Interstitial Hyperthermia Using Magnetite Cationic Liposomes Inhibit to Tumor Growth of VX-7 Transplanted Tumor in Rabbit Tongue

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Abstract: We previously developed a new system of magnetic induction interstitial hyperthermia, implant heating system (IHS), and applied to oral cancer control. To overcome the limitations of this system, the present study dealt with new magnetic induction hyperthermia using Magnetic Cationic Liposomes (MCLs) which are injected into a tumor. We prepared a tongue tumor model using the transplantable rabbit VX-7 cells. MCL was injected into the tumor margins to heat the tumor selectively and induce blood vessel damage by heating. Two separated MCL injection sites were made by using Micro syringe pump and sandwiched the tumor on both sides.

The rabbit in group I (n = 4) were performed hyperthermia, while those in group II (n = 4) were not subjected to magnetic field irradiation as the control. In the experimental rabbits, the temperature in the tumor was as high as 43.0°C. The tumor volumes of irradiated rabbits were significantly smaller than those of the non irradiated ones. The transplanted tumor of experimental rabbits almost completely disappeared following 18 day after treatment. The tongues, removed from irradiated rabbits on 28th day after irradiation, showed no tumor cells and fibrous tissue replacement completely occurred in the implanted tumor area. Tongue muscle adjacent to the MCL injection sites did not exhibit any evidence of inflammation or necrosis. The present study confirmed the effectiveness of interstitial hyperthermia using MCLs against oral carcinoma.

Key Words: Interstitial Hyperthermia, Magnetic Cationic Liposomes (MCLs), Experimental tongue, VX-7 tumor, Rabbit
Introduction

Hypertermia induced by radio frequency (RF) has previously been used to treat head and neck cancer \(^1\)–\(^3\). However, these studies have focused to treating metastasis to lymph nodes and not the primary lesion because of the difficulty in heating oral tumors \(^4\). We have explored for effective therapeutic modality to control malignant diseases, and previously developed a new system of magnetic induction interstitial hyperthermia, implant heating system (IHS), using ferromagnetic implants \(^5\)–\(^6\). However, there were two significant limitations of this system, if the long axes of the implants were not aligned parallel to the direction of the magnetic field, heat production would be limited, and surgical procedure was required for insertion of numerous implants into the tumor. The development of more excellent therapeutic modality for cancer control was thus needed to overcome these limitations.

The present study describes the use of Magnetite Cationic Liposomes (MCLs) for hyperthermia. MCLs are colloids in which colloidal magnetites \((\text{Fe}_3\text{O}_4 : \text{average particle size 10nm})\) are coated with a phospholipid double membrane and have a positive charge on the surface. They readily bind to the negatively charged cell membrane due to electrostatic interaction \(^7\). They attach directly to tumor cells with high affinity and remain within the tissue for relatively long time. To test the effectiveness of new interstitial hyperthermia using MCLs against oral malignancies, we prepared a tongue tumor model with the use of transplantable rabbit VX-7 cells.

Materials and methods

Rabbit and VX-7 transplantable tumor

Female Japanese white rabbits \((n = 8)\) weighting about 2.0Kg were used and rabbits were purchased from Chubu Kagaku Kogyo, (Nagoya, Japan). Animals were maintained under healthy conditions for 1 week after purchase, based on Nagoya University guidelines for experimental animals.

The VX-7 cells were employed as transplantable tumor in the rabbit tongue. VX-7 is a papilloma virus-induced squamous cell carcinoma, purchased from Chubu Kagaku Kogyo. The cells were cultured at 37°C in 5% CO\(_2\) atmosphere in tissue culture media consisting of Eagles minimal essential medium (Gibco Grand Island, N.Y.USA) and supplemented with 10% fetal bovine serum, 5mM nonessential amino acids, 100u/ml of penicillin, and 100u/ml of streptomycin.

Preparation of tumor-bearing rabbits

Under pentobarbital anesthesia (Nembutal \(^\text{®}\); Abbott Lab, North Chicago, Illinois. USA), VX-7 cell suspensions (approximately \(1 \times 10^7\) cells) in 100\(\mu\)l of phosphate buffered saline \((0.05M \text{ Na phosphate and 0.15M NaCl, ph7.4})\) were injected into the left side of the tongue at the lingual margin. The tumors were allowed to grow for 9 days without any treatments.

Magnetite Cationic Liposomes (MCLs)

MCLs were prepared the method as described by Shinkai \(^7\). MCLs are coated colloidal magnetites \((\text{Fe}_3\text{O}_4 : \text{average particle size 10nm})\) with a liposomal membrane diolauroylphosphatidylcholine \((\text{DPPC})\): dioleoylphosphatidylethanolamine \((\text{DPPE})\): \(N\)-(\text{a-trimethylammonio-acetyl}) didodecyl-D-glutamate chloride \((\text{TMAG})\) in the ratio 2 : 2 : 1 (Fig. 1). The MCL solution was adjusted for both pH and salt.
concentration (pH7.4, 0.05M Na phosphate+0.15M NaCl) by the addition of 10 fold concentrated phosphate-buffered saline maintained at 4°C until use.

![Diagram of MCL](image)

**Fig.1.** Schematic representation of MCL
- Cationic lipid (TMAG): N-(a trimethylammonioacetyl) didodecyl-D-glutamate chloride molecules
- Phospholipid DPPC: Dilauroylphosphatidylycholine molecules
- Phospholipid DPPE: Dioleoylphosphatidylethanolamine molecules

**Heating system**

An alternating magnetic field was created (118KHz, 3840e) by using a horizontal coil (inner diameter: 70mm) with a transistor inverter (LTG-100-05; Dai-ichi High Frequency Co., Ltd., Tokyo, Japan) (Fig. 2).

![Diagram of heating system](image)

**Fig.2.** Schema of the transistor inverter LTG 100-5
**MCL injection**

MCL injection was carried out when tumor mass had grown to a size of about 10×10mm. MCL solution was injected into each of two regions of the left tumor mass for a total of approximately 3.3 mg of MCLs in the longitudinal direction from the tumor edge, and the needle left in each place within the tumor mass for 15 min during the injection (Fig. 3A, B). As a result, two separated MCL injection sites were made and sandwiched the tumor on both sides. Micro syringe pump (KD scientific Co., Ltd., USA) was used to perform the injections.

**Hyperthermia in Rabbit tongue carcinoma**

The rabbits (n = 8) were then separated into two groups. Group I (n = 4) received hyperthermia and Group II, did not as control. The tissue was irradiated 5min after MCL injection. The tongue of the injected rabbit was placed at the center of the coil and irradiated for 30 min. The temperature within the tumor was measured using two thermometer probes (Anritsu Meter Tokyo, Japan) inserted into the center and margin of the tumor. (Fig. 3C).

**Measurement of antitumor effects**

The antitumor effect was evaluated by measuring the tumor volume and histologic observation. The sizes of the tumors were measured under pentobarbital anesthesia every 3 days. The volume was determined with the following formula:

\[
\text{Tumor volume} = 0.5 \times (\text{length} \times \text{width}^2)
\]

where the measurement were in millimeters. The antitumor effect was evaluated by comparing the two groups of rabbits. Tumor volume was compared between the treated (Group I) and control (Group II) groups using the X² test. A p value of less than 0.05 was regarded as significant.
Histological examination

Twenty-eight days after the MCL injection, the rabbits were sacrificed under pentobarbital anesthesia, and the tongue, sentinel lymph nodes, heart, lung, spleen, liver, and kidney were examined. Tissues were fixed with 10% formalin solution. Longitudinal sections of the tongue were prepared. The specimens were embedded in paraffin, and sections (4μ) were stained with hematoxylin and eosin for histopathologic examination.

Results

1. Heat generation by MCLs in an alternating field

As shown in Figure 4, the temperature in the center of the transplanted tumors increased rapidly and reached 43°C after 3 min. In contrast, it took at least 5 min until the tumor margin was heated to 43°C. These temperatures were maintained during irradiation. After irradiation, the temperature in the tumors recovered gradually, returning to base line within 5 min.

![Figure 4. Temperature in the tumor during irradiation.](image)

The temperature in the center of the VX-7 tumor increased rapidly and reached 43°C after starting irradiation 3 min. In contrast, it took 5 min until the tumor margin was heated to 43°C. The temperature was maintained during irradiation. Data points are the means of 3 independent experiments. Symbols: ○ center of the tumor ■ tumor margin

2. Tumor growth after hyperthermia

There were significant differences in the volumes of the experimental (irradiated) tumor compared to those of the control on the 3th day after irradiation. In irradiated rabbits, the tumor of each rabbit almost completely disappeared until 18th day after irradiation (Fig.5). No functional disorders were observed and irradiated rabbits maintained their weight (data not shown). In contrast, in control rabbits, tumor volume increased gradually, and all rabbits were dead by 12th day after irradiation. Figure 6 shows a typical tongue from each group.
Fig. 5. Time course of tumor growth after MCL injection.
In group I rabbits with irradiation, the tumor in each rabbit almost completely regressed. In contrast, in group II, the tumor volume of all rabbits increased gradually, with no evidence of regression. Data points are the means of 4 independent experiments.  ρ value<0.01 Tumor Volume Ratio=Tumor Volume / Tumor volume at the time of MCL injection Symbols: ● group I (irradiated) □ group II (control)

Fig. 6. Photographs of a typical rabbit in experimental and control
(A) A rabbit from irradiated (group I)
(B) A rabbit from control (group II)
3. Histological findings

The tongues, obtained from the irradiated rabbits on 28th day after irradiation, showed no tumor cells and fibrous tissue completely replacing the implanted VX-7 tumor. Tongue muscle adjacent to the MCL injection sites did not exhibit any evidence of inflammation or necrosis.

The MCL were very localized and remained in the region of the MCL injection (Fig.7A). The tongues of the control rabbits without irradiation were replaced with enlarged tumors. Tumor margins were well-vascularized and VX-7 invaded into the tongue muscle (Fig.7B).

Tongues in the control rabbits 24h after the MCL injection had high accumulation of MCLs. The normal tissue adjacent to the MCL injection sites did not exhibit any evidence of inflammation or necrosis.

In sentinel lymph nodes, macrophages containing MCL granules were observed in the cortex and the lymphatic sinus. Little or no MCLs was detected in the red pulp of the spleen or Kupffers cells of the liver. There were no MCLs in other organs (data not shown).

Discussion

It has been described that magnetic induction hyperthermia therapy is effective to control malignant lesions\(^9\). This therapy takes advantage of the fact that, under a high-frequency magnetic field, fer-
romagnetic implants in the tumor generate heat due to eddy currents. Interstitial hyperthermia using ferromagnetic implants has been shown to have a favorable antitumor effect on rabbit tongue tumor. However, one disadvantage of this system is that numerous ferromagnetic implants must be inserted into the tumor to achieve this effect. In addition, the long axes of the implants must be parallel to the direction of the magnetic field for maximal heat production. Therefore, since tumor margins are well-vascularized, and nearly all vessels can be destroyed in murine tissues by heating to 43°C for 30 min, we tried to make two separated MCL injection sites and sandwiched the tumor on both sides. As a result, the target tissue was heated effectively using an alternating magnetic field (118Hz, 3840e). The temperature in the MCLs injected tumor increased rapidly, and uniform heating to the desired temperature was achieved without damaging the adjacent normal tissue. Moreover the present study showed that irradiated tumors revealed significant damage and they finally disappeared in MCLs injected tumor area. This therapy overcame some of the complications of the former method and reduced the surgical invasion of the tissue.

The first attempt at using submicron particles has made by Gilchrist, who has used Fe₂O₃ powder. However there has been no specific affinity of the powder for the tumor cells. To increase its affinity and dispersion within the tumor tissue, magnetite cationic liposomes, which consist of colloidal magnetites (Fe₃O₄) coated with a liposomal membrane were developed by Shinkai. The MCLs had such a high affinity for the cell membrane that they nearly all remained in the spleen and liver. These results corresponded with the findings in the rat liver by Gregoriadis. MCLs which are taken into the systemic circulation are thought to be ultimately trapped by the lymphatic system.

The toxicity of MCLs has been examined. In this study, there also was no evidence of any systemic problems such as inflammation or necrosis near the sites of the MCL injections, and any swelling of the cervical lymph nodes into which some MCLs were taken. The present study confirms the effectiveness of interstitial hyperthermia using MCLs in oral cancer therapy.

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References

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Magnetite Cationic Liposome (MCLs) を用いた組織内加温療法は
家兎 VX-7 移植舌腫瘍の増殖を抑制する

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要 旨：我々は先に、新しい磁場誘導組織内加温療法 (Implant Heating System) を開発し、口腔癌治療への応用を試みた。本実験ではこの治療法の限界を克服すべく、Magnetite Cationic Liposome (MCLs) を用いた磁場誘導組織内加温療法について検討した。実験では、家兎 VX-7 細胞を用いて舌腫瘍モデルを作成した。腫瘍を選択的に加温し、腫瘍被着血管をも障害すべく、腫瘍マージン部のみに MCLs の局所注入を行った。注入にはマイクロシリジンポンプを用い、注入部位を 2 個所とし、これらの領域が腫瘍を両側より挟み込む形とした。家兎は 2 つのグループに分け、グループ I を加温を行う治療群、グループ II を加温を行わないコントロール群とした。実験の結果、加温した舌の腫瘍内温度は 45℃に達した。また、治療群の腫瘍の大きさはコントロール群に比べ有意に縮小し、治療より 18 日目にはほとんど消失した。さらに 28 日目に摘出した舌では、腫瘍細胞は全く認められず、以前の腫瘍部位は完全に纖維組織に置換していた。一方、MCLs 局所注入部位に接する舌筋組織において炎症反応や壊死変性は認められなかっ
た。以上より、MCLs を用いた組織内加温療法が口腔癌に一層有用であることが確認できた。