原　著

Electrochemical Cancer Therapy Induces Apoptosis in SCC-7 Tumor of Mice

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Abstract

Electrochemical Cancer Therapy (ECT), called low level direct current treatment is known as one of therapeutic potentials to many diseases in the field of cancer treatment. This study was basically designed to elucidate the relationship between ECT and the prevalence of apoptosis in tumor cells using histopathology.

For the experiment, squamous cell carcinoma (SCC)-7 strain was transplanted into right upper thigh of C3H mice, and the considerable growth of the tumor was confirmed. ECT was done to the tumors and cellular changes were observed microscopically by examining the effect of the ECT with different direct current doses. *In situ* nick end labeling method was employed for the analysis of apoptosis.

A significant difference was found between the above 5 Coulomb treatment groups and the control group ($p<0.05$). In terms of tumor necrosis scores, there were significant differences between the 5 Coulomb treatment group and the 10 Coulomb treatment group, and the control group 24 hours after ECT ($p<0.05$, $p<0.05$). In the 5 Coulomb or the 10 Coulomb-treated group, there were noticeable increases in the number of apoptosis comparing the control group ($p<0.01$, $p<0.01$; $p<0.01$, $p<0.01$). Twenty-four hours after ECT, apoptosis of all treatment groups were significantly decreased more than in the control group ($p<0.05$, $p<0.01$, $p<0.01$).

These results showed that the ECT could delay tumor growth of SCC-7. Particularly in the groups treated with 5 and 10 Coulomb of ECT, the number of apoptosis in tumor mass was significantly increased more than in the control group during the period of experiment ; and it suggests that the apoptosis is directly related with the histopathologically destructive changes of tumor cells after ECT.

INTRODUCTION

Electrochemical treatment (ECT) is also called low level direct current treatment and has been applied in cancer treatment in recent years despite the fact that it has been examined by electrobiologists for long time. The report in 1895 by Golsinger who observed histopathologic changes along with cell death in tissues around the single electrode applying 20 mA direct current to the cerebrum was the first time in ECT history[1)-3)]. After Nordenstrom had reported excellent results by applying ECT for the first time in actual clinical settings in patients with lung cancer, the clinical effectiveness of ECT was reported after it was applied in various countries including US, Japan, Sweden, and China[4)-7)].

Apoptosis is programmed cell death, which is one of the essential physiological processes needed for normal development of organs and maintaining homeostasis in living organisms[8)-11)].

Unlike cellular necrosis, cellular apoptosis is known to accompany unique morphology and biochemical characteristics. Necrosis seen when cells are severely damaged accompanied by the destruction of cellular membrane and expansion and lysis of the cell.

On the other hand, cellular apoptosis accompanies cell shrinkage due to fast dehydration of cells, vacuolization of cell membrane, condensation of DNA, and formation of apoptotic body[12)-15)].

Abnormal cellular apoptosis is related with the development of tumors, immune deficiency, and various viral diseases. According to recent studies, cellular apoptosis is important in the development, growth and metastasis of tumor in most cases. The fact, that apoptosis would increased when tumor growth is suppressed, is indirectly suggests this importance of cellular apoptosis[16),17)].

Although histopathologic changes such as cellular necrosis coming from ECT due to pH changes and tissue dehydration are known, no study has been conducted on the effect of ECT on apoptosis. Upon performing ECT after inducing squamous cell carcinoma (SCC)-7 tumor on the skin of C3H, we confirmed histopathologic changes and observed the degree of apoptosis to determine the mechanism of tumor cell destruction due to ECT[18)-20)].

MATERIAL AND METHODS

1. Animals

The animal used in this study was 4-5 weeks-old male and female C3H mice (15-20g). After the mice were placed in the lab cages for one week for them to adapt to the lab environment, only those healthy mice were selected and used for the study. While allowing the mice to feed freely, they were kept in the sterile lab where the temperature sterile was 22±1°C and the humidity was maintained at 55-60%.

2. Study groups

The mice were divided into control group (40 mice that were not treated with ECT) and experimental group (120 mice that were treated with ECT). The mice in the experimental group were further divided into 3 subgroups according to direct current used. The basic unit of electric charge, 1 Coulomb, was defined as the 1000 mA of electric charge flowing for 1 sec in this study. The subgroups included 1 C group in which the mice were treated with 10 mA of 1 Coulomb direct current for 100 sec, 5 C group in which the mice were treated with 10 mA for 500 sec, and 10 C group in which the mice were treated with 10 mA for 1000 sec. After

giving ECT for 14 days, tumor growth was observed by comparing 10 mice in each group. Histopathologic changes and cellular apoptosis were compared in 10 mice in each mice by 6 h, 12 h and 24 h after ECT.

3. Tumor transplantation and ECT

The mice were used for the experiment after their tumor reached 1 cm in diameter by intramuscularly injecting 1×10^6 cells/0.05 ml of SCC-7 (Tohoku University Cancer Center, Japan) into the right hip for 2 weeks. The experiment was conducted in a sterile lab. ECT was performed using an electrochemical therapy equipment (Sunny Health Co, Tokyo, Japan) (Fig. 1). The voltage, current, and electric charge could be adjusted in the equipment, which was equipped with an automatic control telling the operator whether the circuit was opened or closed. With this equipment, the range of electric current, voltage, and maximum electric charge were 0-150 mA, -20 V, and 1000 C, respectively. Constant-electric current and constant-voltage modes were also possible. It was also equipped with an automatic alarm system and auto-circuit so that electric current could flow constantly. The electrodes were placed along the longitudinal axis of the mouse on top of the tumor while maintaining 10 mm between the anode and cathode. Based on the tumor size, 2 electrodes were used. The voltage used did not go over 3 V, and the electric charge used was defined according to each group. The electrode was made from platinum (Suzuka University, Japan), which was 100 mm long with 0.5 mm diameter. One end of the electrode was inserted into the tumor through a trocar and the other end was connected to the equipment. The electrode was sterilized before use to prevent infection. ECT was performed after anaesthezing the mouse using pentobarbital sodium (2 mg/kg) (Fig. 2).

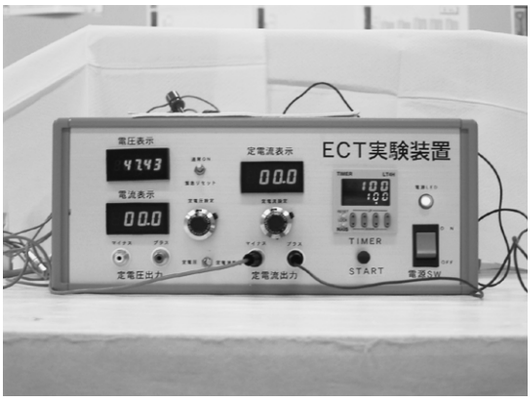

Fig. 1. Electrochemical therapy equipment (Sunny Health Co, Tokyo, Japan).

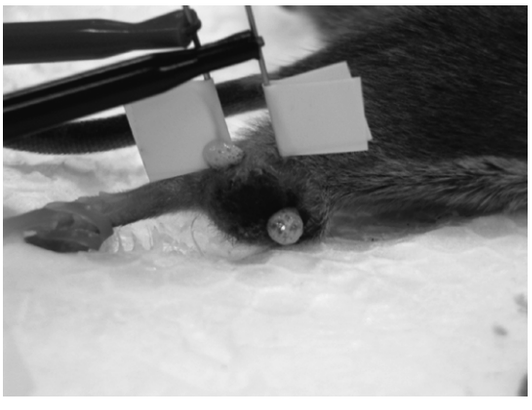

Fig. 2. Case of electrochemical therapy in the right upper thigh mass. There is 1 cm distance between the electrodes.

4. Measurement of tumor size and weight

The tumor size was measured every day for 14 days in the control and experimental 1 C, 5 C, and 10 C group to confirm any change in tumor growth. After checking tumor size, the tumor volume was determined using the following formula.

Tumor volume (mm^3) = $(ab)^2$, (a : length, b : width)

After each mouse in the control and experimental groups was sacrificed by cervical dislocation 14 days after ECT, the tumor was extracted by resect-

ting the femur and weighed for comparison.

5. Histopathology and apoptosis

For microscopic study, the tumors taken from the control and experimental groups by 6 h, 12 h, and 24 h after ECT were fixed in 10% formalin for 6 h as soon as it was taken out, treated with alcohol, embedded in paraffin, cut into 5 um in thickness, stained with Hematoxylin-Eosin (H&E).

Histopathologic findings were used to objectively evaluate the degree of tumor necrosis. Without understanding the process of this study, a pathologist evaluated the degree of necrosis into the following 4 grades by observing the center and each of 4 corners of the sample slide under 400x after confirming the presence of necrosis in each sample under a low power microscope. Each area of observation was classified into "1" when there is no necrosis, "2" when necrosis was slight with less than 30% of the microscopic view showing necrosis, "3" when necrosis was present somewhat between 30-60%, and "4" when necrosis was present severely with more than 60% of the microscopic view showing necrosis.

The presence of apoptosis was determined according to the TUNEL method using ApopTag ®. After preparing paraffin-embedded tissue into 5 μm slices, the sample was treated in xylene 3 times for 5 min each remove paraffin, anhydrous alcohol 2 times for 5 min each, 95% alcohol 3 min, 70% alcohol for 3 min, PBS for 5 min, and proteinase K for 15 min at room temperature to digest protein. The sample was treated with TdT enzyme for 1 h in humid environment at 37°C by covering the sample with a plastic cover. After removing the plastic cover, it was then treated with warm stopping buffer for 10 min, reacted in 70% alcohol at -20°C for 12 h, washed 5 min for 3 times in PBS. The sample was then treated with anti-digoxigenin-peroxidase solution and left at room temperature for 30 min by covering the sample with the plastic cover. After removing the plastic cover, the sample was treated in PBS 3 times for 5 min each and treating the sample with 3,3-DAB containing peroxides developed color. The sample was washed with distilled water 3 times for 1 min each, 1 time for 5 min, and counterstained with Harris hematoxylin for 10 min.

The accuracy of staining for apoptosis was determined using the tumor samples from control C3H mice. Apoptosis was defined in those cells with dark nucleus under an optical microscope using ApopTag®. The number of positive cells under 400x magnification was determined by 6 h, 12 h, and 24 h after ECT. In order to compare the degree of apoptosis with control samples, the number of cells stained positive to its antibody using the point-counting method. The number of cells showing positive response was counted 3 times and the average of 3 counts was used.

6. Statistical analysis

All measurements made in this study were shown in average±SD. SAS was used for statistical analysis. Parametric ANOVA was used to verify differences in the tumor volume and weight and the number of apoptotic cells in each group. P values less than 0.05 were considered significant.

RESULTS

1. Tumor volume and weight

Although no significant difference was seen in tumor volume between control group and 1 C group starting 1 day after ECT, a statistically significant difference was seen in 5 C group and 10 C group compared with control group ($p<0.05$, $p<0.05$).

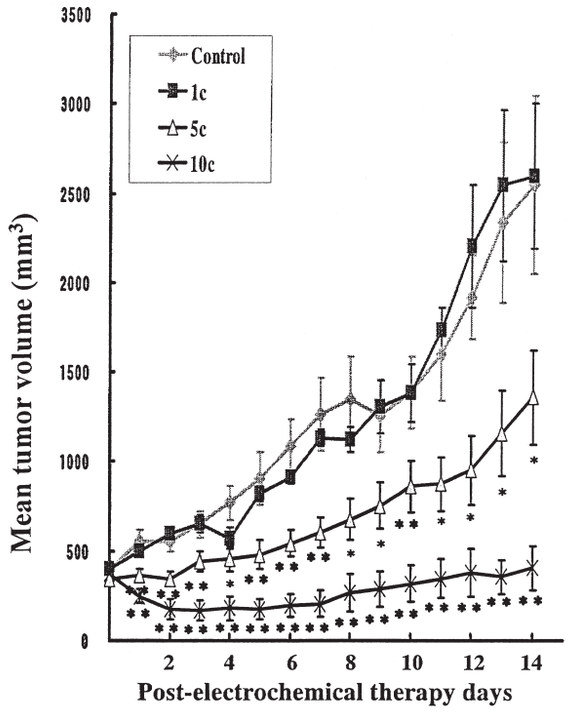

Fig. 3. Changes of the tumor volume after SCC-7 inoculation in the groups of control, 1 C, 5 C, and 10 C (*p<0.05, **p<0.01).

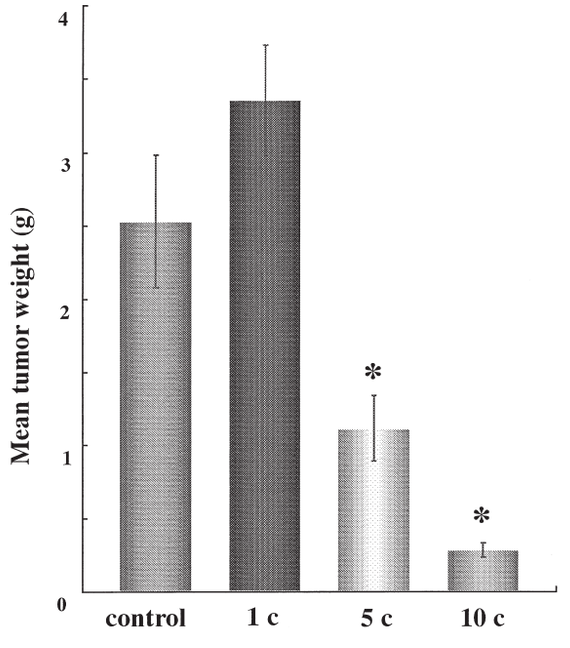

Fig. 4. Weight of extracted tumor 15 days after electrochemical therapy (*p<0.05).

The tumor volume was especially small in 10 C group compared with control group, showing a definite effect of ECT on tumor growth (Fig. 3).

The average and standard deviation weights of tumors extracted 14 days after ECT in control and experimental groups were compared. The average weight of tumors extracted from control mice was 2.53±0.45 g and that from 1 C group mice was 3.36±0.38 g, showing no significant difference. On the other hand, the average weights of tumors extracted from 5 C group mice and from 10 C group mice were 1.11±0.23 g ($p<0.05$) and 0.28±0.05 g ($p<0.01$), respectively, showing significant decreases compared with that in control mice (Fig. 4).

2. Tumor necrosis

The average degree of necrosis in control group 6 h, 12 h, and 24 h after ECT was 1.2±0.3. By 6 h after ECT, no significant difference was seen in 1 C, 5 C and 10 C groups compared with control group, with the average degrees being 1.3±0.6, 1.6±0.5, and 1.8±0.6, respectively. Also, no significant difference was seen 24 h after ECT in 1 C, 5 C and 10 C groups, with the values being 1.4±0.5, 1.8±0.7, and 1.9±0.8, respectively. Although no difference was seen in 1 C group by 12 h after ECT at 1.4±0.3, significant differences were seen in 5 C and 10 C group with the values being 2.8±0.7 and 2.9±0.8, respectively ($p<0.05$, $p<0.057$) (Fig. 5).

Various histopathologic changes were observed by 6 h, 12 h, and 24 h after ECT. Typical cellular biologic characteristics of squamous cell cancer were confirmed in control samples (Fig. 6A). Although similar findings were present in 1 C group samples as in the case of control samples (Fig. 6B), necrosis was present partially in 5 C group samples 6 h after ECT and necrosis tumor cells including

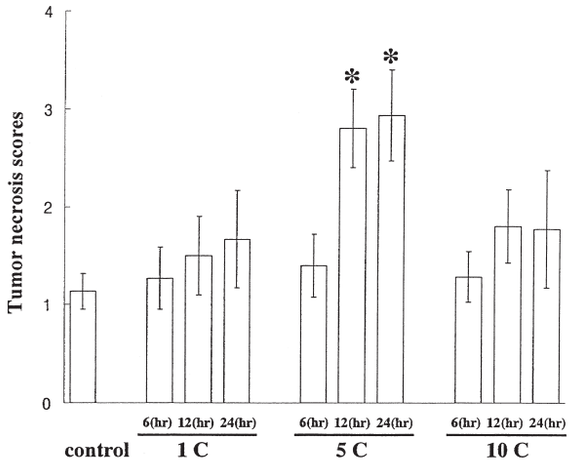

Fig. 5. Tumor necrosis scores after electrochemical therapy in each treatment and control groups (*$p<0.05$).

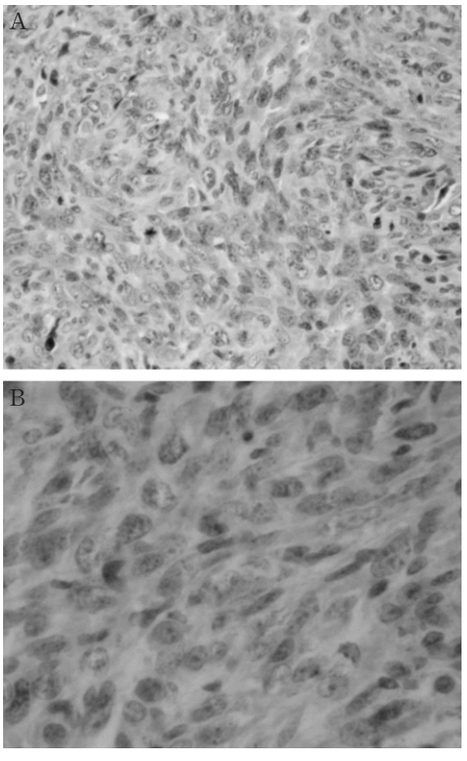

Fig. 6. Microscopic finding of squamous cell carcinoma (1×1 cm) occurring on the right upper thigh after inoculation of SCC-7 in control group (A) and 1 C group (B). A: There are mainly well- differentiated spindle cells with whirled solid pattern and some mitotic figures (H & E, × 200). B: Tissue section taken 6 hours after treatment in experimental group received 1 coulomb. There are fully differentiated spindle cells without difference comparing control (H & E, ×400).

moderate fragmentation of cells by 24 h after ECT, showing the effectiveness of ECT (Fig. 7A, 7B). In 10 C group, the condensation of nucleus was seen by 6 h and 12 h after ECT and cell fragments formed from many tumor cell destroyed were well visible by 24 h after ECT (Fig. 8A, 8B, 8C).

3. Cellular apoptosis

The average number of apoptotic cells in control group by 6 h, 12 h, and 24 h was 7.0±1.0. The number of apoptotic cells in 1 C group by 6 h after ECT was 11.7±1.2, showing no significant difference from that of control group; however, the average numbers of apoptotic cells in 5 C group and 10 C group were 222.0±17.8 and 240.3±8.5, respectively, showing significantly high numbers compared with that of control group ($p<0.01$, $p<0.01$). Compared with control group, the number of apoptotic cells by 12 h after ECT in 1 C group was slightly higher but at no significant difference. The numbers of apoptotic cells in 5 C group and 10 C group were 210.7±15.1 and 261.2±9.9, showing significantly high number compared with that of control group ($p<0.01$, $p<0.01$). By 24 h after ECT, these numbers in 1 C group, 5 C group, and 10 C group were 87.3±3.5, 134.0±5.7, and 216.3±13.9, respectively, showing significant differences from that of control group ($p<0.05$, $p<0.01$, $p<0.01$) (Fig. 9).

Although apoptosis was not confirmed in control group using ApopTag® (Fig. 10A), it was shown at the site of electrodes in the experimental groups. In 1 C group, apoptosis was observed in some areas by

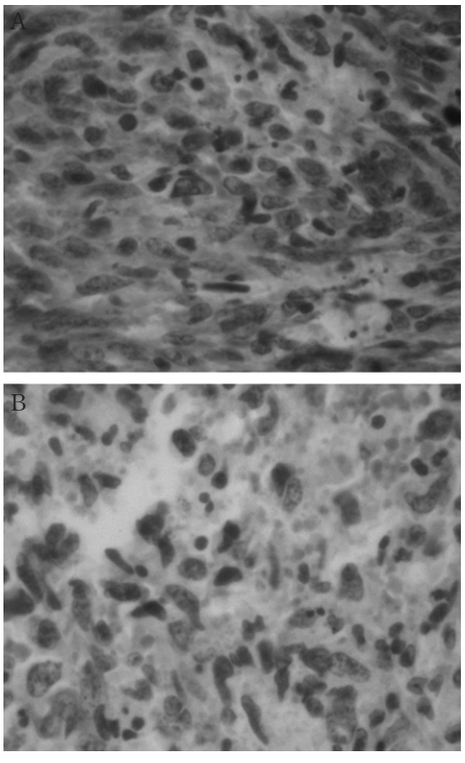

Fig. 7. Microscopic finding of squamous cell carcinoma (1×1 cm) occurring on the right upper thigh after inoculation of SCC-7 in 5 C group. A: Tissue section taken 6 hours after treatment. There are focal cellular apoptotic body of SCC (H & E, ×400). B: Tissue section taken 24 hours after treatment. There are destructive change including moderately fragmented and necrotic neoplastic cells (H & E, ×400).

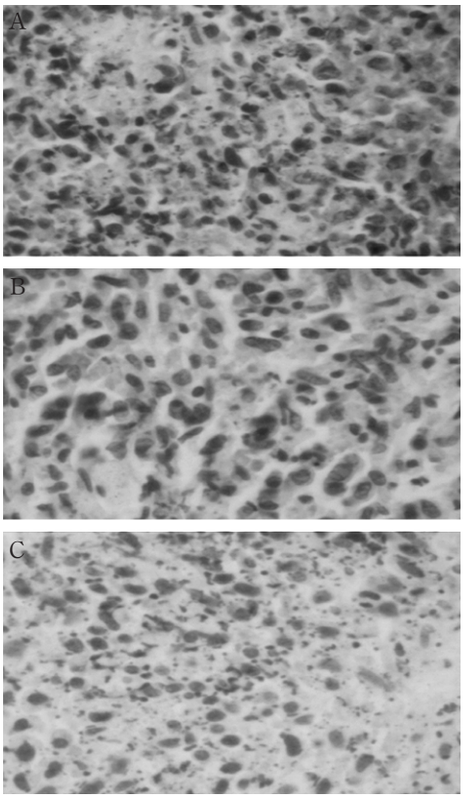

Fig. 8. Microscopic finding of squamous cell carcinoma (1×1 cm) occurring on the right upper thigh after inoculation of SCC-7 in 10 C group. A: Tissue section taken 6 hours after treatment. There are many pykontic neoplastic cells and also show some typical spindle cells with mitosis (H & E, ×400). B: Tissue section taken 12 hours after treatment. There are many destructive neoplastic cells (H & E, ×400). C: Tissue section taken 24 hours after treatment. There are diffuse, numerous cellular debri derived from neoplastic cells (H & E, ×400).

6 h and 12 h after ECT (Fig. 10B) and partially by 24 h. In 5 C group and 10 C group, apoptosis was seen significantly by 6 h, 12 h and 24 h after ECT (Fig. 10 C, 10D).

Discussion

It is known that cellular living environment would change significantly when slight electric current is applied in living tissue. In other words, the theoretical basis of ECT started from the idea that cellular apoptosis could be induced by inhibiting tumor cell growth by applying direct current to tumor cells, which are more sensitive to the surrounding environment compared with normal cells, to bring about electrochemical and electrophysiological changes in micro-electric circuit around tumor cells[21]. Through several years of study, Nordenstrom proposed that vascular inter-

stitial closed circuit (VICC) system that would maintain normal metabolism with electrochemical energy developed in blood and lymph and biologically closed electric circuit (BCEC) system that controls difference in electrochemical energy developed from normal metabolism by tissues and organs exist in the body. Furthermore, Nordenstrom claimed that tumor development results from damage to normal tissue so that direct current could be used to correct electrochemical unbalance around normal tissues to maintain balance in electrochemical energy, proposed the electric circuit hypothesis of VICC and BCEC systems, and reported that direct current would aid in tumor treatment[2)-3)].

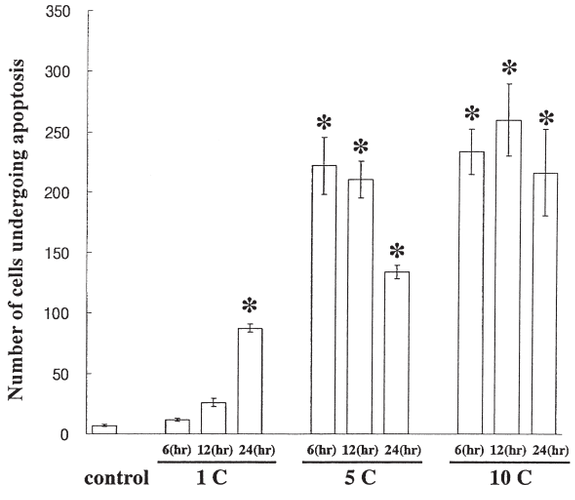

Fig. 9. The number of cells undergoing apoptosis after electrochemical therapy in each treatment and control groups (*p<0.05).

Based on this electric circuit hypothesis, Yen *et al*. examined the relationship between the amount of electric current and tumor treatment[22)]. After

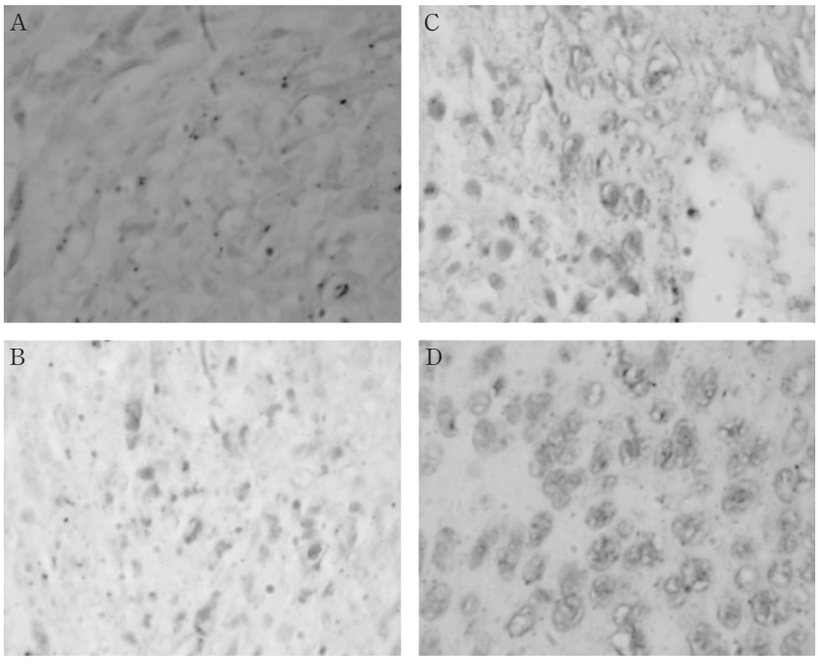

Fig. 10. Findings of apoptosis occurring in SCC-7 of C3H mouse after electrochemical therapy. ApopTag® (Harris hematoxylin stain. A: Control group, scattered with few apoptotic cells (×200). B: 1 C group with mild degree of apoptosis 6 hours after electrochemical therapy (×400). C: 5 C group with moderate degree in apoptosis 6 hours after electrochemical therapy (×400). D: 10 C group showing marked apoptosis 6 hours after electrochemical therapy (×400).

growing human KB cells *in vitro*, they performed ECT to examine the degree of cytotoxicity and reported that tumor growth is inhibited as the time depending upon ECT and the amount of electric charge. Furthermore, Turler *et al*. performed ECT after inducing metastasis in rats by injecting human rectal cancer cells into the liver and observed that treatment response was directly related with the amount of electric charge[23]. Also according to this study, the amount of electric charge was related with the rate of tumor growth. In other words, the inhibitory effect on tumor growth was not significant after ECT in 1 C group compared with control group, whereas it was significant in 5 C group and 10 C group even immediately after ECT. Despite the fact that a difference was present after ECT in 5 C group and 10 C group, the inhibitory effect on tumor growth was persistent for about one week after ECT but tumors grew gradually afterwards, suggesting that most tumor cells would undergo apoptosis by ECT but tumors would grow again due to viable tumor cells present out of the electrode's reach.

Upon examining histopathologic changes by 1 h and 24 h after ECT in mice injected with fibro sarcoma cells, Chou *et al*. reported various morphologic findings including unclear borders of cell membrane and nucleus, dilated blood vessels, and irregular nuclei by 1 h after ECT[24]. By 24 h, they reported structural damage to cells, vacuole formation in the nucleus and cytoplasm, the local condensation of chromosome, and loss of nucleus, showing the effectiveness of ECT within a short period of time. Also in this study, no significant difference was present between control group and 1 C group by 6 h, 12 h, and 24 h after ECT. In 5 C group on the other hand, tumor necrosis was confirmed with various changes by 6 h after ECT and fragmentation of cells progressed further and tumor necrosis was observed compared with 24 h after ECT. In 10 C group, the condensation of nucleus was present by 6 h and tumor cells were destroyed tovarious cell fragments seen by 24 h. In addition, no difference was seen in the degree of tumor necrosis by 6 h and 24 h after ECT. By 12 h on the other hand, no difference was seen in 1 C group but significant difference was seen in 5 C group and 10 C group with various tumor cells that underwent necrosis, showing the effectiveness of ECT.

It has been known that the histopathologic mechanism involved tumor cell destruction by ECT is the mechanism of tumor destruction by necrosis. Once electric current is applied to cells, elcetroanlysis to tissue fluid and electro osmosis of moisture occur in cells around the electrodes at the same time. pH around the cathode would go down to 1–2 and that around the anode would show strong basic environment with pH increased to 11–13. By doing so, chloride ions would be evolved from the cathode and hydrogen ions from the anode. The permeability of cell membrane would change in cells around the electrodes, destroying the living environment of tumor cells[25)–27)]. Due to the change in the living environment inside tumor cells, protein tissues within cells would undergo necrosis and the destruction of tumor cell nucleus and condensation necrosis of nucleus protein occur at the same time. H_2O and Na^+ would migrate from the cathode to the anode due to electro osmosis within cells, developing edema in tissues around the anode and dehydration around the cathode. Tumor cells would eventually undergo apoptosis through this process.

Apoptosis, another mechanism involved in the mechanism of tumor cell destruction, is natural cell death coming from the physiologic phenomenon that does not accompany the destruction of cell

membrane with a decrease in cell size and the condensation of nucleus. Apoptosis occurs even in healthy cells when they are exposed to the factors such as radiation and chemotherapy, and cytotoxic substances that destroy DNA. Indirect evidence suggests that apoptosis is increased when tumor growth is inhibited. The direct methods of determining the development of apoptosis in tumor cells include the observation under an optical microscope and an electron microscope of cell shrinkage, DNA condensation and fragmentation of nucleus and indirect methods include electrophoresis and TUNEL method[28]. We used TUNEL method in this study that is relatively simple and easy for clinical application.

According to the results of the present study related with apoptosis, we confirmed that apoptosis occurred mainly around the electrodes and that electric current directly damaged tumor cells according to histopathologic findings. Compared with control group, more apoptosis was seen in 1 C, 5 C, and 10 C groups as the amount of electric charge increased. Apoptosis was increased especially in 5 C and 10 C groups starting immediately after ECT, suggesting that electric current is directly related with tumor cell destruction and changes in tissue necrosis.

From the molecular biologic point of view related with cellular apoptosis, major changes coming from cytotoxic substances include DNA damage, single strand break or double strand break due to dissociation DNA-DNA or DNA-protein bridge and DNA phosphodiester bone frame[29),30)]. Thus, it would be important to determine the most crucial damage due to apoptosis after ECT. Although we could not determine differences in the frequency and the degree of expression of various changes after ECT due to apoptosis observed in this study, we find that the frequency of apoptosis was significantly increased in 5 C group and 10 C group within short period of time after ECT compared with control group. The fact that histopathologic findings of apoptosis matched with the degree of apoptosis frequency suggests that apoptosis coming from ECT is responsible for the mechanism of tumor cell destruction. Other than necrosis according to histopathologic findings from the previous study, the results of the present study on apoptosis probably are only the beginning. Additional molecular biologic studies are needed on the mechanism of cellular apoptosis due to ECT.

These results confirm the inhibitory effect of ECT on tumor growth in SCC-7 tumor and suggest that ECT is involved in the mechanism of tumor cell destruction by increasing apoptosis significant.

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