Experimental Studies of Anti-tumor Effect Induced by Microwave Tumor Coagulation

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Introduction

Microwave has been utilized for medical diathermy. Recently, a microwave tissue coagulator (MTC), which is an apparatus used for coagulating tissues by converging the energy of microwaves in as small an area as possible, was developed27. One of the characteristics of MTC is non-carbonizing effect on tissues. Since MTC is favorably compared with an electrocautery and a laser with regard to hemostatic performance, it is useful for operation, particularly on parenchymal organs such as the liver28,29,30 and spleen31, and an increasing number of surgeons have become attentive to its clinical usefulness. In the treatment of bleeding or stenosis from gastric, rectal or esophageal cancer, endoscopically guided microwave irradiation done to destroy tumor tissues in a focal region assures us of a satisfactory result1,15,32. Furthermore, microwave coagulation therapy intended for tumor tissue destruction has begun to be attempted as one of multidisciplinary treatments of unresectable malignancies involving the digestive tracts and liver metastases.

In the present experiment, the anti-tumor effect induced by microwave coagulation therapy was studied.

Materials and Methods

APPARATUS

The apparatus used for microwave coagulation therapy was composed of 1) the generator which produced microwaves of 2,450 MHz in frequency and 12 cm in wavelength, 2) the coaxial cable which transmitted microwaves so produced, and 3) the operating monopolar antenna of 10 mm in diameter at the base and 7.8 mm in length.

ANIMALS

Seven to nine-week-old male inbred BALB/c mice were offered for the experiments.

TUMORS

The tumors used in the experiments were as follows; Meth A18, a transplantable fibrosarcoma.

Key words: Microwave Coagulation Therapy, Anti-tumor Effect, Microwave Tumor Coagulation, Microwave immunotherapy.

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induced in a BALB/c mouse by 3-methyl-cholanthrene and Crocker tumor detected in albino mouse and generally known as Sarcoma 180 (S-180). Tumor cells were maintained by serial passage in ascitic form in BALB/c mice, and were used after complete removing of red blood cells.

EVALUATION OF ANTI-TUMOR EFFECT

Living tumor cells (1 × 10⁶ cells) were injected into the abdominal cavity of treated or immunized mice for challenge, and the anti-tumor effect was evaluated in consideration of the 90-day-survival rate or survival period.

PREPARATION OF MICROWAVE-TREATED TUMOR CELLS

A dish of 3 cm in diameter holding 6 ml of a Meth A (3 × 10⁷ cells) suspension in Roswell Park Memorial Institute Medium 1640 (RPMI-1640) (Nissui Pharmaceutical Co., Tokyo, Japan) or phosphate buffered saline (PBS) was buried in heat insulating material, and the monopolar antenna was immersed in the suspension (Fig. 1.). Microwave-treatment was performed while the antenna was being moved gently. The electric output was 20 watts. The degree of treatment was assessed on the basis of the viability of Meth A microscopically determinanced by the use of 0.5% trypan blue.

FRACTIONATION OF MICROWAVE-TREATED CELL SUSPENSION

The microwave-treated suspension was centrifuged at 1,000 rpm for 5 minutes. The resultant supernatant was taken as a liquid fraction. The precipitate as a cellular fraction was washed 3 times with RPMI-1640 or PBS, and was used after addition of an appropriate amount of RPMI-1640 or PBS to regain the original volume.

PREPARATION OF SPLENIC LYMPHOCYTES

The mouse spleen excised under an aseptic condition was swollen by infusion of cooled RPMI-1640, and the spleen was pecked scrupulously with dental tweezers to obtain splenic cells. The suspension of those cells was aspirated into a syringe and poured out 3 to 4 times, so that individual cells came apart. Then, a suspension of splenic lymphocytes was prepared by use of Cedarlane lympholyte-M cell separation medium.

![Fig. 1. Preparation of microwave-treated tumor cells](image)
TREATMENT WITH ANTI-Thy 1, 2 ANTIBODY AND COMPLEMENT

The suspension of splenic lymphocytes, the antibody, and the complement were diluted with RPMI-1640 containing 25 mM HEPES buffer (pH 7.3, GIBCO) and 0.3% bovine serum albumine (Sigma Chemical Co., Ltd.). The diluted suspension of splenic lymphocytes (1×10⁷ cells/ml) was incubated at 4°C for 60 minutes after addition of a 1/20 volume of anti-Thy 1,2 monoclonal antibody (Cedarlane Laboratories, Ltd.). The mixture was incubated at 37°C for 60 minutes, and was washed a time, and then was incubated at 37°C for 60 minutes after addition of a 1/10 volume of Low-Tox-M RABBIT Complement (Cedarlane Laboratories, Ltd.), and was washed 3 times with PBS. Thereafter, dead cells were removed by the use of Cedarlane lympholyte-M cell separation medium.

WINN ASSAY

According to the method of WINN⁴, splenic lymphocytes were mixed with Meth A (1×10⁴ cells) in 0.2 ml PBS (lymphocyte: Meth A=23 : 1). The mixture solution was immediately injected subcutaneously into the flank of BALB/c mice. The weight of a tumor mass was measured 14 days after the tumor cell transplantation. The anti-tumor activity was expressed in terms of the percent growth inhibition of the tumor.

Results

1) ANTI-TUMOR EFFECT INDUCED BY MICROWAVE COAGULATION FOR SOLID TUMOR¹⁷

A solid tumor was produced by transplanting Meth A(4×10⁶ cells) into the subcutis of a mouse hind limb. Fourteen days after transplantation, microwave coagulation treatment (20 watts, 4×15 sec) was performed (microwave group) (Fig. 2.), or the tumor-bearing limb was amputated (amputation group). These groups were compared with the control group (without microwave coagulation or amputation) in the following items:

A) Anti-tumor effect 2 weeks later: The 90-day-survival rates of experimental animals

![Figure 2. Microwave coagulation treatment (20 watts, 15 sec) for solid tumor (from four directions and four times in total)
were 32% and 27% in the microwave group and the amputation group, respectively. The former rates was significantly higher than that (0%) in the control group (p<0.05, by $\chi^2$-test), but the latter was not (Fig. 3).

B) Anti-tumor effect in the presence of many residual tumor cells (ex. peritoneal carcinomatosis): The survival time of experimental animals (in days) was significantly longer in the microwave group than that in the control group (p<0.05, by Mann-Whitney test). By contraries, there was no significant difference in the survival time between the amputation group and the control (Fig. 4.).

2) TRANSPLANTING RATE OF MICROWAVE-TREATED TUMOR CELLS (safety of those cells used for immunization)
The suspension of microwave-treated Meth A (1 × 10^6 cells, 0.2 ml) was injected intraperitoneally, and the survival rate (as the transplanting rate) was determined 90 days after the injection. In case the viability of injected Meth A was suppressed below 48% by microwave irradiation, all mice concerned could survive more than 90 days (tumor transplanting rate: 0%). This may be construed as meaning that tumor cells (Meth A) whose viability is lower than 48% can be used safely for immunization (Table 1).

The effect of temperature on the viability of microwave-treated tumor cells was studied. When the temperature of the suspension of tumor cells (Meth A) rose to 50–60°C under microwave irradiation, their viability was lowered sharply. When it was about 1%, the suspension showed its temperature of about 63°C (Fig. 5).

3) CANCEROCIDAL EFFECT OF INTRAPERITONEAL IMMUNIZATION WITH MICROWAVE-TREATED TUMOR CELLS AND HEAT-DENATURALIZED TUMOR CELLS

In Group 3-A, Meth A (1 × 10^6 cells, cell viability: 0.9%, RPMI-1640 suspension: 0.2 ml, temperature: about 63°C) treated with microwaves in a dish was infused intraperitoneally. In Control 3-A, RPMI-1640 (0.2 ml) treated with microwaves for the same period of time was used instead of Meth A. In Group 3-B, Meth A (1 × 10^6 cells, viability: 1%, RPMI-1640 suspension: 0.2 ml, temperature: about 62°C) subjected to hot water bathing a test tube was infused. In Control 3-B, RPMI-1640 (0.2 ml) subjected to hot water bathing was used.

In Group 3-A, the 90-day-survival rate was 27% and the survival time was significantly longer than that in Control 3-A (p<0.01, by Mann-Whitney test). There was no significant

<table>
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<tr>
<th>Viability of Transplanted Meth A (%)</th>
<th>Number of Mice</th>
<th>Survival Rate of Mice more than 90 Days (%)</th>
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<tr>
<td>85</td>
<td>10</td>
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difference in the survival time between Group 3-B and Control 3-B (Fig. 6).

4) CANCEROCIDAL EFFECT OF INTRAPERITONEAL IMMUNIZATION WITH FRACTIONS OF MICROWAVE-TREATED TUMOR CELL SUSPENSION

The microwave-treated Meth A suspension (cell viability: 0.9%) was separated into a cellular fraction and a liquid fraction. These fractions were adjusted to the cell count of $1 \times 10^6$ and/or the volume of 0.2 ml, and both or either of them were injected intraperitoneally. Immunization was performed with both fractions in Group 4-A, with the liquid fraction alone in Group 4-B, and with the cellular fraction alone in Group 4-C. On the other hand, two control groups were provided, one called Control 4-A/4-B(control for Group 4-A or Group 4-B) and the other called

![Fig. 5. Effect of temperature on viability of microwave treated tumor cells.](image)

**Fig. 5.** Effect of temperature on viability of microwave treated tumor cells.

![Fig. 6. Cancerocidal effect of one intraperitoneal immunization with microwave-treated tumor cells and with tumor cells subjected to hot water bathing.](image)

**Fig. 6.** Cancerocidal effect of one intraperitoneal immunization with microwave-treated tumor cells and with tumor cells subjected to hot water bathing. On the 21st day after immunization, living tumor cells ($1 \times 10^6$) were injected intraperitoneally (Challenge).

*Survival time (in days) was significant at $p<0.01$ by Mann-Whitney test as compared with control group.*
Control 4-C (control for Group 4-C). In Control 4-A/4-B, RPMI-1640 (0.2 ml) treated with microwaves for the same period was used and, in Control 4-C, untreated RPMI-1640 was used.

In Group 4-A, the survival time was significantly longer than that in Control 4-A/4-B (p<0.01, by Mann-Whitney test). In Group B, no significant difference was observed by Mann-Whitney test, but such a difference was detected by Student t-test (p<0.05) or Kolmogorov-Smirnov test (p<0.05). In Group 4-C, the survival time was not significantly different from that in Control 4-C (Fig. 7.).

5) TRANSPLANTING RATES OF INDIVIDUAL FRACTIONS OF MICROWAVE-TREATED TUMOR CELL SUSPENSION

Both or either of the cellular fraction and the liquid fraction, which were adjusted to the cell count of $1 \times 10^6$ and/or the liquid volume of 0.2 ml, were injected into the flank subcutis. In the mice given the liquid fraction alone (n=10) or both fractions (n=10), no tumor mass was formed. In contrast, tumors were formed in 80% of the mice given the cellular fraction alone (n=10). Thus, the liquid fraction exerted an inhibitory effect on tumor growth.

6) CANCEROCIDAL EFFECT OF INTRAPERITONEAL IMMUNIZATION WITH LIQUID FRACTION OF MICROWAVE-TREATED TUMOR CELL SUSPENSION

The liquid fraction (0.2 ml) of a microwave-treated Meth A suspension in PBS (cell viability: about 1%) was intraperitoneally injected once a week and four times in total for immunization.

![Graph representing survival rates](image)

**Fig. 7.** Cancerocidal effect of intraperitoneal immunization with fractions of the microwave-treated tumor cell suspension.

On the 21st day after immunization, living tumor cells ($1 \times 10^5$ cells) were injected intraperitoneally (Challenge).

*Survival time (in days) was significant at p<0.01 by Mann-Whitney test as compared with control group.

**Survival time (in days) was significant at p<0.05 by Student t-test and Kolmogorov-Smirnov test as compared with control group.
Once week after the last injection, immunized mice were challenged by living Meth A or S-180. Control animals received PBS only.

The 90-day-survival rate after challenge with Meth A was 78%. The survival time after the same challenge was significantly longer than that in the control group (p<0.01, by Mann-Whitney test). The survival time after challenge with S-180 was not significantly different from the control value (Fig. 8).

7) INDIRECT IMMUNOFLUORESCENCE TEST FOR DETECTION OF ANTI-TUMOR CELL ANTIBODY IN SERUM

The liquid fraction (0.2 ml) of a microwave-treated Meth A suspension in PBS (cell viability: about 1%) was intraperitoneally injected once a week and four times in total for immunization. Serum was obtained a week after the last injection and was subjected to heat inactivation at 56°C for 30 minutes. The serum so inactivated was used as primary serum. Anti-mouse-γ-globulin conjugation FITC (BEHRING INSTITUTE) served as secondary serum. Living Meth A cells were employed as target cells.

The primary serum was examined for antibody to target cells by means of indirect immunofluorescence using the primary serum diluted twice and the secondary serum diluted ten times. As a result, no fluorescent cell was detected at all. In other words, it could not be demonstrated anti-tumor antibody in the serum from those mice which acquired such a strong immunity against tumors as in experiment 6.

8) ANTI-TUMOR IMMUNITY OF SPLENIC LYMPHOCYTES

The anti-tumor immunity, represented by growth inhibition, of splenic lymphocytes was studied by Winn assay. In the immune lymphocyte group, the liquid fraction (0.2 ml) of a microwave-treated Meth A suspension in PBS (cell viability: about 1%) was intraperitoneally injected once a week and four times in total for immunization, and splenic lymphocytes were

![Fig. 8](image-url)  
Cancerocidal effect of intraperitoneal immunization with the liquid fraction of microwave-treated tumor cell (Meth A) suspension.  
On the 7th day after last injection (injection once a week and four times in total for immunization), living tumor cells (Meth A or S-180) were injected intraperitoneally (Challenge).  
*Survival time was significant at p<0.01 by Mann-Whitney test as compared with control group.
obtained a week after the last injection. These lymphocytes and Meth A (1×10⁶ cells) were transplanted. In the normal lymphocyte group, PBS (0.2 ml) was used in place of the liquid fraction. The other procedures were the same as those in the immune lymphocyte group. In the treated immune lymphocyte group, immune lymphocytes prepared by the above mentioned method were treated with anti-Thy 1,2 antibody and complement (cell viability after treatment: 68%). After removing dead lymphocytes, living cells were transplanted together with Meth A. In the control group, only Meth A was transplanted.

Fourteen days after transplantation, the tumor weight was 22±19 mg (mean±S.D.) in the immune lymphocyte group, 207±72 mg in the normal lymphocyte group, 244±138 mg in the treated immune lymphocyte group, and 327±220 mg in the control group, respectively. There was a significant difference in tumor weight between the immune lymphocyte group and each of the other three groups (p<0.01, by Student t-test). No significant difference was recognized among the normal lymphocyte group, treated immune lymphocyte group, and control group.

Namely, immune lymphocytes inhibited tumor growth, but this inhibitory activity was impeded by treatment with anti-Thy 1, 2 antibody and complement. Normal lymphocytes (not immunized) exerted no inhibitory effect (Fig. 9.).

Discussion

Microwave are electromagnetic waves ranging from 1 to 100 cm in wavelength and from 300 to 30,000 MHz in frequency. The medical use of microwaves of 2,450±50 MHz in frequency
ANTI-TUMOR EFFECT INDUCED BY MICROWAVE TUMOR COAGULATION

and 12 cm in wavelength is permitted internationally. Biologically, microwaves are said to exert a thermal, a specific thermal, and a non-thermal effect\(^{22,24,39}\). Although the thermal effect of microwaves has been well established, there are still controversies about the specific and the non-thermal effect\(^{4,24}\). When microwaves are applied to living tissues, they mainly act upon watery component, which is abundant in a living body, and produce dielectric heat, which is utilized in surgery or for some other therapeutic purposes.

The MTC converges microwaves of 2,450 MHz in frequency and 12 cm in wavelength to a small area to coagulate living tissues. In a canine experiment, liver resection using MTC was reported\(^{12}\). The changes observed on the liver stump immediately after resection may be understood from the following descriptions: A carbonized layer was unseen and a vacuolated layer, a coagulative necrosis layer, a liquefactive necrosis layer, and a healthy area, arranged in increasing order of depth, were recognized. The depth from the vacuolated layer to the healthy area was 5 mm or more. In the healing process, the layer between the coagulative necrosis layer and the healthy area, i.e., the liquefactive necrosis layer charged into a fibrotic layer of 3 mm in width\(^{12}\).

When tumor cells were subjected to the microwave treatment in a dish, the cell membrane, nucleus, and intracellular granules were maintained morphologically, even in case the cell viability was lost completely. In addition, the number of cells was not changed by microwave irradiation even though irradiation time was extended considerably.

It was reported that when rabbit liver tissues were irradiated by microwaves (50–60 watts) emitted from the monopolar antenna connected to MTC, the maximum temperature of tissues 3 mm away from the focus of irradiation was 65°C, though temperature measurement was not possible in an area nearer to the focus\(^{27}\). When the tumor cell suspension in a dish of 3 cm in diameter was subjected to the microwave treatment (20 watts) by immersing the tip of the antenna into the suspension, its temperature did not rise above 90°C irrespective of the treatment time.

The characteristic of microwave coagulation using MTC is that, though its thermal effect on living tissues is comparatively mild, even the tissues far from the antenna are degenerated. Many authors who described the anti-tumor effect of microwaves only reported general or regional hyperthermia\(^{10,21,29}\). However, it has not been described yet whether or not microwaves converged in a small area have an anti-tumor effect.

Cytotoxic T-lymphocytes (CTL)\(^{5,16,33,37,41}\) and natural killer (NK) cells\(^{6,13,37,41}\) immunologically reveal cytotoxicity to tumor cells. Furthermore, antibody dependent cell mediated cytotoxicity (ADCC)\(^{37,39}\) and activated macrophage mediated cytotoxicity\(^{36,37,38}\) are known. Complement exerts a cytolytic action\(^{44}\), but it has a decisive weak point that the nuclear membrane of tumor cells is free from this action. Thus, only cell-mediated cytotoxicity seems to be promising.

In this study, microwave treated Meth A produced an anti-tumor effect, which was mainly Thy 1+ cell dependent. ADCC was not considered to have a notable anti-tumor effect, because the antibody concerned was not detected in the serum. Among Thy 1+ cells, CTLs and NK cells are recognized as those which bring about a strong anti-tumor effect. The Thy 1 antigen of NK cells is weak. Thy 1+ NK cells are said to account for 35–50% of total NK cells\(^{7,14}\) and
approximately 3% of splenic lymphocytes. The loss of NK cell activity by one-time treatment with anti-Thy 1, 2 antibody and complement is unlikely to be large.

The inhibition of tumor growth determined by Winn assay in syngeneic tumor bearing mice well reflected the immune resistance of the hosts. It has been described that the presence of T-cells among donor lymphocytes is essential for growth inhibition.

However, it is said that effector T-cells derived from the spleen are often immature, so that they do not act directly on target cells. In this connection, the following two mechanisms have been suggested: 1) Effector T-cells, which are memory T-cells, produce an anti-tumor effect only after reaction with immune cells of a recipient. 2) Effector T-cells, which are immature cytotoxic T-cells, are differentiated into mature CTLs after coming in contact with tumor cells.

In future, it will have to be clarified, in details, how the final effector cells play a role in strengthening immunological resistance to tumor cells with microwave treatment.

Generally speaking, the antigenicity of tumor associated antigen (TAA), which the immunological response of a host to tumor cells is based, is so weak that the host response is hardly induced. It is desirable to enhance the antigenicity of tumor cells in order to take part in immunoreaction, on the one hand, and increase the immunological offensive power of a host, on the other.

Meth A antigen in the liquid fraction of a microwave treated Meth A cell suspension was examined by the indirect immunofluorescence method. The serum, obtained from rabbits sensitized by intravenous injection of the liquid fraction, was heat inactivated, and was subjected to absorption treatment with the homogenates of BALB/c mouse spleen and liver tissues. This serum was used as primary serum, and anti-rabbit γ-globulin conjunction FITC(Hoechst) was employed as secondary serum. Living Meth A cells and acetone fixed Meth A cells served as target cells. As a result, both target cells showed ring-form fluorescence.

Meth A possess two kinds of TAA, one in the cytoplasm (tumor associated transplantation antigen) and the other on the cell membrane (tumor specific surface antigen). These antigens are different from each other in their antigenicity.

These results suggest that tumor associated antigen on the cell membrane might have been detached by microwave treatment into the liquid fraction, which induced an anti-tumor effect. It is true that when tumor cells are exposed to microwaves, antigen is released from them, but it is still unclear what mechanism works to cause this phenomenon. At least, the possibility that it may be due to the thermal effect of microwave treatment can be excluded, because hot water bathing failed to induce an anti-tumor effect.

There is a possibility that antibody to the normal tissue or autoantibody may be produced by microwave treatment, though no evidence supporting autoimmunoreaction was obtained in mice, which underwent microwave coagulation of normal tissues.

Conclusions

In the basic experiments using BALB/c mice and ascitic type tumor cells (Meth A, S-180), the anti-tumor effect induced by microwave treatment with MTC was studied. The results are summarized, as follows:
1) The anti-tumor effect induced by microwave coagulation treatment for solid tumor was stronger than that of surgical resection.

2) In an in vitro system, microwave treatment exerted a potent anti-tumor effect without the necessity of using an adjuvant.

3) This effect of microwave treatment was thought to be non-thermal effect.

4) It was suggested that this effect of microwave treatment was due to Thy 1+ cell (T-cell and/or NK cell) dependent anti-tumor immunity and ADCC did not play an important role in mediating this immunoreaction.

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References


和文抄録

マイクロ波腫瘍凝固による抗腫瘍効果に関する実験的研究

和歌山県立医科大学消化器外科（主任：藤見正治教授）
野口 博志

マイクロ波は、従来より理学的療法として医療に用いられてきたが、最近、マイクロ波のエネルギーをできるだけ小領域に集中させることにより組織を凝固させる装置（Microwave Tissue Coagulator）が開発された。この装置を用い、実験的に腫瘍（Meth A Sarcoma）を処置し、抗腫瘍効果に関して以下のような成績を得た。

(1) 固型腫瘍にマイクロ波凝固を行なうと、腫瘍組織を外科的に切除して得られる抗腫瘍力より強い抗腫瘍力が得られた。

(2) 腫瘍細胞を直接生体外で Microwave Tissue Coagulator を用い処置すると、特に Adjuvant の必要なしに、強い抗腫瘍力が得られた。

(3) 腫瘍細胞を Microwave Tissue Coagulator で処置して得られる抗腫瘍力はマイクロ波の non-thermal 効果によることが示唆された。

(4) この抗腫瘍力は、主として、Thy1+の細胞（T-細胞あるいはNK細胞）依存性的抗腫瘍免疫により生じるものであり、ADCCは主要な働きをしていないことが示唆された。