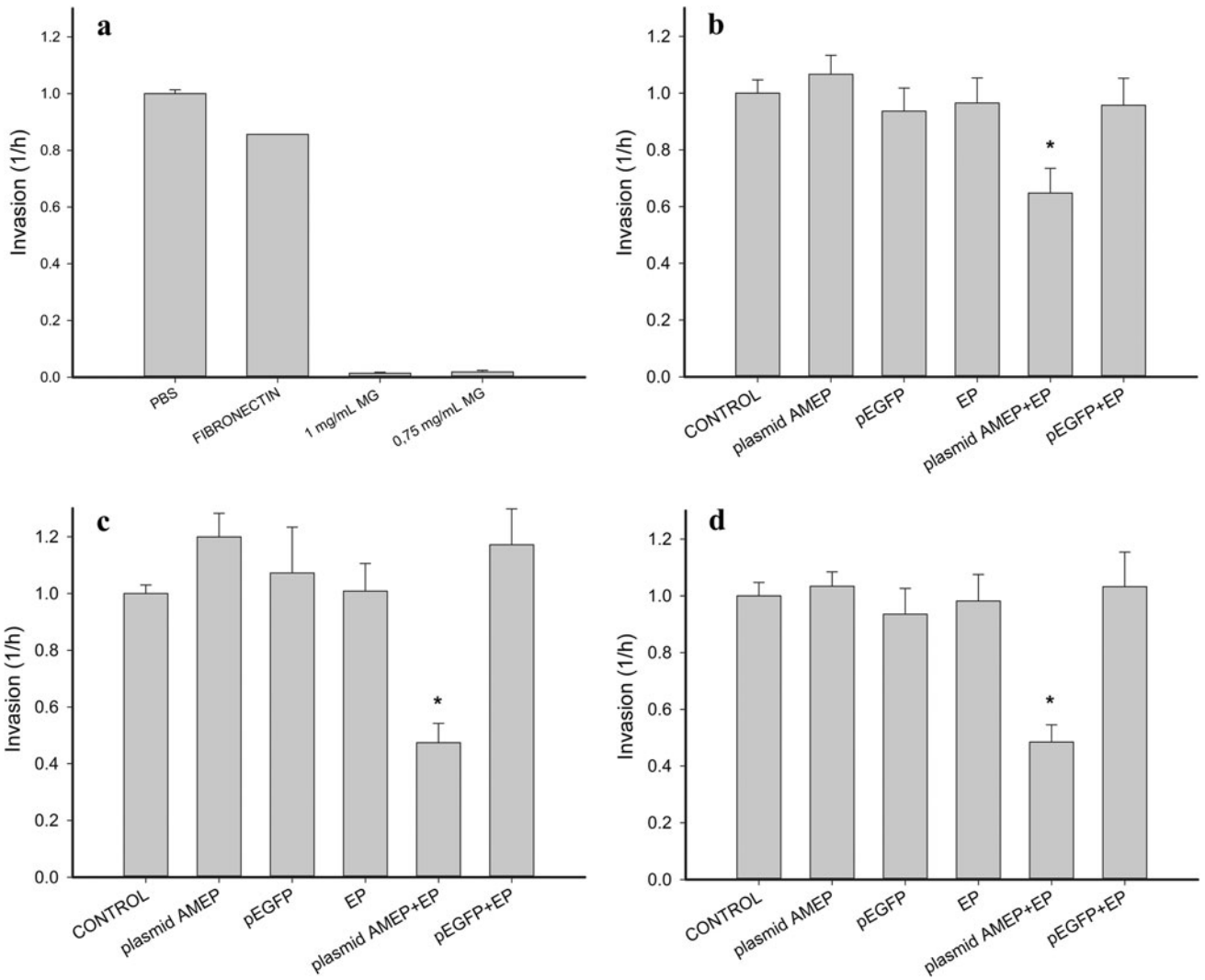

Fig. 5 Gene electrotransfer of plasmid AMEP reduced cell invasion of human endothelial and murine melanoma B16F10 cells. The invasion of untreated control B16F1 cells was too low to perform further experiments. B16F1 barely crossed through Matrigel^{TM} (0.75 or 1 mg/ml of Matrigel^{TM} (MG)) coated wells, whereas crossing through fibronectin coated or uncoated (PBS) wells was observed (**a**). The invasion of melanoma B16F10 (**b**) or endothelial HUVEC (**c**) and HMEC-1 (**d**) cells in untreated cells alone (CONTROL) or in combination with electric pulses (EP) and after the addition of plasmid AMEP or pEGFP alone (plasmid AMEP, pEGFP) or in combination with electric pulses (plasmid AMEP + EP, pEGFP + EP) were also observed. For the B16F10 melanoma cell line 0.75 mg/ml Matrigel^{TM} was used, whereas for human endothelial cell lines 1 mg/ml Matrigel^{TM} was used. The invasion of cells in each experimental group was normalized to the untreated control group. *$P < 0.05$ versus untreated control cells (B16F1, B16F10, HUVEC, HMEC-1)

Gene electrotransfer of plasmid AMEP into human endothelial HUVEC and HMEC-1 cells statistically significantly inhibited tube formation in both cell lines (Table 2). The analysis of binary images showed that in HUVEC cells plasmid AMEP statistically significantly decreased the total length of tubular complexes for ~15 % and the total size of tubular complexes for ~20 %. The reduction of the total number of junctions for ~20 % was not statistically significant (Fig. 7). In HMEC-1 cells the total length of tubular complexes was statistically significantly decreased for ~30 %, the total size of tubular complexes for ~40 % and the total number of junctions for ~40 % (Fig. 8). All three determined parameters in HMEC-1 cells were statistically significantly reduced. The difference in the effects is clearly illustrated on Figs. 7 and 8, where the disruption of tube formation in HMEC-1 cells is more pronounced than the one in HUVEC cells. Gene electrotransfer of pEGFP did not affect any of the parameters.

Integrin Presence in Cell Lines

As AMEP biological responsiveness could be linked to αvβ3 and α5β1 integrins overexpression, melanoma and endothelial cell lines were characterized by flow cytometry

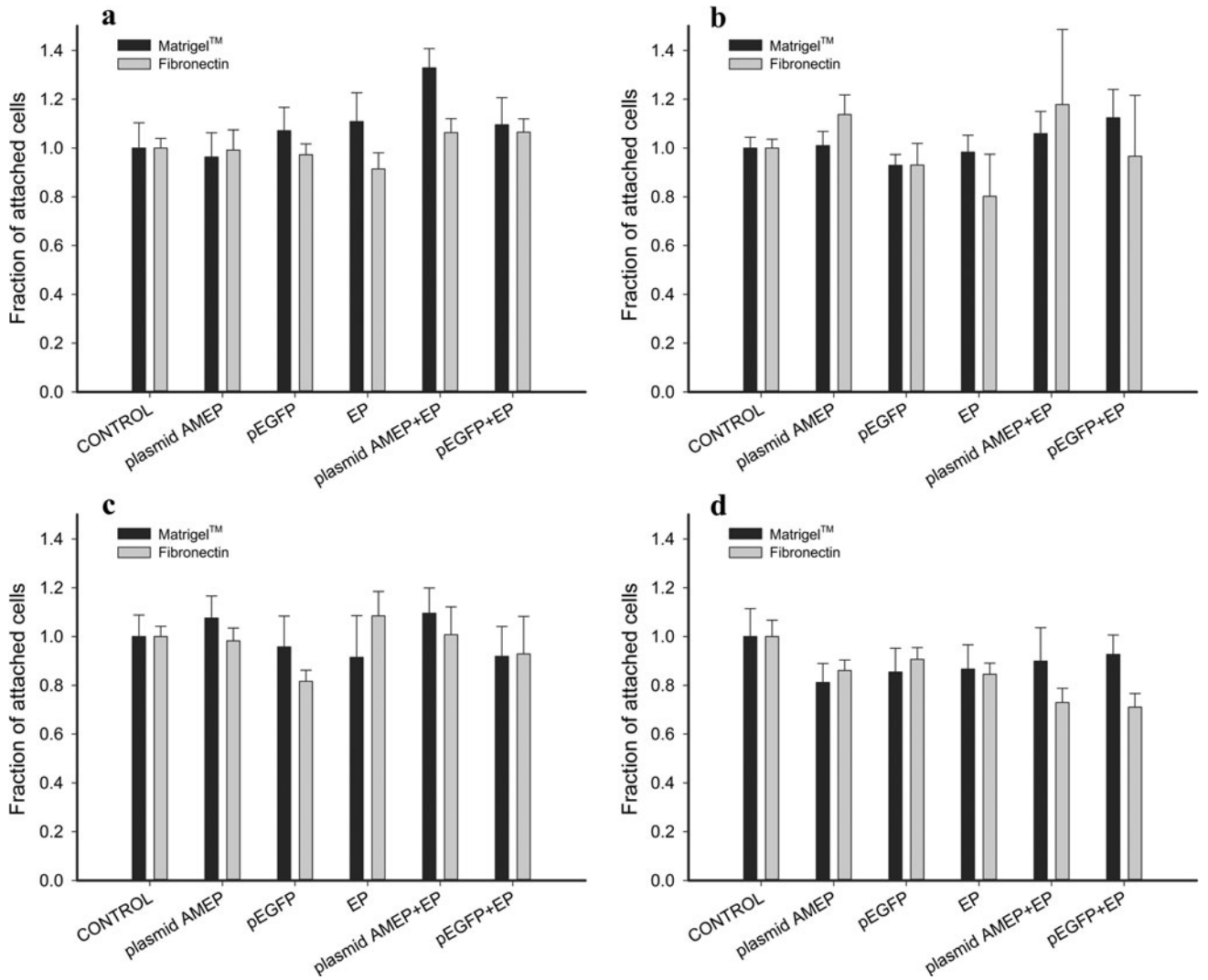

Fig. 6 Gene electrotransfer of plasmid AMEP did not affect cell attachment on Matrigel™ or fibronectin. Adhesion of melanoma B16F1 (**a**) and B16F10 (**b**) or endothelial HUVEC (**c**) and HMEC-1 (**d**) cells in untreated cells alone (CONTROL) or in combination with electric pulses (EP) and after the addition of plasmid AMEP or pEGFP alone (plasmid AMEP, pEGFP) or in combination with electric pulses (plasmid AMEP + EP, pEGFP + EP). Adhesion of cells in each experimental group was normalized to the untreated control group. There was no statistically significant change in cell adhesion neither on Matrigel™ nor on fibronectin in all four cell lines

for their level of expression of these two integrins. The tested cell lines express different amounts of integrins on their cell surface as shown in Table 3. Two parameters were followed: % of gated cells, which indicates the population of cells that express specific integrin, and median fluorescence intensity, which indicates how many of the integrins are located on a single cell. Integrins are known to be overexpressed on melanoma cells compared to normal melanocytes and also on endothelial cells involved in angiogenesis (Albelda et al. 1990; Brooks et al. 1994; Kuphal et al. 2005). For melanoma cell lines, we focused on α5β1 presence as no antibody directed against the murine heterodimer αvβ3 was available. For human endothelial cells, αvβ3 was the main integrin of our interest. Although α5β1 is ubiquitous on all cell lines and also on endothelial cell lines, αvβ3 plays a key role in endothelial cell processes such as cell survival, migration and invasion during angiogenesis (Desgrosellier and Cheresh 2010; Danhier et al. 2012).

Results showed that both melanoma cells expressed α5β1 integrin at a similar level (Table 3). Staining of melanoma cells with antibodies directed against β3 or αv revealed that both cell lines expressed these integrin monomers, suggesting that B16F1 and B16F10 cells could also have overexpressed heterodimer αvβ3 integrin (data not shown). For endothelial cells, HMEC-1 cells expressed αvβ3 integrin at slightly higher level compared to HUVEC cells. Additional staining suggested that HUVEC largely expressed β1 integrin, which was absent from HMEC-1 cells (data not shown).

Table 2 Determined parameters of tube formation assay

Cell line Groups	No. of complexes Avg ± SE	Total length Avg ± SE	Total size Avg ± SE	Total junctions Avg ± SE
HUVEC				
CONTROL	1.00 ± 0.06	1.00 ± 0.02	1.00 ± 0.02	0.98 ± 0.03
Plasmid AMEP	1.13 ± 0.11	0.99 ± 0.02	1.02 ± 0.03	0.96 ± 0.04
pEGFP	1.72 ± 0.27	0.91 ± 0.02	0.93 ± 0.03	0.86 ± 0.03
EP	1.41 ± 0.30	0.94 ± 0.02	1.02 ± 0.02	0.87 ± 0.02
Plasmid AMEP + EP	3.98 ± 0.65	0.84 ± 0.02	0.82 ± 0.02	0.78 ± 0.03
pEGFP + EP	0.99 ± 0.21	0.93 ± 0.03	1.01 ± 0.03	0.84 ± 0.04
HMEC-1				
CONTROL	1.00 ± 0.12	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.02
Plasmid AMEP	1.56 ± 0.08	0.96 ± 0.03	0.91 ± 0.03	0.99 ± 0.05
pEGFP	1.38 ± 0.16	0.98 ± 0.02	0.91 ± 0.03	1.02 ± 0.04
EP	1.46 ± 0.16	0.97 ± 0.03	0.87 ± 0.04	1.01 ± 0.06
Plasmid AMEP + EP	22.82 ± 1.42	0.67 ± 0.03	0.58 ± 0.03	0.59 ± 0.04
pEGFP + EP	2.14 ± 0.19	1.02 ± 0.03	0.87 ± 0.05	1.13 ± 0.05

Tubular parameters, in untreated cells alone (CONTROL) or in combination with electric pulses (EP) and after the addition of plasmid AMEP or pEGFP alone (plasmid AMEP, pEGFP) or in combination with electric pulses (plasmid AMEP + EP, pEGFP + EP), were compared in HUVEC and HMEC-1 endothelial cells. The determined parameters of tube formation assay of each experimental group were normalized to the determined parameters of tube formation assay of the untreated control group

Discussion

In this study, plasmid AMEP gene electrotransfer has proven to have a significant effect on the biological responsiveness of murine melanoma and human endothelial cells, with antiproliferative, antimetastatic and antiangiogenic properties. We have shown that the limiting factor of plasmid AMEP biological response is not the expression level of AMEP after gene electrotransfer of plasmid AMEP in particular cell line, but mainly the quantity of integrins on the cells.

Antitumor effectiveness of AMEP as a recombinant protein or after AMEP coding plasmid gene electrotransfer has already been demonstrated (Trochon-Joseph et al. 2004; Daugimont et al. 2011). The antitumor effectiveness was tested on B16F10 tumors, after intratumoral electrotransfer, leading to significant dose-dependent effect (BioAlliance communication). However, there are limited studies in vitro on melanoma cells, especially comparing cells with differential metastatic potential, such as B16F1 and B16F10 murine melanoma cells. Table 4 provides the summary of the in vitro results, and demonstrates that electrotransfer of plasmid AMEP into B16F1 cells had a stronger cytotoxic and antiproliferative effect than in B16F10 cells. However, the influence of plasmid AMEP on metastatic potential of either of the cell lines was difficult to assess; inhibition of cell migration was more pronounced in B16F1, whereas inhibition of cell invasion was pronounced in B16F10 but not evaluable in B16F1 because they do not exhibit any in vitro invasiveness. Furthermore, there was no difference in cell attachment either on Matrigel™ or on fibronectin after plasmid AMEP gene electrotransfer.

The biological response of murine melanoma and human endothelial cell lines after gene electrotransfer with plasmid AMEP did not correlate with its expression levels in these cells. Although B16F10 cells had several fold higher expression level of AMEP compared to B16F1 cells (or any other examined cell line), the AMEP biological efficiency was not higher in B16F10 cells. Measurement of the presence and quantity of integrin receptors in these cells demonstrate that more B16F1 melanoma cells express integrins, compared to B16F10 cells, which indicates that most probably integrin receptor quantity is the limiting factor for biological response of cells to AMEP. Our data on direct cytotoxic effectiveness of plasmid AMEP gene electrotransfer on melanoma cells in vitro, might also indicate that potent antitumor effectiveness of AMEP, in vivo, is being dependent on the quantity of integrins, and not the level of the AMEP expression. However, it is hard to predict the antimetastatic effects of AMEP in vivo based on in vitro data. Further studies are needed to elucidate this issue, and to clarify whether AMEP also has an antimetastatic effect in vivo.

The antiangiogenic effects of AMEP, measured in human endothelial cells, were also demonstrated. Strong antiangiogenic effect was previously demonstrated with recombinant disintegrin domain of metargidin in different endothelial cell lines, including HUVEC and HMEC-1 cells, but with different assays than we used (Trochon-Joseph

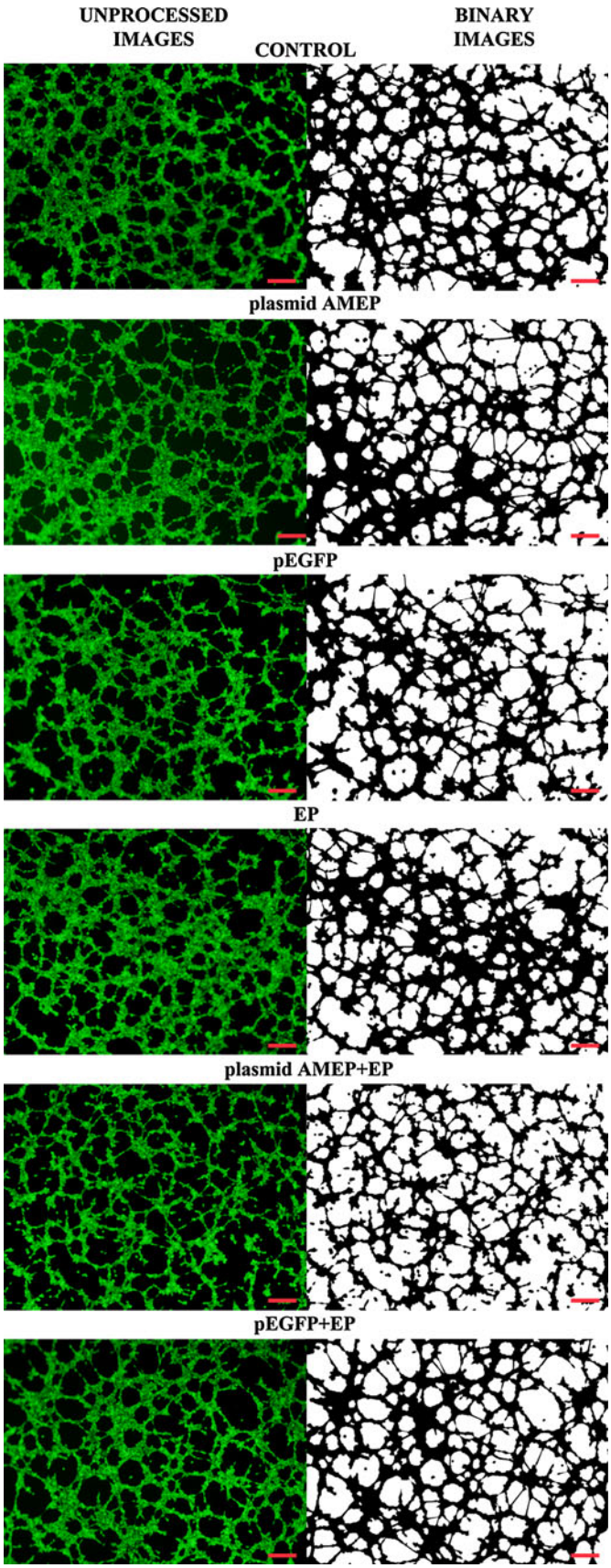

Fig. 7 Plasmid AMEP gene electrotransfer reduced tube formation in HUVEC cells. Formation of capillary like structure in vitro, in untreated cells alone (CONTROL) or in combination with electric pulses (EP) and after the addition of plasmid AMEP or pEGFP alone (plasmid AMEP, pEGFP) or in combination with electric pulses (plasmid AMEP + EP, pEGFP + EP). *Scale bar* = 200 μm

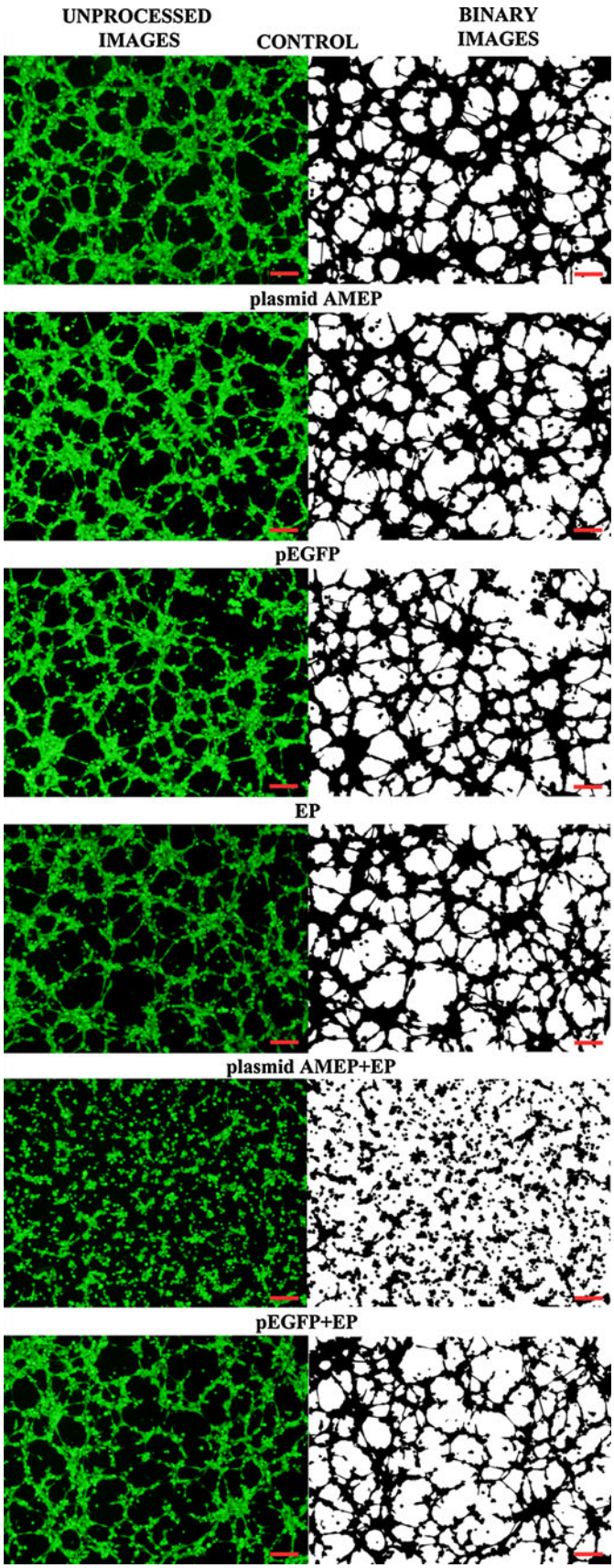

Fig. 8 Plasmid AMEP gene electrotransfer reduced tube formation in HMEC-1 cells. Formation of capillary like structure in vitro, in untreated cells alone (CONTROL) or in combination with electric pulses (EP) and after the addition of plasmid AMEP or pEGFP alone (plasmid AMEP, pEGFP) or in combination with electric pulses (plasmid AMEP + EP, pEGFP + EP). *Scale bar* = 200 μm

Table 3 Presence of integrins in melanoma and endothelial cell lines

Characteristic	Melanoma cell lines		Endothelial cell lines	
	B16F1	B16F10	HUVEC	HMEC-1
Integrin	α5β1	α5β1	αvβ3	αvβ3
(%) gated	81.64	71.69	38.34	50.06
Median fluorescence intensity	6.73	6.04	28.84	10.05

The presence of integrins is given as a result of 2 parameters: percentage of gated cells, which indicates the population of cells that express specific integrin; and median fluorescence intensity, which indicates the quantity of integrins located on a single cell

Table 4 Summary of study results

Characteristic	Melanoma cell line		Endothelial cell line	
	B16F1	B16F10	HUVEC	HMEC-1
Cytotoxicity (μg plasmid AMEP)	1	25	1	5
IC_{50} (μg/μl plasmid AMEP)	0.2	2	NA	NA
Inhibition of:				
Proliferation (%)	30	25	40	40
Migration (%)	55	35	50	30
Invasion (%)	–	35	50	50
Adhesion	NS	NS	NS	NS
Tube formation (length/size/junctions)	NA	NA	15/20/20 (NS) %	30/40/40 %
Increase in expression of AMEP (×)	283	4,407	40	248
Integrin	α5β1	α5β1	αvβ3	αvβ3
(%) gated	81.64	71.69	38.34	50.06
Median fluorescence intensity	6.73	6.04	28.84	10.05

The effects of plasmid AMEP after gene electrotransfer in murine melanoma and human endothelial cell lines are shown, including the expression levels of AMEP in different cell lines and the quantity of integrins on those cells

NS not statistically significant, *NA* not applicable

et al. 2004). Our study also demonstrated the antiangiogenic effectiveness of AMEP, but after plasmid AMEP gene electrotransfer into HUVEC and HMEC-1 cells. Our data demonstrated in both human endothelial cell lines 40 % inhibition of proliferation, 30–50 % decrease in migration, and 50 % decrease of invasion. Furthermore, the tube formation was more affected in human microvascular endothelial cells, HMEC-1, than in human umbilical vein endothelial cells, HUVEC, which might not represent the best model for assessment of effect on tumour vessels. Again, similar to melanoma cells, the effect of AMEP on the antiproliferative and antimigratory biological response of endothelial cells correlated well with integrin quantity on the cells, but not with the expression level of AMEP. Also in previous research, when the equal amount of recombinant AMEP protein was added to HMEC-1 and HUVEC cells, the inhibition of proliferation was higher in HUVEC cells than in HMEC-1, but the reason for that observation was not discussed (Trochon-Joseph et al. 2004). However, another mechanism might be involved in tube formation assay in which we observed that reduction of tube formation correlated with AMEP expression, which was much higher in MHEC-1 cells than HUVEC. Namely even though HUVEC cells had higher integrin level, the reduction of tube formation was less pronounced.

Furthermore, the dose-dependent antitumor effectiveness of gene electrotransfer of plasmid AMEP was demonstrated in melanoma B16F10 in vivo (BioAlliance Pharma communication). Therefore, we speculate on the basis of our in vitro data that dose-dependent antitumor effectiveness of AMEP in vivo is due to AMEP antiangiogenic effect rather than to its cytotoxic effect in melanoma tumors. Further in vivo investigations with tumor models are needed to investigate if in vitro antiangiogenic effects are reflected in reduced tumor blood vessel density.

Electroporation was used as a delivery system for plasmid DNA uptake into the cells (Daud et al. 2008; Gehl 2008; Cemazar et al. 2010; Sedlar et al. 2012). Another application of electroporation is electrochemotherapy, which is used for the treatment of superficial and deep seated tumors (Sersa et al. 2008; Testori et al. 2010; Campana et al. 2012; Linnert et al. 2012; Markelc et al.

2012; Miklavcic et al. 2012; Mali et al. 2013). AMEP has recently been tested for toxicity and effectiveness in a first-in-man phase I clinical trial after intratumoral gene electrotransfer of plasmid AMEP into melanoma metastases. It has been shown that intratumoral gene electrotransfer of plasmid AMEP has favorable toxicity profile and some local antitumor effectiveness (Spanggaard et al. 2012), whereas intramuscular gene electrotransfer is expected to be systemically effective, having effects on local tumor control and on micrometastases. In addition, the combined modality treatment approaches need to be investigated, to bring this melanoma targeted gene therapy approach by AMEP into wider clinical applicability.

In conclusion, our study provides the first evidence that AMEP antitumor effects after plasmid AMEP gene electrotransfer are exerted by direct cytotoxic and antiproliferative effects on melanoma and endothelial cells. The effects on tumor cells strongly correlate with integrin receptor quantity on the cells, but not with the AMEP expression level. Antiangiogenic effects are on one hand dependent on integrin presence on endothelial cells; again affecting antiproliferative and antimigratory effects. On the other hand, in tube formation, which is also a model for angiogenesis, a correlation between the AMEP expression and tube formation effect was observed. This hypothesis needs further evaluation in in vivo tumor models.

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