Hyperthermia, Tissue Microcirculation, and Temporarily Increased Thermosensitivity in VX2 Carcinoma in Rabbit Liver

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ABSTRACT

The role of microcirculatory factors within and around liver tumors after heat treatment was investigated in a rabbit model of liver cancer (VX2 carcinoma). As a physiological factor of the microenvironment, regional blood flow (RBF) was measured by the hydrogen clearance method, and a histopathological study was done. Local hyperthermia was administered directly to the liver tumor via a 915-MHz microwave. Hyperthermia produced a temporary reduction of RBF in both the tumor and the surrounding normal liver tissues, and the histopathology revealed congestion, petechiae, and thrombosis. After hyperthermia at 43.0°C for 20 min, RBF in the tumor rapidly decreased to a minimum of 40% of the pretreatment level during 0–12 h after the treatment and increased gradually to the pretreatment level at 2 days. RBF in the normal liver also decreased rapidly after hyperthermia, to a minimum of 30% of the pretreatment level, 1–12 h after treatment. In the case of treatment at 42.5°C for 20 min, RBF in the tumor also rapidly decreased to a minimum of 40% of the control level, at 4 h after the treatment, and recovery was within 2 days. However, RBF in the surrounding normal liver decreased to 80% of the control level, at 2 h after the treatment, and then increased more rapidly to reach levels seen in the controls. Thus, the latter condition of heat treatment was considered to be favorable for therapeutic gains. Based on the results of these sequential microcirculatory changes, the effects of continuous and intermittent hyperthermia were studied in groups given various treatments. In a group treated with intermittent hyperthermia at 4-h intervals, the antitumor effects determined by tumor growth retardation were significantly greater, as compared with findings in the group given treatment without an interval and that given at a 24-h interval. In the tumor at 4 h after hyperthermia, the increased thermosensitivity was considered to surpass the developing thermotolerance. Thus, the antitumor effect of hyperthermia in vivo greatly depends on the microcirculation. The most efficacious mode for application of hyperthermia must be vigorously examined if a clinical relevance is to be gained.

INTRODUCTION

The antitumor effects of hyperthermia have been demonstrated in vitro (1) and in vivo (2). Tumor cells in vitro are not always more sensitive to heat than normal cells (3). Other studies showed that malignant tissues become selectively sensitive to heat only when maintained within in vivo conditions (4). The innate poor blood flow of tumors in vivo, relative to normal tissues, plays a key role in selective heat sensitivity. A sluggish blood flow will not dissipate the added heat and the temperature will rise above that of the surrounding healthy tissue. On the other hand, hyperthermia induces an even further decrease in tumor blood flow by a direct detrimental effect on the microvasculature. This phenomenon creates a situation in which the tumor is exposed to high acidity and low perfusion (5) and such a state significantly contributes to the antitumor effect and enhances the effectiveness of subsequent hyperthermic treatments.

The current study was done to assess sequential physiological changes in the microcirculation and morphological alterations occurring in the microvasculature of VX2 carcinoma and normal liver tissue of rabbits following microwave-induced hyperthermia. The augmented antitumor effect of intermittent versus continuous hyperthermia was also given attention.

MATERIALS AND METHODS

Animal Model

Japanese white rabbits, weighing 2.0 to 3.0 kg, were anesthetised by giving an i.v. injection of pentobarbital sodium (30 mg/kg). Using a sterile technique, an 1-mm2 fragment of VX2 carcinoma was inoculated under the capsule of the left anterior lobe of the liver of each rabbit, using a trocar needle. All studies were conducted 14 days after the inoculation when the diameter of the tumor had reached 1.0 to 1.5 cm. Within this period, no part of the tumor was necrosed and the blood supply was rich.

The VX2 tumor utilized in these experiments has a high vascularity and is susceptible to heat treatment (6–8).

Hyperthermia

At laparotomy, the tumor bearing hepatic lobe was exteriorized and local hyperthermia was administered using microwave radiation at 915 MHz (Microwave System S/N 004; Microwave Associates, Inc., MA). The applicator of this generator was especially modified to fit the subcapsular liver tumor in rabbits, using a rubber frame and iodine containing gel (Isodine Gel; Meiji Seika Co., Ltd., Japan), as a bolus (Fig. 1A). Temperatures were measured during switch-off of the generator, using a copper/constantan thermocouple (Bailey Instruments Co., Saddle Brook, NJ).

In a pilot experiment, we measured the time required for this heating system to raise the lobe to the intended level (Tables 1). To elevate the main thermocouple up to 42.5°C, 189 s were required, and 229 s were needed in the case of 43.0°C (Table 1). We measured radial and axial temperature distributions in and around the tumor. While the temperature of the main thermocouple was controlled at the intended level, 3 thermocouples covered with a Teflon tube were inserted along the X, Y, and Z axes, and the temperature profile was recorded with the aid of the thermocouple withdrawn along each axis. When the temperature of the main thermocouple fluctuated between 42.5 and 42.6°C, the temperature inside of the tumor ranged from 42.0 to 43.5°C. In case of fluctuation between 43.0 and 43.1°C, the range was from 42.8 to 44.2°C. There was no evidence that the temperature in the adjacent normal liver parenchyma exceeded the control levels (Table 1).

Measurement of Regional Blood Flow

The hydrogen clearance method (9) was used to measure RBF3 of the tumor and of the adjacent normal liver. RBF of the nonheated middle lobe was also measured as a control (Fig. 1B). The rabbits were anesthetized with pentobarbital sodium and respiration was maintained using a positive pressure (respirator, Model SN-480-3; Shinano Manufacturing Co. Ltd., Japan) through an endotracheal cannula. Ringer's lactate was infused continuously at the rate of 10 ml/kg/min via an ear vein to prevent hypovolemic malcirculation during laparotomy and...
consisted of tissue platinum electrodes, 0.3 mm in diameter, connected to a male cannula. The circuit used to measure the hydrogen concentration was made and a histopathological study was done on the tumor tissues and adjacent normal liver and in the middle lobe of the liver. Controlled at the required temperature. In B, measurement of regional blood flow during measurement of RBF. Hydrogen gas was administered for 1-2 min at 0.1-0.2 liter/min through the side port attached to the endotracheal cannula. The circuit used to measure the hydrogen concentration consisted of tissue platinum electrodes, 0.3 mm in diameter, connected to PHG-201 (Unique Medical Co., Ltd., Japan). The clearance curve for hydrogen was recorded and the RBF was calculated as:

\[ \text{RBF} = \frac{k \times 69.3}{t_{\text{1/2}}} \text{[ml/min/100 g tissue]} \]

where \( k \) is the tissue/blood partition coefficient, determined as 1.0 by Auckland et al. (9), and \( t_{\text{1/2}} \) is the time in min required for the tissue hydrogen concentration to be reduced to one-half. This was readily obtained from the slope of the tissue desaturation curve, plotted on a semilogarithmic scale. The tip of each of 3 electrodes was placed in the tumor, in the adjacent normal liver to be heated and in the normal liver of the middle hepatic lobe not to be heated. Depth was 3 mm beneath the capsule of the liver (Fig. 1B). Thus, mean values of more than three measurements in different positions were taken. Although the RBF measured by the hydrogen clearance method may reflect only hepatic arterial liver perfusion rather than portal or total hepatic blood flow (10), it is considered suitable to assess regional perfusion in the peripheral normal liver and in the tumor supplied mainly from the hepatic artery (8).

**Histopathological Examination**

Tissue for histological examination was collected by careful dissection of all liver lobes immediately after killing the animals. The tumor bearing lobes were processed using routine paraffin fixation and hematoxylin and eosin stains.

**Experimental Design**

Experiment 1. Sequential physiological and morphological alterations that occurred in the microvasculature of the tumor and adjacent normal tissue following hyperthermia at 43.0°C for 20 min (Experiment 1A) and at 42.5°C for 20 min (Experiment 1B) were investigated. RBF was measured before hyperthermia, as a control, just after hyperthermia, at 1 h, 2 h, 4 h, 6 h, 12 h, 1 day, 2 days, 4 days, and 7 days after the treatment. After each of these time intervals, histological specimens of all tumor-bearing hepatic lobes were collected for morphological examination. In addition, sequential alterations of RBF in the case of treatment twice with hyperthermia at 42.5°C for 20 min spaced 4 h apart (Experiment 1C) were also measured. A total of 83 rabbits were used.

Experiment 2. The augmented antitumor effect of intermittent hyperthermic treatment was studied in different groups. The 50 treated rabbits were assigned to 5 groups of 10 each and treated as follows: Group 1, 1 unit of hyperthermia, i.e., 42.5°C for 20 min; Group 2, 2 units of hyperthermia applied continuously, i.e., 42.5°C for 40 min; Group 3, 2 units of hyperthermia given at a 4-h interval; Group 4, 2 units of hyperthermia given at a 24-h interval; Group 5, no hyperthermia, i.e., laparotomy only.

Seven days after the treatments, tumor volume was measured and calculated according to the formula (11)

\[ V = ab^{1/2} \]

Here, \( a \) is the longest and \( b \) the smallest diameter of the tumor in vivo.

Tumor growth ratio, defined as tumor volume 7 days after treatment/tumor volume at each treatment, was measured and group comparisons were made. Statistical analysis was carried out using Student's \( t \) test. After assessment of the tumor growth ratio, each tumor bearing hepatic lobe was collected and the liver surrounding the tumor was cut into 2-mm-wide sections, embedded in paraffin, stained with hematoxylin and eosin, and studied histologically and detrimental effects in the normal parenchyma induced by heat treatment were investigated. Fisher's exact probability test was used to determine the statistical significance.

**RESULTS**

**Blood Flow Measurements**

Of 413 measurements, 32 were omitted due to extremely low saturation heights caused by a worn-out electrode or poor general condition of the animal or due to swoops caused by possible placement of electrode tips within blood vessels. Blood flow measurements were calculable on the semilogarithmic scale and entered into the blood flow analysis (381 data points were obtained).

**Experiment 1**

Experiment 1A: Hyperthermia at 43.0°C for 20 min. Before treatment, RBF was 25.6 ± 10.3 (SD) ml/min/100 g in the tumor and 50.0 ± 9.9 ml/min/100 g in the liver adjacent to the tumor. After hyperthermia, RBF in the tumor rapidly decreased
to a minimum of 40% of the level before treatment (range, 10.9–14.2 ml/min/100 g) during 0–12 h after treatment and then increased gradually to the pretreatment level at 2 days (Fig. 2). RBF in the normal liver also decreased rapidly after hyperthermia, to a minimum of 30% of the pretreatment level (range, 14.2–19.2 ml/min/100 g) 1–12 h after treatment and then increased gradually to the control level 2 days after the treatment. The nonheated RBF in the middle hepatic lobe showed a stable time course during these procedures (range, 47.2–57.7 ml/min/100 g).

In the histopathological studies, alterations in the microvasculature after the hyperthermia consisted mainly of congestion, petechiae, and thrombosis in both the tumor and the surrounding normal liver (Fig. 3, A to C). The severity was graded and the time course following hyperthermia was closely related to the time course of RBF (Table 2). The antitumor effect of hyperthermia, as revealed histologically, showed necrosis of the tumor which was prominent in areas distant from tumor vessels (Fig. 3D), and reproliferation of tumor cells was evident 2 days after the treatment. Pathological changes in the nonheated middle lobe of the liver were absent.

Experiment 1B: Hyperthermia at 42.5°C for 20 min. Before treatment, the RBF was 30.8 ± 4.3 ml/min/100 g tissue in the
The detrimental effect induced by hyperthermia is more selective in the tumor at 42.5°C than at 43.0°C.

Table 2: Sequential changes of histopathological findings after hyperthermia at 43.0°C for 20 min

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Pathological findings</th>
<th>HTM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Congestion</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Bleeding</td>
<td>±</td>
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<td></td>
<td>Thrombosis</td>
<td>±</td>
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<tr>
<td>Tumor (vascular change)</td>
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<td></td>
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<tr>
<td></td>
<td>Congestion</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Bleeding</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Thrombosis</td>
<td>+</td>
</tr>
<tr>
<td>Tumor (cell damage)</td>
<td>Necrosis</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Reproliferation</td>
<td>±</td>
</tr>
</tbody>
</table>

* HTM, hyperthermia.
* Grading of the histopathological findings: ++, prominent; +, moderate; ±, mild; -, none.

Table 3: Sequential changes of histopathological findings after hyperthermia at 42.5°C for 20 min

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Pathological findings</th>
<th>HTM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Congestion</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Bleeding</td>
<td>±</td>
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<tr>
<td></td>
<td>Thrombosis</td>
<td>±</td>
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<tr>
<td>Tumor (vascular change)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Congestion</td>
<td>++</td>
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<td></td>
<td>Bleeding</td>
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<td></td>
<td>Thrombosis</td>
<td>+</td>
</tr>
<tr>
<td>Tumor (cell damage)</td>
<td>Necrosis</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Reproliferation</td>
<td>±</td>
</tr>
</tbody>
</table>

* HTM, hyperthermia.
* Grading of the histopathological findings: ++, prominent; +, moderate; ±, mild; -, none.

Fig. 4. Time course of regional blood flow in the tumor and in the adjacent normal liver, before and after hyperthermia (HTM) at 42.5°C for 20 min. The detrimental effect induced by hyperthermia is more selective in the tumor at 42.5°C than at 43.0°C.

Fig. 5. Time course of regional blood flow in the tumor and in the adjacent normal liver and before and after intermittent hyperthermia (HTM), at 4-h intervals. Decrease in regional blood flow was prolonged by the second treatment.

Table 4: Effects of different heat treatments on mean tumor growth ratio

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rabbits</th>
<th>Before treatment</th>
<th>After treatment</th>
<th>Tumor growth ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HTM for 20 min only</td>
<td>10</td>
<td>0.81 ± 0.66</td>
<td>7.14 ± 2.76</td>
<td>11.59 ± 4.11*</td>
</tr>
<tr>
<td>2. HTM for 40 min continuously</td>
<td>10</td>
<td>0.88 ± 0.61</td>
<td>6.69 ± 4.33</td>
<td>7.72 ± 2.64*</td>
</tr>
<tr>
<td>3. HTM for 20 min in 2 fractions (4-h intervals)</td>
<td>10</td>
<td>0.83 ± 0.30</td>
<td>2.19 ± 1.04</td>
<td>2.69 ± 1.33*</td>
</tr>
<tr>
<td>4. HTM for 20 min in 2 fractions (24-h intervals)</td>
<td>10</td>
<td>0.81 ± 0.39</td>
<td>6.38 ± 1.76</td>
<td>8.07 ± 2.84*</td>
</tr>
<tr>
<td>5. Sham operation</td>
<td>10</td>
<td>0.81 ± 0.35</td>
<td>9.93 ± 3.93</td>
<td>12.85 ± 3.95</td>
</tr>
</tbody>
</table>

* Mean ± SD. 
* Geometric tumor volume 7 days after treatment/before treatment.
* HTM, hyperthermia at 42.5°C.
* P < 0.01 compared with Group 5.
* P < 0.05 compared with Group 1.
* P < 0.001 compared with Groups 2 and 4.

At 42.5°C for 20 min produced no further decrease in RBF. From 6 h after the second treatment, RBF in the tumor increased gradually reaching a pretreatment level 2 days after the second treatment (Fig. 5). However, RBF in the adjacent normal liver was little influenced by the second treatment.

Experiment 2

Table 4 shows tumor growth ratios of the five groups. There was no statistical difference in tumor volume in each group at the initiation of treatment. With regard to tumor growth ratio, hyperthermia at 42.5°C for 20 min (Group 1) led to no statistically significant reduction, compared with findings in the nonheated rabbits (Group 5). However, hyperthermia for 40...
min (Group 2) resulted in a statistically significant reduction in tumor growth ratio, compared to findings in Group 5 ($P < 0.01$). Furthermore, intermittent heat treatment at 4-h intervals (Group 3) led to an augmented and statistically significant reduction in tumor growth ratio, compared with findings in the continuously treated Group 2 ($P < 0.001$) and with that of the intermittent treatment at 24-h intervals (Group 4) ($P < 0.001$).

Concerning side effects, fibrosis or necrosis in the surrounding normal liver (Fig. 6), as related to the hyperthermic treatment, was seen in 8 of 10 rabbits treated continuously (Group 2), as compared with the findings in Group 3 treated intermittently at 4-h intervals, 4 of 10 ($P = 0.17$, Fisher’s exact probability test), and with Group 4 treated at 24-h intervals, 0 of 10 ($P < 0.01$). In Group 3, there was no significantly increased frequency, compared with findings in Group 4 ($P = 0.09$). In Group 1, exposed to hyperthermia for only 20 min, and in Group 5, given no treatment, side effects were not observed (Table 5).

**DISCUSSION**

One of the essential conditions of hyperthermia for treating a malignancy is a selective toxicity to the tumor tissue. This selective heat sensitivity of the tumor became evident, using the succinate dehydrogenase inhibition test and fresh specimens of gastrointestinal carcinoma (12). While the succinate dehydrogenase inhibition test reflects *in vitro* factors, factors *in vivo* which play a key role in the selective cytoidal effect on the tumor must be examined. In the present experiment, we studied sequential changes in both physiological and morphological parameters and evaluated the temporarily increased thermosensitivity (thermosensitization) caused by the first treatment of fractionated hyperthermia.

Nilsen (13) and Lefor et al. (14) studied endothelial changes and microvascular leakage due to hyperthermia and obtained histopathological evidence of congestion, thrombosis, and hemorrhage, apparently due to vessel wall injury, as mentioned by other investigators (15–18). The RBF measured by the hydrogen clearance method reflects the capacity of physiological perfusion, and decrease in this capacity is related to acidosis, hypoxia, nutrition-deprivation, and elevation of temperature. The time course of RBF showed a relative decrease in the tumor tissue, as compared to findings in the normal liver, during all processes. Such a selectivity developed after hyperthermic treatment at 42.5°C for 20 min and the reversion was gradual. Furthermore, the second challenge of hyperthermia, at the same dose and at 4 h after the initial treatment, led to a prolonged hypoperfusion in the tumor, despite good preservation of the adjacent normal liver.

We estimated the blood flow by the hydrogen clearance method and from thermal washout curves and noted a good correlation (0.99). We think the former method is more appropriate for our experimental set-up in which we obtained sequential data on a precise tissue in a living animal. With the latter method, the two electrodes are placed some distance apart (electrode for a heater and a sensor); hence only a relative value can be obtained.

Despite numerous studies on thermotolerance (19), *in vivo* studies on fractionated treatments of hyperthermia showed that splitting of a heat treatment into two fractions at short intervals may even improve the overall effect, as compared to cases of a single application (20, 21). In our present *in vivo* study, because of the increased thermosensitivity of the tumor tissue as the result of heat treatment, the 4-h fractionated treatment of hyperthermia had an excellent antitumor effect.

Hyperthermia increases blood flow in the skin and muscle and flow in the tumor tissue decreases (5, 22). In our current study, however, regional blood flow in both the tumor tissue and normal liver decreased after the heat treatment. Liver tissue is considered to be thermosensitive (23) and heat-related damage to normal liver tissue must be avoided. The damage to the surrounding normal liver after heat treatment for 40 min was reduced by splitting the duration of treatment into two fractions, a finding apparently linked to the microcirculation in the normal liver. Hence, microcirculation and/or the tissue envi-

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**Table 5 Frequency of fibrosis or necrosis in normal liver tissue**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rabbits</th>
<th>No. of rabbits with damage to normal liver*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>4*</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>4*</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

* Verified by fibrosis or necrosis.

\* $P = 0.17$ compared with Group 3; $P < 0.01$ compared with Groups 1, 4, and 5.

\[c P = 0.09\] compared with Groups 1, 4, and 5.

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Fig. 6. Histopathological study 7 days after hyperthermia at 42.5°C for 40 min. Note the necrosis ($A, \times 72$) or fibrosis with the proliferation of small bile ducts ($B, \times 72$).
environment no doubt play a key role in therapeutic gain.

We found that 4 h after the application of hyperthermia at 42.5°C for 20 min: (a) RBF in the tumor is at a minimum and acidity is expected to be maximum; (b) RBF in the surrounding normal liver is recovering; (c) thermotolerance of the tumor cell is not likely to be prominent in this period, and applying heat at this interval resulted in a low ratio of tumor growth. Presumably, there is an optimum for the duration of each treatment as well as for the net number of treatments, and these may differ between histologically different tumors and the host tissue. A definite protocol, standard regimen, and unit of thermal dose have apparently not been setup in any country. Therefore, further experiments should be directed to determining the exact mechanism by which hyperthermia selectively destroys tumor cells and to the most efficacious mode of application. The latter is most important if hyperthermia is to gain clinical relevance.

ACKNOWLEDGMENTS

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