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Change in Fibrinogen Turnover in Tumors by Hyperthermia*

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Abstract—*The effect of hyperthermia on the turnover of fibrinogen and albumin in the SCK tumor of A/J mice was studied. Immediately after heating at 43.5°C for 30 min, the content of ^{131}I-labeled fibrinogen in the tumors was about 2.7-fold that in the control tumor and remained elevated for 24 hr. On the other hand, the content of ^{125}I-labeled serum albumin in the tumor immediately after heating was only 1.7-fold that in the control tumor and started to decrease soon after heating. The greater increase in the accumulation and the longer retention of the labeled fibrinogen as compared with the labeled albumin in the heated tumors appeared to be related to the heat-induced vascular damage accompanied by vascular occlusion, fibrination and subsequent thrombus formation.*

INTRODUCTION

A NUMBER of recent laboratory investigations as well as clinical experiences have demonstrated that hyperthermia used alone or in combination with radiation or drugs causes greater damage in tumors than in normal tissues, although the intrinsic cellular thermosensitivity of tumor cells may not be different from that of their normal counterparts [1–5]. One of the causes for this preferential effect of hyperthermia on tumors appears to be the difference in the vascular function and related physiological factors in the tumors and normal tissues [6–12]. It has been suggested that the tumor blood perfusion, particularly in the larger tumors, tends to be sluggish, and thus the dissipation of heat by blood flow during heating in the tumors is slow relative to that in the normal tissues. As a consequence, the temperature in the tumors rises higher than that in the normal tissues during heating, leading to greater damage in the tumors. Furthermore, indications are that the vascular beds in tumors are more vulnerable to heat than that in normal tissues. In fact, severe vascular damage has been observed in a number of experimental tumors with no evident damage in the vasculature in the adjacent normal tissues [11, 12]. It has also been demonstrated that the intrinsically acidic intratumor environment becomes further acidic and also becomes hypoxic and nutritionally deprived after heating, probably because of the heat-induced vascular occlusion and an increase in the synthesis and accumulation of acidic metabolites [13–17].

Although the mechanism of heat-induced impairment of blood flow in tumors is unknown, it would not be unreasonable to suspect that fibrination is involved as in the vascular occlusion and clotting of blood elements. It has been known that the fibrin content in the tumors is much greater than that in the normal tissues. Dvorak *et al.* [18] attributed the deposition of fibrin in tumors to an inflammatory reaction of the host tissues and to the injury of blood vessels of supporting stromata. Copeland [19] and Copeland and Michaelson [20] observed that the accumulation of [^{131}I]fibrinogen in the Walker tumor was intrinsically greater than that in the muscle and that heating at 45°C increased the [^{131}I]fibrinogen accumulation in the tumor without a significant increase in the muscle. These investigators thus concluded that the Walker tumors were much more susceptible to heat treatment than the muscle. It should be pointed out that in these studies mentioned above an increase in [^{131}I]fibrinogen accumulation in the Walker tumor occurred only when the temperature was raised above 45°C. Interestingly, we [10] observed that the blood flow in the Walker tumor remained intact at 43°C but decreased after heating at 45°C, indicating that the heat-induced increase in fibrinogen accumulation and vascular damage might be closely related. We have extensively studied the heat-induced vascular damage in

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the SCK tumor of A/J mice [9, 10, 13]. In the present study we investigated the possible relationship between the heat-induced increase in fibrinogen or fibrin deposition and vascular damage in the SCK tumor by comparing the turnover of [^{131}I]fibrinogen and [^{125}I]albumin in the tumors.

MATERIALS AND METHODS

Animal and tumor

Male A/J mice of 10–12 weeks of age were used. The tumor used was SCK tumor, a mammary carcinoma of A/J mouse, which arose spontaneously in our laboratory and was adapted to grow both *in vitro* and *in vivo* [9, 13]. The tumor cells in exponential growth phase in culture were harvested by trypsin treatment, washed and about 5×10^4 cells in 0.02 ml of culture medium were injected, s.c., into the right thigh of each mouse. When the tumors grew to 7–9 mm in diameter 8–10 days after the inoculation, the effect of hyperthermia on the turnover of [^{131}I]fibrinogen and [^{125}I]albumin in the tumors was compared. The mice were given potassium iodide drinking water (62 μM of KI solution) in order to reduce the uptake of inorganic ^{125}I and ^{131}I by the thyroid. Eight mice were used for each data point.

Heating

Mice were taped onto heating jigs, and the tumor-bearing leg was immobilized by anchoring a toe with thread to a supporter attached to the jig [9, 13]. The tumors were heated by immersing the leg in 43.5°C water for 30 min. The mice were not anesthetized during the heating. The tumor temperature, measured with a 29-gauge thermocouple, was 43.2–43.4°C during the heating. The mice with control tumors were also placed in the heating jig, but the tumors were not heated.

Albumin and fibrinogen turnover study

Mouse serum albumin was purchased from Miles Laboratory (Code No. 82–351, Fraction V) and fibrinogen was prepared from the blood of A/J mice with the use of glycine method [21]. The clottability of the fibrinogen was over 90%. The albumin and fibrinogen were labeled with ^{125}I and ^{131}I, respectively, with the iodine monochloride method [22].

Immediately before the heating of the tumors, each animal was intravenously injected with 0.1 ml of a mixture of 18.2 μCi of [^{125}I]albumin (96 μg) and 14.0 μCi of [^{131}I]fibrinogen (78 μg). At various times during 0–72 hr post-heating, the animals were lightly anesthetized with ether, bled by cardiac puncture and killed. Tumors were excised and the radioactivities of ^{131}I and ^{125}I in the tumor and blood were counted with a well-type gamma counting system (Beckman 7000). The tumors were weighed after drying at 70°C overnight and the amount of labeled albumin and fibrinogen in 0.1 g of tumor was calculated as % of injected dose.

Electron microscopic observation

In order to examine the effect of hyperthermia on the fibrin deposition in the tumor by an electron microscope, the animals were killed by cervical dislocation 6 hr after heating at 43.5°C for 30 min, and the tumors were excised immediately. After fixing in cold cacodylate buffer solution, the tumors were washed in phosphate buffer, dehydrated in graded ethanol and embedded in Epon. Thin sections were mounted on copper grids and treated with anti-A/J mice fibrinogen–rabbit immunoglobulin and then with anti-rabbit IgG–goat Ig-peroxidase conjugate (Cappel Laboratories). The tissue sections were subsequently stained with uranyl acetate, and examined with a JEOL electron microscope. The anti-A/J mice fibrinogen–rabbit immunoglobulin used in the above process was prepared as follows. An emulsion of A/J mice fibrinogen and Freund's complete adjuvant was injected intramuscularly into New Zealand white rabbits once a week for a total of three injections. Two weeks after the last injection, the rabbits were bled and the anti-A/J mice fibrinogen–rabbit immunoglobulin was precipitated by saturated ammonium sulfate (33% (v/v).

RESULTS

Figures 1 and 2 show the time course of changes in the content of [^{125}I]albumin and [^{131}I]fibrinogen, respectively, in the tumors after heating at

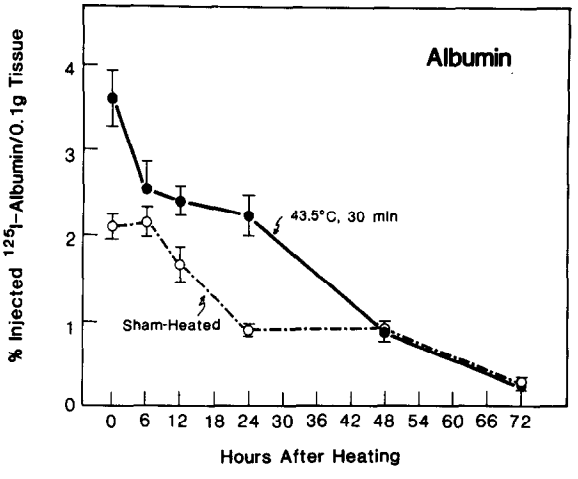
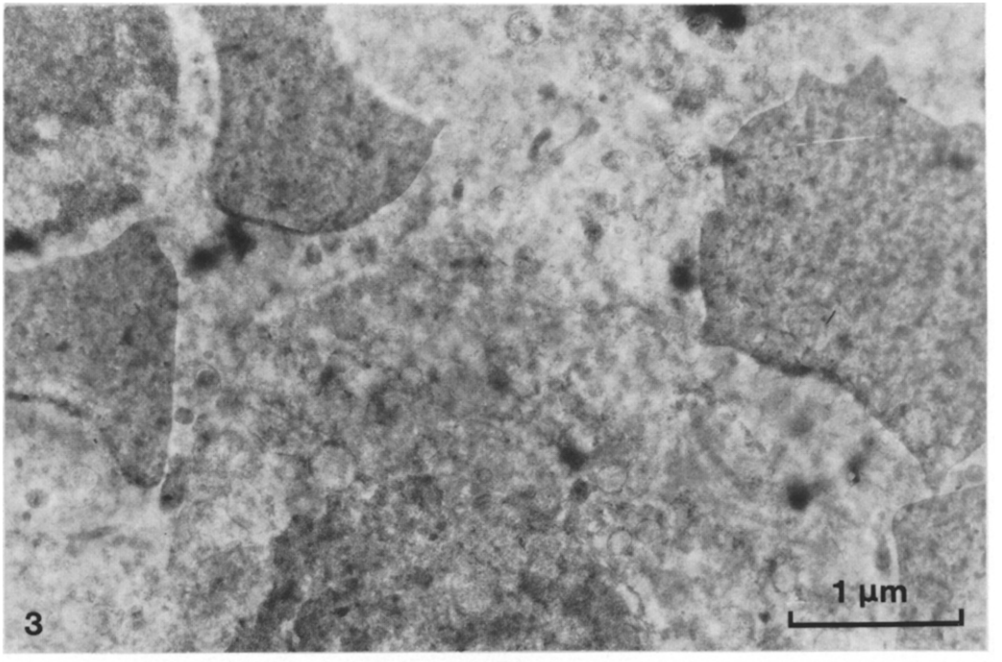


Fig. 1. Changes in radioactivities in SCK tumor after i.v. injection of [^{125}I]*albumin immediately before heating at* 43.5°C *for* 30 min *or sham-heating. The radioactivities were expressed as % of injected dose/*0.1 g *of dried tumor. Each data point is the average of eight mice. The bars show the S.E.M.*



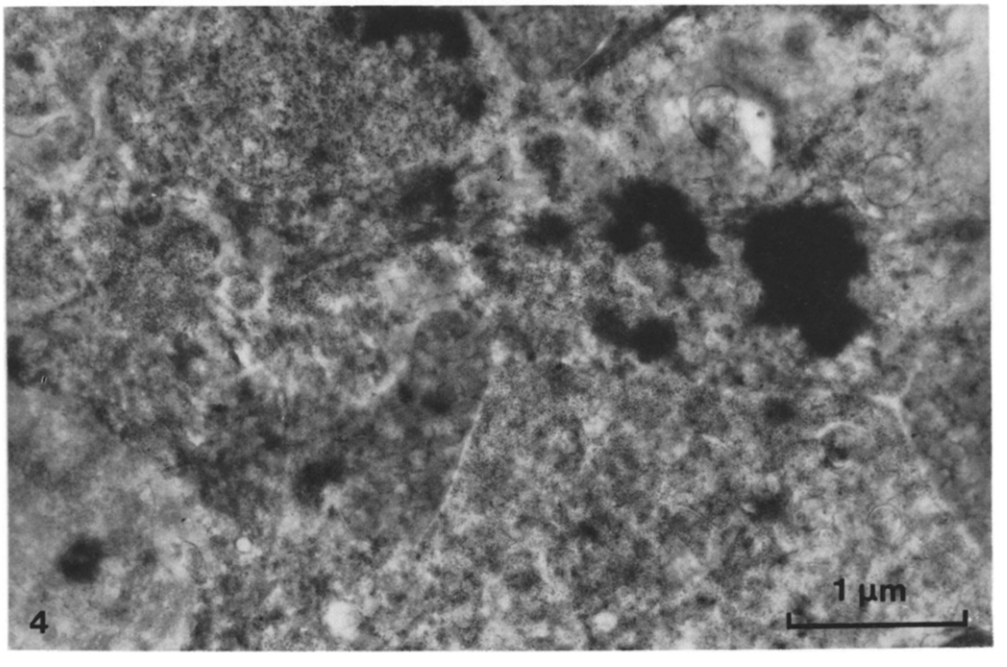


Fig. 3. Electron microscopic demonstration of sham-heated SCK tumor. Fine electron-dense peroxidase granules (fibrin) can be seen.
Fig. 4. Electron microscopic demonstration of heated SCK tumor. The electron-dense peroxidase granules (fibrin) are more abundant and clumped than those in the sham-heated tumors.

43.5°C for 30 min. Since the labeled albumin and fibrinogen were injected immediately before the tumors were heated for 30 min, the 0 hr in Figs 1 and 2 correspond to 30 min after the injections. In the sham-heated control tumors, the content of [^{125}I]albumin at 0 hr (30 min after injection) was 2.10 ± 0.14%/0.1 g of injected dose. The content of [^{125}I]albumin stayed at almost the same level for 6 hr and then started to decrease. The [^{125}I]albumin content at 24 hr was 0.90 ± 0.08%/0.1 g and only a small fraction (0.26%) of the injected [^{125}I]albumin was detected in the tumor when measured at 72 hr. The accumulation of [^{125}I]albumin immediately after heating was 3.60 ± 0.33%/0.1 g of injected dose, which was 1.7 times greater than that in the sham-heated control tumors. However, the content of labeled albumin in the heated tumor rapidly decreased during the first 6 hr post-heating, and then somewhat slowly during the next 2 days, reaching the control level at 48 hr. At 72 hr after heating the content of labeled albumin in the tumors was only 0.23%/0.1 g of injected dose.

As shown in Fig. 2, the content of [^{131}I]fibrinogen in the sham-heated control tumors was 3.01 ± 0.43%/0.1 g of injected dose at 0 hr (30 min after injection). It increased to 7.50 ± 0.85%/0.1 g at 6 hr, and then decreased to 3.43 ± 0.35%/0.1 g at 24 hr. In the heated tumor the content of [^{131}I]fibrinogen at 0 hr was 8.00 ± 0.36%/0.1 g of injected dose, which was about 2.7 times that in the sham-heated control tumors, and it further increased to 11.93 ± 1.04%/0.1 g at 6 hr. Whereas the [^{131}I]fibrinogen content in the sham-heated tumor began to decrease after the increase during the first 6 hr, as described above, it remained elevated for 24 hr in the heated tumors and then decreased, reaching the control level at 48 hr. At 72 hr the labeled fibrinogen content in the control and heated tumors was 1.68%/0.1 g and 1.66%/0.1 g of injected dose, respectively.

The electron microscopic examination of sham-heated tumors demonstrated that fine electron-dense peroxidase granules, presumably fibrin, scattered throughout the control tumors (Fig. 3). In the heated tumors (Fig. 4) the electron-dense materials were more clumped, larger and abundant, particularly in the hemorrhagic area, as compared with those in the control tumors (Fig. 3).

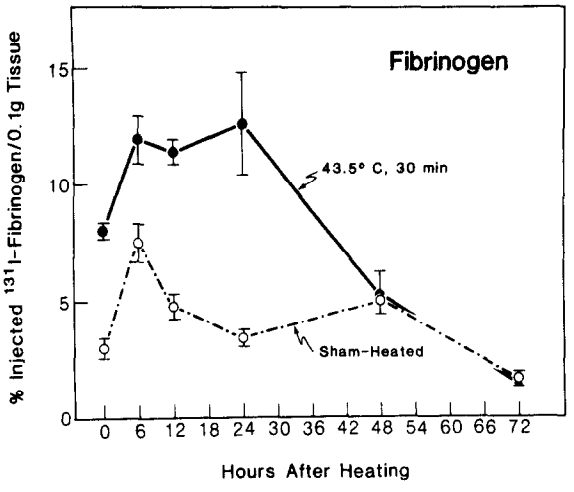


Fig. 2. Changes in radioactivities in SCK tumor after i.v. injection of [^{131}I]*fibrinogen immediately before heating at* 43.5°C *for* 30 min *or sham-heating. The radioactivities were expressed as % of injected dose/*0.1 g *of dried tumor. Each data point is the average of eight mice. The bars show the S.E.M.*

DISCUSSION

The present study demonstrated that the turnover rate of fibrinogen and albumin in the SCK tumor is quite different, particularly after heating. As shown in Fig. 1, the initial accumulation of [^{125}I]albumin in the heated tumors was greater than that in the control tumors. This increased accumulation of [^{125}I]albumin in the heated tumor could be attributed to either an increase in vascular permeability or a leakage of plasma protein, including albumin, as a result of rupture of vessels. It should be noted that while the initial accumulation of [^{131}I]fibrinogen and that of [^{125}I]albumin were similar in the sham-heated tumors, the initial accumulation of [^{131}I]fibrinogen was greater than that of [^{125}I]albumin in the heated tumors in terms of % injected dose/0.1 g of tissue. In this regard, it should be pointed out that fibrinogen denatures at 40°C, while albumin does not. The greater increase in accumulation of fibrinogen relative to albumin may be attributed, in part, to the heat-induced denaturation of fibrinogen and accumulation of the altered fibrinogen during heating. The content of [^{131}I]fibrinogen further increased during the first 24 hr post-heating, while that of [^{125}I]albumin soon declined in the heated tumors, suggesting that certain mechanism(s) more than a simple and passive increase in extravasation of plasma protein is involved in the turnover of fibrinogen in the tumors. The accumulation of fibrinogen and albumin in the tumor is a net product of extravasation and removal of these materials. When tumors are heated, the transport of fibrinogen and albumin across the vascular wall in the tumor would be increased; the tumor vessels are known to be devoid of basement membrane, and are thus rather leaky. Under severe heat stress, hemorrhage may occur due to rupture of vessels. It is likely that whereas most of the extravasated albumin soon returns to circulation, the extravasated fibrinogen undergoes conversion to fibrin followed by polymerization and gelation of the fibrin in the presence of thrombin [18]. The cellular consti-

tuents of extravasated blood at the damaged vessel site would inevitably be aggregated in the thrombus. We previously reported that heating at 43.5°C induced severe vascular occlusion in the SCK tumor [10–12]. It may be concluded that an increased accumulation of fibrinogen and probably its conversion product, fibrin, in the SCK tumors after heating at 43.5°C for 30 min we observed in this study is related to the vascular damage accompanied by occlusion of vasculature. In this connection, it is of interest that we observed a decrease in blood flow after heating at 45°C and not at lower temperatures in the Walker tumor [10], and that Copeland [19] and Copeland and Michaelson [20] reported that an increase in [^{131}I]fibrinogen localization in the Walker tumor occurred at 45°C and not at lower temperatures.

As shown in Fig. 2, the ^{131}I content in the heated SCK tumor declined from 24 hr after heating, probably because fibrin clots are dismantled by a fibrinolytic process and removed from the tumors. The time of this event appears to coincide with the beginning of recovery of vascular function in the heated SCK tumor as previously reported [10–12].

Copley *et al.* [23] described a process by which fibrinogen aggregates inside the blood vessels without the presence of thrombin, which is somewhat different from the generally believed process of fibrin deposition in the damaged tissues. It was suggested that fibrinogen is adsorbed first at the damaged site in the wall of blood vessels, which leads to further adsorption of fibrinogen and other plasma proteins layer upon layer. As a consequence, the lumen of the vessel is partially or completely blocked by the progressively growing protein lump, and subsequently, coagulation of fibrin as well as aggregation of platelets and blood cells takes place. Copley *et al.* [23] further suggested that the above-mentioned intravascular fibrinogen clotting and occlusion of vessels may occur even in the absence of vascular damage when the pH value at close proximity to the endothelium is low. In this regard, it should be mentioned that the intratumor environment is intrinsically acidic and it becomes further acidic when the tumors are heated [13–17]. The pH in the SCK tumor is about 6.92 and it decreases to 6.71 after heating at 43.5°C for 30 min [13]. Perhaps both the classical thrombin-mediated fibrination as a result of vascular damage and the coagulative accumulation of fibrinogen in the acidic milieu inside and outside the vascular lumen without vascular damage may be responsible for the increased accumulation and retention of fibrinogen in the heated SCK tumor.

The electron microscopic study (Figs 3 and 4) showed that the fibrinogen or fibrin was more abundantly deposited in the heated tumors than in the control tumors, which was in agreement with the increased radioactivity in the heated tumors after i.v. injection of [^{131}I]fibrinogen. Furthermore, the fibrin appeared to be clumped and concentrated in the hemorrhagic area in the heated tumors as compared with sham-heated tumors.

In conclusion, hyperthermia at 43.5°C for 30 min significantly enhanced the accumulation of fibrinogen and/or its conversion product, fibrin, in the SCK tumor. These changes in the fibrinogen and fibrin turnover appear to be related to the heat-induced vascular damage in the tumors.

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