HEPATOLOGY

Novel tumor-ablation device for liver tumors utilizing heat energy generated under an alternating magnetic field

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Abstract

Background and Aims: We have developed a novel tumor-ablation device for liver tumors utilizing heat energy induced by magnesium ferrite (MgFe₂O₄) particles under an alternating magnetic field (AMF) produced by electric currents. This novel device can repeatedly heat liver tumors at lower temperature than usual heating devices, such as radiofrequency ablation therapy, with slight infliction of pain. This study assesses its heating effect on rat liver tumors as local therapy.

Method: The small needle was manufactured from MgFe₂O₄ particles by sintering at 1100°C. After a MgFe₂O₄ needle was inserted into liver tumors comprising of dRLh-84 cells, the tumors were heated for 30 min under an AMF. We examined cellular activity by using nicotinamide adenine dinucleotide (NADH) diaphorase staining and terminal deoxyribonucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) staining, and evaluated the effect of suppressing tumor growth by sequentially comparing the tumor diameter with that of the control group.

Results: The mean temperature of the heated tumors was 60.2 ± 1.8°C. The tumor cells were constricted, and chromatin of nuclei had shrunk immediately after heating. The heat-injury area that contained the tumors was negative for NADH diaphorase activity. After 3 days, the tumor cells in the heat-injury area became positive for TUNEL staining, which detects cell death. At 7 days, the mean tumor diameters were significantly smaller in the heating group than in the control group (6.15 ± 0.47 mm vs 16.89 ± 2.69 mm; \( P < 0.05 \)).

Conclusion: This device, utilizing heat energy induced by ferromagnetic metal under an AMF, appears useful as local thermotherapy for human liver cancer.

Key words
liver cancer, magnesium ferrite, magnetic field, thermotherapy.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide.¹-³ Minimally-invasive treatment is highly desirable, not only for patients with unresectable HCC, but also for patients with small HCC, as most patients with HCC display impaired liver function, including chronic hepatitis or liver cirrhosis.⁴-⁶ Against such a background, radiofrequency ablation (RFA) by dielectric heating, which generates heat using an alternating current of 470 kHz, has been developed and is widely performed as minimally-invasive local treatment for liver tumors. However, rapid heating is required for RFA to complete treatment in a short time, as the alternating current from the percutaneously-inserted needle electrode runs continuously in the body toward the counterelectrode during treatment. Accuracy confirming the ablation effect is difficult due to bubbles arising in the target region under ultrasonographic guidance.⁷ In addition, severe complications caused by rapid rises in temperature have been reported, such as thrombosis, injury of the bile duct, and tumor explosion resulting from increased intratumoral pressure leading to tumor dissemination.⁸-¹⁰

To compensate for these disadvantages, we started to develop a novel ablation therapy preserving the effectiveness of RFA. We therefore developed a heating device that utilizes heat energy generated in a magnetic metal within an alternating magnetic field (AMF) at a lower temperature zone than RFA. The heat energy using commercially-available magnetic metal particles, such as ferrite (Fe₃O₄) under an AMF, is too small to heat and causes necrosis of tumor cells in vivo. Maehara et al. reported that among various magnetic metals under an AMF, magnesium Fe₃O₄ (MgFe₂O₄) induces heat energy most effectively.¹¹ We therefore manufactured a small needle by sintering MgFe₂O₄ particles at 1100°C, and used this needle to correctly target liver tumors and obtain improved heating effects. We have previously reported that
this device heats normal rat liver tissues at 60.7 ± 1.1°C and leads to hepatocyte cell death. The present study assessed the heating effect on liver tumors by examining cellular activity in the heat-injury area and sequentially evaluating the tumor diameter after heating.

**Methods**

**Animal and tumor model**

Donryu male rats (8 weeks old; Charles River Japan, Yokohama, Japan) were kept separately during the experiment, with 12 h/day 12 h/night and ad libitum access to standard laboratory diet and tap water. Maintenance and care of all experimental animals used in this study followed the guidelines of the Animal Studies Committee at Ehime University, Ehime, Japan. During the surgical procedures, the rats were anesthetized with an intraperitoneal injection of 40 mg/kg sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL, USA). The dRLh-84 hepatoma cells used for the hepatic tumor model were purchased from the Institute of Development, Aging and Cancer at Tohoku University, Sandai, Japan. The cells were cultured in Iscove’s modified Dulbecco’s medium (Sigma, St Louis, MO, USA) under 5% CO2 at 37.0°C. A suspension of dRLh-84 hepatoma cells was prepared at a concentration of $5 \times 10^6$ cells/mL and 1 mL was injected into the femoral subcutaneous tissue of the Donryu rats. Two weeks later, the subcutaneous solidified tumor was removed and subsequently cut into a 1 ¥ 1 ¥ 1 mm cube to be used in further experiments. A small incision on the left lateral lobe of the liver was made in a different rat, and the 1 mm³ of tumor was gently inserted into the incision. Based on preliminary observations, we decided to conduct subsequent experiments 10 days following tumor implantation.

**Preparation of sintered MgFe$_2$O$_4$ needle and an AMF application**

MgFe$_2$O$_4$ particles (mean particle size, approximately 1 μm; Wako Pure Chemicals, Osaka, Japan) were forced into a thin quartz tube before sintering at 1100°C for 27 h. The completed MgFe$_2$O$_4$ needle was 1.0 mm in diameter and contained 2.21 mg MgFe$_2$O$_4$/mm (Fig. 1a). The entire manufacturing process was performed at the Department of Materials Science, Faculty of Engineering, Ehime University.

An AMF was created using an external coil comprising of six turned loops (19 ¥ 16 cm; length, 7 cm) of copper pipe (internal diameter, 6 mm) and was cooled by water to ensure constant temperature and impedance. The coil was connected to a power supply (T162–5712B; Thamway, Shizuoka, Japan) and a transistor inverter (Dai-ichi high frequency, Tokyo, Japan) through an impedance tuner (FG-281; Kenwood TMI, Yokohama, Japan) (Fig. 1b). This system induced a magnetic field with a maximum magnetic flux density of 4 kA/m using an alternating electric current of 540 kHz and 2 kW. These conditions have been demonstrated previously to be the most effective in generating heat energy in MgFe$_2$O$_4$ particles. The device was produced by the Department of Physics, Faculty of Science, Ehime University.

**Heating treatment using a MgFe$_2$O$_4$ needle under an AMF**

The tumor-implanted rats were anesthetized, and 10 mm of the sintered MgFe$_2$O$_4$ needle was inserted into the center of the hepatic tumor after laparotomy. The rats were placed under an AMF at the center of the coil for 30 min at 2 kW of current output. Temperatures at the tumor core in contact with the needle were measured using an optical fiber probe (AMOTH FL2000, FS100-M; Anritsu Meter, Tokyo, Japan).

**Experimental design**

A total of 48 rats underwent hepatic tumor implantation and were randomly allocated to either the heating group or the control group to investigate the influence of heating on tumor growth. In the heating group, the rats were heated for 30 min under an AMF after needle insertion, while in the control group, a needle was inserted into each rat, but not heated on the center table.
Six rats in each group were killed after hepatectomy of the tumor-implanted liver immediately after heating at 3, 7, and 14 days. The tumor-affected liver tissues were removed from the dead rats. The liver tissues were cut into 2-mm-thick slices; the two specimens with the maximum area of macroscopic color changes were then selected. One specimen was fixed in 10% formalin for 24 h before being embedded in paraffin. The paraffin-embedded sections were cut into 4-μm-thick slices, dewaxed, dehydrated, and stained with hematoxylin–eosin (HE) and terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) staining. Another specimen was used for the enzyme histochemical examination.

**Histochemical evaluation of enzyme activity using nicotinamide adenine dinucleotide diaphorase staining**

In the present study, nicotinamide adenine dinucleotide (NADH) diaphorase staining was used as a definitive early marker of tissue viability. For the histochemical evaluation of enzyme activity using NADH diaphorase staining, another specimen was fixed in 4% formaldehyde for 4 h and embedded in optimum cooling tissue medium before being frozen and stored at −80°C. Cryostat sections (10 μm) were mounted onto glass slides. The cut sections were incubated for 30 min in a humidified chamber at 37°C with a test solution containing 1 mL reduced NAD (2.5 mg/mL), 2.5 mL nitroblue tetrazolium chloride, 1 mL phosphate-buffered saline, and 0.5 mL Ringer’s solution. The sections were rinsed with distilled water, covered with a permanent aqueous mount, and dried at room temperature for 20 min. The sections were then mounted in DePeX and cover-slipped for optical clarity. Cells that exhibited mitochondrial activity had blue staining.

**TUNEL examination**

Tumor cell death was evaluated using TUNEL staining. Serial sections were prepared in the same manner as used for HE staining. DNA strand breaks were detected with an *in situ* cell death detection kit (Boehringer Mannheim, Mannheim, Germany). The specimens were pretreated for 20 min at 37°C in 20 μg/mL proteinase K (Boehringer Mannheim, Germany) in tris-hydrochloric acid HCl buffer (pH 7.6). A mixture of terminal deoxynucleotidyl transferase solution and fluorescein-labeled d-uridine triphosphate was applied to the slides for 60 min at 37°C in a humidified chamber after endogenous peroxidase had been blocked with 0.3% H2O2 in methanol for 30 min at room temperature. The slides were then incubated with an anti-fluorescein antibody conjugated for 1 min at room temperature in diaminobenzidine solution. All of the sections stained using HE, the enzyme histochemical method, and the TUNEL method were examined using a digital microscopic camera (BX51; Olympus, Tokyo, Japan) by a pathologist who was blinded to the treatment status.

**Measurement of tumor size**

We examined the maximum cross-sections of the hepatic tumors stained with HE using the digital microscopic camera and a computer (MacBook Pro; Apple Computer, Cupertino, CA, USA);

**Statistical analysis**

Values of tumor temperature and tumor diameter are expressed as mean ± SD. Comparisons of tumor diameter were made using the Mann–Whitney *U*-test. Values of *P* < 0.05 were considered statistically significant. Data were analyzed using Stat View J-5.0 software (Abacus Concepts, Berkeley, CA, USA).

**Results**

**Tumor temperature**

The mean temperature curves for the liver tumors in the heating and control groups, as determined by the temperature probe, are shown in Figure 2. In the heating group, temperature rose rapidly for approximately the first 5 min of heat treatment and then increased gradually. In contrast, the mean temperature decreased very slowly (closed triangle).

**Histological findings**

On the cross-sections of the liver tumor, the liver tissue surrounding the tumor was macroscopically discolored, and recognized as
a heat-injury area immediately after heating (Fig. 3a). In the central area around the needle on HE section, the tumor cell cytoplasm was spongy and the chromatin of nuclei shrunk immediately after heating (Fig. 3c). At 3 days after heating, the tumor cells with swelling nuclei were also observed (Fig. 3d). At 7 days, coagulative necrosis was observed (Fig. 3e). At 14 days, coagulative necrosis had spread massively in the heat-treated tumor tissues (Fig. 3f).

In the adjacent non-tumorous liver tissues, congested sinusoids and hepatocytes with nuclei with hyper-chromatin were observed immediately after heating (Fig. 4a). At 3 days after heating, the granular leukocytes infiltrated (Fig. 4b). At 7 days, the granular leukocytes infiltrated massively (Fig. 4c). At 14 days, phagocytosis was observed (Fig. 4d).

**Enzyme histochemical examination for NADH diaphorase activity**

The cytoplasm of the live cells was stained blue by NADH diaphorase staining. Immediately after heating, the heat-injury area that contained the tumor tissues was not stained, whereas live cells in the unheated area were (Fig. 5a). At 3 days after heating, the tumor cells and hepatocytes inside a fibroblast layer were not stained (Fig. 5b). Similarly, tumor cells were also not stained at 7 and 14 days after heating.

**TUNEL examination**

The nuclei of tumor cells did not stain TUNEL positive immediately after heating (Fig. 6a). At 3 days after heating, a number of tumor cells with brown-stained nuclei were observed as TUNEL-positive cells in the heat-injury area (Fig. 5b). At 7 and 14 days after heating, the tumor cells were not stained (Fig. 6c,d).

**Tumor growth**

To examine the differences in tumor growth between the heating and control groups, tumor diameters were measured immediately after heating and again at 3, 7, and 14 days after heating (Fig. 7). Immediately after heating, the mean diameter of the tumors was similar in the heating group (6.35 ± 1.34 mm) and control group (6.72 ± 1.30 mm). At 3 days, the mean diameter of the tumors was 6.28 ± 1.04 mm in the heating group and 7.67 ± 1.03 mm in the control group. At 7 and 14 days, the mean tumor diameter was significantly smaller in the heating group than in the control group.
Discussion

Magnetic field hyperthermia, in which magnetic particles themselves mainly induce heat energy under an AMF by hysteresis loss effects, was pioneered by Gilchrist in 1965 and further developed by Gordon.\(^{13,14}\) This heating phenomenon of inductive heating appears to be an ideal hyperthermia to treat cancer, as the magnetic material itself is heated rather than the surrounding water molecules generating heat as in dielectric heating by RFA. However, this system is difficult to use as a practical tool for human cancer, because the heat energy generated by metal particles, such as Fe\(_3\)O\(_4\) particles, in conventional studies of magnetic-induced heating has been too small for tumor ablation.\(^{15-17}\) In addition, the clinical use of this technique has been hindered by the technical limitations of delivering metal particles to the targeted tissues and maintaining concentrations of the particles.

MgFe\(_2\)O\(_4\) was recently reported to generate heat energy most effectively under an AMF among various biocompatible magnetic metal particles.\(^{11}\) We therefore developed a new needle made from MgFe\(_2\)O\(_4\) particles by sintering at 1100°C to concentrate heat energy into the tumor. We have previously reported that the use of this needle under an AMF safely enables heating of a normal rat liver to a temperature of 60.7 ± 1.1°C.\(^{12}\) In the present study, heat treatment using a sintered MgFe\(_2\)O\(_4\) needle could safely heat rat liver tumors to a mean temperature of 60.2 ± 1.8°C. Because metal particles in a sintered MgFe\(_2\)O\(_4\) needle adhere mutually, the induced heat energy could effectively concentrate into the liver tumor. Therefore, this heating device could heat liver tumors at temperatures above 60°C in live rats.

Generally, DNA is directly damaged, and cellular death occurs in cell cultures at temperatures exceeding 43°C.\(^{18}\) Classical hyperthermia treatment has previously relied on temperatures of 42–45°C for periods of 30–60 min to cause irreversible cellular damage.\(^{19,20}\) However, tissue temperatures must be elevated above 60°C to cause immediate cell death in vivo, because animal studies...

Figure 4  In the adjacent non-tumorous liver tissue on the hematoxylin–eosin section. (a) Congestion of sinusoids and hepatocytes with a hyper-chromatin of nuclei was observed immediately after heating (×20). (b) At 3 days after heating, granular leukocytes infiltrated and the nuclei of hepatocytes disappeared (×20). (c) At 7 days, granular leukocytes infiltrated massively (×20). (d) At 14 days, phagocytosis by fibroblasts was observed (×20). T, tumor.

Figure 5  (a) Immediately after heating, the heat-injury area that contained the tumor tissue was not stained by nicotinamide adenine dinucleotide diaphorase staining, whereas live cells in the unheated area was stained (×20). (b) At 3 days after heating, the tumor cells and hepatocytes inside a fibroblast layer were not stained (×20). T, tumor.
have shown that protein denaturation occurs at approximately 60°C. In the present study, histopathological examination of HE-stained specimens immediately after heating revealed cell shrinkage and hyper-chromatic nuclei in tumor cells around the needle. These histopathological findings indicate that heat stimulus induced by this device would damage subcellular organs, such as nucleic acid, the cell membrane, and cytoskeleton.

An enzyme histochemical examination was used to assess functional damage. NADH diaphorase is an enzyme of mitochondria in which the biochemical processes of respiration and energy production occur. The enzymic inactivity of NADH diaphorase would imply functional cellular death in the heat-injury area containing tumor tissues immediately after heating.

Indirect heat damage following direct heat damage is one of factors determining the expansion of thermal injury. Depending on the model and heating application used, indirect heat stimulus generally continues from 24 h to 7 days after heating. In our previous study, heat stimulus by this heat device worked for normal liver tissues up to 3 days after heating. The appearance of a fibroblast layer surrounding the heat-injury area and the results of the TUNEL examination suggest that heat stimulus tissues worked up to 3 days after heating in the present study.

In the present study, this heating device significantly suppressed tumor growth at 7 and 14 days. The histological findings by HE sections supported the results of tumor suppression. Also, the results of the enzyme histochemical and TUNEL examinations suggest that heating damage would start immediately after heating and cell death would occur for 3 days as result of heat energy. Evaluation of the effects on tumors 3 days after heating is very important for this heating device. If initial treatment does not heat the entire tumor and achieve cell death as confirmed by a computed tomography scan or a magnetic resonance imaging scan at 3 days postoperatively, the patient can be painlessly retreated.

Ferromagnetic metal containing magnesium can generate heat energy by hysteresis loss effects and can maintain this temperature under an AMF. This is explained by Curie’s law, which states that magnetic susceptibility is inversely proportional to temperature. This heating device would enable heating of liver tumors at mild temperature without repeated overheating once the needle has been inserted into the body, offering very low invasive treatment for patients with liver cancer. Although problems exist with

Figure 6 (a) Nuclei of tumor cells were not stained by terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) examination immediately after heating (x40). (b) At 3 days after heating, a number of tumor cells with brown-stained nuclei were observed as TUNEL-positive cells in the heat-injury area (x40). (c) At 7 and (d) 14 days after heating, the tumor cells were not stained (x40).

Figure 7 Temporal changes in the mean tumor diameter for heating (gray) and control groups (white). At 7 and 14 days after heating, the mean tumor diameter was significantly smaller in the heating group than in the control group (*P < 0.05).
removing the needle from the liver after treatment to ensure patient safety, this new heating device has various useful applications in the treatment of liver tumors, such as adjuvant therapy to enhance the antitumor effects of chemotherapy or radiotherapy.

This is the first time that magnetic heating using ferromagnetic metal under an AMF has been used to heat liver tumors beyond 60°C, damaging tumor cells sufficiently to cause cell death. In future, to assess the suitability of this system as a clinical treatment for human cancer, data will need to be evaluated for large tumors with greater blood flow in large animal models. We believe that this device will prove useful for the local heat treatment of human liver cancer in the future.

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References