Electroporation of human microvascular endothelial cells: evidence for an anti-vascular mechanism of electrochemotherapy

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Summary Recent studies have indicated that the antitumour effectiveness of electrochemotherapy, a combination of chemotherapeutic drugs with application of high voltage electric pulses applied to the tumour nodule (electroporation), result in a significant reduction in tumour blood flow and may therefore be mediated by an anti-vascular mechanism. The aim of this study was to evaluate the cytotoxicity of electroporation with bleomycin or cisplatin on cultured human microvascular endothelial cells (HMEC-1). The sensitivity of HMEC-1 cells to a 5 min treatment by electroporation with bleomycin or cisplatin (8 electric pulses, pulse duration 100 μs, frequency 1 Hz, electric field intensity 1400 V cm^{-1}) was compared to the sensitivity of cells treated continuously for 3 days with drugs alone. HMEC-1 cells were moderately sensitive to continuous exposure to cisplatin, but showed greater sensitivity to bleomycin. Combination of a 5 min drug exposure with electric pulses increased cytotoxicity of cisplatin by ~10-fold for cisplatin and ~5000-fold for bleomycin. The electroporation of HMEC-1 cells with bleomycin for a 5 min exposure was ~250-fold better than a continuous exposure to the drug alone. The results of this study indicate that the anti-tumour action of electrochemotherapy is likely to be due, in part, to the highly sensitive response of vascular endothelial cells. Further studies are necessary to identify the determinants of endothelial response and its relationship to the anti-vascular action of electrochemotherapy in vivo. © 2001 Cancer Research Campaign   htt://www.bjcancer.com

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Electrochemotherapy is a combined modality treatment using chemotherapy and electroporation (Mir and Orlowski, 2000). Electroporation is a physical method, performed by application of high voltage direct current electric pulses to cells in vitro or tissues in vivo, to increase cell uptake of molecules such as DNA, antibodies, enzymes, dyes and drugs by permeabilization of the plasma membrane (Mir and Orlowski, 2000; Sersa, 2000). In electrochemotherapy, the optimal antitumour effectiveness is achieved when treatment is given at the time of the highest extracellular concentration of hydrophilic chemotherapy drugs, thereby increasing transport through plasma membrane towards the intracellular targets for cytotoxicity (Mir and Orlowski, 2000). The chemotherapeutic drugs, bleomycin and cisplatin, have proven to be effective in electrochemotherapy of experimental tumour cells, both in vitro and in vivo (Mir and Orlowski, 2000; Sersa, 2000) and also in electrochemotherapy of accessible tumour nodules of various malignancies in cancer patients (Heller et al, 2000). Neither drugs alone nor high voltage electric pulses as single treatment affected tumour growth (Heller et al, 2000; Mir and Orlowski, 2000; Sersa, 2000).

Antitumour effectiveness of electrochemotherapy is primarily due to the increased uptake of the chemotherapeutic drugs into the tumour cells, caused by electroporation. The increased accumulation has been confirmed for platinum (component of cisplatin) and radioactively labelled bleomycin into tumour cells in vivo after treatment with electrochemotherapy (Belehradek et al, 1994; Cemazar et al, 1999). However, several other mechanisms have been demonstrated to be involved in tumour response to electrochemotherapy, e.g. involvement of immune system and changes in tumour blood flow (Mir et al, 1995; Sersa et al, 1997a,b). The vascular targeting action of electrochemotherapy was only speculated. The immune status of experimental mice was shown to be positively correlated with the incidence of complete responses of tumours treated with electrochemotherapy (Sersa et al, 1997a) and potentiation of tumour response to electrochemotherapy was achieved by adjuvant immunotherapy, either with IL-2 or TNF-α (Mir et al, 1995; Sersa et al, 1997b). We have previously shown that the application of electric pulses alone result in substantial, but transient, reduction of tumour perfusion (Sersa et al, 1999a) and furthermore that electrochemotherapy with bleomycin causes rapid cessation of tumour perfusion (Sersa et al, 1999b). In addition, electrochemotherapy prolonged entrapment of cisplatin for up to 8 hours within the tumour (Cemazar et al, 1999). We hypothesize that the anti-tumour action of electrochemotherapy may be partly due to its antivascular action. It is our hypothesis that damage to the endothelial cells, which are electroporated during application of electric pulses, leads to the reduction of tumour perfusion, which contributes to the antitumour effectiveness of electrochemotherapy.

The aim of the present study was to investigate the role of endothelial cells in the antivascular action of electrochemotherapy. The sensitivity of human microvascular endothelial cells, HMEC-1, to electroporation with cisplatin or bleomycin was determined

in vitro and compared to sensitivity of the cells to the treatment with the drugs alone.

MATERIALS AND METHODS

Chemicals

Cisplatin (Platamine, Pharmacia & Upjohn, Milan, Italy) was dissolved in sterile H_2O to obtain a concentration of 6.7 mM. The final concentrations were prepared in Dulbecco's modified Eagles medium (D-MEM; Gibco Life Technologies, Paisley, UK). Bleomycin (Bleo-Kyowa, Kyowa Hakko, Slough, UK) was dissolved in D-MEM at a concentration of 10 mM. All further dilutions were also prepared in D-MEM. Propidium iodide (Sigma-Aldrich Company Ltd, Poole, UK) was prepared in phosphate-buffered saline in a concentration of 0.1 mM.

Cells

Human dermal microvascular endothelial cells (HMEC-1) were generously provided by Dr FJ Candal (Center for Disease Control, Atlanta, USA). This cell line was immortalized by transfection with a plasmid containing simian virus 40A gene product, large T antigen (Ades et al, 1992). HMEC-1 cells were grown as monolayer in D-MEM supplemented with 10% fetal calf serum (FCS, Sigma) in a humidified incubator at atmospheric oxygen supplemented with 5% CO_2 at 37°C. They were routinely subcultured twice per week. When grown in monolayer the cells exhibited typical cobblestone morphology.

Determination of electro-permeabilization and electro-sensitivity

Electro-permeabilization of plasma membrane was measured by cellular uptake of propidium iodide. Electro-sensitivity (survival of cells treated with electric pulses) was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The cells were prepared from exponential growth phase, trypsinized and washed twice, first in the D-MEM supplemented with 10% FCS for inactivation of trypsin (Sigma), and then in the serum-free D-MEM. Cell suspensions (2.2×10^7 cells ml^{-1} in 90 µl) were prepared containing 10 µl propidium iodide (100 µM) for permeability assay or with D-MEM for MTT cytotoxicity assay. The cells were placed between the two parallel stainless-steel electrodes (length 6 mm, width 6 mm, gap 2 mm) connected to the electroporator (Jouan GHT 1287, France) and subjected to 8 square wave electric pulses (pulse width 100 µs, repetition frequency 1 Hz) of different electric field intensities, ranging from 400 to 1800 V cm^{-1} (Figure 1). After exposure to electric pulses, the cells were incubated for 5 min at room temperature (24°C). This incubation time was shown to be the optimal, since it allows resealing of plasma membrane and it does not affect cell viability due to the evaporation of medium and lack of nutrients (Rols and Teissie, 1998). To measure the propidium iodide uptake, 25 µl of cell suspension was resuspended in 1 ml of 0.01 M phosphate buffered saline (pH 7.4) and analysed immediately by FACSort (Becton Dickinson, Mountain View, CA, USA). The percentage of stained cells was determined in comparison to the control cells that were not subjected to electric pulses. The electro-sensitivity of cells was determined by MTT assay performed 3 days after plating in 96-well microtitre plates. Briefly, at the end of incubation time
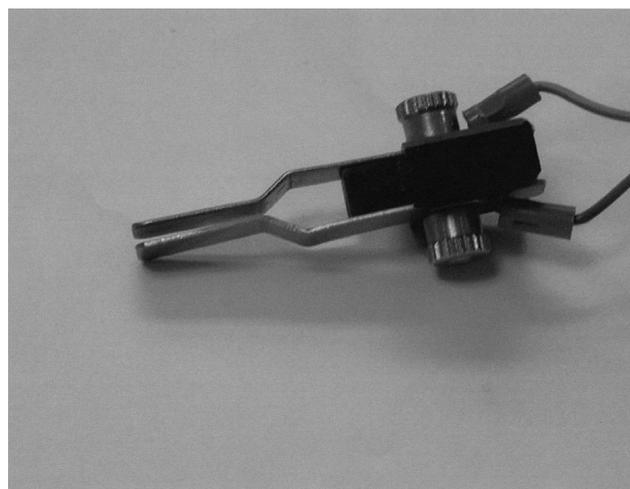

Figure 1 Photograph of the electrodes used in the experiments. The gap between the electrodes was 2 mm and 50 µl of cell suspension was put between the electrodes using an automatic pipette

MTT solution (25 µl of 5 mg ml^{-1} solution) was added to each well and the microtitre plates were further incubated for 3 h at 37°C. The formed formazan crystals were dissolved in 100 µl of dimethyl sulphoxide (Sigma). The microtitre plates were shaken for 99 s to ensure adequate solubilization and the absorbance of the resulting solution was measured at 570 nm using an Anthos microplate reader (Anthos Labtec, Salzburg, Austria). The survival of cells treated with electric pulses was presented as a percentage of the absorbency obtained from control untreated cells.

Cytotoxicity assay for continuous exposure of cells to drugs and combination of electroporation and drugs

To determine the sensitivity of HMEC-1 cells to continuous (3 days) exposure to cisplatin or bleomycin, the cells were plated in 96-well microtitre plates and incubated with cisplatin (1.67 to 16.7 µM) or bleomycin (0.1 nM to 10 µM). The sensitivity of the cells to combined treatments of cisplatin or bleomycin with electric pulses (electroporation) was determined as described above for electro-sensitivity, except that the cells were mixed with cisplatin or bleomycin solution instead of the serum-free D-MEM at the time of electroporation. One half of the cell suspension was exposed to electric pulses (1400 V cm^{-1}) and the other half served as a control for cisplatin or bleomycin treatment alone. The cisplatin concentrations used were ranging from 16.7 to 670 µM and the bleomycin concentrations from 0.1 nM to 100 µM. The cells were exposed to the drugs for 5 min, since this incubation time was shown to be the optimal, since it allows resealing of plasma membrane and it does not affect cell viability due to the evaporation of medium and lack of nutrients (Rols and Teissie, 1998). After that, cells suspension was diluted with D-MEM supplemented with 10% FCS and plated in 96-well microtitre plates. The survival of cells treated with electroporation after exposure to drugs was normalized to electric pulses treatment alone. The IC_{50} value (the concentration of cisplatin or bleomycin that causes 50% reduction in cell survival) was determined for each treatment group.

Statistical analysis

Data were tested for normality using Kolmogorov-Smirnov test. Data were normally distributed and are therefore represented as an arithmetic mean ± standard error of the mean. Statistical analysis was done using SigmaStat statistical software (SPSS Inc).

RESULTS

Electro-permeabilization and electro-sensitivity

In order to determine the optimal electric field intensity, without significant cytotoxicity, both electro-permeabilization (propidium iodide) and electro-sensitivity (MTT assay) of HMEC-1 cells were measured over a range of electric field intensities, which is the critical parameter for obtaining electro-permeabilization (Figure 2). Electro-permeabilization of HMEC-1 cells, at an intensity of 1000 V cm^{-1}, was found to induce 30% of cells to be permeabilized. At the highest intensity of 1800 V cm^{-1} only 85% of cells were permeabilized. This indicates a relatively high cellular resistance to electroporation although the survival of cells was reduced to 57% at this intensity. Therefore, 1400 V cm^{-1} was selected for the subsequent electrochemotherapy experiments. At this electric field intensity, only 60% of cells were permeabilized, but the survival of cells was still approximately 80%.

Cell survival after continuous exposure of cells to drugs and combination of electroporation and drugs

To determine the sensitivity of HMEC-1 cells to cisplatin alone, cells were continuously exposed to the drug and cell survival determined after 3 days. Continuous exposure of HMEC-1 cells to cisplatin resulted in significantly reduced cell survival at doses higher than 0.67 μM. The IC$_{50}$ value for cells to cisplatin was 1.8 μM. In experiments using the combination of electroporation and drugs, the cells were exposed to cisplatin for only 5 min. The IC$_{50}$ value for cells treated with cisplatin alone was 180 μM (Figure 3). Exposure of cells to electric pulses increased cisplatin cytotoxicity 10-fold (IC$_{50}$ value reduced to 18 μM) compared to the IC$_{50}$ value of cells exposed to the cisplatin for 5 min. In comparison to cell survival after continuous exposure to cisplatin, the IC$_{50}$ value for

Figure 3 Cell survival after 5 min. (●) exposure of HMEC-1 cells to cisplatin. In electrochemotherapy protocol (1400 V cm^{-1}), cells were exposed to the drug for 5 minutes (O). Data are mean ±1 standard error of the mean pooled from 3 independent experiments

Figure 4 Cell survival after 5 min. (●) exposure of HMEC-1 cells to bleomycin. In electrochemotherapy protocol (1400 V cm^{-1}), cells were

the combination of electroporation and drugs (5 min) treated cells was ~5-times higher.

The survival of HMEC-1 cells after continuous (3 days) exposure to bleomycin was reduced at the doses higher than 10 nM. The IC$_{50}$ value of HMEC-1 cells continuously exposed to bleomycin was 1 μM. The IC$_{50}$ value cells treated with bleomycin for 5 min was 20 μM (Figure 4). The potentiation of bleomycin cytotoxicity, when cells were exposed to electric pulses was very high, ~5000-fold, when compared to the IC$_{50}$ value of cells exposed to the bleomycin for 5 min. The IC$_{50}$ value of cells treated with combination of electroporation and bleomycin was 4 nM, which was ~700-fold lower than compared to cells that were continuously exposed to bleomycin.

DISCUSSION

The results of this study demonstrate that electroporation with cisplatin or bleomycin significantly enhances the cytotoxicity to
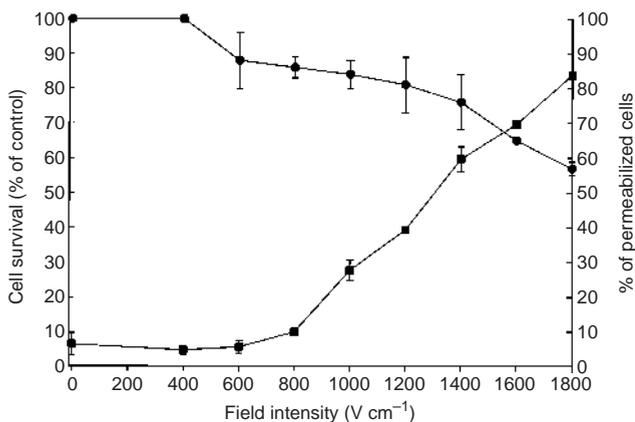

Figure 2 Incidence of electro-permeabilization (■) and electro-sensitivity (●) in HMEC-1 cells by increasing electroporation field strength. Electro-permeabilization was measured by propidium iodide uptake, electro-sensitivity by MTT assay. Data are mean ±1 standard error of the mean pooled from 3 independent experiments
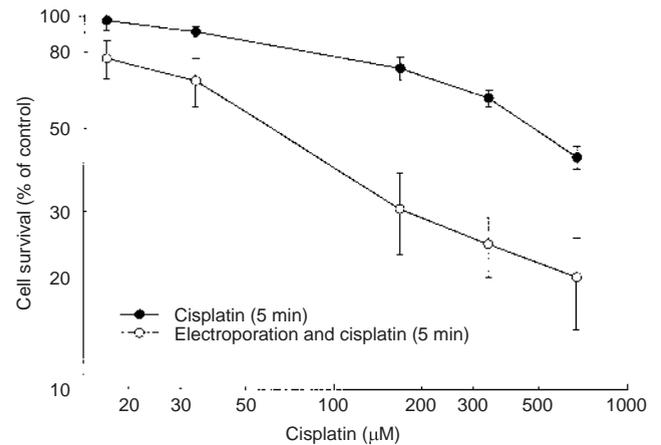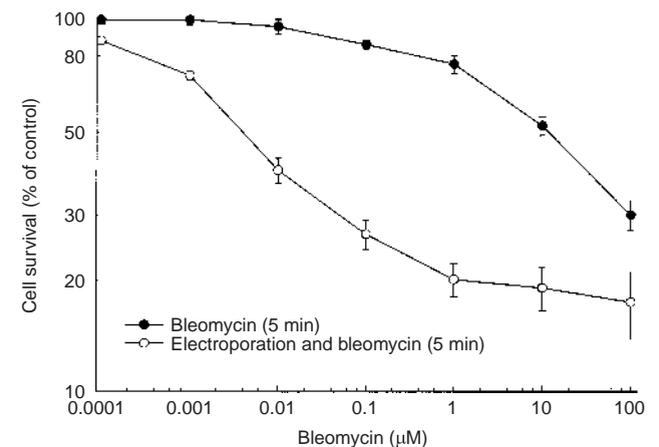

exposed to the drug for 5 minutes (O). Data are mean ±1 standard error of the mean pooled from 3 independent experiments

human endothelial HMEC-1 cells, even after very short drug exposure of cells. It is therefore probable that in vivo electrochemotherapy, especially with bleomycin, may directly damage the vascular endothelium and account for its antivascular actions, such as reduced tumour blood flow and infiltration by host blood cells (Sersa et al, 1999a, b). The antivascular action of electrochemotherapy would be in addition to other, non-vascular, mechanisms previously reported (Sersa et al, 1997a, b; Cemazar et al, 1999).

Continuous exposure of human endothelial cells to bleomycin or cisplatin reduced cell survival substantially in a dose-dependent manner although this effect was more pronounced with bleomycin than with cisplatin. In comparison to the IC_{50} values for several human and murine tumour cell lines, treated with either of these drugs (Tables 1 and 2), the values for HMEC-1 cells appear relatively resistant, especially to cisplatin (Lazo et al, 1989; Hait et al, 1994; Kambe et al, 1996; Dirix et al, 1997; Cemazar et al, 1998a,b). This is consistent with a report of relatively little antivascular action of cisplatin in vivo (Clements et al, 1999). In contrast, the present result for bleomycin indicates that HMEC-1 cells show an equal, or enhanced, sensitivity to that reported for tumour cells with the maximum value of ~ 200-fold more sensitive than tumour cell lines (Table 2) (Hait et al, 1994; Dirix et al, 1997; Cemazar et al, 1998a). At present, only Dirix et al have reported on the sensitivity of two different endothelial cell lines to bleomycin (Dirix et al, 1997). In that study, the response of human umbilical vein endothelial cells (HUVEC) were compared to HMEC and demonstrated a dose-response in survival with bleomycin dose. However, the effect was marginal and the IC_{50} for both HUVEC and HMEC cells were in the same range shown by the most resistant tumour cell lines (Table 2). These observations are in contrast to the present results and probably due to the difference in endothelial cell lines and also to the reduced proliferation status (semi-confluent and confluent) of the cultures used to assess bleomycin cytotoxicity (Dirix et al, 1997) in comparison to the exponential growth phase used in the present study.

In the present study, electric pulses were used as a drug delivery system for bleomycin and cisplatin, which both are reported to have restricted transport through plasma membrane (Belehradek et al, 1994; Cemazar et al, 1999). Increased drug uptake and potentiation of cytotoxicity has been demonstrated in vitro for several tumour cell lines treated with cisplatin, bleomycin, peplomycin and others, following exposure to electric pulses (Orlowski et al, 1988; Sersa et al, 1995; Kambe et al, 1996; Cemazar et al, 1998a;

Kuriyama et al, 1999). When used in combination with application of electric pulses the drug dosage can be greatly reduced for equivalent or higher cytotoxic effect, compared to drug treatment alone (Orlowski et al, 1988; Sersa et al, 1995; Kambe et al, 1996; Cemazar et al, 1998a; Kuriyama et al, 1999). When electrochemotherapy is performed in vivo, the tumour perfusion and intravascular concentration of the drug are additional factors to be considered in achieving optimal tumour cytotoxicity. However, electroporation of solid tumours will also involve treatment of non-tumour cells within the treatment field, including the endothelial cells lining the tumour vasculature. Our in vitro results demonstrate that endothelial cells are electroporated at the same field intensity previously shown to be effective for tumour cytotoxicity in vivo (Sersa et al, 1995; Miklavcic et al, 1998). However, compared to the tumour cells in vitro they appeared to be slightly more resistant to electric pulses (Cemazar et al, 1998c). The reasons for this are currently unclear. To our knowledge, the present study is the first report demonstrating the high sensitivity of human endothelial cells to electroporation with bleomycin and to lesser extent to electroporation with cisplatin compared to the tumour cell lines used in the electrochemotherapy protocol (Tables 1, 2). Although the direct comparison of cell sensitivity to the combination of electroporation and bleomycin or cisplatin would require that the experiments would be done in the same study, according to those studies there is some evidence for endothelial cells being more sensitive to bleomycin than tumour cells. When electroporation was combined with bleomycin, cell cytotoxicity was assessed using MTT assay, while clonogenic assay was used in most of the studies testing combination of electroporation and cisplatin (Tables 1, 2) (Sersa et al, 1995; Kambe et al, 1996; Cemazar et al, 1998a).

The present in vitro data for endothelial cell cytotoxicity are consistent with our previous demonstration that tumour blood flow is completely shut down 12 hours after electrochemotherapy with bleomycin (Sersa et al, 1999b). The severity of the overall antivascular action of electrochemotherapy is likely to be due to a combination of actions mediated at different stages of the cytotoxic mechanism. For example, the increased uptake of drugs, following electroporation of plasma membrane, has been demonstrated in tumour cells, by increased uptake of radioactively labelled bleomycin and by increased DNA platination (Belehradek et al, 1994; Cemazar et al, 1999). It is unlikely to be cell-specific in vivo and may account, in part, for the antivascular action of electrochemotherapy. Secondly, the infiltration of host immune cells

Table 1 IC_{50} values of tumour and endothelial cell lines treated with cisplatin (continuous exposure) or combination of electroporation and cisplatin

Cell line type	Cell line (assay[a])	IC_{50} (μM) cisplatin	IC_{50} (μM) combination	Reference
Tumour	EAT (MTT)	0.4	ND[b]	Cemazar (unpub.)
	SA-1 (clonogenic)	0.9	20.0	Cemazar (unpub.)
	TBL.C12 (clonogenic)	0.18	7.0	Cemazar (unpub.)
	TBL.C12 Pt (clonogenic)	1.5	7.0	Cemazar (unpub.)
	B16 (clonogenic)	1.6	11.0	Sersa et al, 1995
	A253 (Coulter counter)	0.13	ND	Lazo et al, 1989
	IGROV 1 (clonogenic)	0.13	20.0	Cemazar et al, 1998b
	IGROV 1/DDP (clonogenic)	2.0	110.0	Cemazar et al, 1998b
	LS174T (MTT)	0.9	40.0	Kambe et al, 1996
Endothelial	HMEC-1 (MTT)	12.0	40.0	Present study

[a]Assay used to assess cell cytotoxicity. [b] Not determined.

Table 2 IC$_{50}$ values of tumour and endothelial cell lines treated with bleomycin (continuous exposure) or combination of electroporation and bleomycin

Cell line type	Cell line (assay[a])	IC$_{50}$ (μM) bleomycin	IC$_{50}$ (μM) combination	Reference
Tumour	SA-1 (MTT)	20.0	0.025	Cemazar et al, 1998a
	EAT (MTT)	200.0	0.7	Cemazar et al, 1998a
	L1210 (clonogenic)	0.9	ND[b]	Hait et al, 1994
	MCF (clonogenic)	17.0	ND	Hait et al, 1994
	9L (clonogenic)	4.2	ND	Hait et al, 1994
	C6 (clonogenic)	29.0	ND	Hait et al, 1994
	DC-3F (clonogenic)	ND	0.0015	Orlowski et al, 1988
	Colon 26 (MTT)	ND	0.01	Kuriyama et al, 1999
	MC38 (MTT)	ND	0.06	Kuriyama et al, 1999
	Colo320 (MTT)	>70.0	ND	Kambe et al, 1996
Endothelial	HUVEC (Alamar Blue)	>86.0	ND	Dirix et al, 1997
	HMEC (Alamar Blue)	76.0	ND	Dirix et al, 1997
	HMEC-1 (MTT)	1.0	0.004	Present study

[a]Assay used to assess cell cytotoxicity. [b] Not determined.

into the tumour has been shown to be responsible for a degree of tumour cytotoxicity, as evidenced by the change in tumour response to electrochemotherapy applied in immunodeficient animals (Sersa et al, 1997a). Overall, the changes in endothelial function and survival are not unexpected, in relation to tumour cell response, and are consistent with the observed changes in tumour blood flow, as measured using [86]RbCl extraction and Patent Blue technique (Sersa et al, 1999a,b).

The finding that electrochemotherapy has an antivascular action is important since it could identify other approaches to increase the effectiveness of electrochemotherapy treatment of tumours. Electrochemotherapy is being investigated in several trials on cancer patients. Clinical studies on accessible tumour nodules of different tumour types, such as malignant melanoma, basal cell carcinoma, squamous cell carcinoma, adenocarcinoma and others have demonstrated that electrochemotherapy with both, bleomycin or cisplatin is very effective, resulting in high incidence of complete responses (Heller et al, 2000). At present, a major limitation to this treatment is its applicable to small (< 10 mm diameter) cutaneous and subcutaneous nodules that can be placed between the electrodes. This limitation can be overcome by either repetitive application so that the whole tumour area is covered, or by repeating the therapy on any residual tumour mass or by the use of needle electrodes that enable better distribution of the electric field within each tumour (Gilbert et al, 1997; Heller et al, 2000).

It is now clear that improvements in outcome of electrochemotherapy could potentially be achieved by exploiting its antivascular action. Electrochemotherapy could be applied to blood vessels that supply the tumours, if accessible, and achieve cessation of blood supply similar to vascular embolization, a technique already in practice (Acunas and Rozanes, 1999). Intra-arterial injection of either bleomycin or cisplatin, into the vessel supplying the tumour, immediately before electroporation of the tumours would spare the systemic cytotoxicity of the drug because the drug dose used for electrochemotherapy could be reduced relative to the intravenous or intratumour dose necessary for the same tumour effect. These two approaches would exploit the advantages associated with anti-vascular strategies; namely, that the incidence of damaged endothelial cells would result in cessation of flow to an entire vessel segment, consequently enhancing cytotoxicity of tumour cells (Denekamp, 1990).

In conclusion, our demonstration that electroporation with cisplatin or bleomycin is highly cytotoxic for a human endothelial HMEC-1 cell provides evidence for a potential mechanism which explains the observed antivascular actions of electrochemotherapy. Future studies to identify the determinants of endothelial response, and their relationship to antivascular action, will result in increased cytotoxicity by electrochemotherapy.

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