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Review paper

Antitumor drug delivery by tissue electroporation

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Tissue electroporation has been explored to enhance the local delivery of chemotherapueutic agents to solid tumors. The technique, known as electrochemotherapy (ECT), uses high-voltage pulses to deliver drugs across cancerous tissues. ECT has been demonstrated to be an effective treatment for cutaneous malignancies. Recent studies also indicate that the applications of ECT can be extended from the treatment of cutaneous cancers to the treatment of tumors of vital organs such as brain, liver, lungs and others. This review also discusses electrogene antitumor therapy.

Key words: Adjuvant immunotherapy, anticancer drug, drug resistance, electrochemotherapy, electrogene therapy, electropermeabilization.

Introduction

The plasma membrane of tumor cells imposes a great barrier to the permeation of antitumor chemotherapeutic agents. Hydrophilic cytotoxic drugs such as bleomycin (BLM), netropsin, actinomycin D and cisplatin [*cis*-diamminedichloroplatinum)(II); CDDP] diffuse poorly through the plasma membrane into tumor cells, and therefore their antitumor effectiveness is low. However, the drug delivery into tumor cells and cancerous tissues can be enhanced by manipulation of the plasma membrane using a high-intensity pulsed electric field. The technique is known as electroporation (or electropermeabilization)[1–7] and has been extensively used in conjunction with conventional chemotherapy to potentiate the antitumor effects of a variety of chemotherapeutic agents.[8] The combination therapy is termed electrical impulse chemotherapy (EIC) or electrochemotherapy (ECT). This novel approach was introduced in cancer chemotherapy by Okino and Mohri[9] and Mir *et al.*[10] It offers an effective antitumor approach for the treatment of many human malignancies by lowering the doses, thus minimizing side effects and systemic toxicity. In addition, it could be used in conjunction with the cytotoxic drug to circumvent drug resistance in drug-resistant tumor cells.[11]

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Procedure

The electropermeabilization of plasma membranes during ECT is accomplished by exposure of cancerous tissues to short, intense electric pulses (EP) ranging from micro- to milliseconds. The pulses, which are applied directly to the tumor, facilitate delivery of drug through the plasma membrane. The procedure consists of systemic administration of a low dose of an antitumor drug such as BLM followed by local delivery of EP on tumor nodules by means of two electrodes (caliper electrodes) located at each side of the cutaneous nodule.[12] Recently, this method was used in a clinical trial to determine the effectiveness of ECT against certain primary and metastatic cutaneous malignancies.[13]

Localized drug delivery

Drug delivery into solid tumors is often limited by the physiological barrier which is composed of three components: (i) higher interstitial fluid pressure within tumor tissue, (ii) poor blood flow compared to normal host tissue and (iii) large diffusional resistance within tumors.[14,15] However, electroporation can be expected to significantly reduce this physiological resistance by decreasing the pressure within tumors, increasing tissue permeability within tumors and increasing blood perfusion within tumors.[16] The

proposed mechanism involved in this process is outlined as follows.

The electropermeabilization of plasma membranes during ECT results in a localized, large electric field developing across the lipid-based barriers within the cancerous tissue and is the basic mechanism of enhanced therapy during ECT. In addition, it can result in the creation of new aqueous pathways across the barrier, just where they are needed in order to achieve localized drug delivery.[3] Not surprisingly, some of these pores are large. The creation of these aqueous pores in lipid bilayers or plasma membranes leads to a transient and reversible increase in plasma membrane permeability without impairing cell viability.[4,17-19] In turn, the tissue pressure may be expected to drop as the outflow occurs through these large pores.[16] Further, a drop in tissue pressure would decrease the diffusional resistance and increase blood perfusion within tumors.[16] This results in increased cell membrane permeability which enables hydrophilic cytotoxic drugs to diffuse into the tumor cells and reach their intracellular targets. Overall, there is an increase in the intracellular accumulation (concentration) of chemotherapeutic agents in tumor cells and resultant cytotoxicity. It is estimated that the process can substantially enhance cytotoxicity of BLM up to 650 000 times at the level required to kill 90% of the treated cells.[17]

The enhanced delivery of cytotoxic drugs is restricted to the area that has been electrically treated.[20] It is also worth mentioning here that relevant barriers during tissue electroporation are not only the single bilayer membranes of cells, but one or more tissue monolayers in which cells are connected by tight junctions (essentially two bilayers in series per monolayer), and the stratum corneum of the skin, which can be regarded very approximately as about 100 bilayer membranes in series.[3] Further mechanistic studies with ECT should provide a better understanding of combined electroporation and anticancer drugs for improved chemotherapy of intact cancerous tissue *in vivo*.

Factors affecting ECT

Studies indicate that ECT is dependent upon the dose of the antitumor drug administered, as well as upon the amplitude of EP. Also important are the sequencing and the interval of drug administration, relative to application of EP.[21] Domenge *et al.*[22] determined that to obtain optimal antitumor effect in patients with head and neck squamous cell carcinoma (HNSCC) and breast adenocarcinoma (BA), EP have to be delivered between 8 and 28 min after the i.v. bolus administration of BLM. Enhancement in cytotoxicity of BLM has been found to be significantly greater when the electric treatment was given after exposure to the drug than when applied pre-exposure.[23] In a recent study done by Serša *et al.*,[21] a good antitumor effect without side effects was obtained with eight EP applied 3 min after i.v. injection of 4 mg/kg cisplatin in mice. EP were applied to the tumors by percutaneously placed electrodes and had a combination of the following electrical characteristics: EP amplitude, 1040 V; repetition frequency, 1 Hz; pulse width, 100 μs; electrode distance, 8 mm; 1300 V/cm. The data indicated that several-fold potentiation of antitumor effectiveness of cisplatin with EP can be obtained, inducing partial or complete responses in tumor growth. In addition, with a higher cisplatin dose (8 mg/kg), some long-term complete responses were obtained (14%) on melanoma B16 tumors.

Recently, Okino *et al.*[24] examined the relationship between the tumoricidal effect of BLM and the electrical variables of *in vivo* EIC in rats bearing s.c. inoculated hepatocellular carcinomas. The EIC treatment consisted of a single high-voltage electrical pulse of varying voltage and duration, given 30 min after an intramuscular dose of BLM. As a result, it was found that the tumoricidal effect (E) increased in proportion to the square of the applied voltage (V), when the voltage was increased from 0 to 5 kV. Also, when the pulse duration (D) was increased from 2.5 to 5.8 ms, the increase in E was in direct proportion to D. A combination of these two relationships resulted into the formula $E=kV^2D$, which indicates that the tumoricidal efficacy was proportional to the applied electrical energy (k stands for a proportionality constant).

In most of the ECT studies published thus far, trains of EP were delivered via percutaneously placed parallel plate electrodes, so that the tumor was situated in between the electrodes. However, recently it has been demonstrated that changing the electrode orientation results in improved antitumor efficacy of the ECT in mice.[25] In this particular study, the train of EP was divided into two trains, the second one oriented perpendicularly to the first one. This changing of the electrode orientation resulted in prolonged tumor growth delay, and a higher percentage of short- and long-term complete responses of the tumors. Furthermore, a possible explanation for the improved efficacy has also been suggested, based on the knowledge of electric field distribution in the tissue and induced transmembrane potential.[25]

Effects of ECT treatment varies as a function of electrode design. In a standard parallel plate electrode design, the direct current pulses are administered using two parallel plate electrodes placed on either side of the tumor. This simple electrode design has been shown to produce high response rates (70–85%) in animal studies and clinical trials. However, parallel plate electrodes are not suitable for all situations. Recently, Gilbert *et al.*[26] conducted a study using five novel electrode designs and compared their effectiveness to a parallel plate design for treating melanoma in mice. Their results for the 2×2 needle array design showed 50% increase in doubling time and complete response rate compared to the standard parallel plate electrode. It is now possible to monitor the effects of ECT as a function of electrode geometry through the use of an electric current density imaging method.[27]

Mode of treatment is another factor shown to affect the response rates of tumors. Currently, ECT is administered as a single treatment. With the single treatment, the complete response rates are high; however, partial responses are obtained in a fraction of the treated tumors. Recently, Zaroszeski *et al.*[20] conducted a study in an attempt to see whether or not multiple treatments result in an improved therapy for these partially responding tumors. Their study utilized s.c. induced murine B16 melanoma tumors in C57B1/6 mice; results showed large tumor volume reductions in multiple treatment groups. In addition, a 2-fold increase in tumor doubling time and greater percentages of complete responses were found as a result of multiple treatments. Such studies could be utilized to augment existing clinical trials, especially retreating tumors that have partially responded to a single ECT treatment.[20]

From a recently conducted study,[28] it appears that route of drug administration would be the most decisive factor to initiate clinical trials in future. The current procedure of ECT includes exposure of tumor cells to EP followed by i.v. injection of the chemotherapeutic agent. However, this procedure is associated with few unresolved issues such as the existence of a narrow, though optimal, time window for effective treatment and the administration of a systemic dose for a localized therapy. The narrow time window limits the number of tumor sites that can be treated. Heller *et al.*[28] addressed these issues and examined the effectiveness of BLM administered by intratumoral injection. Their results indicated drastic reductions in tumor volume for treated groups. In addition, an increased survival was observed in ECT-treated groups over control groups. The results demonstrate that intratumoral injection of BLM in combination with EP is effective, compared to ECT utilizing its i.v. injection.

Overview of *in vitro* and *in vivo* studies, and clinical trials

ECT has been shown to have a potent antitumor effect in a variety of *in vitro* studies with specific cell lines, *in vivo* studies with various animal tumor models, as well as in clinical trials on patients with HNSCC and BCCs.[29] In all cases, it has been shown that uptake of various drugs by the tumor cells can be increased markedly by EIC/ECT.

In cell culture, the *in vitro* cytotoxicity of anticancer drugs such as BLM, netropsin, actinomycin D and cisplatin can be potentiated several-fold by exposing cells to short, intense and non-cytotoxic EP.[17,23,30–32] In the study by Mir *et al.*[10] nude or conventional mice bearing s.c. transplanted tumors were treated with intramuscular doses of BLM followed by local delivery of EP similar to those used *in vitro*. Tumors were reduced and even eradicated after the ECT. Similar results were obtained by Yamaguchi *et al.*[33] when C3H mice bearing s.c. transplanted mouse bladder carcinoma (MBT-2) were treated with an intramuscular injection of BLM followed by local delivery of EP at the tumor site. The tumors markedly reduced and even disappeared for several days after this treatment. Further, neither EP nor BLM administration alone showed a significant inhibitory effect on the tumor growth. A recent study employing nude mice bearing s.c. transplanted tumors indicated that tumor reduction and its complete disappearance after 12 days of ECT treatment was possible even with non-effective i.p. doses of BLM followed by local delivery of EP.[23] Tada *et al.*[34] investigated the effects of ECT on the growth of colon 26 cells inoculated s.c. into the backs of BALB/c mice. Their results demonstrated significant inhibition of growth of colon 26 tumors in the ECT group, without causing any remarkable adverse effects. Dev and Nanda[35] indicated the possibility of using ECT to treat human pancreatic tumors, derived from a poorly differentiated cell line (Panc-3), xenografted s.c. onto the flanks of nude mice.

ECT appears to be similarly efficient in spontaneous tumors as in aforementioned studies of transplanted and inoculated tumors.[36] Belehradek *et al.*[36] studied C3H/Bi mice with spontaneous mammary carcinomas. The treatment protocol included weekly injections of 50 μg BLM followed by EP 30 min later. All the 38 tumors treated exhibited at least a partial regression, 23 complete remissions were observed and three of which were cures. Their study also indicated one difficulty in assessing the cure rate in such a model that frequent parallel or sequential tumors cause early death. The effectiveness of ECT has also been

determined for the treatment of melanoma B16 tumors using BLM[37] and cisplatin[31] in mice. *In vitro* and *in vivo* studies on s.c. SA-1 (sarcoma) and EAT (Ehrlich ascites tumor) with cisplatin in mice have shown that ECT with cisplatin offers an approach to making chemotherapy with cisplatin more effective.[21] Such studies demonstrate that the antitumor effects of BLM and cisplatin in mice can be considerably potentiated by local EP, suggesting that ECT with these drugs holds promise for treatment of various cancers.

The effects of ECT on *in vivo* growing tumors are highly promising.[9,38,39] Recently, Salford *et al.*,[40] using a different protocol, have demonstrated enhanced cancer chemotherapy by electroporation for the treatment of brain tumors in rats. Zaroszeski *et al.*,[41] in a similar study, examined the effects of ECT in a hepatoma model. The induced rat hepatomas were treated with a 0.5 unit intratumor BLM dose followed by rectangular direct current pulses. During the treatment, six pulses were administered using a needle array electrode that rotated the applied electric field around the tumors. A 84.6% objective response rate resulted for tumors that received both BLM and EP. On the other hand, control groups that received partial or no treatment showed less than 10% objective response rates. Such studies encourage that the applications of ECT can be extended for the treatment of brain, liver and other internal tumors.

The effects of BLM-mediated ECT have been recently documented for the treatment of various primary cutaneous malignancies, including malignant melanoma[13] and several basel cell carcinomas (BCCs).[13,42] In the study of Heller *et al.*[13] two of three melanoma patients had objective responses. In those two patients, five of six treated tumors decreased in size and three completely responded. On the other hand, untreated tumors displayed continuous growth. Objective responses were also observed in both BCC patients. One patient had partial responses in both treated tumors. In the other patient four primary BCC responded completely and the remaining three responded partially.

The first clinical trial of ECT documents the applications of ECT for patients with permeation nodules of HNSCC.[43] The ECT procedure consisted of delivering EP to the tumor a few minutes after an i.v. injection of BLM. Treatment was well tolerated by patients with no serious side effects and a clear antitumor efficiency was obtained. Variants of this protocol have been tested to determine the limitations of ECT using small external electrodes while treating large permeation nodules of HNSCC, or salivary or BA.[22] Results indicated that although relatively efficient ECT can be performed for large and thick nodules, the effectiveness on large nodules was lower than small nodules, probably due to external electrode inadequacy which warrants new electrode designs. ECT has also been used as an effective method for the local treatment of metastatic cutaneous malignancies. In a recent clinical study, patients with metastatic BA showed complete responses in treated nodules after ECT.[13] Glass *et al.*[44] conducted a study that first documents the antitumor effect of ECT in patients with metastatic malignant melanoma. In a group of five patients, 23 lesions of metastatic melanoma were treated with intralesional BLM sulfate followed by delivery of EP. Complete responses were observed in 18 tumors (78%) and partial responses were seen in four (17%). Furthermore, no responses were seen in lesions treated with either pulses or BLM alone. Their findings suggested that ECT, although not a cure, may be an effective alternative to palliative surgery or irradiation in these patients.

Adjuvant immunotherapy

Preclinical data indicate that the host's immune response plays an important role in the curative therapy of tumors.[45] In other words, immune responsiveness of the organism is important for obtaining cures of the tumors after ECT. Recently, a study was conducted in an attempt to investigate how ECT affects the immune system of the organism.[46] More specifically, the aim of the study was to determine the effects of ECT with BLM on natural resistance and immune responsiveness. Natural resistance was evaluated by phagocytic and intracellular killing activity (oxidative burst) in monocytes and polymorphonuclear granulocytes from venous blood, and immune responsiveness by blast transformation of spleen mononuclear cells to mitogens. The percentage of monocytes in venous blood able to elicit oxidative burst was significantly increased 7 days after the ECT and returned to normal values after 14 days. In addition, increased blast transformation of spleen mononuclear cells by stimulation with concanavalin A (seven lymphocytes activity) was found 14 days after ECT treatment.

Through the application of genetic engineering, it is now possible to produce human interleukin-2 (hIL-2) locally at the tumor site by histoincompatible cells. This has been shown to produce a strong immune response in various murine models leading to tumor growth inhibition or rejection.[47] It has been demonstrated that ECT followed by injection of a low dose of IL-2[45] or IL-2 secreting cells,[48,49] both allogeneic as well as xenogeneic, gives better results than ECT

alone. There appears to be a systemic effect and a strong indication that an immune response may be elicited by this adjuvant treatment.[29] It is suggested that ECT combined with such cellular immunotherapy might be a useful approach for the metastasizing cancers.[49] Recently, the safety and therapeutic potential of this strategy has also been demonstrated for gene therapy in canine and feline models with spontaneous metastatic and non-metastatic tumors.[47] This study employed dogs with highly metastatic melanoma and cats with low metastatic fibrosarcoma. Results demonstrated that both cats and dogs when treated by tumor surgery, radiotherapy and repeated local injections of xenogeneic Vero cells secreting high levels of hIL-2 relapse less frequently and survive longer than control animals treated by surgery and radiotherapy alone. Increased anti-tumor effectiveness on SA-1 tumors has also been observed after combining tumor necrosis factor (TNF)-α, injected either intratumorally or peritumorally, with ECT using a suboptimal dose of BLM.[50] It was suggested that adjuvant immunotherapy with TNF-α might be immunomodulatory, thereby potentiating the antitumor activity of ECT.

Biomedical applications

Electroporation leads to reversible breakdown of cell membranes caused by a high-voltage discharge.[51] Therefore, it serves as a rapid, simple and efficient transfection method for introducing foreign DNA into mammalian cells.[52] It has been used for transferring DNA into rat and human brain tumor cell lines of glial and neuronal origin.[53] The uptake and expression of SV40 DNA following electroporation of a human carcinoma-derived cell line, HEp-2, has also been studied.[54] It was demonstrated that to obtain maximal (optimum) DNA transfer by electroporation, one must optimize the electronic parameters such as peak voltage and fall time of the voltage discharge waveform of the instrument for electroporation. Recently, a novel method for high efficiency and region-controlled *in vivo* gene transfer has been developed.[55] The method, known as electrogene therapy (EGT), combines *in vivo* electroporation and intra-arterial plasmid DNA injection. The method does not require viral genes or particles, and allows genes to be transferred and expressed in desired organs or tissues. Studies also indicate that gene transfer by electroporation *in vivo* may avoid anatomical constraints, low transfection efficiency and low gene expression that are commonly associated problems with *in vivo* targeted gene transfer using non-viral vectors.[56] It is anticipated that biomedical applications of electroporation will add great value to ECT and may lead to the development of a new type of highly effective gene therapy for cancer.[57]

Reversal of multidrug resistance

The development of tumor drug resistance is the major obstacle to successful systemic chemotherapy. In recent years, several strategies have been developed for reversing drug resistance that could lead to significant improvements in cancer treatment. A partial reversal of tumor drug resistance has been achieved by the use of competitive inhibitors for the function of glycoprotein P-170 or by the inhibition of glutathione (GSH) synthesis; however, this strategy has not been substantially successful for improving the response of human tumors to clinical therapy.[11] Electroporation, being a highly controllable technique, can lead to permeabilization of tumor cells with an effective bypass of efflux pumps and could offer a broadly successful strategy for killing drug-resistant cancer cells. Recently, it has been used, in conjunction with the cytotoxic drug, cisplatin, in an attempt to circumvent drug resistance in cisplatin-resistant mouse tumor cells.[11] Electroporation plus cisplatin treatment increased the intracellular drug concentration and reversed cellular resistance to cisplatin-induced cell killing.[11]

Advantages and futuristic predictions

Electroporation offers a number of advantages over contemporary methods of antitumor treatment. It may be safe for clinical use under appropriate conditions. Mir *et al.*[10] and Yamaguchi *et al.*[33] applied high-intensity electric impulsions to mice without any lethality or important skin damage. Partial and complete responses of the tumors have been observed without any damaging side effects, provided the field strength is kept sufficiently low.[29] The ECT treatment remains safe even when a large number of EP are delivered and a patient can be treated several times, even if each individual treatment requires a high number of EP.[22] In addition, the combination chemotherapy could lower the systemic toxicity. For example, BLM chemotherapy is usually associated with the risk of dose-dependent pulmonary toxicity which ultimately results in pulmonary fibrosis and limits the scale of application. However, EP could possibly obviate the pulmonary toxicity and result in

an improved chemotherapy.[58] Interestingly enough, the treatment requires such a low amount of drug, e.g. BLM, that it is ineffective without EP and does not induce side effects.[36,38,40,59,60] Only minimal local or systemic side effects are noted in response to the therapy. The only noticeable side effect is the occurrence of erythema and slight edema at the site of the treated areas;[60-62] however, these symptoms are transient and disappear in less than 24 h.[60]

The treatment (application of EP, 100 μs, 1300 V/cm) in combination with chemotherapy has been well tolerated by patients,[13,43,60] with no residual effects from the electric pulses.[13] However, vital signs should be closely monitored during application of the EP and minimal side effects, local as well systemic, should be noted. It might be predicted that, in future, electroporation as a method of controlled drug delivery in cancer therapy may bring about a much awaited revolution in assuaging the patients plagued by side effects of cancer chemotherapy. The overall developments in ECT,[63] and its future prospects for both drug delivery and gene therapy have been described.[29,64]

Concluding comments

ECT appears to be a promising technique for treating cancer, relevant to some precise clinical situations, and offers an effective approach for the local treatment of various cutaneous and s.c. nodules. However, the approach is investigational at the present time and has not been fully understood to realize its clinical potential. More trials remain to be done before electroporation devices (electroporators) become handy for use in cancer clinics.

The results obtained in animal models and clinical trials are very encouraging, and the studies are being continued to find a decisive cure of cancer. Some of the current studies are ongoing with intralesional BLM during ECT to see whether additional antitumor effects can be produced in patients by electroporation.

Lastly, ECT might be revolutionary in the treatment of cancer, including modulation of multidrug resistance. Increased use of electroporation for solid tumor chemotherapy implies that a much better mechanistic understanding of electroporation will be needed to obtain FDA approval.

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