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SHORT COMMUNICATION

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Bleomycin-mediated electrochemotherapy in mouse NR-S1 carcinoma

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Abstract *Purpose*: To determine whether or not low-voltage electrochemotherapy has cell killing effects. *Materials*: Dorsally transplanted NR-S1 carcinomas in mice were stimulated with electric pulses (40 V/cm) after bleomycin (1 μg/g) had been injected around them. The tumors were fixed with a forceps electrode and electroporation was carried out three times a day for 4 days per week for 2 weeks. *Results*: After 8 weeks of experimentation, the tumor had disappeared in four of the ten mice. The cell killing effects were mainly apoptosis and necrosis. *Conclusion*: Electroporation should be clinically introduced into the cosmetic and functional treatment of the head and neck region. Further investigation is also necessary to determine suitable carcinostatic agents and clarify the electric pulse conditions.

Keywords Electroporation · Electrochemotherapy · Cancer therapy · Apoptosis · Transmission electron microscope (TEM)

Introduction

The ability of tissue electroporation to enhance the local delivery of chemotherapeutic agents to solid tumors has been explored. The technique, known as electrochemotherapy (ECT), uses high-voltage pulses to deliver the drugs across cancerous tissues. However, voltages of 1000 V/cm [5] or more are dangerous for clinical use. Low-voltage ECT (40 V/cm) with bleomycin (BLM) was shown to clear cancer in mice and induced apoptosis. These findings are reported after a review of the relevant literature.

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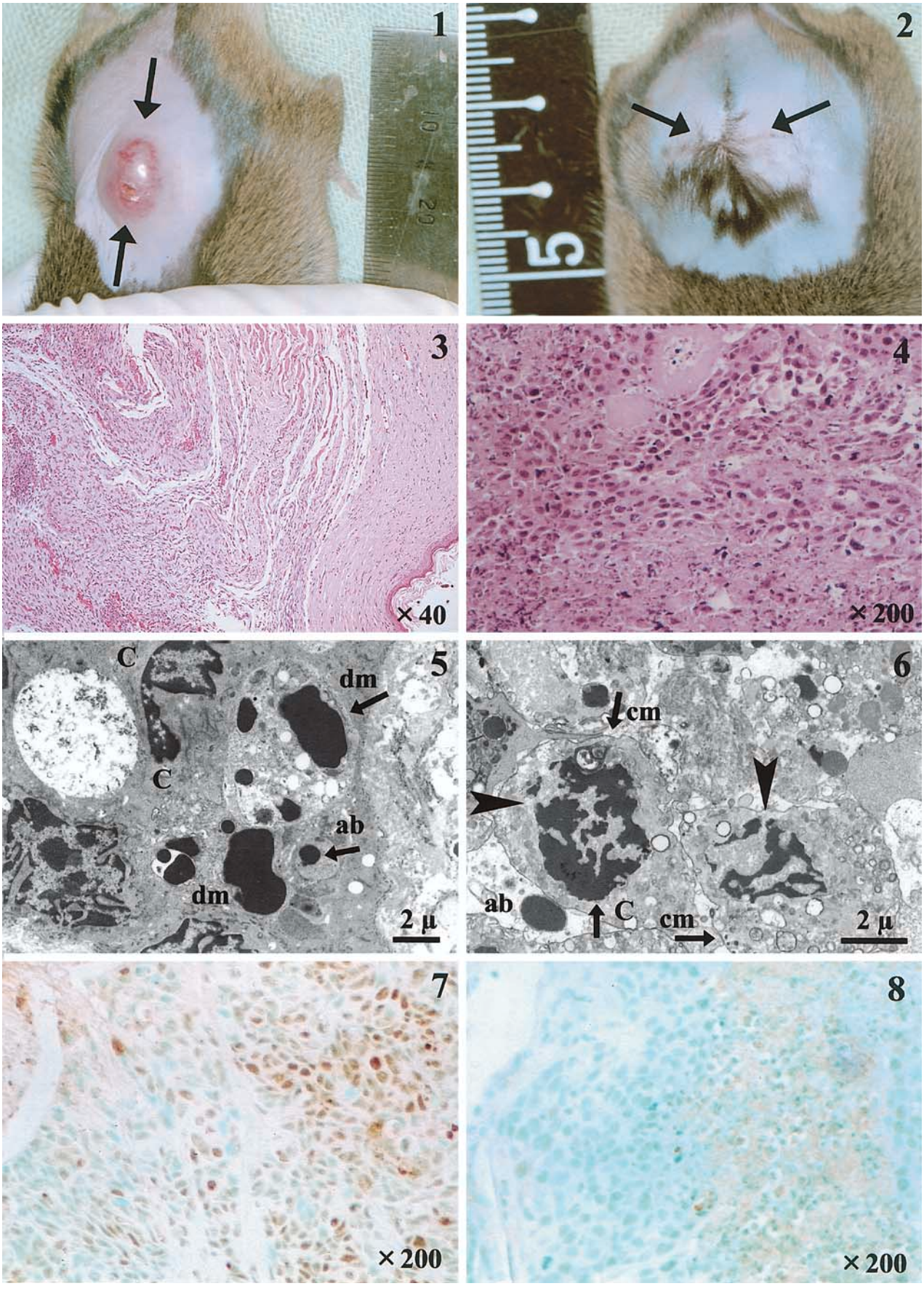
Materials and methods

Male C3H/HeNCrj mice (Charles River, Osaka, Japan) at 6 weeks of age, each weighing 24–26 g, transplanted with NR-S1 mouse squamous carcinoma cells (National Institute of Radiological Science, Chiba, Japan) into the thigh were prepared for the experiment. The hair of the dorsal thigh were shaved and suspended NR-S1 tumor cells were transplanted subcutaneously. Mice were housed in a temperature-controlled room with a 12-h light/12-h dark schedule and fed chow and water ad libitum. Tumors that had grown to 7×7 mm or larger in size by 7 days after transplantation (Fig. 1) were used in these experiments.

For local electroporation a CUY21 electroporator system (Tokiwa Science, Japan) was used. BLM was administered and 10 min later the tumor was picked up with the electrode and electric pulses were given. The ratio of the applied voltage for each pulse to the electrode spacing was 40 V/cm. The electric field was applied for a duration of 50 ms eight times per second for 1 s. The polarity was then reversed and eight more pulse of 50 ms each were given in the next second. The whole process was repeated two more times at intervals of 1 h. These three sets of electric stimulation were continued for 4 days per week for 2 weeks.

A total of 29 mice were divided into four experimental groups, six each for the control (C), electric pulse (E) and BLM (B) groups, and 11 for the E with BLM (B + E) group. BLM (25 μg) diluted in 0.2 ml normal saline was injected around the tumor just before the experiments. The mice were kept under observation for 6 weeks after each experiment. One mouse in each group was killed by inhalation of ether 1 week after the beginning of the experiment. The rest of the mice were killed at 8 weeks from the beginning of the experiment. Tumor and scar tissues were dissected and divided into two groups. The tissues of one group were embedded in paraffin after treatment with 10% formalin in 0.1 *M* phosphate buffer solution, pH 7.3. The tissues of the other group were fixed in solution described by Kalt and Tandler [7] and 1% osmium tetroxide and embedded in Epon 812 using standard procedures.

Sections of thickness 2 μm were taken on silane-coated glass slides and stained with hematoxylin and eosin (HE) for histological examination. To detect nuclei with DNA fragmentation, TUNEL staining [3] was performed using an ApopTag in situ apoptosis detection kit (Oncor). Before inspection by TEM (H-800 Hitachi, Japan), the trimmed block of Epon was orientated and stained with toluidine blue for light microscopy. The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Figs. 1–8 Before experiment (*1*) tumors had increased in size to more than 7 mm (*arrows*) by 7 days after transplantation of NR-S1 carcinoma cells. In a mouse treated with E + B (*2*) the tumor had disappeared and was replaced by crest-like formations (*arrows*) by 8 weeks from the start of the experiment. The specimen from this mouse showed newly formed epithelial tissue underneath which were collagen fibers (*3*). In another mouse treated with E + B killed 1 week after the start of the experiment (*4*), the tumor cells show dense chromatin and necrosis. The TEM findings in the same mouse (*5*, *6*) show chromatin uniformly compacted (*C*) or changed into dense masses (*dm*) and the formation of apoptotic bodies (*ab*). The cell membrane (*cm*) is preserved. The nuclear membrane has lost its integrity (*arrowhead*). *7* TUNEL-positive tumor cells with condensation of nuclei are apparent in the same specimen as in *4*. In a control specimen at 1 week (*8*), TUNEL-positive cells are scant

ultrathin sections with a silver to gold interference color were picked up on a nickel grid and stained with uranyl acetate and lead nitrate in the usual manner.

Results

In the C group of mice transplanted with NR-S1 tumor cells the size of the tumors had increased to 30–35 mm or more after 8 weeks. Tumors in the E and B groups decreased in size during the experiment, but again increased in size after the end of the experiment. All the tumors in the E + B group decreased in size and crest-like formations were seen 1 week after the end of the experiment. The tumor had disappeared completely in four of the ten mice in this group and no recurrence was seen during the 8-week observation period (Figs. 2 and 3). The other six tumors showed a similar increase in size to the tumors in the other groups.

In the tumor of the mouse treated with E + B and killed 1 week from the start of the experiment, the size of the nuclei and chromatin staining were different from the findings in mice of the other groups. Necrosis appeared in the tumor area (Fig. 4). TEM showed necrosis and apoptosis. The nucleus of the tumor cells showed chromatin compacted and changed into a dense mass. Apoptotic bodies were seen among the cells. The cell membrane was preserved but that of the nucleus had lost its integrity (Figs. 5 and 6). TUNEL-positive tumor cells with condensation of nuclei are shown in Fig. 7 and are from the same specimen as shown in Fig. 4, and are shown in comparison with the control (Fig. 8).

Discussion

A novel approach to cancer treatment has emerged recently in the form of ECT [2] which combines conventional chemotherapy with exposure of cancerous tissue to pulses of electricity or electroporation. The basic mechanism appears to be by increasing cell membrane permeability and thus intracellular access [4].

We chose BLM as it has been shown to be most effective in ECT [10]. This drug is a highly cytotoxic molecule that is also hydrophilic. The pores to the cell membrane remain open for the order of minutes [12]. Molecules that normally are unable to enter a cell can be absorbed passively when a cell is in the porous state [9]. Electroporation is a means of circumventing the resistance imposed by the cell membrane to allow a drug to diffuse passively into the internal cellular environment [8].

A complete response was seen in four of ten mice in this experiment. The mechanism of cell death in tumors treated with B + E is thought to be inhibition of DNA replication in actively dividing cells [1]. Necrosis and apoptosis occurred in a large area of the tumors treated with the electric field. The voltage used in other experiments has been more than 1000 V/cm [6], a voltage harmful not only to mice but also to humans. Electric burns or shock could occur in patients, especially those with cardiac pacemakers. The method using a low voltage for the electric stimulation would minimize these problems.

The cytotoxic effects of a chemotherapeutic agent are increased especially in rapidly growing tumor such as NR-S1. This may permit surgical procedures that are less radical than those required normally. In addition ECT can be used in areas in which surgery could lead to great disability [8] or in areas in which the tumor is not accessible to surgery such as the head and neck.

Future studies will focus on determining the optimal drug dose and electric field strength for effective treatment of carcinoma [11]. Although to our knowledge animal studies and clinical investigations published to date have not shown any adverse effect on normal tissues, future studies will focus on this issue as well because it is critical to bringing the treatment closer to clinical practice. In addition, evidence that the host immune system plays a role in this type of treatment suggests that it may be possible to add a systemic component to ECT. This will involve combining ECT with biological response modifiers such an interleukin-12 for the treatment of metastatic disease [13].

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