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Tumour Growth Delay, Cell Inactivation and Vascular Damage Following Hyperthermic Treatment of a Human Melanoma Xenograft*

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Abstract—*The effect of hyperthermia at* 42.5°C *on a human melanoma xenograft in athymic mice was studied. The tumours were heated* in vivo *in a water-bath. Tumour growth delay and single-cell survival* in vitro *were used as endpoints. Qualitative information regarding heat-induced vascular damage was obtained from microangiographic analysis. Tumour growth delay after a given treatment was considerably longer than that expected from the cell survival measured* in vitro *immediately after treatment. Experiments in which removal of the tumours was delayed revealed that tumour cells were continuously dying for at least* 24 hr *after heat treatment. The volume of the tumour vasculature was considerably reduced after treatment, suggesting that the delayed cell death was attributed to vascular occlusion which resulted in an insufficient supply of oxygen and nutrients and an increased tumour acidity. The present work indicates that at least two mechanisms may be involved in heat-induced cell inactivation in our xenograft: firstly, direct cytotoxic effect of heat; secondly, indirect effect following heat-induced vascular damage.*

INTRODUCTION

THE POTENTIAL of hyperthermia as a cancer treatment modality has been investigated in cultured cells and experimental tumours [1, 2]. Furthermore, progress in the technology for local heating and temperature measurement in cancer patients [3, 4] has stimulated clinical investigations with hyperthermia, either used as a single modality or in combination with radiation or cytotoxic drugs [5–8].

In contrast to earlier conclusions that malignant or transformed cells are more heat sensitive than their normal counterparts [9], recent works have demonstrated no systematic difference in heat sensitivity between malignant and normal cells [10, 11]. However, studies *in vitro* have revealed that the effect of hyperthermia is enhanced in poor nutrition media [12] and at low pH [13, 14]. Although early reports suggested that hypoxic cells are more heat sensitive than well-oxygenated cells [15, 16], recent reports indicate that hypoxia has no effect on heat sensitivity [17]. Since tumours are often inadequately vascularized, they may contain a considerable number of cells which are at low pH, nutritionally deprived and/or hypoxic [18, 19]. A therapeutic gain of hyperthermia is therefore expected on the basis of differences in physiology between tumours and normal tissues.

In addition, hyperthermia shows different effects on the vasculature in tumours and normal tissues. The blood flow in normal tissues is profoundly enhanced by heat treatment at the temperatures commonly used in clinical hyperthermia [20–23]. The effects of hyperthermia on the tumour vasculature vary considerably among different tumour lines. In some tumours, especially after mild heat treatment, the blood flow is unchanged or enhanced [24–26]. In others, heat treatment leads to considerable vascular damage and reduced blood flow [27–31], which may result in secondary tumour cell death [32–35].

The effects of hyperthermia on human tumour xenografts in the athymic nude mouse have been extensively studied at our institute. The vascular

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network of such xenografts is of murine origin, while the parenchymal tumour cells are of human origin [36]. Previous work has shown that, when tumours were heated *in vivo*, the tumour cells were more heat sensitive than the same cells heated *in vitro* under aerobic conditions at pH 7.4, indicating that the physiological conditions in the xenografts might have potentiated heat-induced cell inactivation [37]. The heat sensitivity of the tumour cells could be enhanced further by artificially occluding the blood supply to the tumours during heating *in vivo* [38]. The purpose of the present work was to investigate whether moderate heat treatments would cause vascular damage which resulted in delayed cell death. A human malignant melanoma was used as a test tumour since the effect of hyperthermia has been clinically investigated on this type of tumour [39–42].

MATERIALS AND METHODS

Mice and tumour

BALB/c/nu/nu/BOM mice of both sexes were used. They were kept under specific pathogen-free (SPF) conditions.

The malignant melanoma was originally derived from a lymph node metastasis of a patient at The Norwegian Radium Hospital. Tumour tissue was transplanted directly into athymic mice without previous adaptation to *in vitro* culture conditions. Histologically, the metastasis was composed of melanin-poor atypical naevus cells growing in large spheres. Cells and nuclei varied greatly in size and shape, and numerous mitoses were seen.

The melanoma was grown serially in athymic mice by implanting tumour fragments, approximately $2 \times 2 \times 2$ mm in size, subcutaneously into the flanks of recipient mice. Passages 41–46 of the melanoma were used in the present work. Small tumour fragments were implanted subcutaneously in the right hind leg. The tumours were exposed to heat when they had reached a volume of 200–500 mm^3. Light- and electron-microscopic examinations showed that the histological appearance of the xenograft was similar to that of the metastasis in the donor patient.

Hyperthermic treatment

The tumours were treated by immersing the tumour-bearing leg into a water-bath. Non-anaesthetized mice were placed in Perspex mouse holders and immobilized by a piston. A hole was cut in each holder through which the leg with the tumour protruded. The leg was loosely fixed with tape without impairing the blood flow. The water-bath temperature was thermostatically kept at 42.7°C. The tumour temperature was measured with a needle thermocouple probe (diameter 0.7 mm) connected to an electric universal thermometer (Ellab A/S, type TE 3-S, Denmark). It reached 42.5 ± 0.1°C in 4 min and remained within this range during the prolonged treatment. The temperature did not vary significantly with the position of the probe in a tumour or among individual tumours.

Measurements of response

Growth curves for treated and untreated tumours were established on the basis of calliper measurements of tumour volume. Two perpendicular diameters (length and width) were recorded and tumour volumes were calculated as $V = 1/2\ a \cdot b^2$, where a and b are the longest and shortest diameter respectively. Since the skin around the tumours was thin, no correction was made for the skin thickness. Growth delay, i.e the time from the day of treatment to the day the mean normalized tumour volume again reached unity, was used as the endpoint.

Single-cell survival was measured *in vitro* by using the soft agar colony assay developed by Courtenay and Mills [43]. Since the yield of morphologically intact cells was higher after mechanical than after enzymatic dispersion of the tumours, single-cell suspensions were prepared without the use of enzymes. The tumours were finely minced by a scalpel and a pair of tweezers in culture medium (Ham's F12 medium with 20% foetal calf serum) containing 250 mg/l penicillin and 50 mg/l streptomycin (Gibco-Biocult, Glasgow). The resulting suspensions were filtered through 30-μm filters (Nytal, Schweizerische Seidengazefabrik AG). The cell concentration was determined by the use of a haemocytometer. Cells having an intact and smooth outline with a bright halo were scored as morphologically intact and counted. The cell suspensions were diluted to appropriate concentrations in culture medium. The soft agar was prepared from powdered agar (Bacto agar, Difco) and culture medium. Erythrocytes from August rats and melanoma cells were added as described previously [44]. Aliquots of 1 ml of the soft agar were seeded in glass tubes. Immediately afterwards, the tubes were flushed with a gas mixture of 5% O_2, 5% CO_2 and 90% N_2 and carefully sealed. The tubes were incubated at 37°C for 3–4 weeks. Culture medium (2 ml) was added 5 days after seeding and changed weekly. The tubes were flushed on each occasion and once between each medium change. Colonies containing more than 50 cells were counted under a stereomicroscope. The plating efficiency of untreated cells was 15–35% and independent of the number of cells seeded in the range of

100–5000 cells per tube. Details of the experimental procedure are reported elsewhere [44].

Vascularization

The vascular system of the tumours was filled with a contrast medium (100 ml 0.9% saline, 5 g gelatin, 50 g Pb_3O_4, 1 ml detergent (Joy/Salo), 5000 U heparin). The mouse peritoneal cavity was opened after an intraperitoneal injection of 0.1 ml heparin. A needle (Terumo 23G 0.6 × 25 mm, No. 10) connected to a syringe by a 20-cm polyethylene tube was inserted into the abdominal aorta in the cranial direction. The contrast medium was then injected (0.5 ml/min) at a low and steady pressure to prevent vascular damage. About 1 ml of the contrast medium was sufficient to fill all vessels in each mouse. The heart of the mouse functioned for about 1 min after the beginning of injection, and thus helped the distribution of the contrast medium in the body. The mice were fixed in 4% formalin for 1–2 weeks before the tumour removal.

The tumours were then frozen in embedding medium (Tissue-Teck II, O.C.T. compound) and cut into 720-μm-thick sections by a freeze microtome. The sections were directly placed on a film envelope tightly covering a film and irradiated by a Siemens 'Mammomat' X-ray unit. The film (Kodak 4489) was developed in Agfa G-170C developer. Further details of the experimental procedure are reported elsewhere [45].

Histological sections, about 3 μm thick, were made for light microscopical examinations. They were stained with haematoxylin and eosin.

RESULTS

Mean normalized tumour volume following heat treatments at 42.5°C for 30, 45 and 60 min is shown in Fig. 1. Heating inhibited tumour growth, but all tumours regrew exponentially.

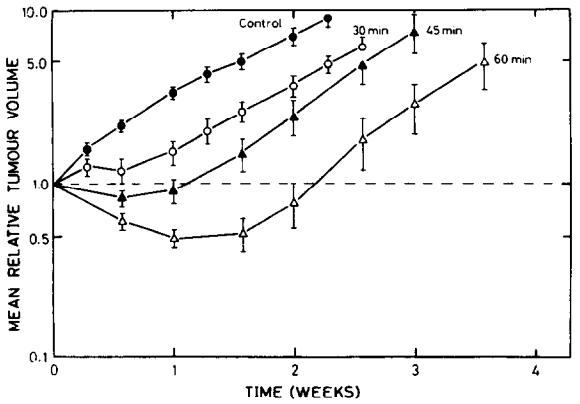

Fig. 1. Mean relative tumour volume for a human melanoma xenograft heated at 42.5°C for 0, 30, 45 and 60 min as a function of the time elapsed after treatment. Each curve is based on 15–20 tumours. The vertical bars represent standard errors.

The exponential portion of the growth curve of each heated tumour was nearly parallel to that of the control tumours, irrespective of length of heating. The response to treatment increased with increasing heating time and was, for a given heating time, independent of the initial tumour volume in the volume range 200–500 mm^3. Growth delay was 15 days after heating for 60 min at 42.5°C.

The survival curve for the melanoma cells heated at 42.5°C as solid tumours *in vivo* is shown in Fig. 2. The tumours were excised immediately after heating and cell survival was assayed *in vitro*. An exponential curve through the origin was fitted to the survival data by linear regression analysis. The surviving fraction after 60 min at 42.5°C was about 0.6, which is surprisingly high compared to the relatively long growth delay caused by the same heat treatment *in vivo*.

In order to explain the apparent discrepancy between the *in vivo* and the *in vitro* data, cell survival was studied at different times after *in vivo* treatment (60 min at 42.5°C). The results are shown in Fig. 3.

The cell yield, i.e. the number of cells obtained per unit tumour volume, was determined for both treated and untreated tumours. All tumour volumes were measured immediately before treatment. The relative cell yield, i.e. the cell yield from treated tumours relative to that from untreated ones, decreased gradually after treatment to a level of about 0.1 in 5 days (Fig. 3a). The cell yield from the untreated tumours and that from the tumours excised immediately after treatment were not significantly different.

The relative plating efficiency, i.e. the plating efficiency of the morphologically intact cells from the treated tumours relative to that from the

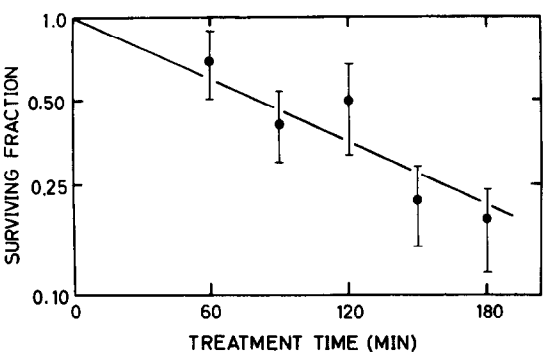

Fig. 2. Survival curve for cells from a human melanoma xenograft heated at 42.5°C as solid tumours in vivo. Each point represents the mean surviving fraction calculated from 4–6 tumours. Standard errors are indicated by vertical bars. The surviving fraction for each tumour was calculated from the mean number of colonies in 4–5 tubes with cells from the heated tumour and 4–5 tubes with cells from an unheated tumour.

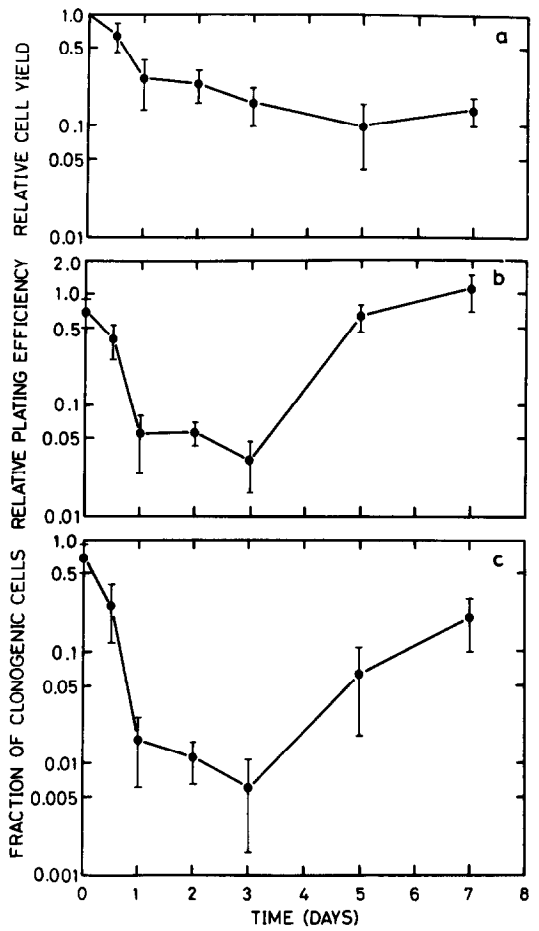

Fig. 3. Cell yield per unit tumour volume (a), plating efficiency (b) and number of clonogenic cells per unit tumour volume (c) for a human melanoma xenograft heated at 42.5°C *for* 60 min *as a function of the time elapsed after treatment. Tumour volumes were measured immediately before treatment. All values are expressed as fractions of the values for unheated control tumours. The fraction of clonogenic cells was obtained by multiplying the relative cell yield and the relative plating efficiency for each tumour. The points and the vertical bars represent mean values and standard errors, based on* 4–6 *individual tumours. The relative plating efficiency for each tumour was calculated from the number of morphologically intact cells seeded and the mean number of colonies in* 4–5 *tubes with cells from heated tumours and* 4–5 *tubes with cells from unheated tumours.*

untreated ones, is shown as a function of the time elapsed after treatment in Fig. 3b. The relative plating efficiency was about 0.6 immediately after treatment, decreased to a minimum of about 5×10^{-2} in 1–3 days after treatment and then approached unity at 7 days. Figure 3b demonstrates that the majority of the cells which were able to form colonies immediately after treatment lost clonogenicity in 1–3 days after treatment.

The relative cell yield (Fig. 3a) and the relative plating efficiency (Fig. 3b) were multiplied to obtain the fraction of clonogenic cells, i.e. the number of clonogenic cells per unit tumour volume relative to that for untreated tumours (Fig. 3c). The fraction of clonogenic cells decreased with time after treatment towards a minimum of about 5×10^{-3} in 3 days, and then increased again. The decrease indicates that a considerable fraction of the tumour cells was killed after the heat treatment. Beyond the third day after treatment the total number of repopulating cells was larger than the total number of dying cells, and the fraction of clonogenic cells increased. Because of this masking effect due to the repopulation, the true surviving fraction was probably somewhat lower than the lowest fraction of clonogenic cells observed, i.e. somewhat lower than 5×10^{-3}.

In order to shed light on the mechanisms behind the delayed cell death, the vascular system of heated tumours (60 min at 42.5°C) was studied. Microangiograms showed that the vascular volume was considerably reduced after heating. Vascular structures were few and scattered in the tumours fixed 0.5–48 hr after treatment. The number of vessels increased thereafter with time (Fig. 4). Panel (a) demonstrates an untreated tumour in which the density of vascular structures is high except in a small central area, where the tumour was confirmed to be necrotic. The vascular density is considerably reduced at 24 hr after treatment (panel b). The vascularized area increased in size by 3 days after treatment (panel c), and by 9 days tumours were well vascularized (panel d).

It should be noticed that the vascular damage was inhomogeneous. Usually, it decreased with increasing depth in the tumours; i.e. it was most pronounced in the upper half and least pronounced in areas adjacent to the muscle tissue (Fig. 5).

The observations made from the microangiograms were confirmed by histological examinations (Fig. 6). Corpuscles in most vessels in heated tumours were densely packed compared to those in unheated tumours, and often appeared rigid and deformed, indicating that the blood flow was occluded. Thromboses and extravasation of erythrocytes were evident in some tumours.

DISCUSSION

The growth delay for the melanoma xenograft after heat treatment at 42.5°C for 60 min was 15 days. The fraction of clonogenic cells after the same treatment was about 0.6 or 5×10^{-3} when measured immediately or 3 days after heating respectively. The median cell-cycle time of the untreated tumour is about 41 hr [46]. Thus the results from the regrowth experiment are consistent with those from the *in vitro* experiment when delayed cell death is taken into account but not when cell survival is measured immediately after treatment. Consequently, conclusions drawn

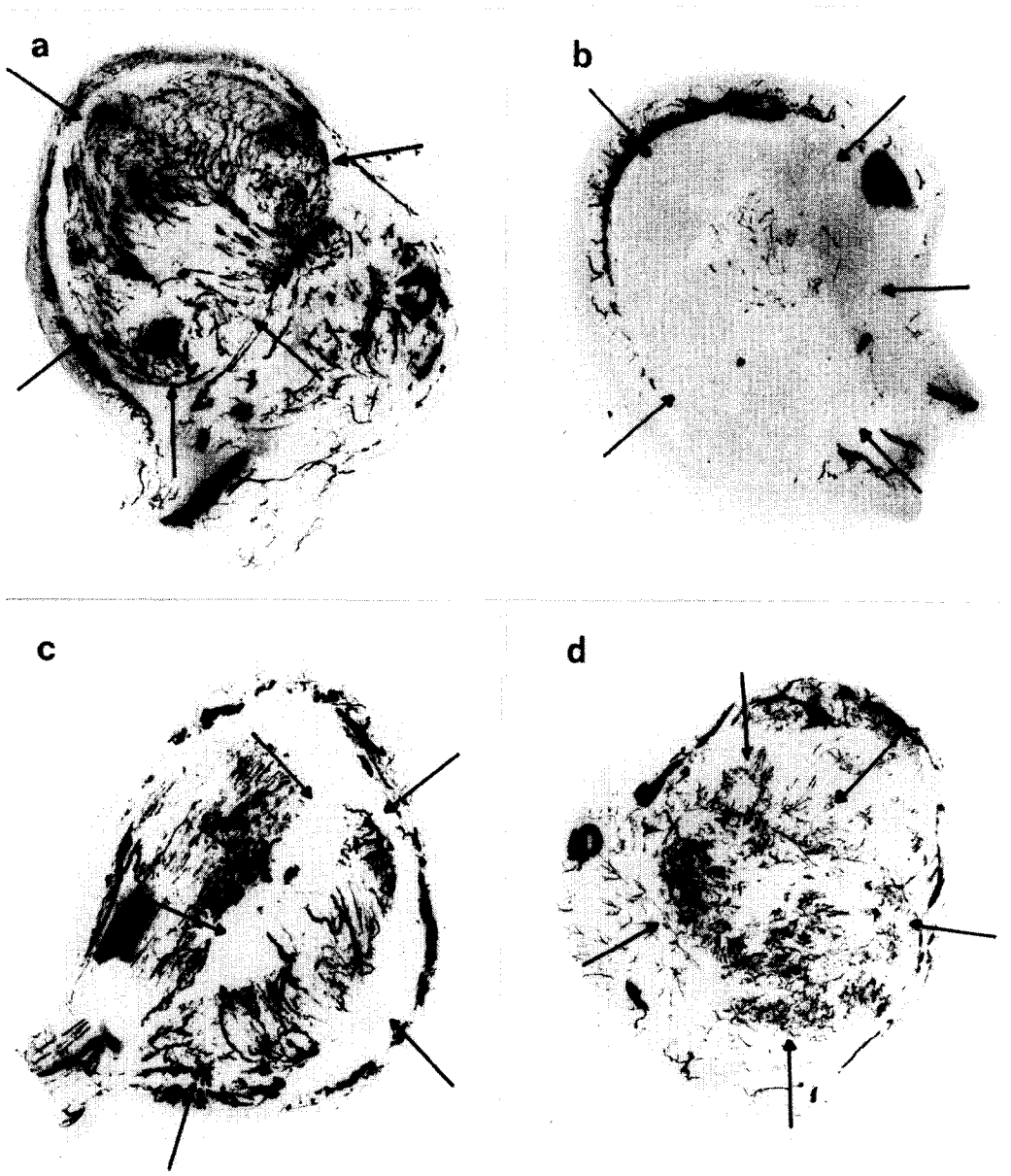

Fig. 4. Microangiograms of 720-μm-thick tumour sections from a human melanoma xenograft heated at 42.5°C *for* 60 min. *An unheated tumour (a) and tumours fixed* 24 hr *(b),* 3 days *(c) and* 9 days *(d) after treatment. All four sections were cut from the deeper third of a tumour, i.e. the third closest to the muscle tissue. The areas circumscribed by the arrow-heads are tumour tissue, whereas the areas outside are normal tissue.*

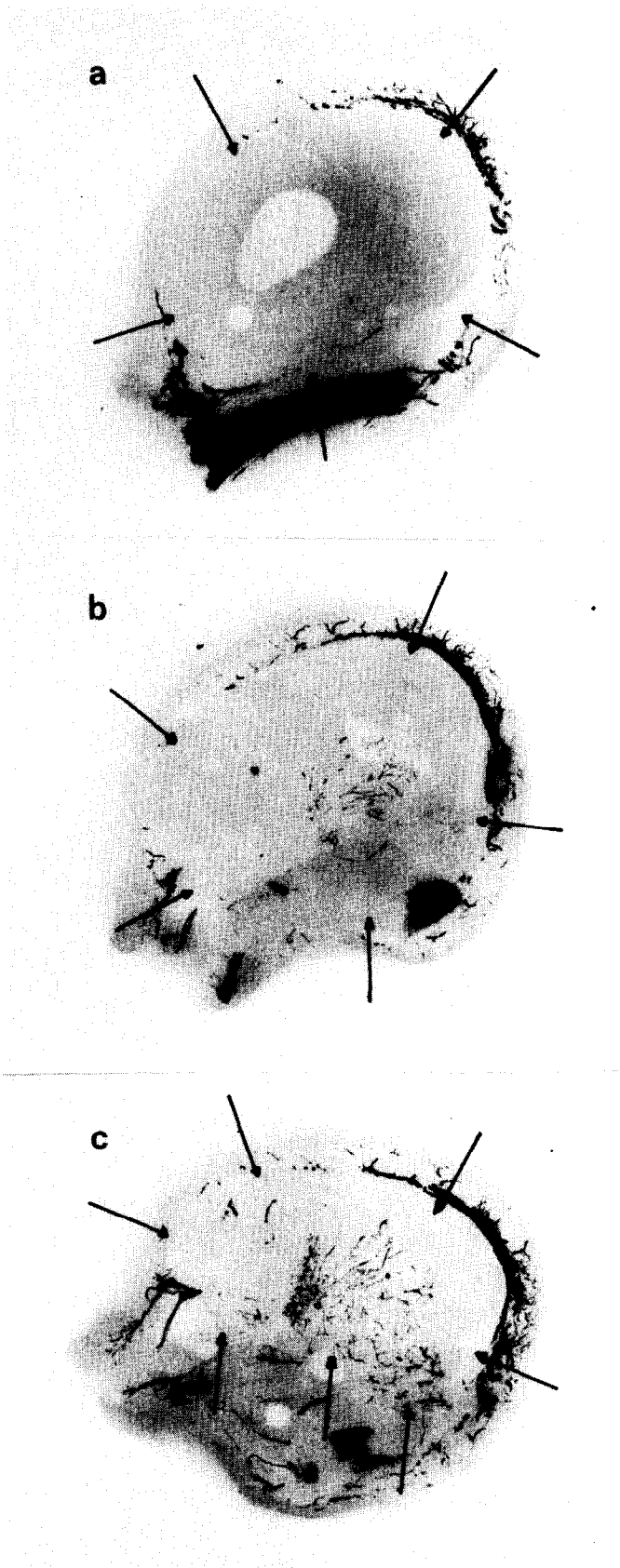

Fig. 5. Microangiograms of 720-μm-thick tumour sections from a human melanoma xenograft heated at 42.5°C for 60 min and fixed 24 hr after treatment. The sections were cut from different depths in a single tumour: the middle (a), the deeper third (b) and the section adjacent to the underlying muscle tissue (c). The areas circumscribed by the arrow-heads are tumour tissue, whereas the areas outside are normal tissue.

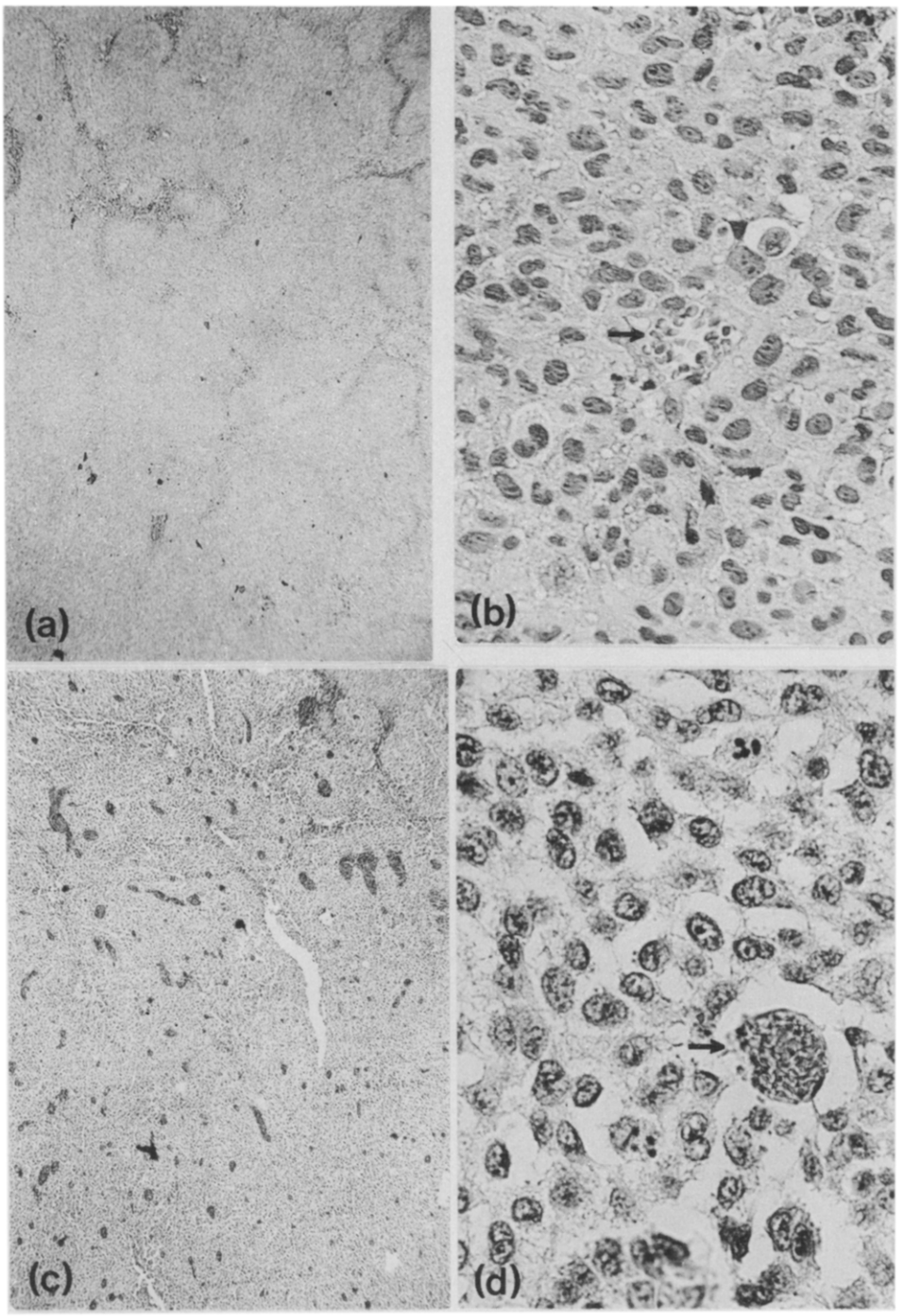

Fig. 6. Light micrographs (40× and 620×) of a human melanoma xenograft: an unheated tumour (a and b) and a tumour fixed 0.5 hr after treatment at 42.5°C for 60 min (c and d). The micrographs illustrate that the density of corpuscles in the vessels of the heated tumour (c and d) is extremely high compared with that in the vessels of the unheated tumour (a and b). The arrows in panels (b) and (d) point at blood vessels.

from hyperthermia experiments in which cell survival is measured *in vitro* immediately after treatment *in vivo* are difficult to use to predict cell killing after clinical use of hyperthermia.

Delayed cell death after heat treatment has also been observed in murine tumours. Song *et al.* [34] and Kang *et al.* [35], who studied the SCK tumour, found that the fraction of clonogenic cells was reduced from about 0.2 to about 6×10^{-3} within 12 hr after exposure to 43.5°C for 30 min. Similarly, Marmor *et al.* [32] and Fajardo *et al.* [33] demonstrated in the EMT6 tumour that the fraction of clonogenic cells decreased from about 1×10^{-2} to about 3×10^{-4} during a 48-hr period after exposure to 44°C for 30 min. Significant delayed cell death was not observed in this tumour after treatment at 43.5°C for 30 min [32]. Results from the same laboratory [1] showed that delayed cell death did not occur in the RIF tumour even after heating at 44°C for 30 min. The present work shows that delayed cell death is induced by lower heat doses in the melanoma xenograft than in the EMT6 and RIF tumours. The results from the melanoma xenograft are similar to those reported for the SCK tumour. Heat doses were comparable, i.e. for 60 min at 42.5°C for the melanoma xenograft and for 30 min at 43.5°C for the SCK tumour, and the magnitude of the delayed cell death was about equal. However, the kinetics of the delayed cell death and the rate of repopulation were different in these two tumours. In the melanoma xenograft about 3 days elapsed until the total number of repopulating cells was larger than that of dying cells, while in the SCK tumour, this increase had already occurred by about 12 hr after treatment. This difference may be explained from differences in the cell proliferation kinetics in the two tumours.

Microangiograms showed that the vascular volume in the melanoma xenograft was considerably reduced shortly after treatment. An increase in the vascular volume, i.e. recovery from the heat-induced damage, was not observed until 3 days after heating. The delayed cell death was therefore probably due to protracted hypoxia, increased acidity, nutrient deficiency and/or accumulation of toxic metabolic products caused by a transient or permanent collapse of the vascular system. This observation is also in agreement with the observation of Kang *et al.* [35] that the delayed cell death in the SCK tumour was preceded by a reduction in the vascular volume as measured by the ^{51}Cr-labelled red blood cell method.

Hyperthermia-induced vascular damage and subsequent reduced perfusion have also been observed in several other experimental tumours [27–31]. The underlying mechanisms for such damage are beyond the scope of the present work. However, our observation from the light microscopical investigation that the lumen of many vessels was densely populated with deformed corpuscles is interesting. Vaupel *et al.* [28] have suggested that although the initial deformation of the shape of corpuscles, which may be due to reduced pH, is reversible, deformed corpuscles tend to stick in tumour microvessels and cause vascular occlusion.

In conclusion, at least two mechanisms are important in heat-induced cell inactivation *in vivo* in our xenograft. Firstly, cells are inactivated due to the direct cytotoxic effect of high temperature *per se*. Secondly, delayed cell death occurs, probably as a result of protracted hypoxia, increased acidity, nutrient deficiency and/or accumulation of toxic metabolic products caused by heat-induced vascular damage.

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