Blood Flow Modifying and Vascular-Disrupting Effects of Electroporation and Electrochemotherapy

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Gregor Serša, Maja Čemažar, and Bostjan Markelc

Abstract

Electroporation/electropermeabilization (EP), achieved by application of electric pulses to cells or tissues, induces reversible permeabilization of cell membranes under suitable conditions, thus facilitating entry of exogenous molecules into cells. EP of tissues in humans is feasible, efficient, and tolerable, and its most advanced routine clinical use is electrochemotherapy (ECT), where cytotoxic drugs are delivered to cells to treat tumors. It is increasingly used in different tumor types, and the technology is being adopted for the treatment of deep-seated tumors like liver, pancreas, and bone metastases and colon tumors. Although the primary mode of action of ECT is the destruction of tumor cells due to the increased cytotoxicity of chemotherapeutic drugs, it also has different effects on the tissue level. It was shown that EP and ECT have blood flow-modifying effects on normal as well as on tumor vasculature. Application of EP pulses to normal blood vessels increases the permeability of affected blood vessels; causes a transient vascular lock, i.e., decrease in perfusion; and modulates the diameter of affected blood vessels. Similarly, in tumors, application of EP or ECT increases the permeability of affected blood vessels and causes a vascular lock. In case of normal blood vessels, these effects are short lived, whereas in case of tumor blood

G. Serša
Department of Experimental Oncology, Institute of Oncology Ljubljana, Ljubljana, Slovenia
e-mail: gsersa@onko-i.si

M. Čemažar
Department of Experimental Oncology, Institute of Oncology Ljubljana, Ljubljana, Slovenia

Faculty of Health Sciences, University of Primorska, Izola, Slovenia
e-mail: mcemazar@onko-i.si

B. Markelc (✉)
ÜRUK/MRC Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, Oxford, UK
e-mail: bostjan.markelc@oncology.ox.ac.uk

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vessels, the effects are long lasting and resolve more than 24 h after ECT. Moreover, ECT has a direct cytotoxic effect on tumor endothelial cells; thus it has a vascular-disrupting effect. This effect is differential, destroying only tumor blood vessels and retaining the functionality of normal blood vessels surrounding the tumor, which is important when ECT is used in well-perfused organs such as the liver.

Keywords

Vascular lock • Vasoconstriction • Permeability • Electroporation • Electrochemotherapy • Vascular-disrupting therapy

Contents

Introduction

Electroporation/electropermeabilization (EP), i.e., application of electric pulses to cells or tissues, induces reversible permeabilization of cell membranes under suitable conditions, thus facilitating entry into the cells of non-permeant or poorly permeant molecules (Rems and Miklavcic 2016). The list of molecules that can be delivered to cells and tissues with EP is ever expanding and covers a broad range of different molecules such as cytotoxic drugs, siRNA molecules, miRNA molecules, plasmid DNA, naked DNA, etc. (Yarmush et al. 2014). If nucleic acids are being delivered into the cells, then the application of EP is termed gene electrotransfer (GET) and can be used for DNA vaccination or in the treatment of cancer (Gothelf and Gehl 2012). However, the most widespread use of EP is to deliver cytotoxic drugs to tumor cells for treatment of cancer, termed electrochemotherapy (ECT). ECT is an effective treatment modality for the treatment of cancer, predominantly melanoma metastases in the skin. It is increasingly used in other tumor types, and the technology is being adopted for the treatment of deep-seated tumors like liver and bone metastases and colon tumors. In the case of ECT, the most commonly used drugs are bleomycin and cisplatin, where the application of EP to cells exposed to the chemotherapeutic drugs potentiates their cytotoxic effect (Yarmush et al. 2014). The majority of ECT in clinical settings is nowadays performed on cutaneous and subcutaneous tumors, where although the target is the tumor, the surrounding skin is also exposed to the electric field (Yarmush et al. 2014). Recently, ECT has also been used in the treatment of visceral and deep-seated tumors such as colorectal cancer and colorectal liver metastases as well as in the treatment of bone metastases (Yarmush et al. 2014).

Although the primary mode of action of ECT is the destruction of tumor cells due to the increased cytotoxicity of chemotherapeutic drugs, it also has different effects on the tissue level. One of the reported effects on the tissue level, regardless of the tissue type, is a blood flow-modifying effect, which was first reported on tumors (Sersa et al. 1998) and later also confirmed in different normal tissues (Gehl et al. 2002).

The growth of solid tumors is heavily dependent on blood supply; thus as the tumor grows, it forms an ever-developing vascular network. The new tumor blood vessels are formed during a process termed angiogenesis, which starts once the tumor reaches a size of 1–3 mm^3 and is responsible for the formation of the majority of tumor blood vessels. The growing tumor mass relies on the newly formed vasculature to supply tumor cells with oxygen and nutrients (Folkman 2007). Due to the rapid growth of tumors, the newly formed tumor vasculature is abnormal and inadequately developed. Compared to normal vasculature, it has several characteristic features: irregular and chaotic microvascular networks and branching patterns, irregular shape and length of capillaries, large intercapillary distances, and shunts and dead ends, all contributing to increased resistance to blood flow and to inefficient and slow blood flow. Furthermore, tumor blood vessel walls are structurally and functionally abnormal. They have a reduced coverage with smooth muscle cells and poor sympathetic innervation leading to a poor local regulation of microcirculatory blood flow. Tumor blood vessel walls are also "leaky" for large molecules, such as plasma proteins, due to an abnormal basal membrane as well as endothelial lining, which have loose intercellular connections and openings between adjacent endothelial cells (Barker et al. 2015). This is further recapitulated in poor perfusion, leading to the formation of hypoxic areas in the tumor, where the local oxygen concentration is below 1%. High tumor cell proliferation rates and the abnormal structure of the tumor vasculature lead to both chronic, diffusion-limited hypoxia and acute, transient, perfusion-limited hypoxia (Barker et al. 2015).

By supplying oxygen and nutrients, the tumor vasculature and angiogenesis are essential for the survival and growth of solid tumors; thus they also present an attractive target for treatment. The rationale behind targeting tumor vasculature comes from the idea that disrupting one vessel can lead to the death of many tumor cells fed by this vessel. Two types of therapies are currently being developed: antiangiogenic therapies that target newly developing tumor vasculature, i.e., angiogenesis, and vascular-disrupting therapies that target established tumor vasculature with vascular-disrupting agents (Clémenson et al. 2013). Another important characteristic of antiangiogenic and vascular-disrupting therapies is that they specifically target tumor blood vessels, leaving normal vasculature intact, thus sparing the functionality of the normal tissue surrounding the tumor (Clémenson et al. 2013).

Nowadays, ECT is performed in clinical settings on cutaneous and subcutaneous tumors, where although the target is the tumor, the surrounding skin is also exposed to the electric field (Yarmush et al. 2014). Moreover, ECT has also been used in the treatment of deep-seated tumors such as colorectal cancer and colorectal liver metastases as well as in the treatment of bone metastases (Yarmush et al. 2014). Although the main mode of action of ECT is the destruction of tumor cells due to the increased cytotoxicity of chemotherapeutic drugs, the effects of EP and ECT on

tumor blood flow may contribute to the overall treatment outcome. The effects of EP on blood flow have been first observed in tumors (Sersa et al. 1998) and later confirmed in various other tissues. Moreover, destruction of endothelial cells and tumor blood vessels after ECT has also been reported (Cemazar et al. 2001; Sersa et al. 2008), implying that ECT may have a vascular-disrupting effect, thus classifying as a vascular-disrupting therapy. In this chapter, the blood flow-modifying effects of EP and the vascular-disrupting effect of ECT, both on normal and tumor tissue, will be discussed.

Effects of Electroporation on Normal Blood Flow

Although EP is a physical method and the response of exposed cells is similar between different cell types (Rems and Miklavcic 2016), there are some effects of EP that can only be observed on a tissue level. After the first observations of blood flow-modifying effects of EP in tumors, liver, and muscle (Sersa et al. 1998; Ramirez et al. 1998; Gehl et al. 2002), more in-depth studies on endothelial cells in vitro and in blood vessels in vivo were performed.

The effects of EP on endothelial cells in vitro were thoroughly investigated using human umbilical vein endothelial cells (HUVECs) (Kanthou et al. 2006) as well as using HMEC-1 cells (Meulenberg et al. 2012). In both cell lines, EP increased the permeability of endothelial monolayers that were grown on 0.4 μm pore size polycarbonate transwell inserts coated with human fibronectin. The increase in the permeability was dependent on the electric field strength. In the study of Kanthou et al. (2006), HUVECs were exposed to 3 square-wave electric pulses (10–80 V/25–200 V/cm; 1 Hz; duration 100 μs; 4 mm distance between electrodes), whereas in the study of Meulenberg et al. (2012), HMEC-1 cells were exposed to 8 square-wave electric pulses (0–500 V/voltage-to-distance ratio 0–685 V/cm; 1 Hz; duration 100 μs; 7.3 mm gap between electrodes). On a structural level, HUVEC and HMEC-1 cells responded to electroporation by dissolution of actin fibers and microtubules, and these effects were evident immediately (within 5 min) after electroporation. Both actin fibers and microtubules were progressively dissociated in response to increasing voltage applications. Additionally, in HUVECs, a loss of VE-cadherin, a key protein responsible for the integrity of cell-to-cell junctions, was evident from the junctions between adjacent endothelial cells immediately after electroporation. When parameters for reversible EP were used, the observed changes were transient in both cell lines and recovered to the pretreatment state within 2 h after EP (Kanthou et al. 2006; Meulenberg et al. 2012). Taken together, these data suggest that electroporation can permeabilize endothelial cells and disrupts the organization of cytoskeletal network. Moreover, EP disrupts the barrier function of the endothelium by interacting junctional integrity of endothelial cell-to-cell junctions.

On the tissue level, the blood flow-modifying effects of EP were predominantly studied in mouse muscle and skin (Gehl et al. 2002; Bellard et al. 2012; Markelc et al. 2012). To determine the effect of EP on blood vessels in mouse muscle, three

different electrode geometries were compared: needle electrodes consisting of two arrays of each four 0.5 mm needles placed 2 mm apart, with 4 mm between the arrays, and two different plate electrodes, both consisting of two opposing metal plates, separated by 4 mm or 5.3 mm. In all experiments, eight pulses with a frequency of 1 Hz were delivered. Pulse duration was varied from 10 to 20,000 μs and applied voltage-to-electrode distance ratio from 100 to 1,600 V/cm (Gehl et al. 2002). A different electrode geometry, two parallel stainless steel rods (length 5 mm, width 1.3 mm) 4 mm apart, was used to study the blood flow-modifying effects of EP on blood vessels in mouse skin. Two different pulse parameters were studied: standard ECT pulses (8 square-wave electric pulses, 100 μs, 1,300 V/cm, 1 Hz) (Bellard et al. 2012) and pulses used for gene electrotransfer, a combination of one short, high voltage (HV) and several long, short voltage (LV) pulses (one pulse (1,000 V/cm, 100 μs) followed by a 1 s lag and 8 pulses (140 V/cm, 50 ms, 1 Hz)) (Markelc et al. 2012). Immediately after EP, a decrease in the diameters of all affected blood vessels was observed that lasted up to 8 min post-EP (Bellard et al. 2012; Markelc et al. 2012). This was followed by an abrogation of blood flow that was demonstrated by a slowed filling up of blood vessels with intravenously (i.v.) injected fluorescently labeled markers (Bellard et al. 2012; Markelc et al. 2012) and by a reduced uptake of ^{51}Cr-EDTA in muscle tissue when it was injected i.v. after EP in comparison to the uptake of ^{51}Cr-EDTA when it was injected before EP (Gehl et al. 2002). A further proof that EP is followed by a delayed perfusion was the observed delay in the time needed for a mouse paw, which was exposed to EP, to turn blue after i.v. injection of a patent violet-blue dye, when compared to the paw that was not exposed to electric pulses (Gehl et al. 2002). If the mice were pretreated with reserpine, the observed effects could be substantially mitigated (Gehl et al. 2002). Reserpine is a drug, which depletes nerve terminals of norepinephrine, and is known to lower blood pressure and heart rate and at the same time diminish regional vasoconstriction mediated by sympathetic fibers acting on smooth muscles. This indicated that local vasoconstriction mediated by the sympathetic nervous system plays a major role in the observed perfusion delays after EP (Gehl et al. 2002). Furthermore, the use of a dorsal window chamber (DWC) model in mice together with fluorescence intravital microscopy revealed that EP increased the permeability of affected blood vessels for macromolecules (Bellard et al. 2012; Markelc et al. 2012). The DWC model is an experimental technique performed in living animals, which enables a direct visual access to the blood vessel network of the skin by intravital microscopy. The DWC consists of two titanium frames that are secured to the double layers of the dorsal skinfold in a mouse with stainless steel screws and sutures. Thereafter, one layer of the skin is excised, exposing the vasculature of the remaining skin layer. When different sizes of fluorescently labeled dextrans are injected i.v., which serve as vascular markers, this approach enables quantification of changes in blood flow and permeability of blood vessels. Immediately after EP, there was an increase in the permeability of blood vessels for all sizes of dextrans (20, 70, and 2,000 kDa) that were used. The increase in permeability after EP was different for different sizes of molecules, with a smaller increase in permeability for 2,000 kDa dextran compared to 70 or 20 kDa and lasted for up to 15 min. However,

it could still be detected even more than 30 min after EP (Bellard et al. 2012; Markelc et al. 2012). The increase in permeability of blood vessels after EP was transient, and the pre-EP state, where blood vessels are impermeable for large macromolecules, was restored.

In summary, EP affects normal blood vessels in several ways. Firstly, immediately after EP, there is a decrease in diameters of all affected vessels which is accompanied by a "vascular lock." This first phase is short lived, and it is resolved within 5 min after EP and was attributed to the permeabilization of endothelial cells/smooth muscle cells which induced a Raynaud-like phenomenon, a reflexory vasoconstriction of afferent arterioles mediated by the sympathetic nervous system. Secondly, EP increases the permeability of blood vessels for macromolecules of various sizes, and this increase in permeability can persist up to 30 min after EP, although it is primarily resolved within 10 min after EP. The resolution of this second phase is supposed to follow the kinetics of membrane resealing after EP (Gehl et al. 2002).

Effects of Electroporation on Tumor Blood Flow

Due to the rapid growth of tumors, the newly formed tumor vasculature is abnormal and inadequately developed. Compared to normal vasculature, it has several characteristic features: irregular and chaotic microvascular networks and branching patterns, irregular shape and length of capillaries, large intercapillary distances, and shunts and dead ends, all contributing to increased resistance to blood flow and to inefficient and slow blood flow. Furthermore, tumor blood vessel walls are structurally and functionally abnormal with a reduced coverage with smooth muscle cells and poor sympathetic innervation. Tumor blood vessel walls are also inherently "leaky" enabling extravasation of large molecules into surrounding tumor tissue (Barker et al. 2015). The differences between the normal blood vessels and tumor blood vessels lead to differences in their response to EP.

The first study to directly show blood flow-modifying effects of EP in tumors used magnetic resonance imaging (MRI) to determine the distribution of i.v. injected albumin-$(Gd\text{-}DTPA)_{30}$ in the tumor after EP. Electric pulses were delivered with stainless steel electrodes 8 mm apart (two stainless steel strips, length 35 mm, width 7 mm, with rounded corners), and the parameters used were eight pulses, voltage-to-distance ratio 1,300 V/cm, 100 μs, and frequency of 1 Hz. Albumin-$(Gd\text{-}DTPA)_{30}$ was i.v. injected 30 min after EP, and MRI revealed a complete abrogation of blood flow in the murine fibrosarcoma SA-1 tumors growing subcutaneously in syngeneic A/J mice. EP had a similar effect on VX2 carcinoma tumors grown in the liver in rabbits, where the transient abrogation of blood flow after EP was determined with i.v. injection of a fluorescent dye fluorescein after EP and illumination of the tumor with a fluorescent Wood's light. In this setting, the EP induced a transient abrogation of blood flow that lasted more than 20 min (Ramirez et al. 1998). Taken together, experiments performed in both studies indicated that in tumors, the transient abrogation of blood flow after EP last more than 30 min (Sersa et al. 1998; Ramirez et al.

1998), contrary to the results obtained on normal blood vessels where it is much shorter lived (Gehl et al. 2002; Bellard et al. 2012).

These intriguing findings were further confirmed in subsequent studies. EP reduced the patent blue stained area of the SA-1 tumors when patent blue was injected i.v. after EP. In this study EP was achieved by 8 square-wave electric pulses, delivered in two sets of four pulses in perpendicular directions, voltage-to-distance ratio 1,300 V/cm, 100 μs, and frequency of 1 Hz, were delivered by two flat, parallel stainless steel electrodes 8 mm apart (two stainless steel strips: length 15 mm, width 7 mm with rounded corners). Within 2 h after EP, the tumor perfusion was reduced to 30% but returned to near pretreatment value within 24 h (Sersa et al. 1999, 2002, 2008; Jarm et al. 2010). A similar observation was made when ^{86}RbCl was used instead of patent blue dye (Sersa et al. 1999). The scale of the reduction in the perfused area stained with patent blue dye after EP was found to be dependent on the amplitude of the electric pulses, with greater reduction observed at higher amplitudes, as well as on the number of the applied electric pulses, with each subsequent electric pulse decreasing the perfused/stained area (Sersa et al. 1999). Using dynamic contrast-enhanced MRI, it was further confirmed that even 24 h after EP, there is still a slight reduction in blood volume and perfusion in tumors that were exposed to EP (Sersa et al. 2002). The initial rapid reduction of blood flow in SA-1 tumors after EP was closely investigated with power Doppler ultrasonographic imaging and laser Doppler flowmetry (LDF) (Sersa et al. 2008). Power Doppler ultrasonographic imaging is an ultrasound-based detection method that can be used to assess the gross tumor blood flow changes when a microbubble contrast agent is injected i.v. before the detection of the signal. LDF can be used to monitor local microvascular blood flow in the tissue by measuring the spread of the wavelengths of photons emitted by a coherent laser source when the photons scatter on moving red blood cells in capillaries (Sersa et al. 2008). With this approach, it was again confirmed that the reduction in blood flow after EP happens immediately after the first pulse and it is further reduced with every subsequent pulse. The rapid and abrupt initial decrease in perfusion of tumors after EP was followed by a gradual but only partial reperfusion that peaked 10–15 min after the delivery of electric pulses and did not continue afterward, during the observation time of 60 min after EP (Sersa et al. 2008). Moreover, the decrease in blood flow was accompanied by a decrease in partial oxygen pressure (pO_2) which was measured by electron paramagnetic resonance (EPR) oximetry. EPR is a noninvasive method (after insertion of the paramagnetic probe), which allows monitoring of pO_2 repeatedly at the same point in the tissue over long periods of time (Sersa et al. 2002, 2008). Using this method, it was shown that after EP, the pO_2 in the tumor immediately decreased to ~20% of the control levels and remained at lower levels for at least 8 h after EP (Sersa et al. 2002, 2008). The reduction in pO_2 after EP recapitulated in an increase in hypoxic area in the tumor (Sersa et al. 2008; Jarm et al. 2010).

A direct visual confirmation of the blood flow-modifying effects of EP on tumor vasculature came from a study using a DWC model in SCID mice where human colon carcinoma HT29 tumors were grown (Markelc et al. 2013). Using 70 kDa fluorescently labeled dextrans, coupled with fluorescent macroscopy, the direct

observation of the effects of EP on tumor and normal blood vessels was achieved. Electric pulses were delivered with two parallel stainless steel plate electrodes (30 mm long, 6 mm wide, 6 mm distance between electrodes), and standard ECT pulse parameters were used (eight pulses, voltage-to-distance ratio 1,300 V/cm, 100 μs, frequency of 1 Hz). EP resulted in an immediate abrogation of blood flow in the tumor, i.e., vascular lock, which lasted for more than 60 min (Fig. 1). Interestingly, the tumor-supplying arterioles responded to EP in the same way as the normal vessels, with rapid vasoconstriction and increased permeability, thus implying that this is the main cause of the immediate vascular lock observed after EP. Furthermore, the blood flow in the blood vessels surrounding the tumor started to return to normal level 10 min after EP, which was in line with a small increase in reperfusion of tumors after EP (Sersa et al. 2008; Markelc et al. 2013). EP also resulted in an increased permeability of tumor blood vessels for macromolecules and a partial long-lasting decrease in perfusion (Markelc et al. 2013). Thus, EP has a differential effect on normal and tumor blood vessels which originates from the differences between normal and tumor vasculature.

Taken together, this data suggests that EP has a differential effect on normal and tumor blood vessels. The extent of the blood flow-modifying effects of EP on tumor blood vessels depends on the number of electric pulses and the amplitude of the pulses. Similar to the response of normal blood vessels, the tumor blood vessels also respond in two distinct phases. Firstly, EP immediately induces a rapid and profound abrogation of blood flow, which is caused primarily by the vasoconstriction of the tumor-supplying arterioles (Jarm et al. 2010; Markelc et al. 2013). After the vasoconstriction of tumor-supplying arterioles is finished, which is ~15 min after EP, there is a small reperfusion of the tumors, primarily on the tumor periphery; however, it does not lead to a complete restoration of blood flow in the tumor. The first phase is immediately followed by a second phase, where the increased permeability of tumor blood vessels for macromolecules causes an increase in the interstitial tissue pressure in the tumor, which coupled with non-normal characteristics of tumor vasculature leads to a prolonged vascular lock effect, which lasts for more than 1 h and is fully resolved only 24 h after EP (Sersa et al. 2008; Jarm et al. 2010). A direct consequence of this prolonged second phase is a decrease in pO_2 and increase in the fraction of hypoxic regions in the tumor (Sersa et al. 2008).

Effects of Electrochemotherapy on Tumor Blood Flow

Nowadays, ECT is performed in clinical settings on cutaneous and subcutaneous tumors, where while the primary target is the tumor, the surrounding skin is also exposed to the electric field (Yarmush et al. 2014). Moreover, ECT has also been used in the treatment of deep-seated tumors such as colorectal cancer and colorectal liver metastases as well as in the treatment of bone metastases (Yarmush et al. 2014). For a tissue to retain its normal functionality, it is essential to have an unperturbed blood flow which supplies the tissue with nutrients and oxygen. Thus, especially when treating well-perfused organs, such as the liver, the effects of ECT on normal

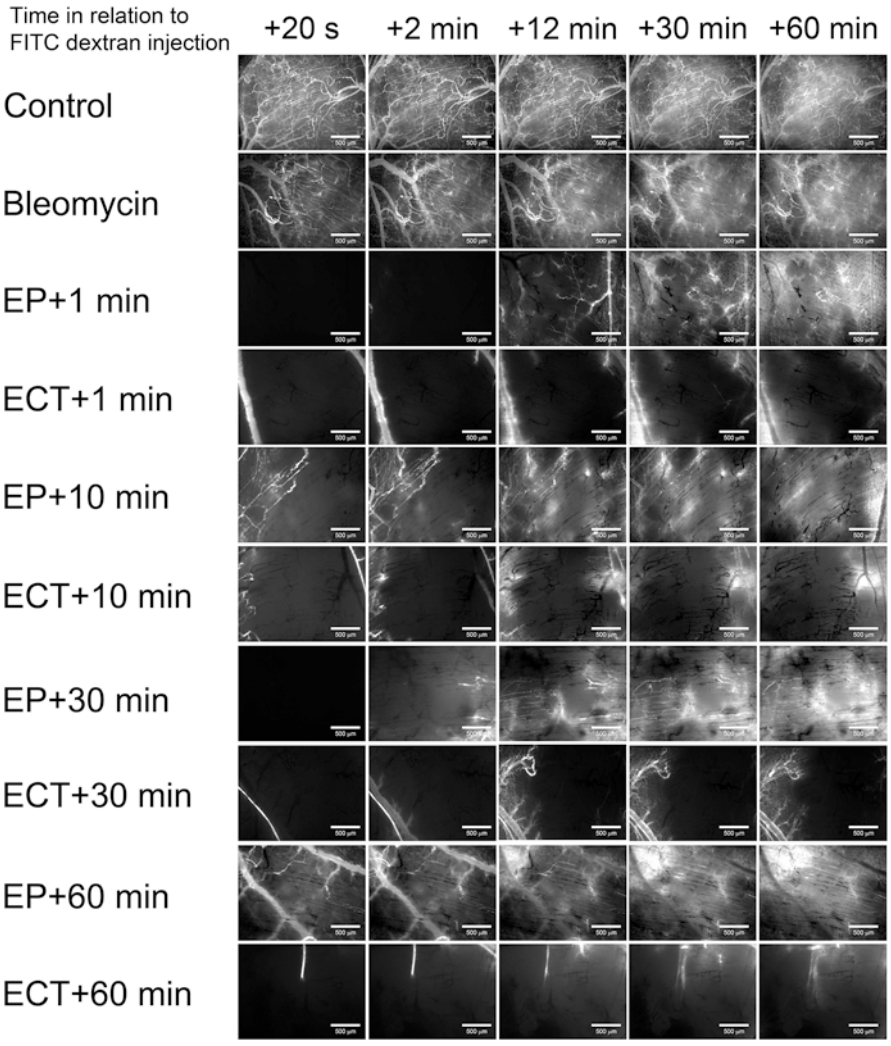

Fig.1 Illustration of a "vascular lock" and reperfusion of tumor blood vessels after EP and ECT. Tumor blood vessels were visualized by fluorescence microscopy at 80× magnification. Control, mice without treatment; bleomycin, mice treated with bleomycin only; EP, mice treated with EP; ECT, mice treated with ECT. 70 kDa fluorescently labeled dextran was injected i.v. at different times after the therapy (1, 10, 30, 60 min), and images were taken at designated times. Scale bar is 500 μm (Reproduced under the terms of the Creative Commons Attribution License from Markelc et al. (2013))

and tumor vasculature are of considerable importance. Although the main mode of action of ECT is the destruction of tumor cells due to the increased cytotoxicity of chemotherapeutic drugs, other cell types within the tumor, as well as in the surrounding normal tissue, are also affected. Moreover, some of the effects of EP and ECT are only detectable on the tissue level and do not exist on a single-cell level. The

destruction of endothelial cells and tumor blood vessels after ECT has been reported (Cemazar et al. 2001; Sersa et al. 2008), implying that ECT may have a vascular-disrupting effect which could contribute substantially to the overall treatment outcome.

In in vitro setting, ECT increased the cytotoxicity of both most commonly used chemotherapeutic drugs, bleomycin and cisplatin in HMEC-1 cells (Cemazar et al. 2001; Meulenberg et al. 2012), thus showing that in in vivo setting, the endothelial cells are also destroyed by ECT. A combination of a 5 min drug exposure with electric pulses increased the cytotoxicity by tenfold for cisplatin and 5,000-fold for bleomycin when compared to drug alone. To achieve electroporation, the cells were placed between two parallel stainless steel electrodes (2 mm distance between electrodes) connected to a Jouan GHT 1,287 electroporator and subjected to 8 square-wave electric pulses (100 μs, 1 Hz) (Cemazar et al. 2001). In HMEC-1 cells, an ECT protocol, combining bleomycin and exposing cells to 8 square-wave electric pulses (0–500 V/voltage-to-distance ratio 0–685 V/cm; 1 Hz; duration 100 μs; 7.3 mm gap between electrodes), showed that within 10 min after ECT, cells appear swollen and the formation of honeycomb-like actin bundles begins (Meulenberg et al. 2012). This was followed by the formation of spindle-like cells 2 h after ECT and more densely packed F-actin and beta-microtubulin, which were dependent on the amount of bleomycin and the electric pulse amplitude (Meulenberg et al. 2012). Moreover, ECT with bleomycin dramatically increased the permeability of endothelial monolayer, when cells were grown on 0.4 μm pore size polycarbonate transwell inserts coated with human fibronectin. The increase in the permeability of the monolayer was dependent on the concentration of bleomycin and electric pulse amplitude (Meulenberg et al. 2012). All of the observed changes had an earlier onset compared to EP alone, and the effect was more pronounced. Contrary to the EP alone, all the observed effects were irreversible, and the majority of endothelial cells were destroyed after ECT with bleomycin (Meulenberg et al. 2012). This implies that ECT with cytotoxic drugs effectively destroys endothelial cells and that in in vivo setting, blood vessels are also a target, making ECT a vascular-targeted therapy.

A mathematical model of electric field distribution in and around blood vessels in a tumor revealed that the endothelial cells lining the blood vessels are actually exposed even to up to 40% higher electric field strength than the surrounding tumor cells (Sersa et al. 2008). Additionally, when chemotherapeutic drugs are injected systemically, then the endothelial cells lining the blood vessel walls are in fact exposed to the highest concentration of the drug, compared to any other cell in the tumor. In several studies, the histological analysis of ECT-treated tumors revealed that tumor endothelial cells are destroyed within 24 h after ECT and the vascular lesion is formed leading to necrotic areas around the destroyed blood vessels (Ramirez et al. 1998; Sersa et al. 2008; Jarm et al. 2010). The first observation of blood flow-modifying and vascular-disrupting effects of ECT with bleomycin (0.5 or 1 mg/kg injected i.v.) was made on VX2 carcinoma tumors grown in the liver in rabbits, where the transient abrogation of blood flow after ECT was determined with i.v. injection of a fluorescent dye fluorescein after ECT and illumination of the tumor with a fluorescent Wood's light. In this setting, the

ECT induced an irreversible abrogation of blood flow in the tumors that later lead to vascular lesions and necrosis (Ramirez et al. 1998). An in-depth investigation of tumor blood flow after ECT with cisplatin (4 mg/kg was injected i.v.) was performed, using patent blue staining as a marker of perfusion on subcutaneous SA-1 tumors in syngeneic A/J mice (Sersa et al. 2002). Patent blue was injected i.v. after ECT. In this study EP was achieved by 8 square-wave electric pulses, delivered in two sets of four pulses in perpendicular directions, voltage-to-distance ratio 1,300 V/cm, 100 μs, and frequency of 1 Hz, were delivered by two flat, parallel stainless steel electrodes 8 mm apart (two stainless steel strips: length 15 mm, width 7 mm with rounded corners). Similar to when only EP was used, 2 h after ECT, the tumor perfusion was reduced to 30%, but contrary to EP, it remained below 40% of the pretreatment value up to the fifth day after ECT (Sersa et al. 2002). When bleomycin (1 mg/kg injected i.v.) was used instead of cisplatin, ECT had the same effect, reducing the perfused area to 20% within 2 h after ECT and remaining under 30% of the pretreatment value for more than 2 days (Sersa et al. 2008). Using dynamic contrast-enhanced MRI, it was further confirmed that 24 h after ECT, there was still a significant reduction in blood volume and perfusion in SA-1 tumors that were treated with ECT with cisplatin (Sersa et al. 2002). Furthermore, when blood flow in the SA-1 tumors treated with ECT with bleomycin was measured with power Doppler ultrasonographic imaging and laser Doppler flowmetry (LDF), it was confirmed that the reduction in blood flow after ECT happens immediately after the first pulse is further reduced with every subsequent pulse (Sersa et al. 2008). Similar to when EP alone was used, the rapid and abrupt initial decrease in perfusion of tumors after ECT was followed by a gradual but only partial reperfusion that peaked 10–15 min after the delivery of electric pulses and did not continue afterward, during the observation time of 60 min after ECT. Interestingly, this approach did not detect significant differences in the tumor blood flow in the first minute after ECT when compared to EP only (Sersa et al. 2008). Similar to when EP alone was used, the pO_2 measured with EPR was reduced to ~20% of pretreatment values immediately after ECT, regardless whether cisplatin or bleomycin was used as the drug (Sersa et al. 2002, 2008). Contrary to when EP alone was used, where the pO_2 values returned to normal within 24 h after EP, ECT had a prolonged effect, and it took more than 48 h for the pO_2 values to return to 100% (Sersa et al. 2002, 2008). The discrepancy between the tumor perfusion determined with patent blue staining, which was still at around 30% of pretreatment value at this time point, and the pO_2 values that have already returned to normal was explained by the reduced number of tumor cells that were killed by ECT, thus reducing the demand for oxygen, which in return increased the pO_2 in the tumor (Sersa et al. 2002, 2008; Jarm et al. 2010). The initial decrease in pO_2 in the tumor also led to an increase in the fraction of hypoxic areas of the tumor 90 min after ECT with bleomycin that returned to the values similar to control tumors within 24 h after ECT (Sersa et al. 2008). A detailed histological analysis revealed rounding up of tumor endothelial cells 1 h after ECT and an onset of their apoptosis 8 h after ECT (Sersa et al. 2008). Regardless whether cisplatin or bleomycin was used as a drug, ECT resulted in a

substantial increase in the necrotic areas of the tumor (Sersa et al. 2002, 2008). Thus, the data suggests that ECT has a vascular-disrupting action and that the destruction of tumor blood vessels leads to a long-lasting perturbation of tumor blood flow leading to tumor necrosis.

Intriguing results were found when using the DWC model in SCID mice where human colon carcinoma HT29 tumors were grown (Markelc et al. 2013). Using 70 kDa fluorescently labeled dextrans, coupled with fluorescent macroscopy, the direct observation of the effects of ECT on tumor and normal blood vessels were achieved. Electric pulses were delivered with two parallel stainless steel plate electrodes (30 mm long, 6 mm wide, 6 mm distance between electrodes), and standard ECT pulse parameters were used (eight pulses, voltage-to-distance ratio 1,300 V/cm, 100 μs, frequency of 1 Hz). ECT resulted in an immediate abrogation of blood flow in the tumor, i.e., vascular lock, which lasted for more than 60 min (Fig. 1). The tumor-supplying arterioles responded to ECT in the same way as the normal vessels to EP alone, with rapid vasoconstriction and increased permeability for macromolecules, which was even higher than when EP was applied (Markelc et al. 2013). Interestingly, although the blood flow in the blood vessels surrounding the tumor started to return to normal levels 10 min after ECT, this was not recapitulated in the tumor. The blood flow in the tumor did not return even 24 h after ECT (Fig. 2) (Markelc et al. 2013). ECT also increased the permeability of tumor blood vessels for macromolecules, which was evident by leakage of trapped 70 kDa fluorescently labeled dextran from the vessels. However, due to the pronounced long-lasting vascular lock after ECT, the pool of molecules did not get renewed; therefore the quantification was not possible (Markelc et al. 2013). Importantly, the normal blood vessels surrounding the tumor were not destroyed after ECT and retained their functionality after the therapy (Markelc et al. 2013).

In summary, this data suggests that ECT has an immediate effect on tumor blood vessels, as well as a differential effect on tumor and normal blood vessels, destroying the first and sparing the latter. This is of a special interest as it implies that ECT is a vascular-disrupting therapy with selective action against tumor blood vessels. Similar to the response of tumor blood vessels to EP, their response to ECT has two distinct phases. Firstly, ECT immediately induces a rapid and profound abrogation of blood flow, which is caused primarily by the vasoconstriction of the tumor-supplying arterioles (Jarm et al. 2010; Markelc et al. 2013). After the vasoconstriction of tumor-supplying arterioles is finished, which is ~15 min after EP, there are a small reperfusion of the tumors and increased permeability of blood vessel for macromolecules. This is limited primarily to the tumor periphery; however, contrary to when EP alone is used, there is no further increase in tumor perfusion. Instead, ECT acts on tumor endothelial cells by direct cytotoxic action, thus destroying them, which leads to the destruction of tumor blood vessels and complete, long-lasting abrogation of blood flow (Sersa et al. 2002, 2008; Markelc et al. 2013). A direct consequence of this prolonged second phase is a decrease in pO_2 and an increase in the fraction of hypoxic regions in the tumor, which in the end leads to tumor necrosis and tumor cure (Sersa et al. 2002, 2008).

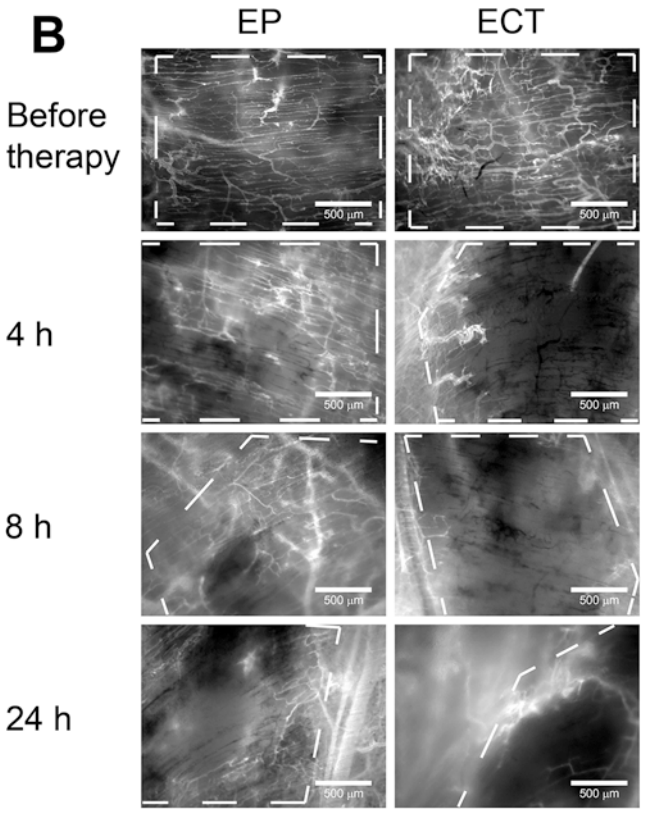

Fig.2 Illustration of reperfusion of tumor blood vessels at 4–24 h after EP and ECT. Tumor blood vessels were visualized by fluorescence microscopy. EP, mice treated with EP; ECT, mice treated with ECT. 70 kDa rhodamine D-labeled dextran was injected i.v. at 4, 8, and 24 h after the therapy, and images were acquired 5 min after the injection at 80× magnification. The images are representative of different tumors. Tumors are marked with a *dashed line*. Scale bar is 500 µm (Reproduced under the terms of the Creative Commons Attribution License from Markelc et al. (2013))

Conclusions

Electroporation (EP) and electrochemotherapy (ECT) have blood flow-modifying effects on normal as well as on tumor vasculature. The mechanisms behind the response of tumor and normal blood vessels to EP or ECT are different, due to different characteristics of tumor blood vessels compared to normal blood vessels. In both types of the vasculature, EP or ECT increases the permeability of affected blood vessels and causes a vascular lock, i.e., decrease in perfusion. In the case of normal blood vessels, these effects are short lived, whereas in the case of tumor blood vessels, the effects are long lasting and resolve more than 24 h after EP or ECT.

Moreover, ECT has a direct cytotoxic effect on tumor endothelial cells; thus it has a vascular-disrupting effect. Importantly, this is a differential effect that destroys only tumor blood vessels and retains the functionality of normal blood vessels surrounding the tumor that are also affected (Markelc et al. 2013). In clinical settings, this phenomenon was also observed as the differential effect on tumor and normal tissues in the terms of toxicity and in even larger normal vessels surrounding tumor that were not damaged after ECT (Gehl and Geertsen 2000; Jarm et al. 2010; Yarmush et al. 2014). This shows that ECT can be safely used even in a situation when large blood vessels are close to the tumor and will be exposed to the electric field during the treatment (Marcan et al. 2015).

Cross-References

- ▶ Antiangiogenic Gene Therapy
- ▶ Effects of Reversible and Irreversible Electroporation on Endothelial Cells and Tissue Blood Flow

References

Barker HE, Paget JTE, Khan AA, Harrington KJ (2015) The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. Nat Rev Cancer 15:409–425. doi:10.1038/nrc3958

Bellard E, Markelc B, Pelofy S, Le Guerroué F, Sersa G, Teissié J, Cemazar M, Golzio M (2012) Intravital microscopy at the single vessel level brings new insights of vascular modification mechanisms induced by electropermeabilization. J Control Release 163:396–403

Cemazar M, Parkins CS, Holder AL, Chaplin DJ, Tozer GM, Sersa G (2001) Electroporation of human microvascular endothelial cells: evidence for an anti-vascular mechanism of electrochemotherapy. Br J Cancer 84:565–570. doi:10.1054/bjoc.2000.1625

Clémenson C, Chargari C, Deutsch E (2013) Combination of vascular disrupting agents and ionizing radiation. Crit Rev Oncol Hematol 86:143–160. doi:10.1016/j.critrevonc.2012.10.002

Folkman J (2007) Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 6:273–286. doi:10.1038/nrd2115

Gehl J, Geertsen PF (2000) Efficient palliation of haemorrhaging malignant melanoma skin metastases by electrochemotherapy. Melanoma Res 10:585–589

Gehl J, Skovsgaard T, Mir LM (2002) Vascular reactions to in vivo electroporation: characterization and consequences for drug and gene delivery. Biochim Biophys Acta 1569:51–58

Gothelf A, Gehl J (2012) What you always needed to know about electroporation based DNA vaccines. Hum Vaccin Immunother 8:1694–1702. doi:10.4161/hv.22062

Jarm T, Cemazar M, Miklavcic D, Sersa G (2010) Antivascular effects of electrochemotherapy: implications in treatment of bleeding metastases. Expert Rev Anticancer Ther 10:729–746. doi:10.1586/era.10.43

Kanthou C, Kranjc S, Sersa G, Tozer G, Zupanic A, Cemazar M (2006) The endothelial cytoskeleton as a target of electroporation-based therapies. Mol Cancer Ther 5:3145–3152. doi:10.1158/1535-7163.MCT-06-0410

Marcan M, Kos B, Miklavcic D (2015) Effect of blood vessel segmentation on the outcome of electroporation-based treatments of liver tumors. PLoS One 10, e0125591. doi:10.1371/journal.pone.0125591

Markelc B, Bellard E, Sersa G, Pelofy S, Teissie J, Coer A, Golzio M, Cemazar M (2012) In vivo molecular imaging and histological analysis of changes induced by electric pulses used for plasmid DNA electrotransfer to the skin: a study in a dorsal window chamber in mice. J Membr Biol 245:545–554. doi:10.1007/s00232-012-9435-5

Markelc B, Sersa G, Cemazar M (2013) Differential mechanisms associated with vascular disrupting action of electrochemotherapy: intravital microscopy on the level of single normal and tumor blood vessels. PLoS One 8, e59557. doi:10.1371/journal.pone.0059557

Meulenberg CJW, Todorovic V, Cemazar M (2012) Differential cellular effects of electroporation and electrochemotherapy in monolayers of human microvascular endothelial cells. PLoS One 7, e52713. doi:10.1371/journal.pone.0052713

Ramirez LH, Orlowski S, An D, Bindoula G, Dzodic R, Ardouin P, Bognel C, Belehradek J, Munck JN, Mir LM (1998) Electrochemotherapy on liver tumours in rabbits. Br J Cancer 77:2104–2111

Rems L, Miklavcic D (2016) Tutorial: electroporation of cells in complex materials and tissue. J Appl Phys 119:201101. doi:10.1063/1.4949264

Sersa G, Beravs K, Cemazar M, Miklavcic D, Demsar F (1998) Contrast enhanced MRI assessment of tumor blood volume after application of electric pulses. Electro Magnetobiol 17:299–306. doi:10.3109/15368379809022574

Sersa G, Cemazar M, Miklavcic D, Chaplin DJ (1999) Tumor blood flow modifying effect of electrochemotherapy with bleomycin. Anticancer Res 19:4017–4022

Sersa G, Krzic M, Sentjurc M, Ivanusa T, Beravs K, Kotnik V, Coer A, Swartz HM, Cemazar M (2002) Reduced blood flow and oxygenation in SA-1 tumours after electrochemotherapy with cisplatin. Br J Cancer 87:1047–1054. doi:10.1038/sj.bjc.6600606

Sersa G, Jarm T, Kotnik T, Coer A, Podkrajsek M, Sentjurc M, Miklavcic D, Kadivec M, Kranjc S, Secerov A, Cemazar M (2008) Vascular disrupting action of electroporation and electrochemotherapy with bleomycin in murine sarcoma. Br J Cancer 98:388–398. doi:10.1038/sj.bjc.6604168

Yarmush ML, Golberg A, Sersa G, Kotnik T, Miklavcic D (2014) Electroporation-based technologies for medicine: principles, applications, and challenges. Annu Rev Biomed Eng 16:295–320. doi:10.1146/annurev-bioeng-071813-104622