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Differences in the Response of the Microcirculation to Hyperthermia in Five Different Tumours

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Abstract—*The response of the microcirculation in five different tumours, growing in 'sandwich' observation chambers in the back of the rat, to hyperthermia was investigated. The tumours investigated encompassed three human xenografted tumours, of which two were carcinomata of the colon and one of the lung, and two isologous rat tumours, the Rhabdomyosarcoma BA1112 and a rat mammary carcinoma. It was concluded* (1) *that the various tumours required significantly different exposure times for inducing* 50% *stoppage of the tumour microcirculation* (ST_{50}). *This seems to indicate that differences in the characteristics of the tumour cells are more important for causing microcirculatory stoppage than is the sensitivity of the cells of the blood vessels.* (2) *An increase in surface (i.e. volume) was observed in all four tumours examined for this phenomenon. The rate of increase (usually* 1–2% *per hour at* 42.5°C) *was, however, significantly different between the various tumours. This rate was higher by higher exposure temperatures* (43 *and* 43.5°C), *but this was only investigated for the Rhabdomyosarcoma BA1112. Extensive statistical analysis of this phenomenon of volume increase could not demonstrate a correlation with any of the circulation parameters.* (3) *The relative velocity of the erythrocytes in selected capillaries in the tumours decreases as a result of the hyperthermic treatment, and is probably related to the tumour-specific* ST_{50}. (4) *A human colon carcinoma xenograft, one of the tumours investigated, showed strong fluctuations in the parameter 'erythrocyte velocity'. The appearance of such fluctuations did not seem to influence the heat-induced stoppage of the circulation. Probably the phenomenon of fluctuations in the velocities of the erythrocytes in the tumour capillaries is a tumour-specific phenomenon.*

INTRODUCTION

HYPERTHERMIA in the treatment of cancer has not only direct effect of cell killing (see for a recent review [1]), but also affects the physiology of tumours. In experimental tumours generally a shut-down of the tumour microcirculation during or after hyperthermic treatment is observed. This has recently been reviewed by several authors [1–6]. In the latter review the question of which tissue component of the tumour is instrumental in causing the stoppage of the tumour microcirculation that is so often observed in experimental tumours following heat treatment was discussed. Is it caused by changes in the physiology of the tumour cell, or is the vascular wall the most vulnerable component? If the dose-limiting tissue component is the endothelial cell, then one should expect a similar response in different types of tumour. This question led us to investigate a number of tumours in the same way. Determinations on some, but not all, parameters reported in this study were available on two different rat tumours. In addition, experiments on human xenografts were performed, in which the 'sandwich' tumours were carried by the same rat strain, but this time under immune suppression. This report deals with the hyperthermia-only experiments, encompassing 119 different experiments.

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MATERIALS AND METHODS

Tumours

Two experimental rat tumours and three human xenografted tumours were used. The latter were maintained in male BALB/c nude mice, and for the purpose of the experiment, transplanted in the 'sandwich' chambers (see below) in immunosuppressed rats. The latter received initially a daily i.m. injection of 10 $mg.kg^{-1}$ cyclosporin A (Sandimmune® [7]). Later this was—with the same immunosuppressive efficiency—changed to four i.m. injections per week, total dose per week 240 mg. All experiments were carried out in the WAG/Rij strain of rats.

The rat mammary carcinoma RMA originated in 1975 in a female WAG/Rij rat, 1 year after a total body irradiation of 4 Gy. The tumour was maintained in its isologous strain, and its vascular system, as observed in the 'sandwich', system was

typically that of a mammary carcinoma. A characteristic of this is the wide sinusoids, which frequently run in pairs [8]. Histologically, it is presently an undifferentiated carcinoma.

The rat Rhabdomyosarcoma BA1112 originated in 1962 in the muscles of the jaw of a WAG/Rij rat, 35 weeks after a total body dose of 8.64 Gy, followed by bone marrow transplantation. The tumour gradually adapted the characteristics of an undifferentiated sarcoma.

The human tumour xenograft XColF is a carcinoma of the colon which originated in 1977 in a 56-year-old female patient. It has since then been maintained in nude mice. Histologically, it is a well- to moderately differentiated mucinous adenocarcinoma.

The human tumour xenograft XColH is a carcinoma of the sigmoid and originated in 1978 in a 81-year-old male patient. It has since then been maintained in nude mice. Histologically, it is a well-differentiated adenocarcinoma, without mucus formation.

The human tumour xenograft LTH40 is a small cell carcinoma of the lung. It originated in 1980 in a 75-year-old male patient and is since then maintained in nude mice. Histologically, it is a a typical small cell carcinoma.

The 'sandwich' tumour system has been described in this journal [9], as well as its use for investigations on the response of the microcirculation to hyperthermia [7, 10]. In short, the tumour is inoculated between two transparent surfaces on both sides of a very thin layer of subcutis of the back of the rat. The thickness is in this way limited to 0.2–0.3 mm, and continuous observation of the tumour microcirculation, with photographic recording, is possible. Also the erythrocyte velocity in one selected capillary can be traced for this purpose. By doing so, no conclusions regarding flow in terms of $ml.g^{-1}.\ min^{1}$ [11] can be made, but changes can be expressed in relative change to the value at time zero [7]. The relative values, derived in this way, can then be averaged. This has been done in the present investigations.

In the course of the investigations (i.e. for the three last tumours investigated, including the human colon xenograft XColH, the experimental Rhabdomyosarcoma BA1112, and the human lung carcinoma xenograft LTH40), a system was used to determine changes in size of the tumours during treatment. This was done by using markers placed in the tumour tissue. These consist of carbon microspheres, 80 μm diameter, which were implanted in the tumour tissue. When the tumour grows, the peripheral carbon microspheres, which were incorporated in the growing tumour mass, are also carried outwards. During an experiment, i.e. before the hyperthermic treatment begins, a series (usually eight) of peripherally positioned microspheres were located, and their place was also visualized with a drawing prism, and traced on a sheet of paper. The recording of the positions of the selected microspheres was repeated (on a new sheet of paper) at regular intervals. Depending on the type of investigation, the length of invervals selected was 5, 10 or 15 min. Later, the points on the paper were connected, and the surface they encompassed was determined by means of a planimeter. Also in this instance the change, occuring during treatment, was expressed as the relative change to time zero.

Hyperthermic treatment

Details have been described before in this journal [10]. In short, the animal was anaesthetized with Hypnorm® (Philips Duphar) with 0.5–1 $ml.kg^{-1}$ i.p., and the tumour-bearing skin flap was inserted into an isolated Perspex box heated by hot air. The temperature of the air in the box was controlled electronically by a small thermocouple junction which was held in place on the coverslip next to the tumour by means of adhesive gum. Another thermocouple, also attached to the glass coverslip next to the tumour, served for independent thermometry. The air temperature in the box was usually about 0.5°C higher than the tumour temperature. The accuracy of this system is ±0.1°C. A standard exposure time of 180 min was maintained. Most experiments were carried out at 42.5°C, but for the rat Rhabdomyosarcoma BA1112 experiments at 33.5 (= control), 43 and 43.5°C were added.

RESULTS

A summary of the findings at 42.5°C exposure (total exposure time 180 min) with the various endpoints is presented in Table 1. The way the endpoints were derived will be discussed in sequence.

The ST_{50} stands for the 50% stopping time, i.e. the time of heating required to stop flow. This determination is based on the moment (i.e. duration of exposure at 42.5°C) that a cessation of the blood flow in an individual tumour is established by (microscopic) observation. The value of 190 min, which is in fact somewhat longer than the actual exposure time, was derived by extrapolation of the Weibull regression [12]. The microscopic examination is typically repeated at exactly 15 min intervals, and the pooled average value, i.e. the ST_{50}, is calculated via the Weibull probability function [13]. This method has been used extensively in our experiments [7, 10, 13, 14] and has been shown to be a reliable parameter [14]. From Table 1 it appears that, for the various tumours investigated, this ST_{50} value for 42.5°C may range from 190 min (95% confidence limits 173–219 min) for a rat

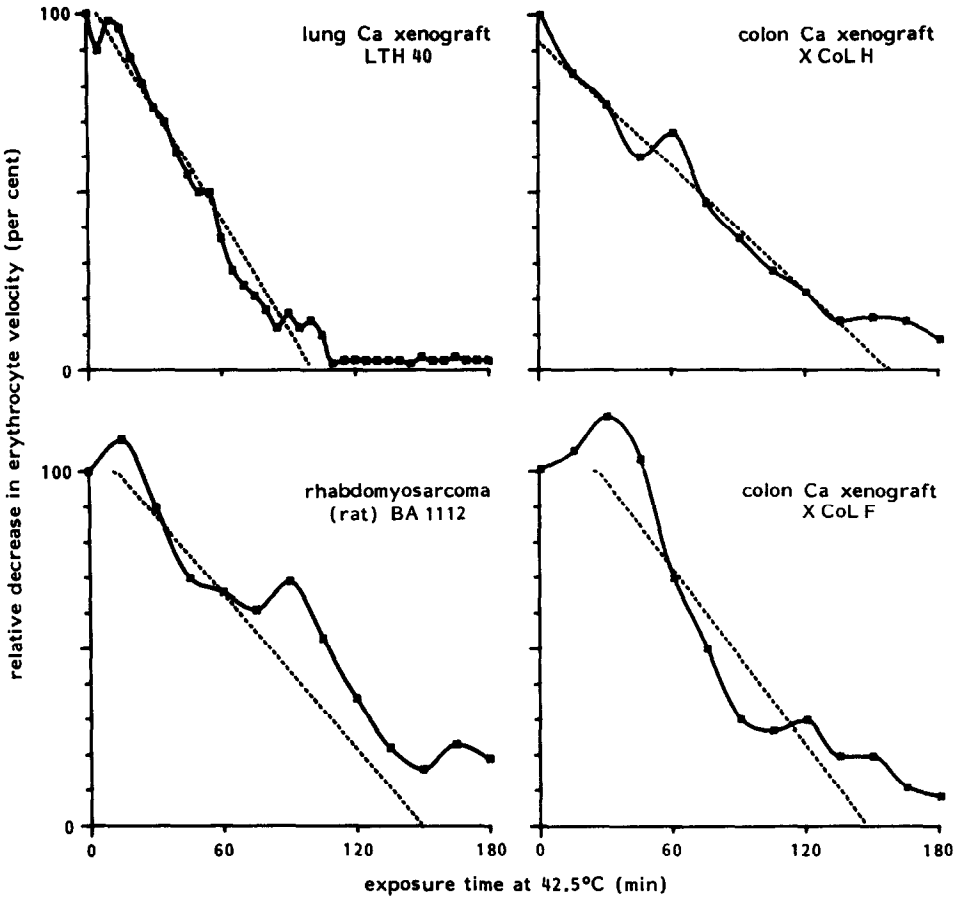

Fig. 1. Decrease in relative erythrocyte velocities in selected capillaries in four different tumour types. Solid lines: average of all *tumours. Dotted lines: linear regression on only those tumours that showed a decrease to zero within the exposure time of* 180 *min. The values of the slopes of these dotted lines are listed in Table* 1.

mammary carcinoma to 102 min (95% confidence limits 96–110 min) for a xenografted human lung carcinoma.

The rate of velocity decrease was measured in one pre-selected capillary per tumour, and the slope of the linear regression represents only those tumours from which that capillary showed a decrease to zero during the 180 min exposure. This is also shown in Fig. 1. In this figure the solid lines represent the average erythrocyte velocity, as measured for all tumours. It can be seen from the 'tail' in these graphs that in some tumours, some capillaries maintained their flow. When these few tumours were deleted, a least-squares linear regression showed in all instances a very satisfactory fit (P values ranging from 0.998 to 1). Although such a process, i.e. decrease in erythrocyte velocity, is not necessarily a linear function with time, its exceedingly good fit provides us with some measure for expressing the rate of decrease, as a percentage per minute exposure. These regressions are depicted in dotted lines in Fig. 1, and comparison with the solid lines, which include also the non-stoppers, show that this approximation provides a reasonable estimate of this value, which is used for tabulation in Table 1.

The third row in Table 1 represents the rate of increase in size, expressed as relative increase in surface area, as discussed in the Materials and Methods. The increase in surface area for the Rhabdomyosarcoma BA1112 is depicted in Fig. 2. Again, such a phenomenon does not necessarily have a linear time–effect relationship, but, as can be seen in Fig. 2, the slope of the line provides a reasonable approximation of a value for comparison. The average slope values with their standard deviation are tabulated in Table 1. They all three differ very significantly ($P < 0.001$).

The bottom row of Table 1 indicates the occurrence of fluctuations in the erythrocyte velocity, as derived from velocity measurements and microscopic examination. An example of this phenomenon is given in Fig. 3. The velocity of erythrocytes is never really constant over a time period of 3 h, as shown in Fig. 3, bottom diagram. In this diagram, both tumours show some fluctuations, but one is obviously much more severe than the other. Indeed, it turned out that, of the three different tumour types investigated, only one showed in virtually all individual tumour preparations an irregularly spaced inhibition in the flux of erythrocytes in the selected capillary to zero. After some time the flow

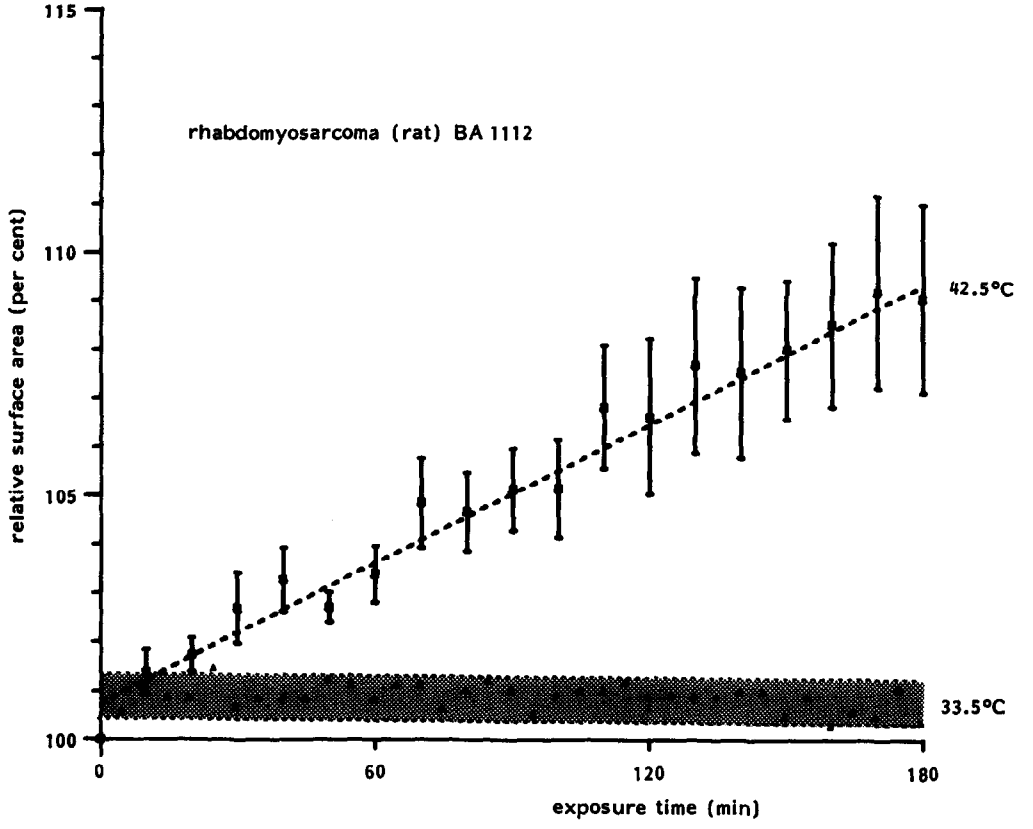

Fig. 2. Relative increase in tumour surface area during hyperthermia in Rhabdomyosarcoma BA1112 during treatment at 42.5 or 33.5°C. Bars indicate S.E.M. Shaded area indicates average S.E.M. for the control determinations at 33.5°C.

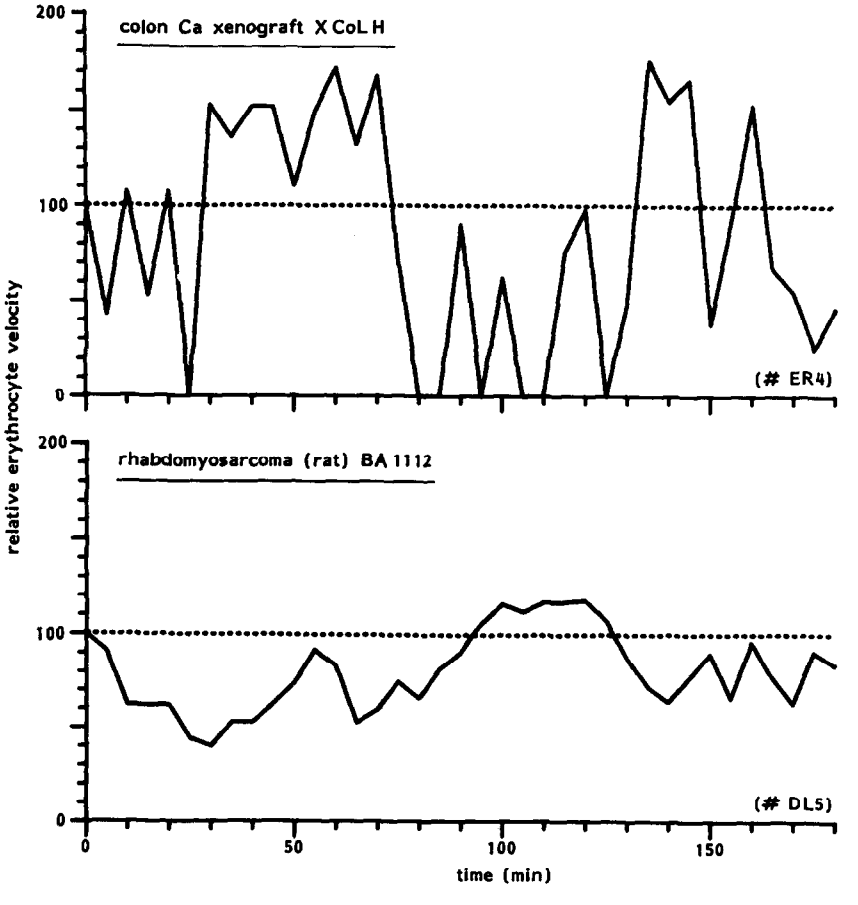

Fig. 3. Examples of fluctuations in erythrocyte velocity in a fluctuating tumour (upper part) and a nonfluctuating tumour at control temperature (33.5°C).

Table 1. *Summary of the microcirculatory determinations at a treatment temperature of* 42.5°C (180 *min*)

	Rat mammary carcinoma RMA	Human colon carcinoma xenograft XColH	Rat Rhabdomyosarcoma BA1112	Human colon carcinoma xenograft XColF	Human lung carcinoma xenograft LTH40
ST50 (min) 95% c.l.	190 173–219	171 138–241	155 151–160	118 107–137	102 96–110
Rate of velocity decrease (%.min^{-1}) ± S.D.	ND*	−0.59 ± 0.025	−0.73 ± 0.015	−0.83 ± 0.095	−1.0387 ± 0.0445
Increase in surface area (%.h^{-1}) ± S.D.	ND*	1.705 ± 0.109	2.861 ± 0.132	ND*	0.61 ± 0.204
Fluctuations in erythrocyte flux	ND*	+	−	−	−

*ND = not determined.

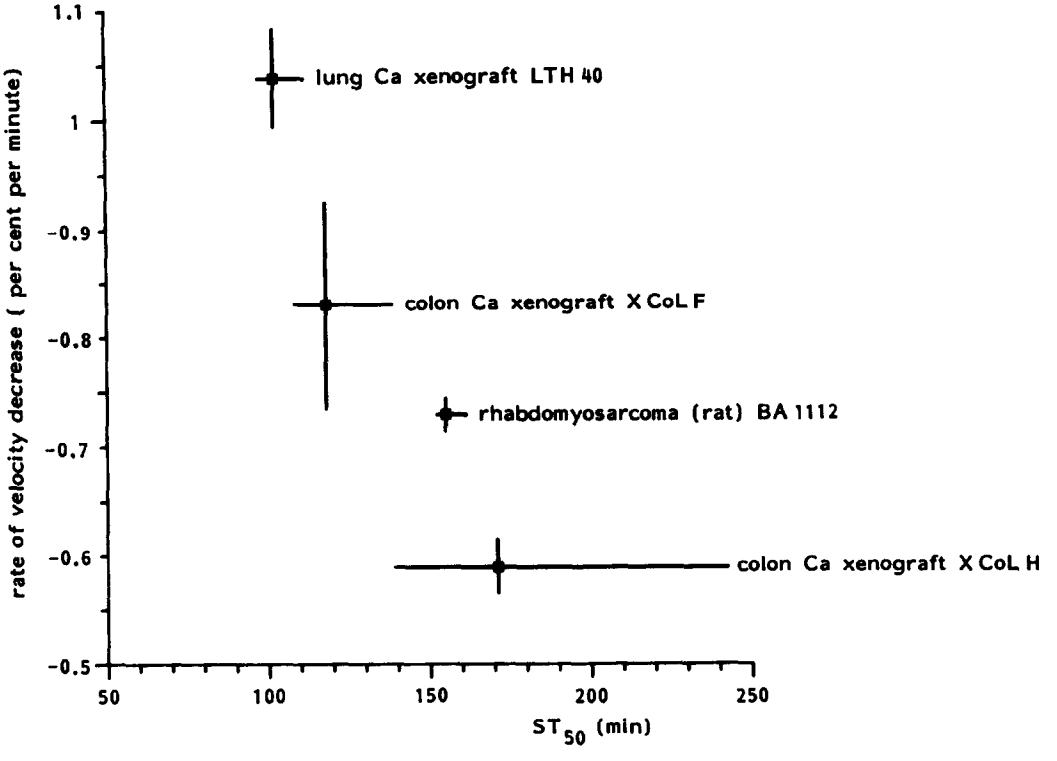

Fig. 4. *Illustration of the* ST_{50} *values of four different tumours, with their* 95% *confidence limits, as well as the rate of velocity decrease for those tumours* (*Fig.* 1) ± *S.D.*

through the vessel started again. This behaviour becomes clear from Fig. 3, upper diagram. After switching to lower magnification, it could mostly be observed that at that moment not only the circulation in the single selected capillary had stopped, but also in the entire tumour. As can be seen in Table 1, only tumour XColH, i.e. one of the colon carcinomata xenografts, showed this behaviour. Phenomena like this can only be demonstrated by showing individual determinations. This is so because averaging the relative values of the velocity of a series of tumours would only result in obscuring the fluctuating pattern by averaging out the randomly occurring peaks and troughs.

DISCUSSION

The data on the ST_{50} values and regression of the erythrocyte velocities with hyperthermia, as shown in the upper two rows of Table 1, indicate a wide range of values. In fact, although both parameters

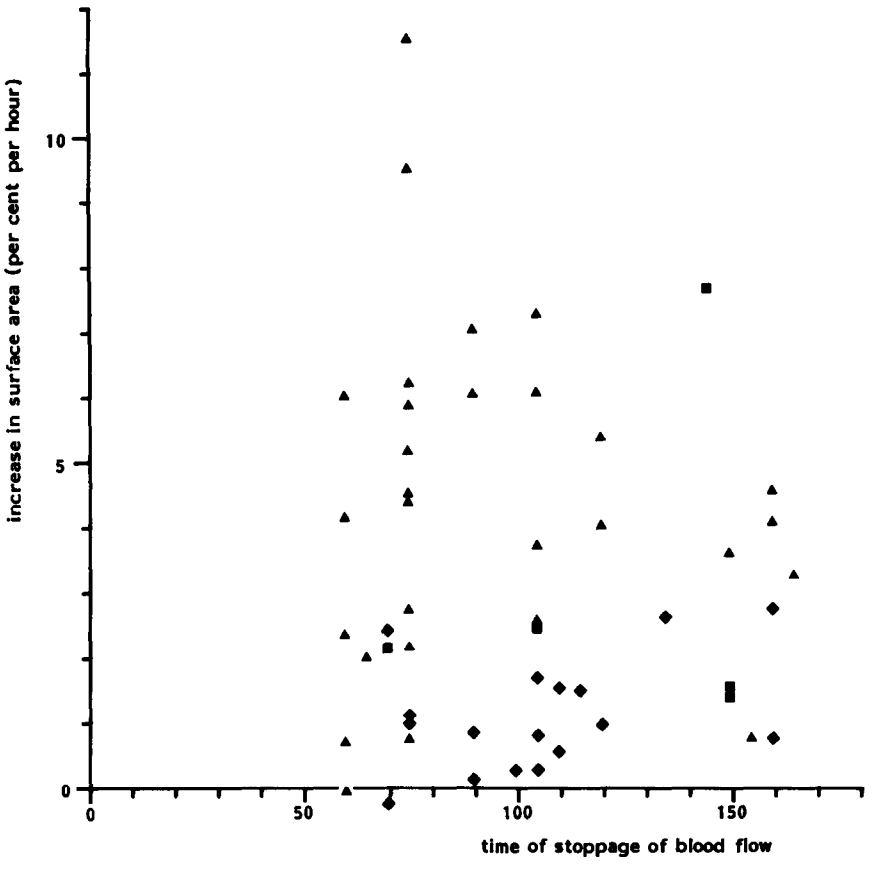

Fig. 5. Scatter diagram for the relationship between the time of stoppage for the individual tumours and the rate of increase in surface area for that tumour. ▲: Rhabdomyosarcoma BA1112; ■: XColH; ♦: LTH40.

do not necessarily indicate the same biological phenomenon, they both represent a heat-induced change in blood flow. It should be noted here that, from our extensive experience on this kind of determination, we may conclude that the stoppage of tumour microcirculation due to hyperthermia is irreversible, and is inevitable followed by necrosis of the affected parts of the tumour. In Fig. 4 the aforementioned values are depicted for comparison. It turns out that the human lung carcinoma xenograft is the most sensitive to heat, and is very different from the human colon carcinomata. The rat mammary carcinoma has the highest ST_{50} value, but is not plotted in Fig. 4 because no velocity data are available. In a recent review [6] a large number of determinations on the tumour microcirculation was listed. Here, with many different assay systems and different tumours, large differences in response were also found. The Walker carcinoma, as investigated by Gullino [15] and Song [16], belonged to the most resistant ones. This is in agreement with the present finding that the rat mammary carcinoma has the highest ST_{50} time. Taking the other values in Table 1 as four points (and not as weighted values), there is an indication for a correlation between the ST_{50} value and the velocity decrease (correlation coefficient 0.96, $2P = 0.042$). More extensive statistics is not indicated, in view of the complex nature of the confidence intervals in this picture.

The interesting point is that different tumours have essentially different stoppage times in their microcirculation. This was also borne out in a log-rank test, which yielded a P of 0.01, indicating that the values obtained from the various tumours did not belong to the same population. This can be interpreted by suggesting that no common factor, like the heat sensitivity of the endothelial cell [17, 18], is the cause of circulation stoppage, but that this represents rather a characteristic of the tumour tissues, or cells, themselves. Here one could think of differences in heat-induced acidity by the tumour cells with its vascular consequences [6] or differences in the chaotic vascular architecture. It should be noted here that the effects of hyperthermia on the tumour microcirculation differ essentially from that of irradiation [23]. Curiously enough, our measurements and observations indicated that the fluctuating pattern of the tumour microcirculation of the human colon carcinoma XColH appeared to be independent—and did not influence—the heat-induced vascular stasis (data not shown). The initial indication of the existence of such fluctuations was found on recordings of the NAD(H) fluorescence in the Rhabdomyosarcoma BA1112 [19] and optical

Table 2. Spearman rank correlation of stoppage time versus relative surface size increase

Tumour	Correlation coefficient	2*P*
BA1112	0.1379	0.4656 = NS*
XColH xenograft	−0.5643	0.2588 = NS*
LTH40 xenograft	0.3015	0.2275 = NS*

*NS = not significant

Table 3. Temperature dependence of the increase in size of Rhabdomyosarcoma BA1112 during hyperthermia (180 min)

Temperature (°C)	Increase in surface (% per hour)	S.D.
33.5 = C	0.01	±0.06
42.5	2.86	±0.13
43	5.01	±0.08
43.5	4.69	±0.15

velocity measurements [20, 25]. In view of the present observations, however (Fig. 3b), this particular tumour seems to have lost this characteristic.

The increase in size in the tumours presents a puzzle. The measurements were started because of the finding by Herman *et al.* [21] that the cell volume of cells in tissue culture increased, when they were rapidly heated to 42.4°C. Our results confirm this finding, and probably the magnitude of the increase is similar. Recently, this was also found by Schiffner *et al.* [22]. However, an increase in size (i.e. surface area in our system) could very well be caused by interstitial oedema, and this might be thought then to be related to heat-induced changes in the microcirculation, like the oedema, noted by Vaupel *et al.* [24]. We therefore undertook an extensive statistical analysis to investigate whether the change in volume could be in any way related to one of the circulation parameters. This is shown in Table 2, in which the Spearman rank correlation coefficient with its significance is tabulated for the three tumours on which all data on the ST_{50} values as well as the rate of increase of size were available. No significant correlations were found. Also in a scatter diagram (Fig. 5) on the time of cessation of the individual tumours against the increase in surface area, no signs of correlation can be discerned. The conclusion therefore is that probably, as in the investigations of Herman *et al.* [21] and Schiffner *et al.* [22] the increase in size during hyperthermia is due to cellular swelling, and is not correlated with changes in the microcirculation. We found some evidence, however, as shown in Table 3, that the increase in size depended to a limited extent on the treatment temperature.

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