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In Vivo Antitumor Effects of Electrochemotherapy in a Tongue Cancer Model

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Purpose: This study investigated the in vivo antitumor effects of electrochemotherapy (ECT) using electroporation and bleomycin in a hamster tongue cancer model to assess its clinical applicability.

Materials and Methods: Twenty animals with chemically induced tongue cancer were divided into four experimental groups designated B−E−, B−E+, B+E−, and B+E+. The B+E+ and B+E− groups received an intraperitoneal injection of 100 μg bleomycin. Fifteen minutes after the injection, the B+E+ animals received electric pulses. The B−E+ group received only electric pulses. The B−E− group received neither bleomycin nor electric pulses. Each group received the same treatment twice. The antitumor effects were assessed based on tumor volume reduction and histologic findings.

Results: The B+E+ group showed remarkable tumor volume reduction, decreasing an average to 8.8% of its original volume 14 days after the treatment. Complete loss of the protruding tumor was observed in two of the five animals. Histologically, the tumors of the B+E+ group consisted of severely degenerated tumor cells and desquamative keratinizing cells. No living cancer cells were detected in three animals. The B+E−, B−E+, and B−E− groups showed progressive tumor growth, exceeding 200% of initial tumor volume during the experimental period.

Conclusion: The current study showed remarkable antitumor effects of ECT with bleomycin in the hamster tongue cancer model. ECT with bleomycin may be clinically applicable to the treatment of oral cancer.

Electroporation, or electropermeabilization, is a process that causes a transient increase in the permeability of cell membranes.[1,2] When a cell is exposed to high-voltage, short, electric pulses, the cell membrane can be opened transiently without causing permanent cell damage. This leads to reversible permeabilization that allows input of chemotheraputic agents[3,4] as well as genetic materials.[5]

Electrochemotherapy (ECT) is a novel method of cancer treatment that consists of the combined use of high-voltage, pulsed, electric fields and a chemotherapeutic agent. High cytotoxicity of bleomycin normally is restricted by a limited capacity to diffuse through the lipid bilayer of the plasma membrane because of its hydrophilic character.[6] In vitro, bleomycin shows much higher cytotoxicity to permeabilized cell than to nonpermeabilized cells.[7] The antitumor effect of ECT using bleomycin as a chemotherapeutic agent (ECT with bleomycin) has been evaluated in animal models with various types of malignant tumors.[8-18] Based on the response rate obtained in these animal studies, preliminary clinical trials have been started.[19-21]

Excellent antitumor effects of ECT have been shown on a variety of tumor types, including melanoma,[9,12,14] hepatocellular carcinoma,[11] mammary tumors,[17] sarcoma,[8] fibrosarcoma,[10,16] colorectal carcinoma,[15] cervical squamous cell carcinoma,[13] and glioma.[18] These studies indicate the efficacy of ECT over a wide histologic spectrum in cancer treatment. However, in most studies, the evaluation was performed on subcutaneously induced tumors. This is because the direct current pulses of ECT are generally administered through two parallel plate electrodes that are placed on either side of the tumor. This type of electrode has been considered to be suitable only for lesions on the

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body surface, typically skin lesions, but it can also allow the delivery of electric fields to the oral cavity. Although ECT with bleomycin has been applied to metastatic skin lesions from head and neck cancer,[19,20] its applicability to the primary site has not yet been studied. We report the efficacy of ECT with bleomycin in the hamster tongue cancer model.

Materials and Methods

CARCINOGENESIS OF TONGUE SQUAMOUS CELL CARCINOMA IN THE HAMSTER

Thirty-five male Syrian golden hamsters aged 5 weeks and weighing 50 to 60 g were obtained from Japan SLC (Hamamatu, Japan). They were maintained under standard laboratory conditions with access to both food and water ad libitum. A 0.5% 9,10-demethyl-1,2-benzanthracene (DMBA)-acetone solution was used as the carcinogen. The DMBA solution was applied to the left lateral border of the middle third of the tongue after scratching the area with a pulp canal cleaner (barbed broach) three times per week under ether anesthesia. The lesions increased in size gradually, and protruding tumors measuring approximately 8 to 12 mm in maximum diameter formed by the end of 20 weeks in 20 of the 35 animals. These 20 animals were used in the current study. The details of the procedure and the histologic characteristics of this tongue cancer model have been previously reported.[22]

EXPERIMENTAL DESIGN

The 20 animals with established tumors were divided into four experimental groups designated B−E−, B−E+, B+E−, and B+E+. The B+E+ and B+E− groups received an intraperitoneal injection of 100 μg bleomycin, which corresponds to roughly one-hundredth of the median lethal dose (LD_{50}). Fifteen minutes after the injection, the B+E+ animals received electric pulses (Fig 1). The B−E+ group received the same electric pulses to the tumor without a bleomycin injection. The B−E− group received neither bleomycin nor electric pulses. Each group received the same treatment twice, the second treatment being performed 48 hours after the first.

Treatment results were assessed based on tumor volume and the histologic findings. The tumor volume was measured 2, 4, 7, and 14 days after the first ECT. The tongue were excised for microscopic examination at 14 days.

ELECTRIC PULSE DELIVERY

Electric pulses were generated by a BTX T820FE generator (Genetronics Inc, San Diego, CA) 15 minutes after bleomycin injection. Electrical energy was transmitted directly to the tumors through a pair of parallel, round, gold-plated, stainless-steel, 10-mm diameter electrodes placed on either side of the protruding tumor (Fig 1). Each electrical treatment regimen consisted of eight triangular direct current pulses with a width of 99 μs and a repetition frequency of 1 Hz. The electric field strength was determined at a voltage-to-electrode spacing ratio of 130 V/mm. The voltage of each pulse was monitored using a graphic pulse analyzer BTX500 (Genetronics Inc).

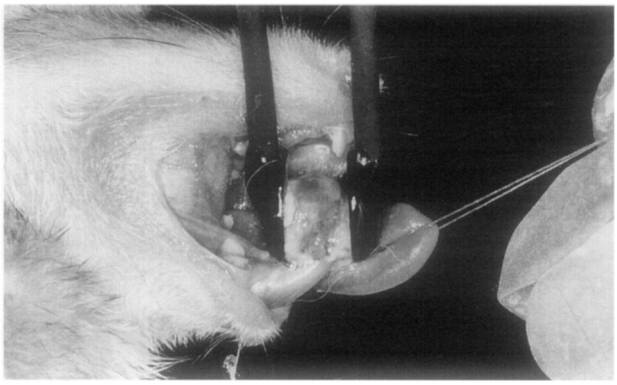

FIGURE 1. Electric energy being transmitted directly to tumors through parallel, round, stainless-steel electric placed on either side of protruding tumor.

MEASUREMENT OF TUMOR VOLUME

Tumor volume was measured 2, 4, 7, and 14 days after the first ECT session as well as immediately before treatment. The volume was calculated using the longest diameter (L-mm) and the shortest diameter perpendicular to L (W-mm) in accordance with Giavazzi's formula[23]: $V = L^*W^2/2$. Relative tumor volume was defined as the tumor volume *n* days after the first ECT session divided by the tumor volume immediately before treatment. The mean relative tumor volume was calculated for each treatment group at periodic intervals during the follow-up period, and the objective response was scored according to World Health Organization (WHO) guidelines as: (1) progressive disease if tumors increased in size, (2) no change if tumor size decreased by less than 50%, (3) partial response (PR) if the tumor size decreased by more than 50%, and (4) complete response (CR) if they became nonpalpable. The term *objective response* has been used to refer to the sum of the complete and partial responses.

HISTOLOGIC EXAMINATION

The animals were killed 14 days after the first ECT by a lethal dose of ether, and the tongue was excised. Tissue specimens were fixed overnight in 10% formalin and then processed for routine microscopic examination. The specimens were dehydrated through a sequence of 50%, 70%, 95%, and 100% ethanol, cleared in xylene, and then embedded in paraffin wax. Hematoxylin and eosin staining was performed on 4-μm sections cut using a microtome.

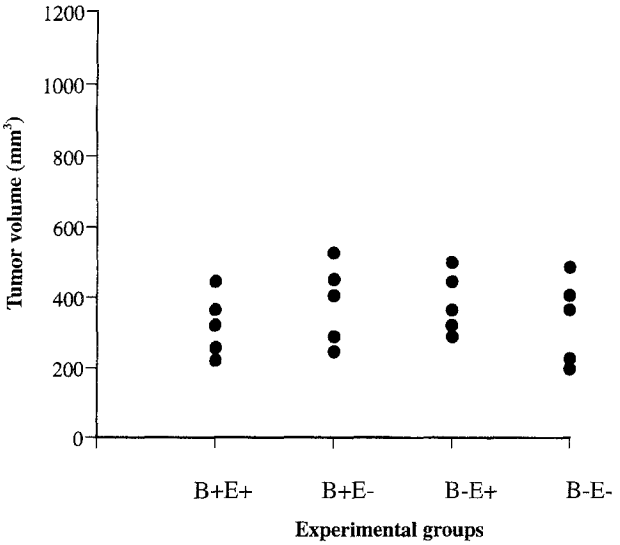

FIGURE 2. Tumor volume in each experimental group immediately before treatment: No significant differences in mean tumor volume among experimental groups.

STATISTICAL ANALYSIS

Significant differences between the mean tumor volume among the experimental groups were determined by a one-way analysis of variance followed by Scheffe's test or Newman-Keuls test for multiple comparisons. A level of $P < .05$ was taken as indicative of significant differences. Analysis was performed using the SPSS software package (SPSS Inc, Chicago, IL).

Results

TUMOR VOLUME

The initial mean tumor volumes ± SE of the B+E+ group, B+E− group, B−E+ group and B−E− (control) group were 321.3 ± 39.8 mm³, 383.15 ± 51.9 mm³, 383.6 ± 39.3 mm³, and 335.6 ± 54.8 mm³, respectively, and there was no significant difference among the four experimental groups (Fig 2).

Tumor volume reduction was observed in all the B+E+ animals. Two days after treatment, the color of the tumor changed from light reddish brown to whitish yellow, and a slight reduction in tumor volume was noted, although the reduction was not statistically significant. Four days after the first treatment, the tumor volume decreased by 45% to 70% (mean, 56.5 ± 5.0%) in the B+E+ group, whereas in the other experimental groups the tumor gradually increased in size. The mean relative tumor volume of the B+E+ group was significantly smaller than that of the other three experimental groups. Seven days after treatment, the tumors in the B+E−, B−E+, and B−E− groups progressively increased in size (Figs 3, 4). However, in the B+E+ group, the five tumors became a flaccid mass, the surface of which was ulcerated and necrotic. In one animal, the protruding tumor almost disappeared (Fig 5). The mean relative tumor volume of the B+E+ group decreased to 24.3%

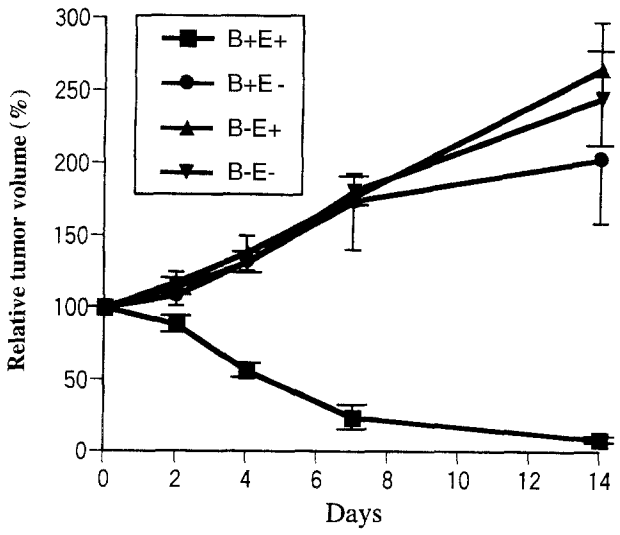

FIGURE 3. Alterations in mean relative tumor volume in each experimental group 2, 4, 7, and 14 days after first treatment. The B+E+ group shows remarkable tumor volume reduction.

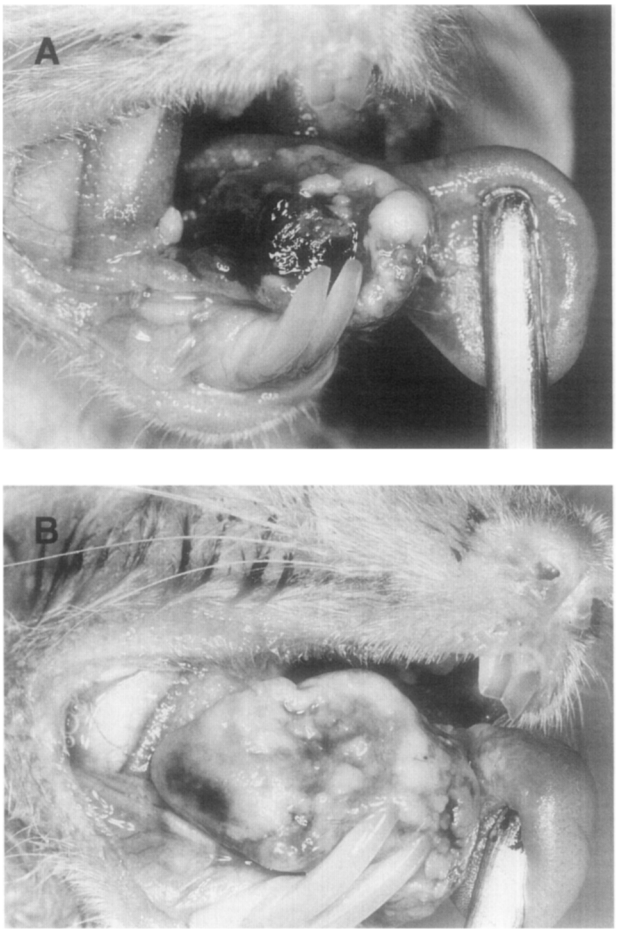

FIGURE 4. Example of B+E− group *A*, immediately before the treatment and *B*, 7 days after first treatment. The tumor has increased in size.

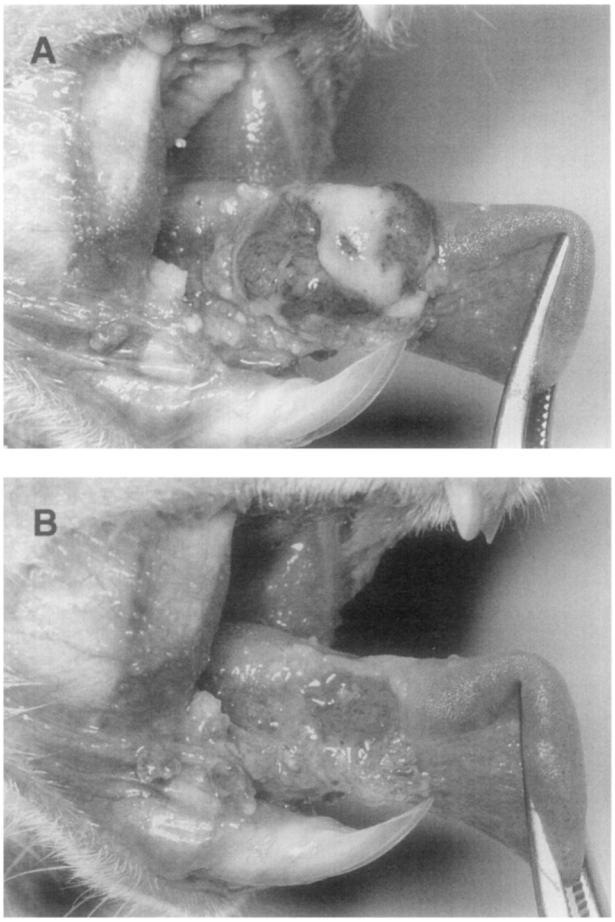

FIGURE 5. Example of B+E+ group *A*, immediately before the treatment and *B*, 7 days after first treatment. The protruding tumor mass almost disappeared.

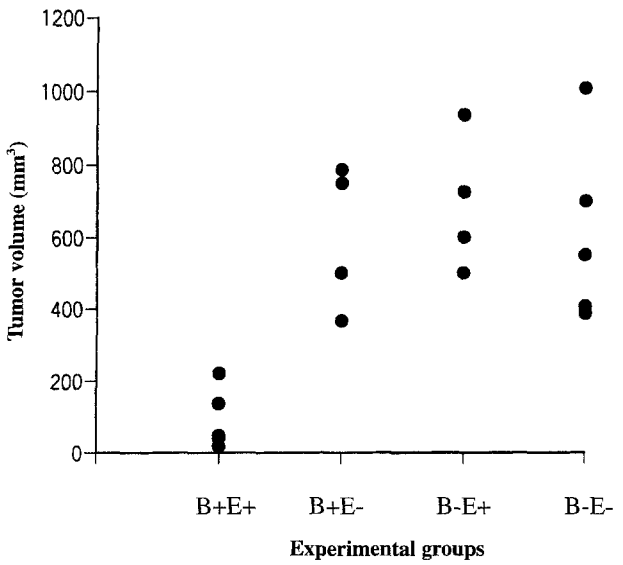

FIGURE 6. Tumor volume in each experimental group 7 days after first treatment: the B+E+ group differs significantly from the other experimental groups.

(Fig 3), which was significantly different from the other three experimental groups (Fig 6). Local edema was observed in the tongue surrounding the tumor in all the B+E+ animals.

Fourteen days after treatment, the tumors in the B+E−, B−E+, and B−E− groups showed excessive growth, and the mean relative tumor volume increased to over 200% (Figs 3, 7A). On the other hand, the B+E+ group showed continuous tumor volume reduction. The visible protruding tumors became ulcerative lesions covered with necrotic debris (Fig 7B). In two animals, the tumors were too small to measure. After completion of the follow-up period, all the B+E+ animals showed an objective response (PR, n = 3; CR, n = 2). All animals in other groups showed a PD rating. Swelling of the anterior third of the tongue because of gradually progressive local edema was evident in all animals of the B+E+ group (Fig 7B). Changes in the mean relative tumor volume for each experimental group 2, 4, 7, and 14 days after the treatment are shown in Figure 3.

HISTOLOGIC FINDINGS

In the B+E−, B−E+ and B−E− groups, the protruding tumors histologically consisted of squamous cell carcinoma in which the degree of keratinization

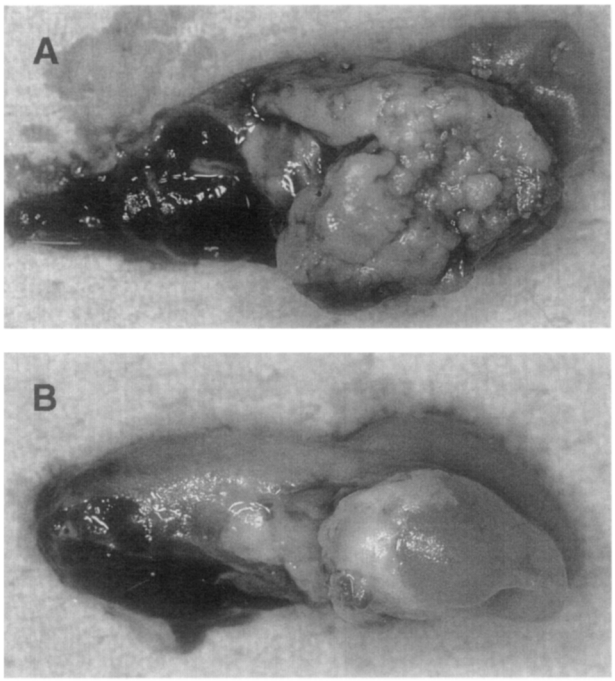

FIGURE 7. *A*, Tongue excised for microscopic examination 14 days after the first treatment in the B+E− group. Note the large protruding tumor with relative tumor volume exceeding 200%. *B*, Tongue excised for microscopic examination 14 days after first treatment in the B+E+ group. The tumor is reduced in size, and the ulcerative lesion is covered with necrotic debris. Diffuse swelling due to local edema is evident in the anterior one third of the tongue.

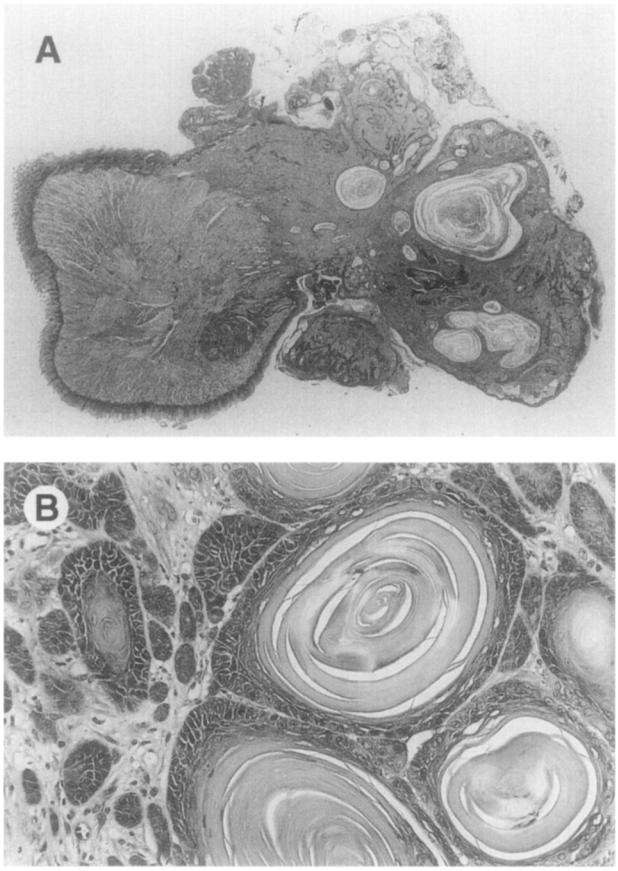

FIGURE 8. *A*, Low-magnification view of large protruding tumor in the B+E− group 14 days after treatment (Hematoxylin and eosin, original magnification ×5). *B*, Higher-magnification view of growing tumor consisting of highly keratinized squamous cell carcinoma (Hematoxylin and eosin, original magnification ×400).

varied from low to high (Fig 8). No antitumor effects were observed in any of these three groups. However, a remarkable antitumor effect was histologically observed in the B+E+ group 14 days after ECT with bleomycin. The flaccid tumor mass consisted primarily of severely degenerated tumor cells and desquamative keratinizing cells showing a severe inflammatory response (Fig 9A, B). Complete disappearance of tumor cells was noted in three animals, and only a small number of living cancer cell nests were observed in the deep layer of the tumors in the remaining two animals (Fig 9C). The muscle tissue surrounding the degenerated tumor was remarkably edematous (Fig 9A, 9D). However, the overlying mucosa, including the tongue papillae, was intact (Fig 9D).

Discussion

The antitumor effects of ECT with bleomycin may vary considerably with 1) electrical treatment conditions, 2) electrode design, 3) treatment times (single or multiple), 4) bleomycin dose, and 5) route of administration.

ELECTRICAL TREATMENT CONDITIONS

In the current study, the electrical treatment conditions applied to the tongue were equal to that administered to subcutaneous tumors in previous studies.[9-17] The electrical treatment conditions are defined according to the strength of the electric field, pulse shape, pulse width, and the number of pulses. These conditions should be achieved in a manner that results in temporary membrane destabilization with minimal cytotoxicity. The electric field strength is defined as the voltage applied to the electrodes divided by the distance between the electrodes. The pulses are usually rectangular. In 1991, Mir et al[8] reported that the minimal efficient field strength required for the treatment of subcutaneously induced LPB sarcoma is 1,100 to 1,200 v/cm through eight pulses of 100 μs in conjunction with administration of bleomycin corresponding to roughly one tenth of the LD_{50}.[8] These conditions have been widely applied to a variety of subcutaneously induced tumors.

ELECTRODE DESIGN

Remarkable antitumor effects were seen in the more superficial regions of the nodules, whereas living cancer cell nests were present in the deeper portions. These histologic features indicated that deeper tumor regions were not subjected to an electric field of sufficient intensity to cause electropermeabilization. This inequality was in part due to the electrode design. Recently, Jaroszeski et al[11] and Gilbert et al[12] used a modified electrode consisting of six needles equally spaced around a 1-cm diameter circle.[11,12] The needle electrode was inserted to a specified depth around the tumor, and six pulses were applied to opposing needle pairs in a manner that rotated the applied electric field around the tumor. This modification resulted in a 97.1% CR rate in the murine B16 melanoma model, compared with a 67.6% for the parallel plate electrode protocol.

TREATMENT TIMES

ECT has been investigated as a single treatment almost exclusively, but multiple drug administration is very common in conventional cancer chemotherapy. Jaroszeski et al[9] investigated the efficacy of a three-ECT treatment protocol administered at 1-week intervals on B16 melanoma tumors induced in mice. They reported a 75.8% CR rate, which was 12 times higher than that for the single treatment.[9] Thus, a multiple ECT treatment regimen may be effective in eliminating residual cancer cell nests.

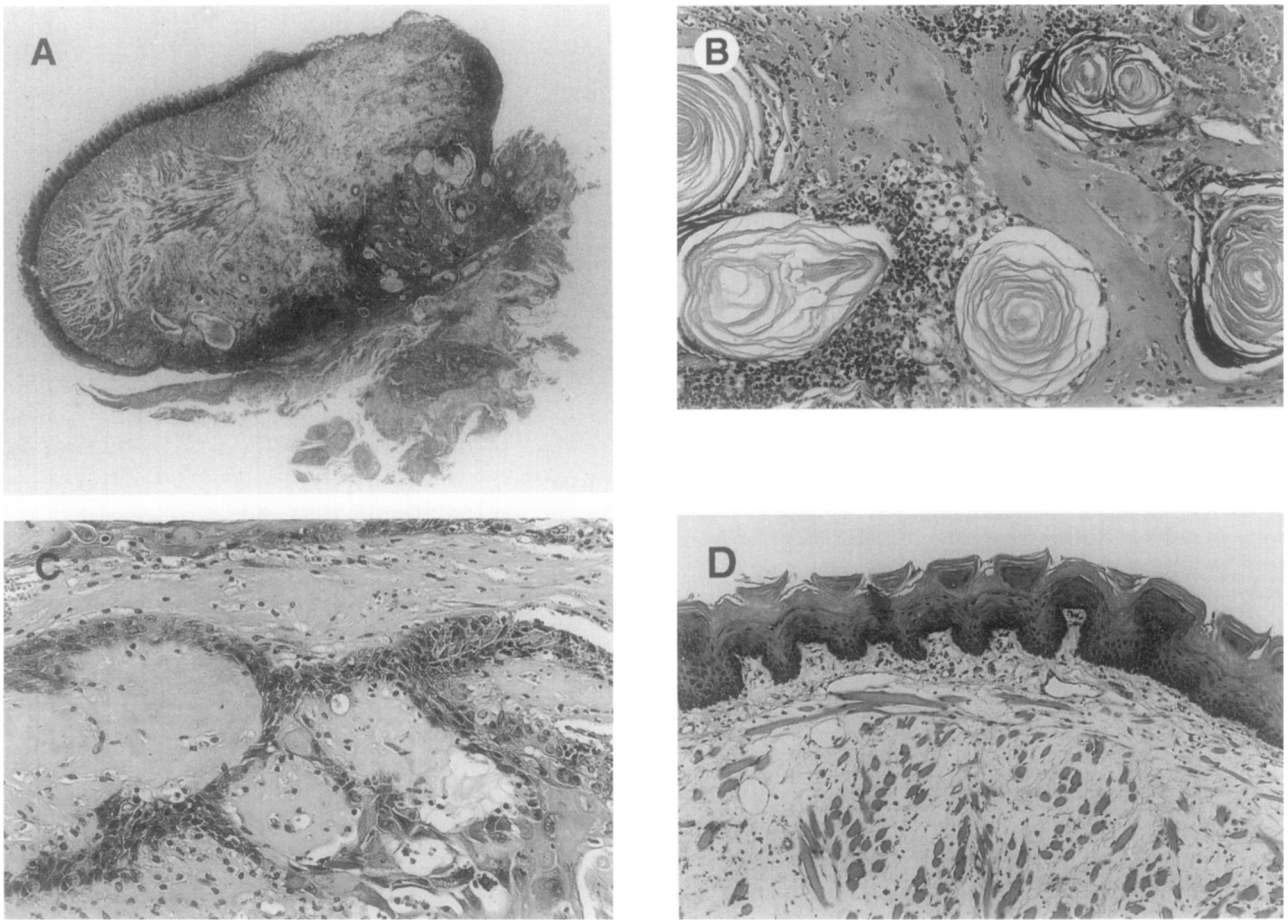

FIGURE 9. *A*, Low-magnification view of severely degenerated tumor mass in the B+E+ group 14 days after ECT. Local interstitial edema is evident in the tongue (Hematoxylin and eosin, original magnification ×5). *B*, Higher-magnification view of tumor predominantly consisting of necrotic debris, with severe inflammation and degenerated keratin pearls. No living cancer cells are seen (Hematoxylin and eosin, original magnification ×400). *C*, Living cancer cell nests observed in the deep layer of the tumor (Hematoxylin and eosin, original magnification ×400). *D*, Higher-magnification view of local edema in the tongue. The overlying mucosa and papillae are intact (Hematoxylin and eosin, original magnification ×100.)

BLEOMYCIN DOSE

The effective dose of a chemotherapeutic agent depends on the sensitivity of the cancer cells to the chemotherapeutic agent. Bleomycin has generally been used as a chemotherapeutic agent against a variety of cancer cell types in ECT studies, because bleomycin shows the highest increase in intracellular concentration and subsequent tumoricidal effects among a variety of chemotherapeutic agents when used in this manner.[7] Experimentally, a bleomycin dose corresponding to roughly one tenth of the LD_{50} has been applied against tumors for which bleomycin sensitivity has not been shown clinically, and the observed objective response rate (the sum of PR and CR) has ranged from 70% to 85% for most cancer cell types. However, in one case, subcutaneously induced cervical squamous cell carcinomas completely disappeared in all six animals 12 days after ECT with bleomycin.[13] In the current study, the electric field strength administered to the tongue tumors was equal to that administered to subcutaneous tumors in the previous studies. However, the administered bleomycin dose, corresponding to roughly one hundredth of the LD_{50}, was relatively low. Nevertheless, a clear antitumor effect was observed in which all animals had an objective response with a CR rate of 40%. The higher response rate in both cervical and tongue squamous cell carcinomas than in other tumor types is likely caused by naturally higher sensitivity of squamous cell carcinoma to bleomycin.

ADMINISTRATION ROUTE

In most studies, including clinical trials, bleomycin is systemically administered through intramuscular, intravenous, or intraperitoneal injection. However, the physical effects of electrical treatment are confined to the tumor between the electrodes, and the effective drug delivery is also restricted to the same region. Therefore, intratumoral injection of a chemotherapeutic agent, which allows much higher concen-

tration within the tumor than systemic injection, may be beneficial in the ECT protocol. In addition, such local drug administration may allow the drug dose to be decreased, thereby reducing any subsequent drug-related side effects compared with systemic drug administration. Heller et al[14] reported approximately equal results for ECT treatment with both administration routes for bleomycin in a murine B16 melanoma model. In contrast, Cemazar et al[24] reported markedly better results for animals treated with an intratumor injection of cisplatin than with an intravenous injection in the mice EAT tumor model. Thus, differences may exist in the optimal administration route according to the chemotherapeutic agent, tumor location, and cancer cell type.

In both clinical and animal studies, electric burns to the skin under the electrode or local edema of the tissue surrounding the tumor are common side effects of ECT with bleomycin.[8,13,19-21] In the current study, neither necrosis nor loss of mucosal epithelium under the electrodes was observed in the B+E+ and B−E+ groups. However, local interstitial edema of the lingual muscles around the necrotic tumor was observed in the B+E+ group after ECT with bleomycin. In contrast, no local edema was detected in the B−E+ group. These results suggest that the local interstitial edema could not be attributed to electric burns. Mir et al[8] reported that the antitumor effect of ECT with 500 μg bleomycin in immunologically reactive C57B1/6 mice bearing LPB sarcomas was apparently greater than that found in immunodeficient nude mice, and that more severe local edema was always detected in the immunologically reactive C57B1/6 mice than in the nude mice.[8] This difference in response suggests that the host immune response could be instrumental in elimination of the tumor cells after massive cell lysis following ECT, and that the local edema may be associated with the host immune response.

Remarkable tumor volume reduction and dramatic histologic changes were observed in the hamster tongue cancer model after ECT with bleomycin. However, the recurrence rate and the effects of the proposed ECT protocol on survival are not yet clear. Ultimate locoregional control and survival effects following chemotherapy are closely associated with a pathologic response. The living cancer cell nests observed histologically in two animals represent a risk for recurrence. To confirm the local recurrence rate and effects on survival, further investigation with a longer follow-up is required. Recent combination chemotherapy for head and neck cancer, particularly cisplatin-based regimens, have resulted in a higher overall response rate at the cost of a significant increase in toxicity. However, none of proposed regimens has provided a survival benefit.[25] Based on the remarkable antitumor effects, ECT with bleomycin may be clinically applicable to the treatment of oral cancer. The results of the current study are promising for the development of an optimal ECT treatment protocol for tongue cancer that maximizes response and minimizes side effects.

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