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Vascular Occlusion and Tumour Cell Death*

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Abstract—*Vascular occlusion has been tested as a means of inducing regrowth delay, local control, reduced cell viability and prolonged alteration of blood flow in mouse tumours. The occlusion has been achieved by applying D-shaped metal clamps across the base of subcutaneously implanted tumours. The period of clamping has been varied from* 30 min *to* 24 hr. *Marked tumour regression, delayed growth and long-term tumour control were seen, with the magnitude of the response being proportional to the duration of clamping. Vessel occlusion for at least* 15 hr *is necessary to achieve local cure of the tumour. The overall effect results partly from an immediate loss of cell viability and partly from a failure of the capillary network to recover its normal perfusion pattern after the clamp has been removed. The implications of this for anti-proliferative endothelial therapy is discussed.*

INTRODUCTION

TUMOUR blood vessels are more disorganised, tortuous and dilated than the blood vessels in normal tissues (see [1, 2] for references). The morphological differences give rise to a difference in the nutrient supply, oxygen tension, pH and patterns of blood flow and heat dissipation in different tumour types. These factors may also change within the life history of a single tumour, since the total vascular network may become depleted of oxygen and other nutrients, or the flow in whole sections may even become static when the tumours reach large sizes [3, 4].

It has long been recognised that the disorganised nature of the tumour vasculature may result in tumour resistance to therapy. Regions at low oxygen concentrations will be protected against radiation injury and nutritionally deprived cells will be resistant to cycle-specific chemotherapeutic drugs.

Many authors have reported the appearance of cords of viable tumour cells around individual blood vessels, with necrosis appearing at 60–150 μm from the nutrient supply (e.g. [5–8]). They have attributed the necrosis to nutrient deficiency and accumulation of metabolites leading to cell death. Thus it appears that tumour cell death by starvation occurs as a natural process in some, if not all, solid tumours. The extent of this cell death, followed by subsequent resorption, may be the underlying cause of the exponential slowing of growth as each tumour enlarges. This growth pattern fits a Gompertz equation [9] and may reflect progressive failure of the longer, more tortuous, deep-lying vessels in large established tumours [4].

Most oncologists and radiobiologists have concentrated on methods of overcoming the resistance created by the poor vasculature. Hyperbaric oxygen, high LET radiation, chemical radiosensitizers and induced re-oxygenation are all possible approaches to overcoming radioresistance resulting from hypoxia. Induced recruitment of cells into the growth fraction during therapy could improve the effectiveness of chemotherapy during fractionated schedules.

Only two therapeutic approaches are designed to use the vascular pathophysiology of tumours as a means of increasing the effect of tumour directed therapy. These are hyperthermia and vascular occlusion by means of surgical intervention. Hyperthermia, with heat applied externally by means of electro-magnetic radiation, is more effective as a cytotoxic agent on tumour cells in solid tumours than on normal tissues. This is because of the low pH, nutrient deficiency and inability to increase blood flow in tumours in order to dissipate applied heat [10–12]. Surgeons and diagnostic radiologists have been interested

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in partial or complete occlusion of the main arterial supply to tumours as a means of downstaging them by starvation therapy. Techniques tried have included simple ligation, injection of mechanical obstructions or thrombogenic agents such as cyanoacrylate glues, autologous thrombin clots and permanent or biodegradable microspheres [13]. These techniques unfortunately require direct access to the major tumour artery, either by open surgery or by catheters. Since not all tumours have single identifiable afferent blood vessels, the approach has been limited mainly to renal and hepatic tumours, as a debulking procedure before radical surgery.

We have proposed that a more sophisticated version of starvation therapy could be possible if specific monoclonal antibodies could be raised against rapidly proliferating endothelial cells [14]. The mean labelling index of endothelial cells after administration of tritiated thymidine has been recently shown to be approximately 50 times higher than that in normal blood vessels, indicating a correspondingly higher proliferation rate [2]. It seems feasible that cytotoxic drugs could be conjugated to an appropriate antibody and thus be targeted to all solid tumours by simple intravenous injection. The hitherto localised approach of 'starvation therapy' could then be made systemic. However, little data exists to indicate how long the starvation would need to be in order to produce extensive tumour cell death.

With this in mind we have undertaken some simple experiments to determine the duration of occlusion necessary before extensive or complete cell destruction is obtained. The vessels to subcutaneous transplanted tumours in mice have been crudely occluded with a clamp for varying periods of time and the regrowth delay or local control has been assessed for tumours left *in situ*. In order to distinguish between lost cell viability within the clamping period and failure to re-establish a blood supply, blood flow measurements have been made by injecting radioactive tracers and cell viability has been tested by excision of tumours and re-implantation into fresh recipients.

MATERIALS AND METHODS

The transplantable tumour SA F was used in the mouse strain CBA/Ht in which it arose spontaneously in 1957. Tumour fragments were implanted subcutaneously on the posterior dorsum under penthrane anaesthesia. When the tumours reached a mean diameter of 5.0–6.5 mm (usually within 10–18 days) the tumours were randomly allocated to different treatment groups. D-Shaped metal clamps were applied to the tumours to induce complete vascular occlusion. Previous studies using a radioactive tracer have shown that less than 0.01% of the tracer enters the tumour over a period of 30 min when such a clamp is in position.

The procedures that were used are summarised in Table 1. The clamp was left in position for periods of time ranging from 0.5 to 24 hr. Some groups of animals were then returned to their cages and observed 3 times a week for tumour regrowth or local tumour control. Other groups were assessed either at 15 min or at 24 or 48 hr after the clamp had been removed for tumour cell viability or for tumour blood perfusion. The perfusion was estimated by injecting ^{86}RbCl into the tail vein of the mouse and excising the tumour at 60 sec after injection. The tumour was dissected free of overlying skin and connective tissue, placed in a double glass vial and counted for 200 sec on a Wallac 80,000 autogamma counter. For assessing cell viability tumours were removed, minced with scissors to form a fine brei and diluted with an equal volume of saline; 0.03-ml aliquots of this suspension were then injected into 4 sites per mouse (axillae and groins). Whilst this does not yield an exact estimate of the surviving fraction it was found to give reasonable dose–response information.

Table 1. Summary of experiments relating to vascular occlusion

	No. of mice per point	Duration of clamping of blood vessels (hr) 0.5	2	4	8	15	18	24	Assayed
Regrowth delay	6	–	1	1	1	–	–	–	*in situ*
Local tumour control	5–9	–	–	–	–	1	3	1	*in situ*
Cell viability assessed by reimplantation	8–16 (× 4 sites)	1	1	1	1	1	1	1	at 15 min
		1	1	1	1	1	1	1	at 24 hr
Vascular perfusion (^{86}Rb extraction)	4–8	1	3	1	2	–	2	1	at 15 min
		1	3	–	2	–	2	1	at 24 hr
		1	1	–	–	–	1	–	at 48 hr

The numbers indicate the number of experiments of each type that were performed.

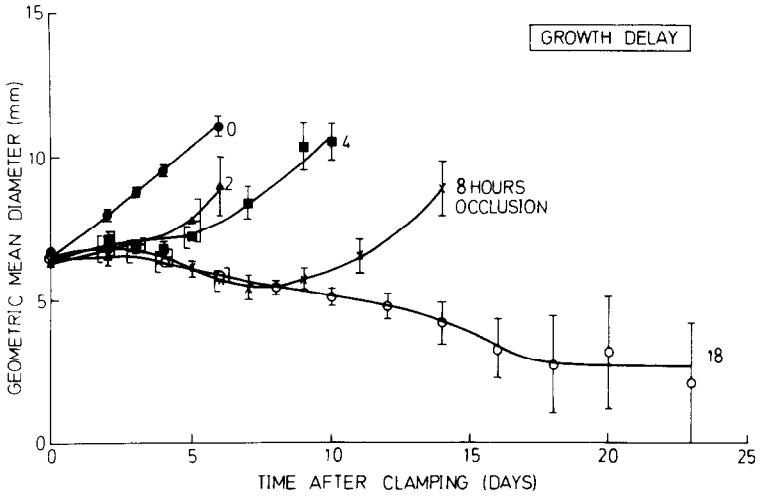

Fig. 1. Growth curves for subcutaneous tumours measured in situ *after no treatment* (○) *or after the indicated period of vascular occlusion. Clamping for periods up to* 8 hr *induced a delay in tumour growth. Longer periods induced permanent disappearance of some of the treated tumours.*

RESULTS

Regrowth delay

Figure 1 shows the regrowth delay induced by various periods of clamping. The tumour took 4.2 days to grow from 6.5 to 11.0 mm mean diameter if it was left undisturbed. Clamping for periods of 2–8 hr induced a progressive delay in the growth of the tumour. Regression of the tumour was followed by regrowth at a time which was related to the period of clamping. Eight hours clamping induced a mean delay of 11 days in the time taken to regrow to 11.0 mm. After 18 hr clamping no tumours regrew within 23 days.

Local control

If the clamping period was longer than 8 hr some of the tumours failed to regrow at all. This is illustrated in Fig. 2, where the percentage of tumours locally controlled at 80 days after treatment are plotted as a function of the clamping time. Fifteen hours clamping caused permanent local contol in ⅙ of tumours and the percentage increased if the occlusion time was extended to 18 or 24 hr. Apparently a period in excess of 24 hr would be needed to result in a complete eradication of every cell in every tumour.

Cell viability

Figure 3 shows the results of an experiment in which tumour cells were re-injected into recipient mice after excising the tumours 15 min or 24 hr after the clamping treatment. For clamping periods in excess of 4 hr a considerable loss of tumour cell viability was detectable, since there was a failure of some of the injected sites to develop a palpable tumour. The tumours excised

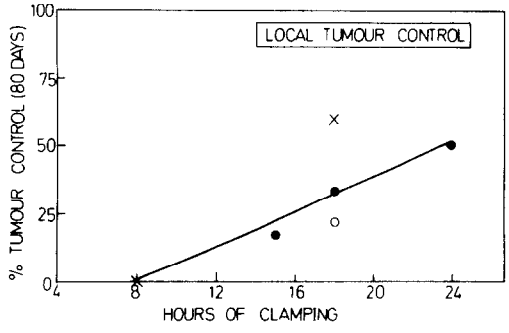

Fig. 2. The percentage of locally controlled tumours as a function of the duration of occlusion of the vasculature by a clamp. Occlusion for 15 hr *or longer resulted in 'cure' of some of the tumours, i.e. no regrowth by* 80 days.

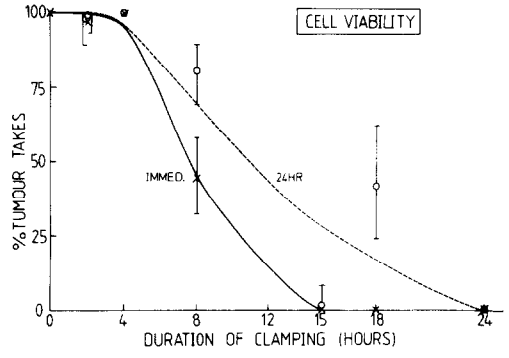

Fig. 3. Cell viability assessed by re-implantation of tumours into recipient mice. The tumours were excised immediately (×) *or* 24 hr (○) *after removal of the clamp. A proportion of the injected sites failed to give rise to palpable tumours if the period of occlusion was* 8 hr *or longer.*

immediately showed lower viability than those excised 24 hr later. This would imply that tumour cells may have recovered or proliferated during

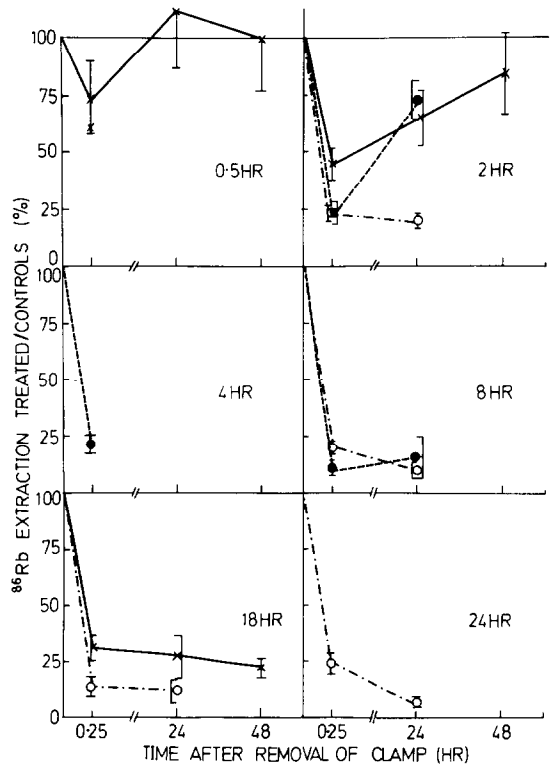

Fig. 4. Capillary perfusion estimated from the uptake of ^{86}Rb *after intravenous injection. The tracer was administered* 15 min–48 hr *after removal of the clamps, which had been left in position for periods ranging from* 0.5 *to* 24 hr. *Clamping for any period induced a transient residual drop in* ^{86}Rb *uptake compared with untreated controls. Clamping for periods in excess of* 2 hr *led to a long-term reduction in blood flow.*

this 24-hr period and that further cell death from a failure of the blood flow to return after clamping was not a major factor, at least after 8 and 18 hr.

Blood flow

The ^{86}Rb extraction technique is a measure of functional perfusion since rubidium is extracted from the blood stream as if it were potassium [15]. The counts per gramme of tumour were related to the counts in untreated control tumours, and the relative extraction is shown in Fig. 4. Six panels are shown for tumours clamped for periods ranging from 0.5 to 24 hr. Most of the groups were tested 15 min or 24 hr after removing the clamp, but in addition several groups were studied at a later time of 48 hr to determine whether recovery to normal levels could be observed.

The blood perfusion was still reduced at 15 min after all the clamping periods tested. This reduction was small and transient for the tumours clamped for 30 min, but was more extensive and long lasting for longer clamping periods. If occlusion of the vessels was maintained for 8 hr or longer the blood flow after releasing the clamp was reduced to between 10 and 30% and showed no sign of recovery at 24 or 48 hr. Thus prolonged clamping appears to result in permanent occlusion and a failure to recanalize some, though not all, of the blood vessels.

DISCUSSION

These preliminary studies indicate that occlusion for periods as short as 2 hr results in significant regrowth delay and in a prolonged depression of blood flow after the clamp has been removed. However, total vascular occlusion for periods in excess of 24 hr would be needed to cause a complete local control in 100% of the animals bearing the implanted sarcomas. Clamping for 8 hr is the longest time that has been shown to give a prolonged regrowth delay without permanent local control. Longer periods resulted in some cures and a reduced ability to produce tumours on re-implantation of a cell suspension. Whilst these data are not precise estimates of surviving fractions of clonogenic cells, they clearly demonstrate the potential effectiveness of vascular occlusion as a form of tumour therapy.

It is difficult to distinguish in these experiments between the permanent vascular damage that has been caused and the cell death resulting from the deprivation of all nutrients solely during the period of the clamping. Figures 3 and 4 show that both factors are probably important. It is obvious, however, that the occlusion must be maintained for periods of hours rather than minutes. This would suggest that the approach of using biodegradable microspheres with half-lives of 15–20 min in human blood would be inadequate [16].

The period of 4–8 hr for causing significant cell kill and 15–24 hr for causing very extensive cell kill is in reasonably good agreement with estimates of the lifetime of hypoxic cells that occur naturally in tumours at the limiting diffusion distance for nutrients from individual blood vessels. Hirst *et al.* [8] used tritiated thymidine to study the proliferation characteristics of tumour cells at different positions around individual cords in three types of mammary carcinoma. They calculated, from the time at which the labelled cells progressed across the cord, that the transit time through a single-cell layer adjacent to necrosis would be 5–11 hr in the 3 tumour types. This estimate of the lifetime of nutritionally deprived cells is close to that in the present study. It is also similar to the period of hypoxia that can be tolerated for certain cell lines maintained *in vitro* with access to all other nutrients but with no access to oxygen (see [17] for review).

These studies demonstrate the feasibility of killing tumour cells by nutrient starvation and

encourage us to seek monoclonal antibodies to proliferating endothelial cells as a means of inducing vascular occlusion of tumour blood vessels by systemic therapy [14]. Since each endothelial cell in a vessel subtends several hundred tumour cells it follows that occlusion of whole capillaries would result in extensive cell death. It seems likely that such an approach would be even more effective if combined with other forms of therapy, e.g. hyperthermia [18, 19]. The recent discovery of a monoclonal antibody that is specific for mouse endothelial cells is encouraging [20] and this may be useful if combined with cell cycle-specific drugs. However, the ideal approach would be to have a proliferating endothelial cell antibody and to use this as a focus for forming a clot, or as a means of transporting potent cell toxins, such as abrin, or short-range emitting radioisotopes directly to the tumour blood vessels.

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