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# 24 Electroporation for Electrochemotherapy and Gene Therapy

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## CONTENTS

24.1	Electroporation: The Phenomenon .....	395
24.1.1	Induced Transmembrane Voltage and Electroporation .....	397
24.1.2	Transport across the Electroporated Membrane.....	398
24.2	Electrochemotherapy: Preclinical In Vitro and In Vivo Studies .....	399
24.2.1	Electrochemotherapy: In Vitro Studies .....	399
24.2.2	Electrochemotherapy: In Vivo Studies .....	400
24.2.3	Electrochemotherapy: Studying in Veterinary Oncology .....	400
24.2.4	Mechanisms of Antitumor Action of Electrochemotherapy.....	401
24.2.5	Vascular Targeted Action of Electroporation and Electrochemotherapy .....	401
24.3	Clinical Applications of Electrochemotherapy .....	402
24.4	Preclinical and Clinical Application of Gene Electrotransfer: Gene Therapy.....	403
24.5	Perspectives .....	406
	Acknowledgments.....	406
	References.....	406

## 24.1 ELECTROPORATION: THE PHENOMENON

The theoretical understanding of the phenomenon of electroporation is crucial for planning and optimization of protocols for drug and/or gene delivery. In the last four decades, a number of tentative theoretical descriptions of this phenomenon have been proposed, assuming either deformation of membrane lipids,<sup>1-3</sup> their phase transition,<sup>4</sup> breakdown of interfaces between the lipid domains,<sup>5</sup> or denaturation of membrane proteins.<sup>6</sup> However, each of these former descriptions has serious flaws,<sup>7</sup> and today, there is broad consensus that electroporation is best described as the formation of aqueous pores in the lipid bilayer.<sup>8-11</sup> This also clarifies the prevalent choice of the term *electroporation*, as opposed to the broader term of *electropermeabilization*, which is also applicable to all the alternative explanations of the phenomenon.

The theory of electroporation is largely based on thermodynamics and describes the initial stage of pore formation by penetration of water molecules into the lipid bilayer of the membrane, forming unstable structures termed *water wires* or *water fingers*. This subsequently causes the adjacent lipids to reorient with their polar heads toward these structures, forming metastable aqueous pores.

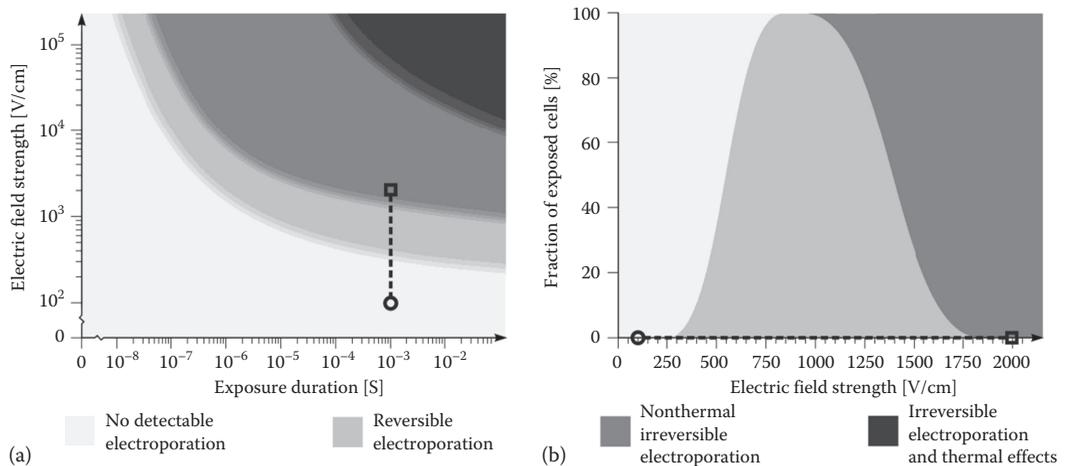
Both the theory and molecular dynamics simulations suggest that small unstable pores are forming and closing within nanoseconds even in the absence of an external electric field, but an exposure of the membrane to an electric field reduces the energy required for penetration of water into the bilayer. As such exposure starts, the external field infiltrates the membrane so that the membrane

field is of the same order of magnitude as the external field, but within less than a microsecond, the external field also causes a polarizing flow of dissociated ions in the media surrounding the membrane, resulting in the gradual buildup (inducement) of transmembrane voltage that amplifies the membrane field by about three orders of magnitude.<sup>12,13</sup>

Exposure of the membrane to an electric field thus increases the probability of pore formation in the membrane's bilayer so that on the average pores form more frequently and with much longer lifetimes than those formed in the absence of the electric field. For transmembrane voltages of hundreds of millivolts, the number of pores and their average lifetime become sufficient for detectable increase in membrane permeability to molecules otherwise unable to cross the membrane.

Metastable aqueous pores in the bilayer are at most several nanometers larger in diameter, which is too small to be observable by optical microscopy, while sample preparation techniques required for electron microscopy of soft matter are too harsh for reliable preservation of metastable formations in the bilayer and often themselves cause pore-like structures in the bilayer. Still there is growing and increasingly convincing indirect support for aqueous pore formation in the form of molecular dynamics simulations. These computational studies largely confirm the theoretically predicted stages of aqueous pore formation, including the strong increase in the rate of pore formation with the increase in the electric field to which the membrane is exposed—first through the direct action of the external field and then augmented by the inducement of transmembrane voltage due to polarization.<sup>13–16</sup>

The characteristics of electroporation and the accompanying phenomena depend on the amplitude and duration of the electric field to which the cells are exposed, and this relation is sketched in Figure 24.1. With low amplitudes and durations of the electric field, there is no detectable effect on the membrane and the transport across it. With moderate amplitudes and durations, electroporation is reversible so that after the exposure ceases, the pores gradually reseal and the cells remain viable. With higher field amplitudes and/or longer durations, electroporation is irreversible, as the transport through the pores—particularly the leakage of intracellular content—is too extensive,



**FIGURE 24.1** Electroporation and thermal effects caused by exposure of cells to electric fields. (a) Reversible electroporation, irreversible electroporation, and thermal damage as functions of electric field strength and duration. (Adapted from Bower, M. et al., *J. Surg. Oncol.*, 104, 22, 2011; Yarmush, M.L. et al., *Annu. Rev. Biomed. Eng.*, 16, 295, 2012.) (b) The fractions of non-electroporated, reversibly electroporated, and irreversibly electroporated cells as functions of electric field strength, for a fixed exposure duration of 1 ms (i.e., along the dashed vertical in panel a). Note that the field scale is logarithmic in panel a but linear in panel b, where it covers a much narrower range. (Reprinted from Delemotte, L. and Tarek, M., *J. Membr. Biol.*, 245, 531, 2012. With permission.)

and the resealing is too slow for the cells to recover, resulting in their death. At still stronger and/or longer exposures, irreversible electroporation becomes accompanied by thermal damage to the cell, as well as to the molecules released from it. Since pore formation is a stochastic process and exposed cells are typically not all identical in size, shape, and orientation, the ranges of no poration, reversible poration, irreversible poration without thermal damage, and irreversible poration with thermal damage partly overlap. The bounds of these four ranges also vary with the type of the cells exposed and by the properties of the medium surrounding the cells. In addition, thermal damage is both organism and molecule dependent, as proteins already start to denature at relatively small temperature increases (at  $\sim 43^{\circ}\text{C}$ – $45^{\circ}\text{C}$  in human cells), DNA melting occurs only above  $\sim 70^{\circ}\text{C}$ , and most lipids and simpler saccharides are not affected even by boiling.

Similarly to pore formation, pore resealing is a stochastic process, but it proceeds on a much longer time scale. Namely, the formation of electropores takes nano- to microseconds, while their resealing—as revealed by the return of the membrane’s electric conductivity to its preporation value and by termination of detectable transmembrane transport—is often completed only within seconds or even minutes after the end of the exposure.<sup>20</sup> More detailed measurements reveal that the resealing proceeds in several stages with time constants ranging from micro- and/or milliseconds up to tens of seconds.<sup>21,22</sup> Unfortunately, neither the existing theory nor the experiments can provide a reliable picture of specific events characterizing each of these distinctive stages, while reliable molecular dynamics simulations, even in their most simplified versions (e.g., coarse-grained), cannot yet cover time scales that are extensive.

### 24.1.1 INDUCED TRANSMEMBRANE VOLTAGE AND ELECTROPORATION

In most applications of electroporation, biological cells to be porated are not brought into direct contact with the electrodes, so that the voltage on the membranes of the exposed cells, termed the induced transmembrane voltage ( $\Delta\Psi_m$ ), represents only a part of the voltage delivered to the electrodes. Unlike with clamped membrane patches, where  $\Delta\Psi_m$  is a constant all over the exposed patch, with cells exposed as a whole in a contactless manner,  $\Delta\Psi_m$  is position dependent; in spherical cells, its spatial variation is described by the steady-state Schwan equation<sup>23</sup>:

$$\Delta\Psi_m = 1.5ER \cos \theta$$

where

$E$  is the amplitude (strength) of the external electric field

$R$  is the radius of the spherical cell

$\theta$  is the angle between the direction of the applied field and the radial line connecting the cell center with the considered point on the membrane

Thus,  $\Delta\Psi_m$  is proportional to the applied electric field and the cell radius, and it varies as  $\cos \theta$ , with extremal values at the two points where the field is perpendicular to the membrane, that is, at  $\theta = 0^{\circ}$  and  $\theta = 180^{\circ}$  (the *poles* of the cell).

The induced transmembrane voltage is typically established within microseconds after the onset of the field. To describe the initial transient behavior, one uses the more general first-order Schwan equation<sup>11</sup>:

$$\Delta\Psi_m = 1.5ER \cos \theta \left( 1 - \exp\left(\frac{-t}{\tau_m}\right) \right)$$

where  $\tau_m$  is the time constant of membrane charging (approximately  $0.5 \mu\text{s}$  under physiological conditions).

Induced transmembrane voltage as a function of position and time can also be assessed for non-spherical cells. For cells resembling a regular geometrical body such as a cylinder (e.g., a muscle cell, an axon of a nerve cell), an oblate spheroid (e.g., an erythrocyte), or a prolate spheroid (e.g., a bacillus), this can be done by means of analytical derivation, solving the Laplace equation in a suitable coordinate system with the appropriate boundary conditions.<sup>24–26</sup> For irregularly shaped cells and cells in dense suspensions or clusters,  $\Delta\Psi_m$  can be computed only numerically, using either the finite-differences or the finite-elements method; the latter is used more frequently and more efficiently both for irregularly shaped cells<sup>27,28</sup> and for clusters of cells.<sup>29–31</sup>

Experimental alternatives to analytical derivation and numerical computation of  $\Delta\Psi_m$  are provided by measurements with microelectrodes and with potentiometric fluorescent dyes. The use of microelectrodes is invasive, characterized by a rather low spatial resolution, and the physical presence of the electrodes distorts the electric field and hence the voltage it induces; these are serious disadvantages. On the other hand, measurements with potentiometric dyes are noninvasive; with no physical disruption of the membrane, they offer higher spatial resolution than microelectrodes, and their presence does not distort the electric field, but such measurements can be taken only on the cells that are visually accessible. Their use in tissues is thus rather limited, but for experiments *in vitro*, potentiometric dyes, such as di-8-ANEPPS,<sup>32,33</sup> RH292,<sup>34</sup> and ANNINE-6,<sup>35</sup> have become established tools for measurements of  $\Delta\Psi_m$ , experimental studies of voltage-gated membrane channels, as well as for monitoring of nerve and muscle cell activity. A potentiometric dye incorporates into the lipid bilayer of the membrane, where it starts to fluoresce with a spectrum dependent on the amplitude of the induced voltage. With a suitable setup comprising a pulse laser, a fast sensitive camera, and a system for synchronization of acquisition with field exposure, these dyes also allow to monitor the time course of  $\Delta\Psi_m$  with a resolution of microseconds, and even nanoseconds for ANNINE-6.<sup>35</sup>

As the pores in the membrane caused by electroporation are not observable directly with the currently available techniques, electroporation can be detected and studied only indirectly, by assessing its larger-scale manifestations—mainly the changes in electrical or optical properties of the membrane resulting from the formation of pores or transport through them. The changes in electrical properties of the membrane can be measured by patch-clamp techniques, and they show that during electroporation, the electric conductivity of the membrane increases by several orders of magnitude, and its dielectric permittivity is also affected.<sup>36,37</sup> In dense cell suspensions, electroporation can also be monitored by measuring the bulk electric conductivity, which increases significantly if a large fraction of the exposed cells is electroporated.<sup>38,39</sup> A similar approach is also used in tissues and can be augmented by measuring the conductivity and permittivity at several frequencies, typically in the kilohertz range, which allows to distinguish between nonporated, reversibly porated, and irreversibly porated tissues.<sup>40,41</sup>

The bulk optical properties of the membrane, particularly light scattering and absorption, are also affected by the reorientation of lipids around the pores, and measurements of these properties can also be used to assess electroporation.<sup>42</sup> Finally, an even more indirect, and perhaps also the most frequently used method of electroporation assessment, is by means of imaging the transport of molecules that cannot permeate an intact membrane, as described in more detail later.

### 24.1.2 TRANSPORT ACROSS THE ELECTROPORATED MEMBRANE

Electroporation-mediated transport across the membrane is strongly correlated with the transmembrane voltage induced by the exposure to the electric field, which is in turn proportional to this field.<sup>20,34</sup> This correlation can be demonstrated particularly clearly by combining potentiometric measurements and monitoring transmembrane transport on the same cell.<sup>21</sup> On the tissue level, this same correlation is reflected in the fact that the tissue regions with the highest local electric field are generally also the regions containing the highest fractions of electroporated cells.<sup>22</sup>

The transport of molecules across an electroporated membrane can be characterized by the Nernst–Planck equation<sup>42</sup>

$$\frac{V}{S} \frac{dc}{dt} = -DE \frac{zF}{\rho T} c - D\nabla c$$

where

$V$  is the volume of the cell

$S$  is the surface area of the electroporated part of the membrane

$c$  is the concentration of molecules or ions transported across this part of the membrane

$D$  is the diffusion coefficient for such transport

$z$  is the electric charge of the molecules or ions

$E$  is the local electric field acting on them

$F$  is the Faraday constant

$\rho$  is the gas constant

$T$  is the absolute temperature

The first term on the right-hand side of the Nernst–Planck equation corresponds to the electrophoretic transport driven by the exposure of the cell to the electric field, and the second term to the diffusive transport that persists until either the concentrations of the transported molecules on both sides of the membrane equalize or all the pores reseal.

During an electric pulse, the electric field is the main source of the driving force acting on charged molecules and ions, and the electrophoretic term dominates the right-hand side of the Nernst–Planck equation. As a pulse ceases, so does the electrophoretic transport, with only the diffusive component persisting. Although diffusive transport proceeds at a much slower rate than electrophoretic transport, complete pore resealing takes seconds or even minutes,<sup>42</sup> while pulses used for electroporation last at most several milliseconds. As a consequence, despite the fast initial rate of electrophoretic transport, the total transport of both ions and small molecules through an electroporated membrane is often predominantly diffusive.<sup>43,44</sup> In contrast, electrophoretic transport can contribute crucially in the transport of macromolecules, particularly DNA, across the electroporated membrane.<sup>45,46</sup> Besides electrophoretic transport for macromolecules, also electroporation-enhanced endocytotic transport of plasmid DNA has been demonstrated.<sup>47</sup>

## 24.2 ELECTROCHEMOTHERAPY: PRECLINICAL IN VITRO AND IN VIVO STUDIES

### 24.2.1 ELECTROCHEMOTHERAPY: IN VITRO STUDIES

Application of electric pulses to the cells *in vitro*, aiming to increase cytotoxicity of chemotherapeutic drug bleomycin, was first described by Orłowski et al.<sup>48</sup> Thereafter, several other chemotherapeutic drugs were tested *in vitro* on cells for potential application in combination with electroporation; among them only cisplatin was the most promising drug. Electroporation of cells increased the cytotoxicity of bleomycin (up to several 1000-fold) and cisplatin (up to 70-fold). The prerequisite for the drug to be effective in combination with electroporation is that they are either hydrophilic or lack transport system in the membrane, since electroporation can facilitate the drug transport through the cell membrane only for poorly or non-permeant molecules.<sup>49–51</sup>

Increased cytotoxicity of cisplatin due to electroporation of cells was demonstrated also in cell lines resistant to cisplatin, however, to a lesser degree than on parental cell line.<sup>52</sup> Furthermore, it was demonstrated that endothelial cells are sensitive to bleomycin and to cisplatin, especially when the drug delivery was increased by electropulsion. These data are important for the explanation of vascular disrupting effect of electrochemotherapy (ECT).<sup>53</sup>

### 24.2.2 ELECTROCHEMOTHERAPY: IN VIVO STUDIES

Bleomycin and cisplatin were tested in ECT protocol on a number of animal models *in vivo*. Extensive studies on different animal models with different tumors, either transplantable or spontaneous, were performed. Antitumor effectiveness of ECT was tested on tumors in mice, rats, hamsters, and rabbits. Tumors treated by ECT were either subcutaneous, grew in the muscle, brain, or liver, and were of different types, for example, sarcomas, carcinomas, glioma, or melanoma.<sup>50,54–58</sup> The studies demonstrated that with drug doses that have minimal or no antitumor effectiveness, high (up to 80%) complete responses (CRs) of the ECT-treated tumors were obtained. The drug doses used were so low to have no systemic toxicity. Route of administration was either intravenously (for bleomycin) or intratumorally (bleomycin and cisplatin). The time interval between drug injection and application of electric pulses is important. The prerequisite is that, at the time of the application of electric pulses to the tumor, a sufficient amount of drug is present in the tumor. Therefore, after intravenous drug administration into small laboratory animals (4 mg/kg of cisplatin or 0.5 mg/kg bleomycin), only a few minutes' interval is needed to reach the maximal drug concentration in the tumors. After intratumoral administration (2 mg/cm<sup>3</sup> of cisplatin and 3 mg/cm<sup>3</sup> of bleomycin), this interval is even shorter, and the application of electric pulses has to follow the administration of the drug as soon as possible (within a minute).<sup>58</sup> Some other well-established drugs or drugs in development were also tested in combination with electric pulses for potential increase in effectiveness. The majority of results showed some potential benefit; however, the results of the studies were not as pronounced as for bleomycin or cisplatin; therefore, further studies were not conducted.<sup>59–63</sup>

The application of electric pulses of suitable parameters to the tumors, which led to adequate and sufficient electric field distribution in the tumor to obtain cell electroporation, had no antitumor effectiveness and no systemic side effects.<sup>64</sup> Local side effects were contractions of the muscles underlying the treated area, but these are present only during the application of electric pulses and were tolerable, so in most cases, anesthesia of laboratory animal was not necessary.<sup>65</sup>

### 24.2.3 ELECTROCHEMOTHERAPY: STUDYING IN VETERINARY ONCOLOGY

In the first veterinary clinical trial, conducted in 1997, 12 cats with spontaneous large soft tissue sarcomas that had relapsed after treatment with conventional therapies were treated with ECT with bleomycin combined with immunotherapy consisting of intratumoral injection of CHO (interleukin-2 [IL-2]) living cells that secreted IL-2, which makes this study substantially different from other studies.<sup>66</sup> In most of the studies on ECT in small animals, cisplatin was used as a chemotherapeutic agent. In these studies, ECT was used as single treatment and not as an adjuvant treatment. It was used for the treatment of dogs, cats, and horses with up to 100% tumor cures.<sup>67–71</sup> Studies using intratumorally injected bleomycin were performed either alone or as an adjuvant treatment to surgery. ECT with bleomycin injected intratumorally was performed in pets with spontaneous tumors of different histological types, and the therapy resulted in good response rate.<sup>71</sup> Comparison of ECT of mastocytoma to surgical excision demonstrated that ECT is equally effective and can represent an alternative to surgery.<sup>72</sup> In the case of adjuvant treatment, ECT proved to be very effective as an adjunct to surgery for the treatment of mast cell tumors and soft tissue sarcoma in dogs and hemangiopericytoma and soft tissue sarcoma in cats.<sup>71</sup> Furthermore, several recent studies evaluated ECT with either bleomycin or cisplatin in cats.<sup>73–77</sup> For example, ECT with bleomycin of superficial squamous cell carcinoma in cats resulted in 82% CR, making ECT as a good alternative option for treatment, especially when other treatment approaches are not acceptable by the owners, owing to their invasiveness, mutilation, or high cost.<sup>77</sup> ECT with cisplatin injected intratumorally was tested in several clinical trials on larger numbers of equine sarcoids. The results of the studies confirmed that ECT with cisplatin is a highly effective treatment with long-lived antitumor effects and good treatment tolerance.<sup>70,71</sup>

#### 24.2.4 MECHANISMS OF ANTITUMOR ACTION OF ELECTROCHEMOTHERAPY

Several mechanisms of antitumor effectiveness of ECT were described. Recently, a lot of studies were devoted to elucidation of vascular targeted action of ECT; therefore, it will be explained in more detail. Nevertheless, the principal mechanism of ECT is increased permeabilization of the membranes of cells in the tumors, leading to increased drug effectiveness by enabling the drug to reach the intracellular targets. In preclinical studies on murine tumors, increase in the uptake of bleomycin and cisplatin in the electroporated tumors was demonstrated compared to those tumors without electroporation.<sup>78,79</sup> Furthermore, twofold increase in cisplatin DNA adducts was determined in electroporated tumors.<sup>79</sup>

Another mechanism involved in the antitumor mechanism of ECT is the *involvement of immune system*. It was demonstrated by the difference in response to ECT of tumors growing in immunocompetent and immunodeficient laboratory mice.<sup>80</sup> The tumors growing in immunodeficient mice did not completely regress after ECT, while tumor growing in immunocompetence mice did. We also demonstrated the increased activity of T lymphocytes and monocytes in tumor-bearing mice treated with ECT.<sup>81</sup> In addition, due to the massive tumor antigen shedding in the organisms after ECT, systemic immunity can be induced and can be upregulated by additional treatment with biological response modifiers like IL-2, IL-12, GM-CSF, and TNF- $\alpha$ .<sup>82-84</sup>

#### 24.2.5 VASCULAR TARGETED ACTION OF ELECTROPORATION AND ELECTROCHEMOTHERAPY

It was shown in preclinical studies that the application of electric pulses to the tissues induces a transient, but reversible, reduction of blood flow. The first study, using albumin-(Gd-DTPA) contrast-enhanced magnetic resonance imaging, has demonstrated that 30 min after application of electric pulses to SA-1 tumors, tumor blood volume was reduced from 20% in untreated tumors to 0% in electroporated tumors.<sup>85</sup> A pharmacological study with <sup>86</sup>RbCl extraction technique in the same tumor model was also done, demonstrating that significant reduction of tumor perfusion (~30% of control) was observed within 1 h following the application of electric pulses to the tumors, which returned to pretreatment value with 24 h. The degree of tumor blood flow reduction was dependent upon the number and amplitude.<sup>86</sup> In subsequent studies, it was demonstrated that the results obtained with the <sup>86</sup>RbCl extraction technique correlated with the Patent Blue staining technique, which is a much more simple method for measuring tissue perfusion,<sup>87</sup> and with tumor oxygenation, which was measured by the electronic paramagnetic resonance technique.<sup>88</sup>

In vitro studies have shown that application of electric pulses to a monolayer of endothelial cells results in a profound disruption of microfilament and microtubule cytoskeletal networks, resulting in increased permeability of endothelial monolayer.<sup>89</sup> Furthermore, mathematical model demonstrated that endothelial cells in the lining of small tumor blood vessels are exposed to an electric field that can increase their permeability and it's higher than in the surrounding tumor tissue.<sup>90</sup> Changes in endothelial cell shape were observed also in histological analysis 1 h after the application of electric pulses. Endothelial cells turned spherical in shape and became swollen, and the lumen of blood vessels was narrowed.<sup>90</sup> The observed effects of tumor blood flow modification after the application of electric pulses were also observed in normal muscle tissue in mice. Similar effects on leg perfusion, measured by Patent Blue, were observed in mice, with a wide variety of electric pulse amplitudes and pulse durations (10–20,000  $\mu$ s and 0.1–1.6 kV/cm).<sup>91</sup> Based on all the gathered information on vascular effects of electric pulses in the tumor, a model of the sequence of changes was proposed.<sup>92</sup>

Compared to vascular changes obtained by the application of electric pulses, the changes observed after ECT were more severe, but depending on the type and the dose of chemotherapeutic drug used.<sup>87,88</sup> Studies on ECT with bleomycin as well as with cisplatin have demonstrated that changes, within 2 h in tumor perfusion and oxygenation, are identical to those observed after the application of electric pulses alone. Immediately after the treatment, tumor perfusion was maximally reduced.

Approximately 30 min later, the tumors started to reperfuse in both groups; in the tumors treated by ECT, the reperfusion leveled after ~1 h and stayed at 20% up to 48 h after the treatment, whereas the tumors treated with the application of electric pulses alone continued to reperfuse. If using low dose of chemotherapeutic drug, gradual reperfusion of the tumors occurred, whereas the higher dose of bleomycin resulted in complete shutdown of tumor perfusion and a high percentage of tumor cures (70%).<sup>87,88,90</sup>

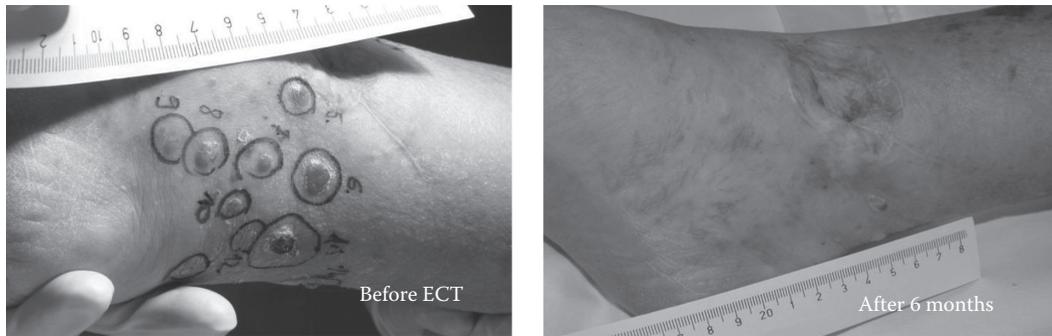
In vitro data supported the observed in vivo effects. It was demonstrated that electroporation of human endothelial HMEC-1 cells, even after short-term drug exposure, significantly enhanced the cytotoxicity of bleomycin or cisplatin<sup>93</sup> and resulted in significant disruption of cytoskeletal network of endothelial cells.<sup>94</sup>

Detailed histological analyses of tumors after ECT demonstrated that the same morphological changes in endothelial cells occurred as after the application of electric pulses to the tumors, endothelial cells turned spherical in shape and became swollen, and the lumen of blood vessels was narrowed. However, apoptotic morphological characteristics were found in some vessels 8 h after ECT. Furthermore, blood vessels were stacked with erythrocytes, and extravasation of erythrocytes was also observed. Apoptotic endothelial cells were not observed in the control group or in tumors treated with either electric pulses or bleomycin alone,<sup>90</sup> while intravital microscopy of tumors in dorsal window chamber also confirmed differential effect: tumor blood vessels were more affected than normal blood vessels surrounding the tumor, which has a significant clinical applicability (significance).<sup>95</sup>

### 24.3 CLINICAL APPLICATIONS OF ELECTROCHEMOTHERAPY

Based on vast preclinical data, ECT soon entered clinical trials. The first clinical study on ECT was published already in 1991 by Mir et al.<sup>96</sup> It has demonstrated the feasibility, safety, and effectiveness of ECT. Soon followed the reports from the group in the United States (Tampa), Slovenia (Ljubljana), France (Toulouse and Reims), and Denmark (Copenhagen) with their own clinical results, confirming the results of the first study.<sup>97–101</sup> The first results were compiled in a mutual paper in 1998, which is still a hallmark of clinical ECT.<sup>102</sup> The development of the field was then marked by the report of the European project called “European Standard Operating Procedures on Electrochemotherapy” (ESOPE). Results from this prospective multicenter study were published in 2006<sup>103</sup> together with the standard operating procedures for ECT using the electric pulse generator CLINIPORATOR (SOP).<sup>104</sup> This was the foundation for wider acceptance of ECT into broader clinical use throughout the Europe. So far, the predominant tumor type was skin metastases of melanoma, along with skin metastases of other tumor types. ECT for skin tumors is predominantly used in palliative intent and also in previously heavily pretreated area (Figure 24.2).

The clinical indications were published in a review paper,<sup>105</sup> along with the compiled results of all published studies till then. Recently, the systematic review and meta-analysis of all clinical data have demonstrated that overall effectiveness of ECT was 84.1% objective responses (ORs), from these 59.4% CRs.<sup>106</sup> Data analysis confirmed that ECT had a significantly ( $p < 0.001$ ) higher effectiveness (by more than 50%) than bleomycin or cisplatin alone. Furthermore, ECT was more efficient in sarcoma than in melanoma or carcinoma tumors. Another recent review and a clinical study suggested that SOP may need refinement since the currently used SOP for ECT may not be suitable for tumors bigger than 3 cm in diameter, but such tumors are suitable for the multiple consecutive ECT treatments.<sup>59,107</sup> In line with these findings, future investigations are needed to focus on the prognostic and predictive markers for the response of the tumors, in order to adjust ECT for the specific tumor type. Several studies are ongoing on superficial tumors, not only on melanoma but also on the treatment of chest wall breast cancer recurrences<sup>108–111</sup> and head and neck cancers,<sup>112</sup> Kaposi sarcomas,<sup>113</sup> and metastatic soft tissue sarcomas.<sup>114</sup> Furthermore, the technology is being adapted also for the treatment of deep-seated tumors, like colorectal tumors, soft tissue sarcomas, and brain, bone, and liver metastases.<sup>117</sup> The first clinical study on liver metastases of colorectal carcinoma has



**FIGURE 24.2** The antitumor effectiveness of electrochemotherapy with intravenously administered bleomycin in skin melanoma metastases. Electric pulses were delivered by plate electrodes, encompassing the nodules. Two electrochemotherapy sessions were performed. Excellent antitumor and cosmetic effects are visible.

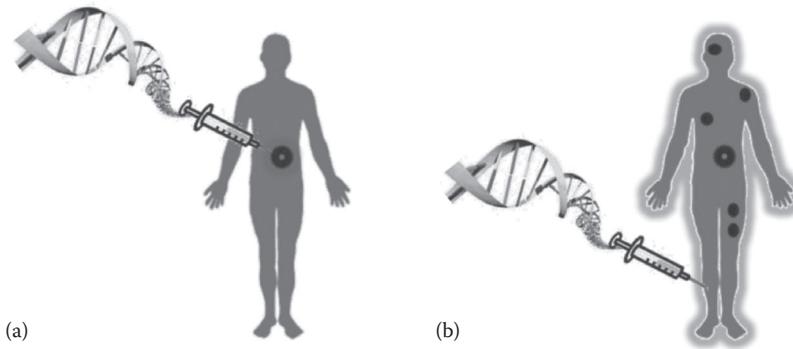
demonstrated feasibility, safety, and effectiveness of ECT.<sup>116</sup> The data indicated again on 84% CR rate of the treated tumors, verified with histology and/or radiology. Specifically, ECT was demonstrated to be effective also in tumors that are close to major hepatic vessels, and not amenable for radiofrequency ablation. This study has set the stage for the use of ECT of other tumors in the liver and also in other organs in the abdomen.

Technology, electric pulse generators, as well as electrodes, were adapted for the treatment of deep-seated tumors. Several different electrode types have been prepared.<sup>117</sup> However, to meet the prerequisite that the whole tumor needs to be covered with sufficient electric field in order to provide good clinical response, for deep-seated tumors bigger than 2 cm, which are being treated with the placement of individual electrodes, treatment planning is recommended.<sup>117</sup> It is similar to the treatment plan that is prepared for radiation therapy,<sup>118</sup> providing the amplitudes of electric pulses that need to be delivered between the pairs of electrodes.<sup>119</sup> The plan that is based on the segmentation of the target tumor with safety margins is then by numerical modeling prepared for the specific tumor, with the placement of the electrodes and the treatment parameters to enable whole coverage of the tumor with the sufficiently high electric field.<sup>120,121</sup>

#### 24.4 PRECLINICAL AND CLINICAL APPLICATION OF GENE ELECTROTRANSFER: GENE THERAPY

Another application of electroporation in biomedicine is gene electrotransfer—electrogene therapy. It can be used either for DNA vaccination against infectious diseases or for the treatment of various diseases, such as cancer, where therapies either are targeted directly to tumor cells or aim to increase the immune response of the organism against cancer cells. *In vivo* gene delivery using electroporation was first performed in the 1990s,<sup>122</sup> and since then, a number of different types of tissues have been successfully transfected using this approach (for instance, tumors, skeletal muscle, skin, and liver).<sup>123,124</sup> Transfection efficiency of electrotransfer is still low compared to viral vectors; yet its advantages, mostly lack of pathogenicity and immunogenicity, make it a promising new method.

Gene therapy can be performed using two different approaches. The first one is *ex vivo* gene therapy, where cells, including stem cells, are removed from patient, transfected *in vitro* with the plasmid or viral vector, selected, amplified, and then reinjected back into the patient. The other approach is *in vivo* gene therapy, where exogenous DNA is delivered directly into host's target tissue, for example, locally to tumor or peritumorally and for systemic release of the therapeutic molecule into skeletal muscle depending on the type of therapeutic molecules and intent of treatment (Figure 24.3).

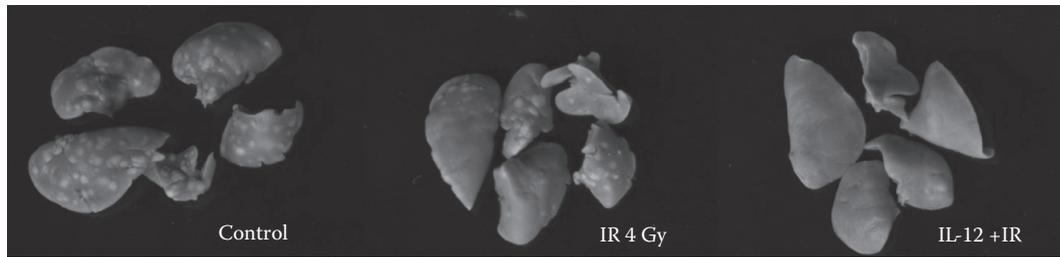


**FIGURE 24.3** (a) Local approach to cancer gene therapy. Injection of plasmid DNA directly into the tumor. (b) Systemic approach to cancer gene therapy by injection of plasmid DNA into the muscle, which then produces therapeutic protein that is distributed throughout the body reaching distant tumors.

Gene electrotransfer of therapeutic genes into tumors facilitates local intratumoral production of therapeutic proteins, enabling sufficient therapeutic concentration and thus therapeutic outcome. This is especially important in case of cytokines, where high systemic concentrations are associated with severe toxicity.<sup>125</sup> Gene electrotransfer can be used as a single therapy or in combination with other modalities for cancer treatment, such as standard treatment options surgery and radiotherapy, but also for example electrochemotherapy.<sup>126,127</sup>

The first evaluation of intratumoral electrogene therapy for cancer treatment was performed on murine melanoma tumor model in 1999 by Niu et al.<sup>128</sup> Since then, not only a variety of therapeutic genes, mostly encoding cytokines, but also tumor suppressor proteins, siRNA molecules against various targets, etc., have been tested in numerous animal tumor models, for example, melanoma, squamous cell carcinoma, sarcoma, and hepatocellular carcinoma.<sup>129,130</sup> Results of preclinical studies indicate that intratumoral therapeutic gene electrotransfer enables efficient transgene expression with sufficient production of therapeutic proteins, which can lead to pronounced antitumor effect on treated tumor (e.g., suppression of tumor growth, partial or complete reduction of tumor nodule), and even induces long-term antitumor immunity in treated animals.<sup>84,131</sup> Interestingly, some of the studies reported that even control plasmid without therapeutic gene can result in CR of the tumors, especially melanoma B16 tumor model. It was demonstrated that the underlying mechanism for this result is multifactorial, including direct toxicity of DNA, selection of electric pulses parameters, and induction of immunity.<sup>132</sup>

Some of the most significant antitumor effect to date in cancer gene therapy have been achieved with the employment of active nonspecific immunotherapy, that is, the use of cytokines. Gene electrotransfer of genes, encoding different cytokines, has already shown promising results in preclinical trials on different animal tumor models. Cytokine genes, which showed the most potential for cancer therapy, are IL-2, IL-12, IL-18, interferon (IFN)  $\alpha$ , and GM-CSF.<sup>129,133</sup> Currently, the most advanced therapy is using IL-12, which plays important role in the induction of cellular immune response through stimulation of T-lymphocyte differentiation and production of IFN- $\gamma$  and activation of natural killer cells.<sup>134</sup> Antitumor effect of IL-12 gene electrotransfer has already been established in various tumor models, for example, melanoma, lymphoma, squamous cell carcinoma, urinary bladder carcinoma, mammary adenocarcinoma, and hepatocellular carcinoma.<sup>133</sup> Results of preclinical studies show that besides regression of tumor at primary and distant sites, electrogene therapy with IL-12 also promotes induction of long-term antitumor memory and therapeutic immunity, suppresses metastatic spread, and increases survival time of experimental animals.<sup>130</sup> Gene therapy with IL-12 was successfully combined also with other therapies, such as ECT and radiotherapy resulting in potentiated effect<sup>126,127,135–137</sup> (Figure 24.4).



**FIGURE 24.4** Effect of combined IL-12 gene electrotransfer and radiotherapy (IR) on lung metastases. Combined therapy resulted in complete eradication of metastases. (From Heller, L. et al., *Cancer Gene Ther.*, 20, 695, 2013.)

Recently, clinical studies performed in patients with melanoma, as well as in veterinary patients, show great promise for further development of this therapy.<sup>135,136</sup> In human clinical study, 24 patients with malignant melanoma subcutaneous metastases were treated three times. The response to therapy was observed in treated as well as in distant nontreated tumor nodules. In 53% of patients, a systemic response was observed resulting in either stable disease or an OR. The major adverse side effect was transient pain after the application of electric pulses. In posttreatment biopsies, tumor necrosis and immune cell infiltration were observed. This first human clinical trial with IL-12 electrogene therapy in metastatic melanoma proved that this therapy is safe and effective.<sup>138</sup> In veterinary oncology, eight dogs with mastocytoma were treated with IL-12 gene electrotransfer. A good local antitumor effect with significant reduction of treated tumors' size, ranging from 15% to 83% (mean 52%) of the initial tumor volume, was obtained. Additionally, a change in the histological structure of treated nodules was seen as a reduction in the number of malignant mast cells and inflammatory cell infiltration of treated tumors. Furthermore, systemic release of IL-12 and IFN- $\gamma$  in treated dogs was detected, without any noticeable local or systemic side effects.<sup>139</sup> Again, the data suggest that intratumoral IL-12 electrogene therapy could be used for controlling local as well as systemic disease.

On preclinical level, gene electrotransfer to tumors was also employed in suicide gene therapy of cancer. The concept of suicide gene therapy is intratumoral transfer of a prodrug-activating gene, which selectively (intratumorally) activates otherwise nontoxic drugs. The most often used strategy in suicide gene therapy is the delivery of gene, encoding herpes simplex virus thymidine kinase (HSV-TK) and prodrug ganciclovir (GCV). HSV-TK activates GCV, which blocks extensions of DNA strands, leading to cell death by apoptosis. Results of several studies show that electroporation-based HSV-TK/GCV gene therapy may provide potentially effective gene therapy for cancer.<sup>140–143</sup>

Another approach in cancer gene therapy, which is currently being widely investigated, is based on the inhibition of angiogenesis of tumors. The basic concept of antiangiogenic gene therapy is the transfection of cells with genes, encoding inhibitors of tumor angiogenesis. Electrotransfer of plasmids encoding antiangiogenic factors (angiostatin and endostatin) was demonstrated to be effective in the inhibition of tumor growth and metastatic spread of different tumors.<sup>144–146</sup> Recently, we showed that the RNA interference approach, using siRNA molecule against endoglin, which is a coreceptor of transforming growth factor  $\beta$  and is upregulated in activated endothelial cells, also resulted in vascular targeted effect in mammary tumors.<sup>130</sup>

Besides tumors, skeletal muscle is an attractive target tissue for the delivery of therapeutic genes, since it is usually a large mass of well-vascularized and easily accessible tissue with high capacity for the synthesis of proteins, which can be secreted either locally or systemically.<sup>147</sup> Furthermore, transfection efficiency in muscle is very high compared to other tissues, especially tumors.<sup>147</sup> Owing to the postmitotic status and slow turnover of skeletal muscle fibers, which ensures that transfected DNA isn't readily lost, it is possible to achieve long-term expression of exogenous DNA, which can last up to 1 year.<sup>147,148</sup> This is due to the dynamics of naked DNA transfer since plasmid does not

integrate into the genome of transfected cell, and thus the duration of exogenous DNA expression in part depends on the lack of cell division. In contrast to muscle cells, in tissues, where cell turnover is much higher, for example, tumors, plasmid DNA is rapidly lost from the cells.<sup>147,149</sup>

Gene electrotransfer into skeletal muscle can be applied for the treatment of various muscle diseases, for local secretion of angiogenic or neurotrophic factors, or for systemic secretion of different therapeutic proteins, such as erythropoietin, coagulation factors, cytokines, and monoclonal antibodies.<sup>147,150–152</sup> In cancer gene therapy, gene electrotransfer of plasmid DNA encoding IL-12, IL-24, and antiangiogenic factors was evaluated with encouraging results. In clinical studies, intramuscular delivery of growth hormone–releasing hormone, human coagulation factor IX, and IL-12 was evaluated.<sup>153–155</sup> Results of our study indicate that in canine cancer patients, intramuscular IL-12 EGT is a safe procedure, which can result in systemic shedding of hIL-12 and possibly trigger IFN- $\gamma$  response in treated patients, leading to prolonged disease-free period and survival of treated animals.<sup>155</sup>

## 24.5 PERSPECTIVES

Electroporation-based biomedical applications, such as ECT and gene electrotransfer, are the most advanced of its applications. Very likely, electroporation will find its place also in vaccination, in the treatment of cancer, and in the delivery of drugs to and through the skin, for local and systemic treatment of diseases other than cancer.

ECT is now on the verge to enter into standard of care in many European Oncology Centers. Its application has spread; experiences are being gained, and further profiling of ECT have begun. The next steps are in the translation of this technology into the treatment of deep-seated tumors. Furthermore, many possibilities exist to combine ECT with other local or systemic treatments, either to potentiate the local effect, that is, radiotherapy, or to augment the systemic response by adjuvant immunotherapy. This will, again, take time, but will broaden current clinical indications of ECT.

In the future for gene therapy, some crucial questions need to be resolved, such as how optimization of treatment protocols for different tumor types should be performed, with respect to defining optimal plasmid dose, number of treatment repetitions, optimal route of administration (intratumoral, peritumoral/intradermal, intramuscular), and effect of combination with other treatment protocols (e.g., local intratumoral plasmid delivery or ECT) in order to achieve effective long-term antitumor effect in cancer patients.

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