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Electrochemotherapy for Colorectal Cancer with Commonly Used Chemotherapeutic Agents in a Mouse Model

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We examined here the usefulness of electrochemotherapy against colorectal cancer (CRC) using a mouse model. Electropermeabilization profoundly increased the sensitivity of murine CRC, Colon 26, and MC38 cells to bleomycin (BLM) but not to 5-fluorouracil (5-FU) or to cisplatin (CDDP) *in vitro*. *In vivo* experiments revealed that electrochemotherapy with 5-FU, CDDP, or BLM was much more effective against CRC compared with the treatment of the drug alone. Electrochemotherapy with BLM or CDDP exhibited profound antitumor effects on subcutaneously established CRC in mice, and complete tumor regression was observed in five and four of eight animals, respectively. Electrochemotherapy with 5-FU also had an impact on CRC development, and complete cure was observed in one of eight animals. Subsequent analyses revealed that electropermeabilization significantly increased intratumoral amounts of BLM and CDDP but not 5-FU. These results indicate that electrochemotherapy may be a promising treatment modality against CRC.

KEY WORDS: electrochemotherapy; electroporation; colorectal cancer; bleomycin; cisplatin; 5-fluorouracil.

Colorectal cancer (CRC) is the fourth commonest form of cancer worldwide with an estimated 678,000 new cases yearly (1). High incidence rates are found in Western Europe, Australia, and North America, accounting for 151,000 new cases and 61,000 deaths yearly in the United States (2). Intermediate rates are found in Eastern Europe and the lowest rates are in sub-Sahara Africa (3). In Japan its incidence has been increasing rapidly over the last two decades, and it is now the second most common malignancy, following gastric cancer, accounting for 30,000 deaths yearly. Surgery will cure approximately 50% of patients, and the overall five year survival of patients with CRC is of the order of 50–55%, with deaths in the main being due to cancer spread (4). The disease is not uniformly fatal, although there are large differences in survival according to the stage of disease. It is estimated that there are nearly 400,000 deaths from CRC worldwide annually (5). Furthermore, there are a considerable number of patients who cannot undergo surgery due to severe complications, such as chronic heart failure, chronic renal failure, and chronic obstructive pulmonary diseases. Therefore, effective chemotherapy against CRC should be established for patients who cannot undergo surgery or have recurrent diseases. Chemotherapy with 5-fluorouracil (5-FU) may be of some value in individual cases but can be limited by side effects when used systemically. Conventional

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chemotherapy of inoperable or recurrent disease is unlikely to have any impact on survival (6, 7), although other forms of treatment, such as gene therapy, are now being intensively explored (8–11). Thus, in spite of intensive efforts for the treatment of CRC, there is still no satisfactory treatment that significantly improves the overall survival rate of patients with CRC.

Electrochemotherapy could provide an innovative therapeutic approach for the treatment of CRC. The combination treatment, which involves the administration of a chemotherapeutic agent followed by the delivery of electric pulses to the tumor, was termed electrochemotherapy by Mir et al (12). Under specific conditions, electropermeabilization is a reversible process that does not impair cell viability (13). The permeabilized state is transient and typically lasts on the order of minutes. The electric pulse permeabilizes, or electroporates, the tumor cell membrane, allowing the chemotherapeutic agent greater access to its intracellular site of action and consistently provides improved responses relative to treatment with drug alone. It has been shown that the *in vitro* cytotoxicity of bleomycin (BLM), netropsin, actinomycin D, and cisplatin (CDDP) was increased several-fold by exposing cells to short, intense electroporation (14–16).

Although the effectiveness of electrochemotherapy on various types of cancer has been demonstrated not only *in vitro* but also in animal models, current ongoing clinical trials of electrochemotherapy are limited to cutaneous and subcutaneous tumors, such as head and neck squamous cell carcinoma, melanoma, malignant lymphoma, and breast adenocarcinoma (17–20). However, electrochemotherapy should, in theory, be effective for the treatment of cancer of any histological type. In the present study, we investigated the usefulness of electrochemotherapy against CRC not only *in vitro* but also *in vivo*. 5-FU and CDDP were selected as chemotherapeutic agents for electrochemotherapy against CRC, because both drugs have been used most frequently in the clinic alone or in combination with other chemotherapeutic agents for the treatment of CRC. We also examined the effectiveness of electrochemotherapy with BLM for the treatment of CRC, because it has been shown that electropermeabilization results in markedly increased susceptibility to BLM in various types of cancer cells not only *in vitro* but also *in vivo* (21–25).

MATERIALS AND METHODS

Cell Culture. The highly metastatic murine colon adenocarcinoma cell line Colon 26 (26), which was originally established from a BALB/c mouse, was generously provided by the Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). The nonmetastatic dimethylhydrazine-induced murine colon adenocarcinoma cell line MC38 (27), which was established from a C57BL/6 mouse, was a generous gift from Dr N. Tomita (Osaka University Medical School, Osaka, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 0.3 mg/ml L-glutamine, 100 units/ml ampicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Animals. Twelve-week-old female BALB/c mice, weighing approximately 254 g, were purchased from Japan SLC, Inc. (Hamamatsu). Mice were kept in a conventional animal colony at 24°C and in a 12-h day–night light cycle. Animal experiments were performed with approved protocols and in accordance with recommendations for the proper care and use of laboratory animals.

Chemotherapeutic Agents. 5-FU and CDDP were purchased from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan) and Nippon Kayaku Co., Ltd. (Tokyo, Japan), respectively. BLM was a generous gift from Nippon Kayaku Co., Ltd. The agents were diluted to an appropriate concentration with phosphate-buffered saline (PBS).

***In Vitro* Cytotoxicity by Electrochemotherapy.** Because exponentially decaying electric pulses are widely used for transferring various materials, such as DNA and antibodies, into cells *in vitro*, we used an electric generator that generates exponentially decaying pulses for *in vitro* electrochemotherapy against CRC. Cells were suspended in the serum-free medium containing various concentrations of 5-FU (10 nM–770 μM), CDDP (0.02 nM–42 μM), or BLM (0.9 nM–67 μM) at a concentration of 2×10^4 cells/ml. Four hundred microliters of the cell suspension was seeded in a well of 48-well tissue culture plates, 400 μl of the medium with 20% FCS was added, and the plates cultured for four days. Four hundred microliters of the cell suspension with various concentrations of 5-FU, CDDP, or BLM was also gently injected into an electroporation chamber (800-μl capacity) with 12- $\times$ 21-mm stainless steel electrodes fixed 4 mm apart (Bio-Rad, Hercules, California). A single-pulse procedure was performed at a voltage of 750 V/cm with high capacity (960 μF) for *in vitro* electrochemotherapy, because in our preliminary experiments this condition was found to cause little cell damage with survival rates of both Colon 26 and MC38 cells being greater than 90%. An electric pulse was generated using a Gene Pulser (Bio-Rad). After the pulsing procedure, 400 μl of the cell suspension was collected from the electroporation chamber, seeded in a well of 48-well tissue culture plates, 400 μl of the medium with 20% FCS was added, and then cultured at 37°C in 5% CO_2 for four days. The number of viable cells was quantitated using an MTT tetrazolium (Sigma, St. Louis, Missouri) conversion assay as described previously (28). The number of viable cells was calculated using curve-fitting parameters based on the Marquardt and Siam method (29). The survival rate was determined by compar-

ing the number of viable cells cultured with and without chemotherapeutic agents.

Quantification of Intracellular Amounts of Chemotherapeutic Agents. Cells were suspended in the serum-free medium containing 154 μM 5-FU, 33 μM CDDP, or 13 μM BLM at a concentration of 1×10^7 cells/ml. Five hundred microliters of the cell suspension was seeded in a well of six-well tissue culture plates, 1.0 ml of the medium with 15% FCS containing 154 μM 5-FU, 33 μM CDDP, or 13 μM BLM was added, and the suspension cultured at 37°C in 5% CO_2 for 60 min. Five hundred microliters of the cell suspension with 154 μM 5-FU, 33 μM CDDP, or 13 μM BLM was also gently injected into an electroporation chamber (800-μl capacity), and a single-pulse procedure was performed at a voltage of 750 V/cm. After the pulsing procedure, 500 μl of the cell suspension was collected from the electroporation chamber, seeded in a well of six-well tissue culture plates, 1.0 ml of the medium with 15% FCS containing 154 μM 5-FU, 33 μM CDDP or 13 μM BLM was added, and then cultured for 60 min. The cells treated with and without the delivery of electric pulses were harvested, washed three times in PBS by centrifugation, and resuspended in 1.3 ml of PBS. The cell number was counted directly by a bright-field microscope. The cell suspensions were sonicated, completely lysed, centrifuged at 14,000 rpm for 30 min at 4°C to remove the cellular debris, and 1.0 ml of the supernatants were collected. 5-FU amounts of the cell lysates were estimated by the high-performance liquid chromatography assay as described previously (30). CDDP determinations of the cell lysates were made by the flameless atomic absorption spectrophotometric assay as described previously (31). BLM determinations were made by the microbiological assay as described previously (32).

***In Vivo* Electrochemotherapy Against CRC.** Because square-wave electric pulses are much more adapted to *in vivo* electrochemotherapy than exponentially decaying electric pulses, we used an electric pulse generator that generates square-wave pulses for *in vivo* electrochemotherapy against CRC. Colon 26 cells were suspended in PBS at a concentration of 2×10^7 cells/ml, and 100 μl of the cell suspension was inoculated subcutaneously into the flank regions of syngeneic BALB/c mice. When subcutaneous CRC tumors reached approximately 8 mm in diameter, animals were randomly separated into the PBS, 5-FC, CDDP, and BLM treatment groups and electrochemotherapy groups with PBS, 5-FU, CDDP, and BLM. Each group consisted of eight animals. Animals treated without electroporation were given an intratumoral injection of 100 μl of PBS, 38 mM 5-FU, 1.2 mM CDDP, or 3.4 mM BLM. These amounts of 5-FU, CDDP, and BLM correspond to approximately one tenth of 50% lethal doses to mice. Animals in the electrochemotherapy groups were given an intratumoral injection of PBS, 5-FU, CDDP, or BLM in the same doses as those in the chemotherapy groups, and received electric pulses 5 min after the injection of the chemotherapeutic agents. Animals were anesthetized with ether and received electric pulses (1000 V/cm, 99 μsec, 1 Hz, 8 pulses) that were delivered through two needle-shaped electrodes inserted subcutaneously on both sides of the protruding CRC tumor using an electropulsator (T820FE; BTX, Inc., San Diego, California) with an optimizer (BTX500; BTX). Tumor size was measured by a caliper every three or four days and tumor volume was estimated according to the formula: $V\ (mm^3) = A\ (mm) \times B\ (mm)^2/2$ (A = largest diameter; B = smallest diameter). Animals were killed when the tumor diameters exceeded 2 cm, when there was large tumor ulceration, or when there were other signs of animal distress.

Quantification of Intratumoral Amounts of Chemotherapeutic Agents. One hundred microliters of Colon 26 cells suspended in PBS (2×10^7 cells/ml) was inoculated subcutaneously into the flank regions of syngeneic BALB/c mice. When subcutaneous CRC tumors reached approximately 8 mm in diameter, animals received an intratumoral injection of 5-FU, CDDP, or BLM as described above. Animals were separated into electroporation-treated and untreated groups, and those in the electrochemotherapy groups received electric pulses 5 min after the injection of chemotherapeutic agents as described above. Each group consisted of four animals. Four hours after the treatment, animals were anesthetized with ether and perfused with 50 ml of PBS from the left ventricle which drained out from the right atrium to completely remove blood. Tumors were then resected, homogenized in 2 ml of PBS, and centrifuged at 14,000 rpm for 30 min at 4°C in a tabletop microfuge to remove the debris. 5-FU, CDDP, and BLM amounts in the supernatants were determined as described above, and standardized based on the protein content in the supernatants using the Bio-Rad protein assay kit.

Statistical Analysis. Values are expressed as means ± SD. All results were analyzed using StatView Software (Carlsbad, California). Paired analysis between the two groups was performed using the Student's t test. Results of the tumor development were analyzed by the analysis of variance (ANOVA) with Fisher's follow-up testing. Survival rates of animals were analyzed using the log-rank analysis of a Kaplan-Meier survival cure. Statistical significance was accepted when $P < 0.05$.

RESULTS

***In Vitro* Cytotoxicity of Electrochemotherapy on CRC Cells.** We first examined whether electropermeabilization enhanced the *in vitro* cytotoxicity of chemotherapeutic agents on CRC cells. Electropermeabilization did not significantly increase the sensitivity of Colon 26 and MC38 cells either to 5-FU or to CDDP. Conversely, electropermeabilization did profoundly increase the sensitivity of both CRC cell lines to BLM. The values of IC_{50}, defined as the concentration that inhibits growth by 50% compared with untreated control cells, for Colon 26 and MC38 cells are shown in Table 1. Although values of IC_{50} to 5-FU for Colon 26 and MC38 cells exhibited a little shift by electroporation, the differences were not statistically significant. Values of IC_{50} to CDDP for Colon 26 and MC38 cells also were not significantly shifted by electroporation. Conversely, the value of IC_{50} to BLM for Colon 26 cells was shifted from 10 μM to 4.6 nM by electroporation and that to BLM for

TABLE 1. *In Vitro* CYTOTOXICITY OF CHEMOTHERAPEUTIC AGENTS WITH AND WITHOUT ELECTROPORATION

Cell line	*Electroporation (EP)*	*5-FU* (nM)*	*CDDP* (nM)*	*BLM* (nM)*	
In vitro cytotoxicity (IC_{50})†					
Colon 26	(−)	480 ± 140	3,700 ± 1,000	10,000 ± 2,300	‡
Colon 26	(+)	330 ± 77	3,100 ± 800	4.6 ± 1.3	‡
MC38	(−)	770 ± 200	1,100 ± 250	12,000 ± 2,500	‡
MC38	(+)	570 ± 130	870 ± 180	3.0 ± 0.8	‡
Therapeutic index§					
Colon 26 EP(−)/EP(+)		1.5	1.2	2,200	
MC38 EP(−)/EP(+)		1.4	1.3	4,000	

*Results are means ± SD of four separate experiments.
†IC_{50} is defined as the concentration required for 50% cytotoxicity.
‡Values are significantly different at $P < 0.001$ using Student's *t*-test.
§Therapeutic index is expressed as IC_{50} for cells without EP/IC_{50} for cells with EP.

MC38 cells was shifted from 12 μM to 3.0 nM by electroporation. The therapeutic indices, expressed as the IC_{50} for CRC cells without electroporation/IC_{50} for CRC cells treated with electroporation, were increased only 1.2- to 1.5-fold by electrochemotherapy with 5-FU or CDDP. Conversely, the therapeutic indices in CRC cells were increased more than 2,000-fold by electrochemotherapy with BLM.

Intracellular Amounts of Chemotherapeutic Agents With and Without Electroporation. It was then examined whether intracellular amounts of chemotherapeutic agents were increased by electroporation. Intracellular amounts of chemotherapeutic agents in CRC cells with and without electroporation are shown in Table 2. Although intracellular amounts of 5-FU in Colon 26 cells were increased from 17.8 to 21.5 ng/10^6 cells by electroporation, the difference was not statistically significant. Conversely, intracellular amounts of CDDP and BLM in Colon 26 were significantly increased by electroporation, resulting in 2.0- and 3.7-fold higher intracellular amounts of CDDP and BLM, respectively.

Antitumor Effects of Electrochemotherapy on CRC Tumors. Effectiveness of electrochemotherapy against CRC was evaluated using a murine CRC model. Syngeneic BALB/c mice were inoculated subcutaneously with Colon 26 cells. When animals developed a subcutaneous CRC tumor reaching approximately 8 mm in diameter, they were randomly separated into various treatment groups as described in Materials and Methods. There were no significant differences in tumor volume at the time of the initiation of the treatment among the groups. Animals that received electric pulses after the intratumoral injection of PBS developed rapidly growing tumors, and there were no significant differences in tumor volume between PBS and PBS plus electroporation groups, indicating that electroporation itself did not have any impact on CRC growth (Figure 1).

Antitumor effects of the chemotherapeutic agent alone and in combination with electroporation were then examined. Although animals in the 5-FU treatment group exhibited a delayed tumor growth and the tumor volume was significantly smaller than that of animals in the PBS treatment group, significant tumor regression was not observed (Figure 2). CRC tumors of animals in the electrochemotherapy with 5-FU group became significantly smaller than those before the treatment and were significantly smaller than those of animals in the 5-FU treatment group (Figure

TABLE 2. INTRACELLULAR AMOUNTS* OF CHEMOTHERAPEUTIC AGENTS WITH AND WITHOUT ELECTROPORATION

Chemotherapeutic agent	*Electroporation*		*Ratio of electroporation(+)/ electroporation(−)*
	(−)	*(+)*	
5-FU	17.8 ± 5.7	21.5 ± 3.2	1.2
CDDP	9.1 ± 0.6†	18.6 ± 3.8†	2.0
BLM	312.6 ± 23.7†	1156.7 ± 137.2†	3.7

*Intracellular amounts of chemotherapeutic agents are expressed as ng/10^6 cells and values are means ± SD of four separate experiments.
†Values between electroporation-treated and untreated groups are significantly different at $P < 0.001$, using Student's *t* test.

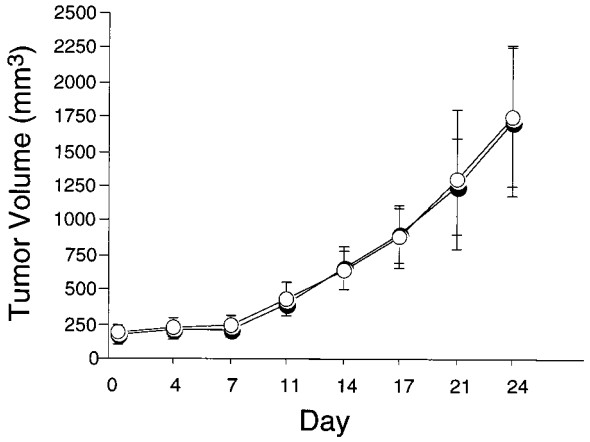

Fig 1. Antitumor effects of electroporation on CRC. Colon 26 cells were inoculated subcutaneously into the flank regions of syngeneic BALB/c mice. When subcutaneous CRC tumors reached approximately 8 mm in diameter, animals were treated with intratumoral injection of PBS alone (open circles) or in combination with electroporation (closed circles) on day 0. Both groups consisted of eight animals. Tumor volume was estimated every three or four days after the treatment. Each data point represents the mean ± SD of eight animals. Data are not shown in the figure after any of animals in either group died or was killed due to the excessive size of the CRC tumor.

2). Although subcutaneous CRC tumors of animals in the CDDP treatment group also exhibited significantly delayed tumor growth compared with those of animals in the PBS treatment group, significant tumor regression was not observed (Figure 3). CRC tumors of animals in the electrochemotherapy with CDDP group became significantly smaller than those before the treatment and were significantly smaller than

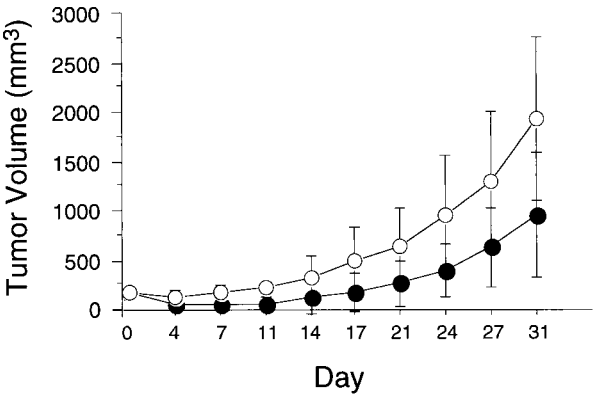

Fig 2. Antitumor effects of electrochemotherapy with 5-FU on CRC. Animals were treated with intratumoral injection of 5-FU alone (open circles) or with electrochemotherapy with 5-FU (closed circles) on day 0. See the legend of Figure 1 for experimental details. Each data point represents the mean ± SD of eight animals. Tumor volume was significantly different between the groups at $P = 0.0155$, using the Fisher's follow-up testing.

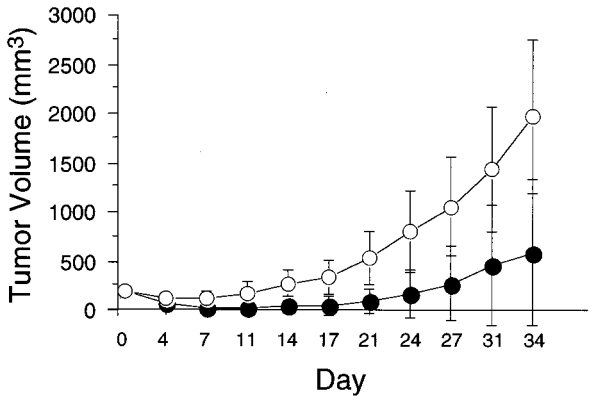

Fig 3. Antitumor effects of electrochemotherapy with CDDP on CRC. Animals were treated with intratumoral injection of CDDP alone (open circles) or with electrochemotherapy with CDDP (closed circles) on day 0. See the legend of Figure 1 for experimental details. Each data point represents the mean ± SD of eight animals. Tumor volume was significantly different between the groups at $P = 0.002$, using the Fisher's follow-up testing.

those of animals in the CDDP treatment group (Figure 3). Although animals in the BLM treatment group also exhibited delayed tumor growth compared with those in the PBS treatment group, there were no significant differences in tumor volume between the PBS and BLM groups (Figure 4). In contrast, CRC tumors of animals in the electrochemotherapy with BLM group regressed significantly compared with those before the treatment and were significantly smaller than those of animals in the BLM treatment group (Figure 4).

Among the 5-FU, CDDP, and BLM treatment groups without electroporation, only the BLM treat-

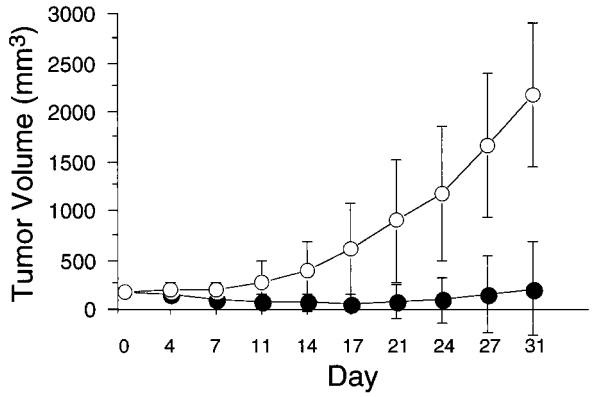

Fig 4. Antitumor effects of electrochemotherapy with BLM on CRC. Animals were treated with intratumoral injection of BLM alone (open circles) or with electrochemotherapy with BLM (closed circles) on day 0. See the legend of Figure 1 for experimental details. Each data point represents the mean ± SD of eight animals. Tumor volume was significantly different between the groups at $P = 0.0004$, using the Fisher's follow-up testing.

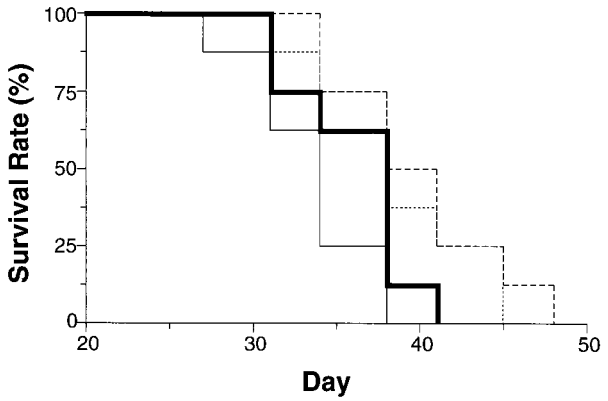

Fig 5. Survival rates of animals with a subcutaneously established CRC tumor treated with an intratumoral injection of PBS, 5-FU, CDDP, or BLM. Animals were given an intratumoral injection of PBS (thin line), 5-FU (dotted line), CDDP (dashed line), or BLM (bold line) on day 0. See the legend of Figure 1 for experimental details. Survival rate of animals in the PBS group was significantly lower than that of animals in the 5-FU and CDDP treatment groups, using the log-rank analysis.

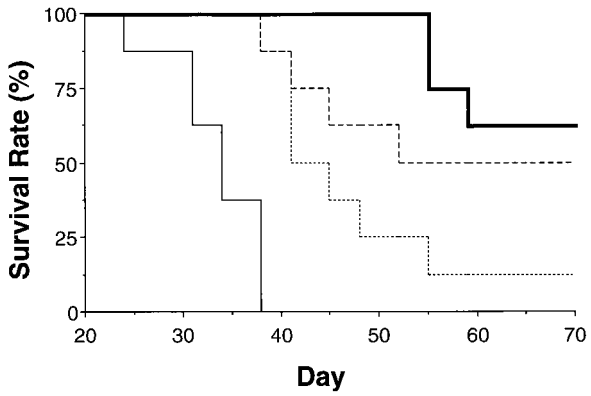

Fig 6. Survival rates of animals with a subcutaneously established CRC tumor treated with electrochemotherapy. Animals were treated with electrochemotherapy with PBS (thin line), 5-FU (dotted line), CDDP (dashed line), or BLM (bold line) on day 0. See the legend of Figure 1 for experimental details. Survival rate of animals in the electrochemotherapy with PBS group was significantly lower than that of animals in the electrochemotherapy with 5-FU, CDDP, and BLM groups, using the log-rank analysis. Survival rate of animals in the electrochemotherapy with 5-FU group was significantly lower than that of animals in the electrochemotherapy with BLM group, using log-rank analysis.

ment group failed to exhibit a significant antitumor effect on CRC tumors compared with the PBS treatment group. The strongest antitumor effect on CRC was observed in the CDDP treatment group. There were, however, no significant differences in tumor volume among these three chemotherapy groups. In contrast, all electrochemotherapy groups exhibited significant antitumor effects on CRC tumors compared with the electroporation with PBS group. Furthermore, antitumor effects induced by electrochemotherapy with 5-FU, CDDP, or BLM were much more profound than those induced by chemotherapy with 5-FU, CDDP, or BLM. Interestingly, the best antitumor effect was observed in the electrochemotherapy with BLM group and the second one was in the electrochemotherapy with CDDP group. Tumor volume of animals treated with electrochemotherapy with BLM was significantly smaller than that of animals treated with electrochemotherapy with 5-FU. Significant differences in tumor volume were not observed between the electrochemotherapy with 5-FU and CDDP groups or between the electrochemotherapy with CDDP and BLM groups.

Survival Rates of Animals with CRC Tumors Treated with Chemotherapy or Electrochemotherapy. Survival rates of animals with CRC tumors treated with intratumoral injections of chemotherapeutic agents are shown in Figure 5. The animal with the shortest survival period treated with an intratumoral injection of PBS died on day 27 after the treatment, and all animals died or were killed due to the excessive size of the subcutaneous CRC tumors within 38 days. Survival rate of animals injected intratumorally with BLM was not significantly different compared with that of animals injected intratumorally with PBS. The animal with the shortest survival period died on day 31, and all animals died or were killed within 41 days after the BLM treatment. Survival rates of animals in the 5-FU and CDDP treatment groups were significantly higher than those of animals in the PBS treatment group. The animal with the shortest survival period in the 5-FU treatment group died on day 31 after the treatment and all animals died or were killed within 45 days. Animals with the shortest survival period in the CDDP treatment group died on day 34, and all died or were killed within 48 days after the treatment. Although the survival period of animals in the CDDP treatment group was the longest, followed by that of animals in the 5-FU treatment group, survival rates of animals among the 5-FU, CDDP, and BLM treatment groups were not significantly different.

Survival rates of animals treated with electrochemotherapy with 5-FU, CDDP, or BLM are shown in Figure 6. The animal with the shortest survival period in the electroporation with PBS group died on day 24 after the treatment and all animals died or were killed due to the excessive size of the subcutaneous CRC tumors within 38 days. Survival rates of animals treated with electrochemotherapy with 5-FU, CDDP, or BLM were significantly higher than those of ani-

TABLE 3. INTRATUMORAL AMOUNTS* OF CHEMOTHERAPEUTIC AGENTS WITH AND WITHOUT ELECTROPORATION

Chemotherapeutic agent	*Electroporation*		*Ratio of electroporation(+)/ electroporation(−)*
	(−)	*(+)*	
5-FU	39.0 ± 11.1	45.5 ± 14.8	1.2
CDDP	4.7 ± 1.3†	18.4 ± 2.7†	3.9
BLM	BT‡	26.3 ± 11.7	NE§

*Intratumoral amounts of chemotherapeutic agents are expressed as ng/mg protein and values are means ± SD of four CRC tumors.
†Values between electroporation-treated and untreated groups are significantly different at $P < 0.001$, using Student's *t* test.
‡BT, below threshold of 0.1 ng/mg protein of BLM.
§NE, not estimable.

mals treated with electroporation with PBS. Survival rates of animals treated with electrochemotherapy with 5-FU, CDDP, or BLM were also significantly higher than those of animals that were given an intratumoral injection of 5-FU, CDDP, or BLM without electroporation. The animal with the shortest survival period in the electrochemotherapy with 5-FU group died on day 38 after the treatment. Complete regression of an established CRC tumor was observed in one of eight animals in the electrochemotherapy with 5-FU group. Although survival rate of animals treated with electrochemotherapy with CDDP was higher than that of animals treated with electrochemotherapy with 5-FU, the difference was not statistically significant. Complete cures of an established CRC tumor were observed in four of eight animals in the electrochemotherapy with CDDP groups. In contrast to the results of chemotherapy without electroporation, animals in the electrochemotherapy with BLM group exhibited the highest survival rate among all groups. The survival rate of animals treated with electrochemotherapy with BLM was significantly higher than that of animals treated with electrochemotherapy with 5-FU, and complete cures were achieved in five of eight animals. There were no significant differences in survival rate between the electrochemotherapy with CDDP and BLM groups.

Intratumoral Amounts of Chemotherapeutic Agents with and Without Electroporation. We then examined whether electropermeabilization increased the intratumoral amounts of chemotherapeutic agents in CRC tumors. Syngeneic BALB/c mice carrying an established subcutaneous CRC tumor were given an intratumoral injection of 5-FU, CDDP, or BLM, and half of the animals were then given electric pulses at the tumor site. Intratumoral amounts of chemotherapeutic agents with and without electroporation are shown in Table 3. Although intratumoral amounts of 5-FU in CRC tumors were increased from 39.0 to 45.5 ng/mg protein by electroporation, the difference was not statistically significant. Conversely, intracellular amounts of CDDP in CRC tumors were significantly increased by electroporation, resulting in a 3.9-fold higher intratumoral level of CDDP. Furthermore, although intratumoral amounts of BLM in CRC tumors were below the threshold of 0.1 ng/mg protein without electroporation, they were increased profoundly by electroporation and the mean level of intratumoral BLM amounts was 26.3 ng/mg protein.

DISCUSSION

We have demonstrated here that the delivery of electric pulses rendered CRC cells much more susceptible to BLM toxicity by increasing intracellular amounts of BLM in CRC cells. Although intracellular amounts of CDDP in CRC cells were also significantly increased by electroporation, electropermeabilization did not significantly increase the *in vitro* sensitivity of CRC cells to CDDP. Furthermore, it was shown that electropermeabilization did not increase intracellular amounts of 5-FU in CRC cells and had no impact on the *in vitro* cytotoxicity of 5-FU to CRC cells. However, *in vivo* results of electrochemotherapy against CRC were more encouraging than *in vitro* results. Although intratumoral amounts of 5-FU were not increased significantly by electroporation, electrochemotherapy enhanced antitumor effects of 5-FU on established CRC tumors. Furthermore, intratumoral amounts of CDDP and BLM were increased significantly by electroporation, and electrochemotherapy with CDDP or BLM exhibited profound antitumor effects on CRC tumors, resulting in complete regression of established CRC tumors in approximately 50% of animals.

Although 5-FU has been the key drug for the

treatment of CRC, it was shown here that electrochemotherapy did not enhance the *in vitro* cytotoxicity of 5-FU to CRC cells. Furthermore, electrochemotherapy with 5-FU on established CRC tumors were not so effective as electrochemotherapy with CDDP or BLM. Recently, gene therapy with suicide genes has been intensively investigated. Among a number of suicide gene/prodrug combinations, bacterial cytosine deaminase (*CD*) gene/5-fluorocytosine (5-FC) is one of the best-characterized systems. The enzyme encoded by the *CD* gene converts relatively nontoxic 5-FC into the toxic metabolite 5-FU. It has been shown that the *CD*/5-FC system can kill not only *CD*-transduced tumor cells but also untransduced neighboring ones. 5-FU generated from 5-FC by *CD*-transduced cells mediated this phenomenon termed the bystander effect. It has been shown that direct cell-to-cell contact is not required for the bystander effect induced by the *CD*/5-FC system (30, 33–35). It is, therefore, considered that 5-FU is freely diffusible across the cellular membrane and spreads from *CD*-transduced cells to adjacent tumor cells that do not express the *CD* gene. This property that 5-FU can readily pass through the cellular membrane may be the reason why electropermeabilization did not increase intracellular or intratumoral amounts of 5-FU and why electrochemotherapy with 5-FU on CRC was not so effective as electrochemotherapy with CDDP or BLM.

It has been shown that CDDP cytotoxicity was potentiated by increasing cellular accumulation of CDDP, because the cellular membrane is a barrier through which CDDP must enter the cells (36–38). It has been shown that electropermeabilization of human cervical carcinoma NHIK 3032 cells immediately before or during exposure to CDDP potentiated CDDP cytotoxicity three-fold (14). Similar results were obtained on CDDP-sensitive and -resistant murine fibrosarcoma RIF-1 cells, where electrochemotherapy with CDDP increased cell killing 1.9-fold in CDDP-sensitive and 2.3-fold in CDDP-resistant cells (39). Furthermore, it has been shown that permeabilization by electroporation of murine melanoma cells potentiated CDDP cytotoxicity up to 70 times *in vitro* (40). We have also demonstrated here that electropermeabilization increased intracellular amounts of CDDP in CRC cells 2.0-fold and intratumoral amounts of BLM in CRC tumors 3.7-fold. Furthermore, it was shown here that electrochemotherapy significantly enhanced the antitumor effects of CDDP on CRC, resulting in complete regression of established CRC tumors in 50% of the animals. These results indicate that electrochemotherapy with CDDP may be of considerable use for the treatment of CRC.

BLM is a water-soluble glycopeptidic antibiotic discovered by Umezawa et al (41) and is currently used in the treatment of head and neck squamous cell carcinoma, Hodgkin and non-Hodgkin lymphomas, and testicular carcinoma. BLM is known to have very few side effects and, unlike many other chemotherapeutic agents, it is devoid of myelotoxicity, which is the most serious side effect in chemotherapy against cancers. However, because of its low effectiveness, BLM has not been administered alone but has been associated with many other chemotherapeutic agents. The major reason for its limited antitumor effectiveness is the hampered transport of BLM through the plasma membrane. However, it has been shown that once inside the cell, BLM possesses a very potent intrinsic cytotoxicity, inducing single- and double-strand DNA breaks (42). To facilitate the entry of BLM into cells, various approaches have been exploited (15, 43, 44). Among them, electropermeabilization has been shown to be effective to enhance the permeability of BLM through the plasma membrane into cells. Use of electropermeabilization to increase BLM uptake into the cells and consequently to increase the antitumor effects has been shown *in vitro*, *in vivo*, and in clinical trials (12, 17–25). Since BLM is a potent cytotoxic drug once inside the cell, it has been shown that, regardless of the histological type, tumors respond very well to electrochemotherapy with BLM. In clinical trials, responses 50–100% were reported in basal cell carcinoma, malignant melanoma, head and neck squamous cell carcinoma, and breast adenocarcinoma tumors treated with electrochemotherapy with BLM (17–20). We have shown here that electropermeabilization profoundly increased not only intracellular amounts of BLM in CRC cells but also intratumoral amounts of BLM in CRC tumors. Importantly, electrochemotherapy with BLM was shown to be most effective for the treatment of CRC not only *in vitro* but also *in vivo*. Antitumor effect of electrochemotherapy with BLM was significantly stronger than that of electrochemotherapy with 5-FU, and the survival rate of animals treated with electrochemotherapy with BLM was also significantly higher than that of animals treated with electrochemotherapy with 5-FU. Although electrochemotherapy with BLM was more effective against CRC tumors than electrochemotherapy with CDDP, the difference was not statistically significant. These results indicate that electrochemotherapy with BLM

may be an extremely promising treatment modality against CRC.

It has already been shown that electrochemotherapy with BLM significantly inhibited the growth of subcutaneously implanted murine and human CRC tumors in mice (45, 46). We have shown here that electrochemotherapy significantly enhanced antitumor effects of the chemotherapeutic agents on CRC, resulting in some cures of established CRC tumors. Although we used a pair of needle-shaped electrodes for *in vivo* electrochemotherapy against CRC, it is known that the electric field generated by two needles is not distributed homogeneously, resulting in the fact that a significant part of the tumor may not be exposed to electric fields with enough intensity. In fact, when we repeated electrochemotherapy with BLM against subcutaneously established CRC tumors, complete cures were achieved in all animals used in the experiment (47). Therefore, the use of optimal electrode geometry for electric field application will result in more effective antitumor effects. These results indicate that electrochemotherapy can be successfully employed for the treatment of internal cancers, including CRC, as well as superficial cancers. Furthermore, although we have already shown that electropermeabilization did not significantly enhance the antitumor effects of mitomycin or adriamycin on CRC *in vitro* (48), these assessments should be reestimated, because there are often discrepancies between *in vitro* and *in vivo* results as observed here in electrochemotherapy with CDDP. Although necessary devices have to be developed to apply electrochemotherapy to the treatment of CRC, it may be achievable by producing a pair of needle electrodes that can be manipulated through a fiberoptic endoscope. Furthermore, electrochemotherapy may be of considerable use for the treatment of patients with CRC who are not eligible for other therapies, including surgery due to their physical status, such as chronic respiratory failure, chronic renal failure, and chronic hepatic failure. Although more investigations have to be carried out to prove the usefulness of this option for CRC therapy, electrochemotherapy may be one of the most promising approaches and may open up new avenues for the treatment of CRC.

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