Abstract: Electrochemotherapy (ECT) is currently receiving much attention as a method that can enhance the delivery of antitumour drugs into tumour cells by electroporation. In this study, we examined the effect of cisplatin on metastatic lesions of hamster oral fibrosarcoma by administering it in association with low-voltage electrical pulses. Oral fibrosarcoma was transplanted submucosally into the cheek pouches of 80 hamsters. After transplantation, when the diameter of the metastatic lesion in the left submandibular lymph node had reached 100 mm, the hamsters were divided into four groups: D+E+ group (cisplatin treatment followed by electroporation); D+E- group (cisplatin treatment); D-E+ group (electroporation without cisplatin treatment); and D-E- group (no treatment). The antitumour effect of cisplatin was marked in the D+E+ group and the metastatic lesion disappeared in some of the animals. These results showed that when ECT consisting of cisplatin plus low-voltage electroporation was applied to metastatic tumour lesions, the antitumour effect of the drug on the lesions was enhanced. Therefore, ECT would seem to be a highly-safe new method that may be of use in the treatment of metastatic tumours.

Key words: Electrochemotherapy, Cisplatin, Low-voltage, Metastatic tumour, Fibrosarcoma

Introduction

Electroporation, an experimental technique used to move molecules through cellular membranes, has recently received much attention. An electroporation method\textsuperscript{1,2} that involves the use of high-voltage electrical pulses forming reversible pores in cell mucosa has been developed as a method for enhancing the intracellular uptake of substances. In addition, use of this technique has been reported to greatly enhance the uptake of drugs within cells\textsuperscript{3,4}. It has also been confirmed that when high-voltage pulses are administered to cells within body tissues, reversible pores are formed in the cell membranes without damaging the cells\textsuperscript{5}. When local electrical pulses were administered to body tissues using this technique, the permeability of cell membranes by antitumour drugs was increased, whereas the intracellular uptake of such drugs is normally poor. This method, known as electrochemotherapy (ECT), was developed in order to increase the efficacy of antitumour drugs, and this effect is now drawing considerable attention\textsuperscript{6-9}. To date, there have been a considerable number of experimental studies on ECT\textsuperscript{10-20}, but most of these have involved bleomycin and the use of high-voltage (1.3 to 3.0 kV) electrical pulses. The use of high-voltage ECT in experimental and clinical studies poses the problem of thermal injury as a possible adverse reaction\textsuperscript{10}. Therefore, many researchers have turned their attention to the use of low-voltage ECT, where adverse reactions such as thermal burns rarely occur\textsuperscript{9,21-24}.

In the past decade, ECT with cisplatin has been demonstrated to be effective in local control of tumour growth. Cisplatin has antitumour efficacy against a very wide range of tumours; however, it is also associated with a high level of renal toxicity\textsuperscript{25}. By using ECT with cisplatin, it is possible to decrease the drug’s dosage, and the clinical application of this approach is already being investigated in the field of dermatology. In this area, good antitumour effects against malignant melanoma\textsuperscript{26-28} and cutaneous lesions\textsuperscript{29} of breast cancer in humans have been reported. The successful treatment of sarcoma in rodents\textsuperscript{14,30,31} has also been reported. However, there is yet no report of the clinical application of this technique orally; only a few experimental studies on ECT in the oral region have been reported\textsuperscript{15,32-34}.

The greatest concern with malignant tumours is metastasis,
and it is not an overstatement to say that the treatment of a metastatic lesion determines the subject’s survival. Because electroporation—via the use of a needle electrode to deliver electrical pulses to cells—enhances the intracellular delivery of an antitumour drug, this therapy may be indicated in the treatment of many superficial tumours. Even in the oral field, ECT will likely be indicated for the treatment of metastatic lymph node lesions as well as other oral tumours.

We have, therefore, examined the antitumour efficacy of cisplatin in the treatment of metastatic tumour lesions in association with low-voltage electroporation, where adverse reactions such as thermal burns do not occur. The metastatic submandibular lymph node lesions of oral fibrosarcoma that developed in the mandibular bone of hamster served as the target of our investigation.

Materials and methods

Transplant tumour tissue

In this study, transplantable hamster oral fibrosarcoma (HOFSC-M), derived from a 9,10-dimethyl-1, 2-benzanthracene-induced fibrosarcoma in the mandibular bone of Syrian golden hamsters, was used. This tumour elicits the formation of metastatic lesions in lymph nodes. In addition, this HOFSC-M has been maintained by serial transplantation into the cheek pouch of hamsters for over 97 generations.

Antitumour agents

Cisplatin (Nippon Kayaku, Tokyo) was used. The cisplatin was dissolved in physiological saline solution and 2 mg/kg equivalent to roughly one-tenth of the median lethal dose (LD$_{50}$) was administered intraperitoneally to the experimental animals.

Experimental animals and study procedures

In this study, 100 three-week-old male golden hamsters weighing 90-95 g, purchased from Japan SLC Co. Ltd (Hamamatsu, Japan), were used. HOFSC-M was transplanted submucosally into the left cheek pouch mucosa of all the animals. After confirming the presence of left submandibular lymph node metastatic lesions, the submucosal tumours in the cheek pouch were isolated. When the tumour diameter of the left submandibular lymph node metastatic lesion had reached approximately 100 mm$^3$, the animals were divided into 4 groups (25 from each group): D+E+ group (cisplatin treatment followed by electroporation); D+E- group (cisplatin treatment without electroporation); D-E+ group (electroporation without cisplatin treatment); D-E- group (no treatment). Ten animals in each group were used to measure tumour volume, and the remaining 15 (5 from each 0, 6, 14 days post-treatment) for tissue examination. In the animals of D+E+ and D+E- groups, 2 mg/kg of cisplatin was administered intraperitoneally, and in the animals of D+E+ and D-E+ groups, low-voltage electroporation was applied. In the D+E+ group, electroporation was performed 30 min after cisplatin administration. Electrical pulses were applied to the tumours using a CUY21 electroporator (TR Tech Co. Ltd, Tokyo, Japan). Rectangular 8 square-wave electric pulses (field strength 50V/cm, each pulse 75 msec duration) were delivered to each tumour.
was repeated three times from three directions at 60° intervals in the same plane under anesthesia.

All experimental protocols were in accordance with the regulations for animal care established by the Institutional Committee for Animal Care at Aichi Gakuin University.

**Tumour assessment**

The tumours in 40 of the hamsters (10 from each group) were measured by three people using a vernier caliper every 2 days after the treatments were administered. To measure tumour volume in each animal group, the method of Auerbach et al. was used. In this method, tumour diameter (mm) was measured at 3 sites (a, b, and c) where a, b and c were three mutually orthogonal measurements of the face of the nodule, and tumour volume was determined using the equation below.

Tumour volume (mm$^3$) = $\frac{abc}{6}$.

In addition, the tumour growth rate was determined using the equation below where $S_0$ is tumour volume at the start of the study and $S_n$ is the $n$th day of volume determination.

Tumour growth rate = $\frac{S_n}{S_0}$.

Figure 3. Histological findings of the lymph node metastatic lesion of tumours on 0 day post-treatment in the no-treatment group (D-E-). The tumour cells infiltrated the lymph node tissues. The tumour cells consisted of round shaped nuclei and spindle-form cytoplasm. Those tumour cells were irregular and closely arranged. NL: normal lymph node tissue, ML: metastasized tumour tissue. HE stain $\times$ 100.

Figure 4. Histological appearance on day 6 post-treatment

a. The no-treatment group (D-E-): Tumour cells, which were spindle shaped and with round nucleus, showed several mitotic figures. HE stain $\times$ 200.

b. The low-voltage electroporation-only group (D-E+): Tumour cells showed the feature similar to those of D-E- group. HE stain $\times$ 200.

c. The cisplatin treatment only group (D+E-): Mixture of apoptotic, necrotic tumour cells and viable tumour cells are seen. HE stain $\times$ 200.

During 1 sec of total treatment time by two parallel stainless steel electrodes (length 15mm, diameter 0.8mm) inserted percutaneously close to, and on both sides of, the tumour. Delivery percutaneously close to, and on both sides of, the tumour. Delivery
Biopsies were taken from five animals from each group 0, 6 and 14 days post-treatment and examined histologically using light microscopy.

**Statistical analysis**

Results were given as the mean±SE, and were compared using the Kruskal-Wallis H test followed by the Student-Newman-Keuls test. A P-value < 0.05 was considered to be statistically significant. In addition, survival data were plotted during 150 days post-treatment as a survival curve using the Kaplan-Meier method.

**Results**

In the D+E-, D-E+ and D-E- groups, tumour volume increased continuously throughout the 14-day period of the study. However, in the D+E+ group, the tumour growth rate was significantly inhibited (p<0.01, Fig. 1). In the D+E+ group, the tumour volume gradually decreased up to 8 days after treatment and then started to increase again. In the D-E+ group, tumour volume increased continuously throughout the experimental period; however, in the D-E+ group, the increase in tumour volume was less than that in the D-E- group but more than that in the D+E- group (Fig. 1).

The Kaplan-Meier survival plot showed that hamsters in the D+E+ group survived significantly longer than those in the other three groups (p<0.01, Fig. 2). In the D+E+ group, 60% of the animals were still alive 75 days after treatment; in the other three groups, all animals died within same days.

Histological examination on the 0 day post-treatment of the metastatic lesions in all groups showed that the tumour cells infiltrated into the lymph node tissue (Fig. 3). In the D+E+ group, highly necrotic or many apoptotic cells were seen on both days 6 and 14 post-treatment (Figs. 4d, 5c, d). In contrast, the untreated or partially-treated animals had well-defined tumours. In the D-E+ group, spindle-shaped tumour cells with oval nuclei were seen throughout the experimental period and mitosis was also commonly seen (Figs. 4a, 5a). The animals of D-E+ group showed almost the same findings as in the D-E- group on day 6 (Fig. 4b), and mixture of apoptotic, necrotic and viable tumour cells were seen in the D+E- group on days 6 and 14 (Figs. 4c, 5b).

Figure 5. Histological findings on day 14 post-treatment

a. The no-treatment group (D-E-): Tumour cells show the feature similar to those of D-E- group on day 6 post-treatment. HE stain × 200.

b. The cisplatin treatment only group (D+E-): Mixture of apoptotic, necrotic tumour cells (right) and viable tumour cells (left) are seen. HE stain × 200.

c. The cisplatin treatment with electroporation group (D+E+): Apoptotic change which shows fragmentation of the nuclei (arrow) occurred in almost all the tumour cells. HE stain × 200.

d. The cisplatin treatment with electroporation group (D+E+): All tumour cells show necrotic figures. HE stain × 200.
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No burns were observed on the animals exposed to electrical impulses; however, they did show signs of oedema for 2 days after the electrical treatment.

**Discussion**

The present study showed that when cisplatin was administered to metastatic lesions of submandibular lymph node in association with ECT, the efficacy of the antitumour drug was enhanced. In our study, this procedure eliminated the metastatic lesions in three of the 10 experimental animals in the D+E+ group. In addition, tumour volume was reduced in all animals. To date, there have been reports on ECT with a variety of antitumour drugs\(^{6-11,14,24,25}\). Among these, there were many in vivo and in vitro studies on ECT with bleomycin\(^{6,8,36,37}\). Among studies of ECT with bleomycin in the oral area, Hasegawa et al.\(^3\) performed single electroporation (1.2 kV/cm, 100 μs, 1 Hz, 8 pulses) on squamous cell carcinoma in rat tongue and reported a decrease in tumour volume in all animals. In addition, Omura et al.\(^4\) performed electroporation (1.3 kV/cm, 99 μs, 1 Hz, 8 pulses) on squamous cell carcinoma in hamster tongue and also reported a decrease in tumour volume. Furthermore, Maeda et al.\(^3\) performed ECT with bleomycin for oral fibrosarcoma, eliminating 30% of the tumours and inhibiting their growth in all animals. These results indicated that ECT with bleomycin produced different responses in various types of tumour cells; however, good antitumour effects were enhanced in almost all instances. This appeared to show that ECT had positive antitumour effects in the treatment of a very wide range of tumours.

In reports on ECT with cisplatin, good antitumour effects have been reported for sarcoma in rodents\(^{14,30,31}\), human malignant melanoma\(^{27,28}\), and cutaneous lesions in human breast cancer\(^{29}\). In addition, in animal studies, Fujimoto et al.\(^6\) performed ECT with cisplatin for oral fibrosarcoma and reported that 30% of the tumours were eliminated and that the growth of all tumours in all animals was inhibited. Even in the present hamster study, the treatment of ECT with cisplatin for metastatic oral fibrosarcoma lesions of submandibular lymph node in hamsters inhibited tumour growth and significantly prolonged the survival of all the animals. This result demonstrated that cisplatin is an effective antitumour agent even against metastatic lesions of fibrosarcoma in the animal model. However, most studies on ECT have involved metastatic lesions of malignant melanoma in human skin\(^{28,38,39}\). Apart from that, ECT in the treatment of squamous cell carcinoma of the upper respiratory tract metastatic to the skin has been reported\(^5\). In sum, all of these reports showed that ECT with bleomycin or cisplatin might be useful in the treatment of metastatic lesions.

The minimal voltage required for electroporation has been reported to be > 900 V/cm\(^2\).\(^3,16\). The use of a higher voltage probably enhanced the formation of reversible pores; on the other hand, it might also be associated with an increased risk of damage, such as thermal injury, to tissue cells. In this regard, recent studies using low-voltage ECT have demonstrated good antitumour efficiency without a significant risk of adverse reactions\(^{21-24,31,40}\). In these reports, ECT was performed using a low voltage of 25-150 V/cm. In the present study, low-voltage pulses of 50 V/cm in combination with cisplatin were used and enhanced antitumour efficacy towards metastatic tumour lesions was achieved. This result demonstrated that the use of low-voltage electroporation prevented tissue damage due to high voltage pulses, particularly thermal injury and discomfort in patients being treated in a clinical application of the ECT technique.

Generally, the antitumour effect will vary widely according to the extent to which the antitumour agent enters the cells. Belehradek et al.\(^3\) used bleomycin radiolabeled with cobalt 57 (\(^{57}\)Co) to examine the intracellular uptake of bleomycin, finding that such uptake in the group receiving ECT was approximately four times greater than that in the no-ECT control group. From the results shown for the D+E+ group in our study, it might be concluded that the formation of reversible pores in the cell membrane by electroporation improved the delivery of cisplatin into the cells and hence effectively reduced tumour growth. Our histological findings showed marked necrosis or apoptosis of the tumour cells in the metastatic lesions of the D+E+ group. This result also suggested that the addition of electroporation in forming reversible pores in the cell membrane increased the cellular permeability of cisplatin and hence the intracellular concentration of the drug, leading to the necrosis and apoptosis of tumour cells.

The application of electrical pulses has been reported to injure the vascular endothelial cells of the tissues surrounding tumours, thus decreasing the flow of blood to them\(^41\). Even in the present study, tumour growth was inhibited and survival prolonged to almost the same extent in the electroporation-only group as in the cisplatin treatment only group. This indicated not only the ECT would enhance the effect of an antitumour drug, but that it would also affect the growth of tumours by reducing their blood supply.

From the results of our study, it became evident that ECT consisting of the administration of cisplatin together with electroporation enhanced the effect of cisplatin on metastatic lesions of tumours. With regard to the voltage of ECT, even when low-voltage electroporation was applied, antitumour effects on metastatic fibrosarcomas were enhanced. This suggested that in the clinical application of ECT, low-voltage electroporation could be expected to prevent damage to tissues, such as thermal injury, and to produce both safe and effective antitumour effects. Furthermore, ECT was easy to apply even on an outpatient basis, thus reducing both the cost of treatment and inconvenience to the patient. These should prove useful for treating both superficial metastatic tumours as well as metastatic lesions of tumours in patients who are not candidates for surgery. Hereafter, further studies on ECT for various types of tumours will be needed to elucidate the clinical application of this form of therapy.
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