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Vascular attack as a therapeutic strategy for cancer

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Summary

The blood supply to all solid tumours consists of parasitized normal vessels and new vessels which have been induced to grow by the presence of the tumour. These vessels are inadequate in many respects, being tortuous, thin-walled, chaotically arranged, lacking innervation and with no predetermined direction of flow. The walls consist of a basement membrane lined with rapidly proliferating immature endothelial cells, and are more permeable than normal vessels. The spacing of the vessels and their average diameters are not optimal for nutrient provision.

This paper focuses on the evidence that many existing therapies may already have, as part of their action, a vascular mediated process of killing tumour cells. This may result from local changes within individual vessels or from systemic alterations in blood pressure, viscosity, coagulability etc. The hallmarks of vascular injury are identified and the dangers of discarding useful anticancer agent by failing to understand their mechanism of action are highlighted.

Introduction

Most cancer research is aimed at killing tumour cells directly, presumably because of differences in their proliferation rates, intrinsic sensitivity, DNA repair capabilities or other features resulting from the malignant transformation. For successful control of the cancer, and hence for a tumour cure, every cancer cell capable of indefinite proliferation must be eradicated. In small tumours of ~ 10 g this may involve the killing of 10^9–10^{10} cells, unless there is a low stem cell fraction, or a host-mediated effect which enhances the cell kill from the therapeutic agent. To explain why existing therapies, e.g. radiotherapy, are as effective as they are, it has been postulated that the proportion of tumour cells capable of indefinite proliferation may be as low as 1 in a 1,000. Alternatively it has been proposed that the natural immune system of the host can dispose of the last few thousand cells if the majority are killed by the therapeutic agent. Recently, a third option has become apparent, in which a different host-mediated effect is involved, namely failure of the tumour microcirculation (Denekamp 1982). This will also amplify the damage by several orders of magnitude since thousands of tumour cells are dependent for their sole nutrient supply on individual blood vessels.

Recently, several lines of research have converged so that vascular attack, taking advantages of the differences between newly formed vessels in tumours and mature vessels in normal tissues, now appears a feasible and attractive approach. Cell kinetic studies have shown that endothelial cell proliferation is rapid in all solid tumours, both in rodents and in man. Studies using hyperthermia as a therapeutic agent have emphasized that poor tumour vasculature may enhance the therapeutic ratio if it is combined with radiation. Electron affinic nitroimidazoles, originally developed as oxygen-mimetic sensitizers have been shown to act also as prodrugs, being metabolically activated to a toxic

product in hypoxic cells. This has led to the development of bioreductive drugs for which transient bursts of hypoxia, induced by manipulating the blood flow, could increase the therapeutic gain. Some of the nitroimidazoles, e.g., misonidazole have been shown to have a vasoconstrictive action themselves, thus inducing the requisite hypoxia for their bioreductive activity and also trapping other cytotoxic drugs within the tumour vasculature. The radioprotector WR2721 is also a prodrug, requiring dephosphorylation before it can enter cells. It seems that this may occur at the endothelial cell membrane in normal vessels but not in neovasculature. Studies with photodynamic therapy indicate that the singlet oxygen damage produced when laser light interacts with haematoporphyrin results in a collapse of the vasculature. The dye may be concentrated in tumour endothelium. The cytokines, e.g., tumour necrosis factor, interleukins, interferon, all show evidence of vascular involvement in the tumour response. A relatively new drug, flavone acetic acid, which has inexplicably high activity in solid murine tumours, has recently been shown to have marked effects on the tumour vasculature in responsive tumours.

All these threads indicate that considerable cell death in tumours may be indirectly mediated by vascular failure after many existing forms of therapy, and after several agents that are the subject of intense experimental interest at present. Some attention must be focused on this mechanism, and its value assessed relative to direct malignant cell kill and immune mediated cytotoxicity, or useful agents may be discarded because their promise from experimental studies does not immediately translate into clinical response.

Vascular differences in tumours and normal tissues

There are many differences between mature vessels and those newly evoked within solid tumours (Table 1) (Gullino 1975; Peterson 1978; Jain 1988). As any focus of tumour cells expands, it initially acquires its nutritional supply from the existing normal vessels. These vessels become incorporated in the expanding mass, but they do not adequately meet the needs of the growing tumour. Metabolites, catabolites and specific angiogenic factors act as stimulants for new vessel formation (Folkman 1975, 1985). Methods of inhibiting angiogenesis are being developed as an approach to prevent tumour expansion, or even cause tumour regression e.g., Folkman *et al.* 1983. This angiogenesis involves dissolution of the basement membrane, migration and proliferation of the endothelial cells to form buds, which finally anastomose to form an expanding network. The process appears to begin on the venous side of the circulation, perhaps of hydrodynamic problems in the fragile capillary buds when the vessel wall is initially breached. The newly formed vessels are usually thin-walled capillaries or

Table 1. Differences between tumour and normal blood vessels

Normal vasculature	*Tumour vasculature*
– Is 3-dimensionally adequate	– Is 3-dimensionally inadequate leads to
– Is innervated	– nutritional deprivation
– Has collateral potential	– hypoxic cells
– Has well-constructed walls	– acidic cells
– Can increase blood flow in response to demand	– areas of necrosis
It is:	*Vessels have:*
– Resistant to radiation	– Poor wall structure
– Can withstand 90% depletion of endothelium (arterioles)	– No innervation
	– Fewer capillaries
Endothelial cells proliferate very slowly	– More sinuses
	– No collateral potential
	– Endothelial cells which proliferate rapidly

sinusoids with little more than an endothelial lining backed by a basement membrane. There is no smooth muscle layer and no innervation. The endothelial cells are often difficult to stain with characteristic histochemical markers which identify differentiated characteristics of endothelial cells, e.g. factor VIII, angiotensin converting enzyme, etc. (Murray *et al.*, 1989a). Thus, they lack the features of mature endothelium which may be so important in controlling or modulating features of immunity, inflammation and coagulation (Fajardo 1989).

The uptake of tritiated thymidine (a precursor of DNA synthesis and hence a marker of proliferation) is much higher in tumour endothelium than in normal vessels (Denekamp and Hobson 1982). This is illustrated in Fig. 1 where the uptake of ^{3}HTdR into endothelial cells is illustrated as a function of exposure to a single flash injection. In normal tissues very few cells take up the label, even after 7 days of labelling (data not shown). This is independent of the proliferation rate of the parenchymal cells, being very low in lung, kidney, heart etc. which have slow parenchymal turnover and also in jejunum and skin which have high rates of epithelial proliferation. By contrast, the uptake into tumour endothelium is very high, being about 50 times higher initially (5–15%) and rising within a few days to 40–60%. Placental vessels, and those in granulation tissues in wounds (not shown) demonstrate even faster proliferation, implying that tumour angiogenesis factors are not eliciting the maximal response. Data from a human tumour, a salivary gland carcinoma labelled by intracarotid infusion, (C. Nervi, personal communication) are remarkably similar in spite of marked differences in size and in gross volume doubling times (Denekamp 1986). This large difference in proliferation rates of endothelium is one of the most dramatic and consistent differences between solid tumours and normal tissues. For this reason it has been postulated that monoclonal antibodies could be produced that would be effective against actively proliferating endothelial cells (Denekamp 1982). It is possible that specific antigens may be expressed on such cells. Several antibodies have been raised against tumour endothelium but so far they do not give the required degree of specificity (Duijvestijn

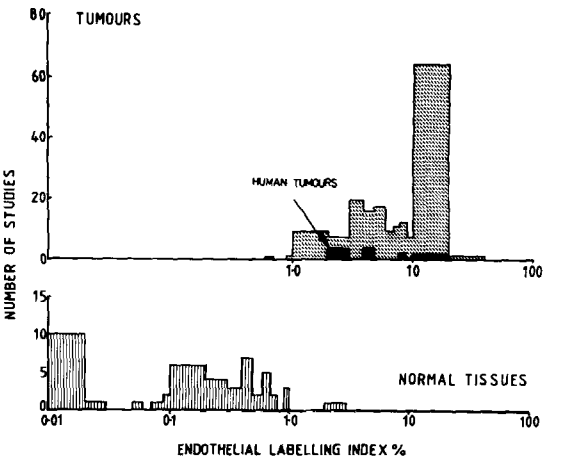

Fig. 1. Labelling index of endothelial cells in tumours (upper panel) and ten normal tissues (lower panel) as assessed in autoradiographs of tissue taken after tritiated thymidine administration. The abscissa is on a logarithmic scale to demonstrate the very large differential. The solid bars represent human tumour endothelium, labelled *in vitro* (from Denekamp 1986).

et al. 1987; Hagemeier *et al.* 1986; Murray *et al.* 1989a; Schlingeman *et al.* 1986). Alternative strategies may be possible using antibody constructs containing one antibody recognising endothelium and another recognising proliferation antigens, e.g., Ki 67. This antibody approach would have the enormous advantage over tumour targeted antibodies that the antigen would be presented directly to the blood stream.

These thin-walled fragile vessels do not have the nervous and muscular components that would allow a normal response to vasoactive stimuli, i.e., for dilatation or constriction. They may also lack the endothelial surface features that act as tissue addressins and influence flow, viscosity and clotting (Ryan and Ryan 1984; Nawroth *et al.* 1988). Tumour vessels are often more permeable to large molecules than normal vessels. The afferent blood may be of poor quality, being depleted of nutrients to values characteristic of venous blood rather than arterial levels and falling within tumour capillaries to extremely low levels (Vaupel *et al.* 1981; Mueller Klieser *et al.* 1981). Finally, the network array and the organisational architecture of a tumour vasculature do not resemble that in any normal tissue. Vessels are often tortuous, with variable diameters, and an intercapillary spacing that is sub-

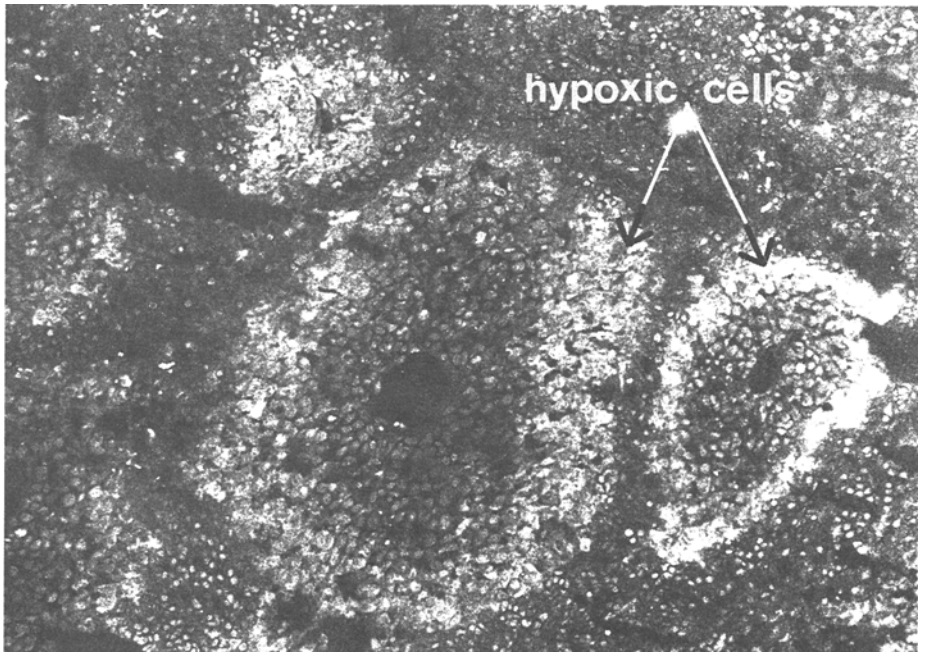

Fig. 2. Photomicrograph of a mouse mammary carcinoma stained with an immunofluorescent technique using an antibody specific for hypoxic cells. These cells metabolise bioreductively a theophylline-nitroimidazole conjugate, which is then bound in the cells. Two cords are seen with a rim of hypoxic cells some 5–7 cell diameters from the central vessel. A third cord is seen which is very small and surrounds an apparently occluded vessel (from Hodgkiss *et al.* 1990).

optimal (Egawa and Ishioka 1979; Reinhold and Visser 1983; Bujaski *et al.* 1989). This sometimes gives rise to the corded structures, described by Thomlinson and Gray (1955) and illustrated in the photomicrograph Fig. 2 and schematically in Fig. 3. More commonly the cords are not obvious, but diffusion limited gradients can be detected with proliferation markers or drugs, e.g., ^{3}HTdR, BrdUdr, adriamycin.

The pattern of necrosis seen around tumour cords is a clear indication of cell death induced by vascular insufficiency. Obviously any cell dependent upon such a central vessel has no alternative source of nutrients. Cell kinetic measurements have shown that proliferating cells are pushed away from the central blood vessels. Cells traverse the boundary layer between apparently viable and obviously necrotic regions in 5–11 hours (Hirst *et al.* 1982). Experiments in which the blood supply to a tumour is temporarily occluded by an external clamp also show that periods of 12–24 hours' total deprivation of blood can lead to extensive cell kill, giving prolonged regrowth delay or even tumour cure (Denekamp *et al.* 1983). By contrast, short occlusions of 1–2 hours are not particularly damaging even if repeated many times.

Vascular damage produced with existing therapies

Figure 4 illustrates the many different forms of therapy that have been reported to cause effects on the tumour vasculature. These agents have a wide spectrum of activities and it is unlikely that they all act via a common initial step. However, changes as varied as endothelial cell death, altered blood viscosity, local coagulation events, changed cardiac output or blood pressure could all result in collapse or occlusion of individual tumour vessels. The net result of this would be to cause nutritional deprivation of the cells around each collapsed or occluded vessel, leading to focal areas of necrosis, whilst

other cords around patent vessels would appear relatively normal. This is in marked contrast to the random cell death that should result from an agent killing cells individually. A brief outline follows of the response to several forms of cancer therapy to illustrate the ways in which vascular mediated injury in tumours has been documented.

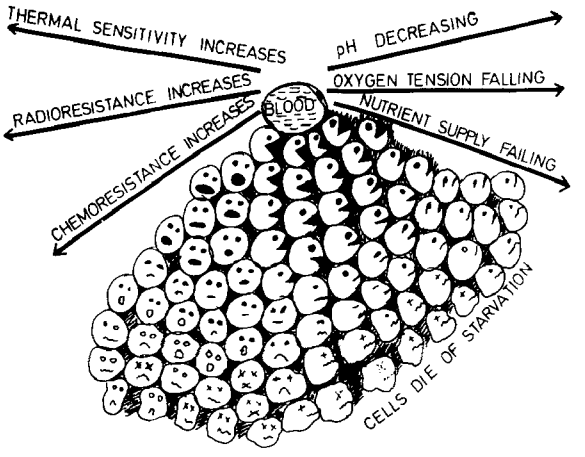

Fig. 3. Schematic figure illustrating the vascular influence on radiosensitivity chemosensitivity and thermal sensitivity.

Hyperthermia

The recognition of the beneficial effect of a poor microcirculation in tumours came first with hyperthermia. The thermal sensitivity of cells *in vitro* was found to be greater if they were nutrient-deprived, or hypoxic. Use of appropriate buffers indicated that the key factor is the lower pH in the extracellular fluid that accompanies changes in oxygenation (Hahn 1982). Higher temperatures have been recorded in tumours than normal tissues when energy is locally deposited by microwaves, ultrasound or radiofrequency. This relates to the inability of tumour vessels, which lack innervation, to vasodilate in response to heating. In normal tissues, hyperaemia is the normal response to applied heat. Vasodilatation and the opening of additional collateral reserve vessels can increase total flow by a factor of 5–10 (Song 1984: 1990). In tumours this response is very limited. At quite moderate temperatures (41° C–44° C for 30 minutes) blood flow progressively decreases in sandwich tumours as individual vessels fail (Reinhold *et al.* 1978). Vascular stasis persists for several days and results in massive necrosis. Occasional vessels survive with a surrounding cord of tumour cells in close proximity to vessels that have obviously failed recently as a result of therapy (Denekamp 1989). Several pathways

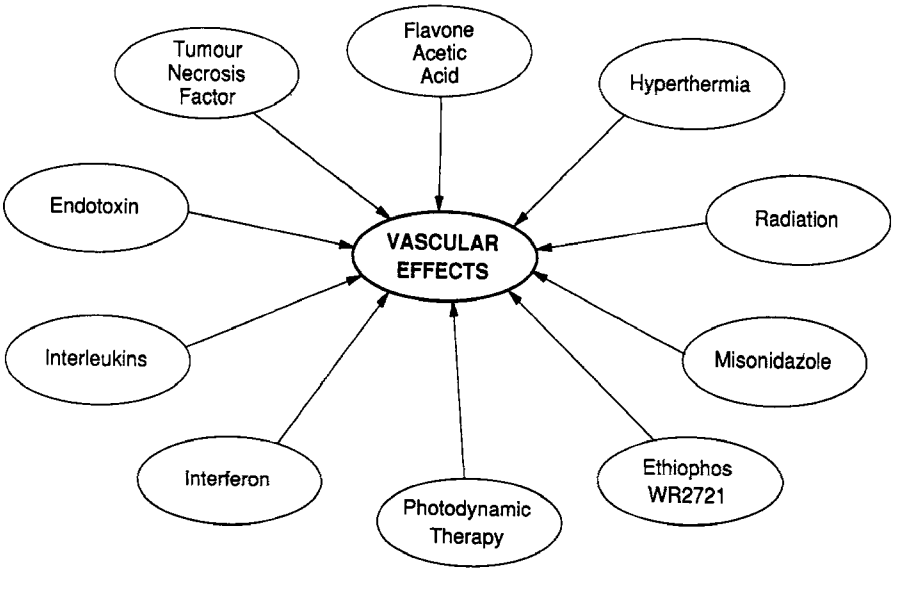

Fig. 4. Therapeutic agents which have been demonstrated to cause effects on the tumour vasculature which may partly account for the tumour response to therapy.

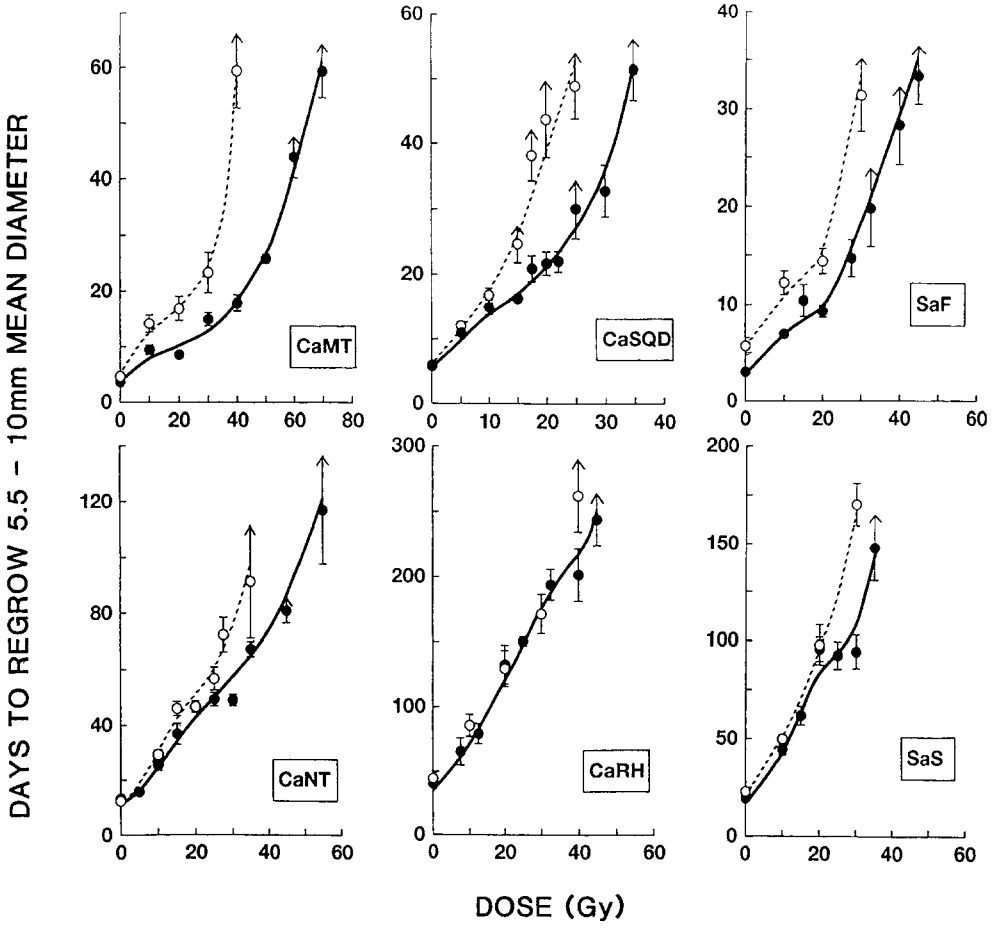

Fig. 5. Growth delay induced by graded doses of X-rays is shown as a function of the radiation dose to the tumour, either with ○ or without ○ adjunctive hyperthermia (42.8° C for 60 minutes). The thermal sensitization varies with tumour type, being generally less in the slow growing CaRH and SaS tumours (from Hill *et al.* 1989a).

which may lead to this vascular collapse have been described. Aggregation of erythrocytes and adhesion of leukocytes to vessel walls has been observed, influencing both viscosity and flow, especially through small vessels (Endrich and Hammersen 1984; Endrich *et al.* 1979). Oedema and thrombosis are also observed in heated tumours, which can both lead to cessation of flow, by increasing interstitial pressure or by occlusion of the vessels (Rappaport and Song 1983; Lee *et al.* 1985). Endothelial cells in culture (both murine and human) have been shown to be more thermosensitive than fibroblasts, and this sensitivity is further enhanced when they are under the influence of mitogenic growth factors (Fajardo *et al.* 1985).

Although the vasculature is clearly involved in the response of hyperthermia, and is likely to be the major reason for a therapeutic benefit in mouse tumours, the effect in human tumours has been less dramatic. One reason for this may be that the required temperature to cause vascular collapse is about 1° C higher in slow growing rodent tumours (Hill *et al.* 1989a). This is illustrated in Fig. 5 where the dose response curves are shown for tumours treated with graded doses of X-rays, either alone, or combined with 1 hour's heating at 42.8° C. A large sensitization (i.e., a shift of the heated curve to the left) is seen in the rapidly growing tumours CaMT, CaSq.D, SaF and Ca NT and a much smaller effect in the slowly growing tumours Ca Rh and Sa S. This range of responsiveness was used to look for a correlation between the thermal enhancement ratio (X-

ray dose needed without or with heat to cause equivalent tumour damage) and the blood flow reduction. A clear, but non-linear, relationship was demonstrated (Fig. 6) when ^{86}RbCl extraction was used as a measure of the relative blood flow.

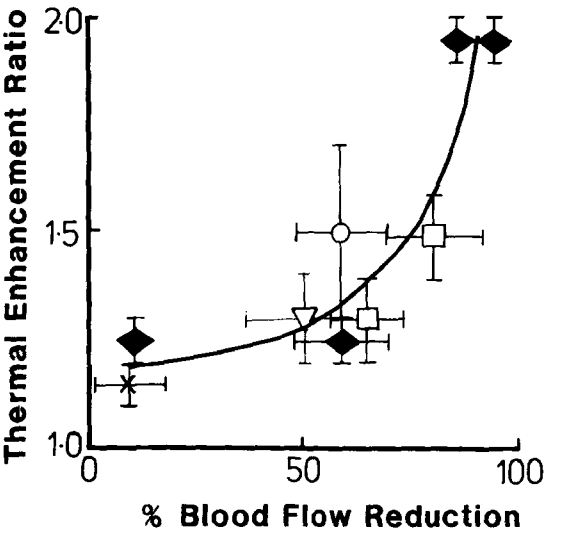

Fig. 6. Correlation between thermal sensitization to X-rays and the reduction in tumour perfusion, as assessed from ^{86}RbCl extraction 24 hours after heating. Each symbol represents a different type of tumour.

Radiation therapy

The main interest in tumour vasculature in radiotherapy is the resistance of hypoxic cells, which exist because of the excessive spacing of capillaries, such that they exceed the oxygen diffusion gradient. This gives rise to the corded structure with hypoxic cells dying at 100–150 μm from the central vessels (Fig. 2). Doomed but still viable hypoxic cells may be resistant to radiation injury and may be rescued from a hypoxic death by a process of reoxygenation. Such cells may then lead to tumour recurrence and failure of treatment. The emphasis in radiobiology has for many years been on the problems posed to the therapist by this inadequate vasculature. Little attention has been paid to the concept that vascular collapse would lead to an enhancement of the hypoxia-mediated killing.

X-rays have been shown to cause vascular collapse, with vessel occlusion by thrombosis being described after single large doses when the tumours had started to regrow (Thomlinson and Craddock 1967). More detailed morphometric studies on xenografts have demonstrated that the occlusion is mainly in the narrower 10 μm nutrient capillaries, with only small effects in vessels of 20–30 μm diameter (Solesvik *et al.* 1984). However, the effect is small and unlikely to account for most of the observed tumour response to radiation. Because radiation injury is characteristically expressed at mitosis, endothelial cell death will not occur immediately, but will be delayed for several days, depending on the endothelial cell turnover rate (Denekamp 1986; 1989). However, it will occur much earlier in tumours than in normal vessels because of the difference in endothelial cell kinetic parameters. An indirect measure of radiation-induced vascular damage influencing tumour growth comes from the Tumour Bed Effect. In such experiments untreated tumour cells implanted into a previously irradiated site grow much more slowly than those in unirradiated animals (e.g. Begg and Terry 1983). The tumour bed effect is dose-dependent, but varies in magnitude with the type of tumour used to elicit the response, for reasons which are at present unclear.

Radiation sensitizers

Chemical radiosensitizers and radioprotectors which preferentially sensitize tumours or protect normal tissues are redox active compounds, believed to interact with radiation-induced chemical species, e.g. free radicals. Misonidazole is the prime example of a radiosensitizer which moved rapidly in the 1970's from experimental studies to worldwide clinical testing (Adams *et al.* 1978). Its radiosensitizing effect is clearly limited to hypoxic cells, and in the presence of the more efficient sensitizer (oxygen), it completely loses its effect (Denekamp *et al.* 1974). *In vitro* studies with miso showed that it could be metabolised reductively to a toxic product in hypoxic cells (Sutherland 1974; Wong *et al.* 1978). This had led to an expanding interest in the development of bioreductive drugs, which should be metabolised from the non-toxic

prodrug to an active toxic metabolite, only within the hypoxic cells that exist in solid tumours. In spite of being developed to overcome the deficiencies of the tumour vasculature, this unexpected action allows a tumour-specific approach to be developed based on the pathophysiology resulting from the poor blood supply rather than on malignant versus normal cell differences.

A further unexpected action of miso was found when blood flow studies were performed on tumour-bearing mice given miso alone, or in combination with melphalan. An enhanced effectiveness of the two drugs had been reported, which had been attributed to independent subpopulations responding, i.e., the cycling cells close to vessels being killed by melphalan and the distant hypoxic cells by miso (Sutherland 1974; Randhawa *et al.* 1984). Miso was shown to cause a massive collapse of the vasculature, detected by morphometric assessments of the structurally defined vascular volume (with an anti-laminin antibody) or by the space filled by the fluorescent dye, Hoechst 33342 (Murray *et al.* 1987). The vascular collapse lasted for 1–2 weeks. The extent of this vascular shutdown was increased even further, and lasted for a longer period of time when the two drugs were given. Thus it appears that the enhanced cytotoxicity of these agents when they are combined may not be simply due to direct cytotoxicity, but may also involve pharmacokinetic changes (trapping of the melphalan within the tumour) and indirectly mediated ischaemic necrosis. This latter effect was completely unexpected.

Radiation protectors

Thiol radioprotectors are assumed to protect by proton donation, to inactivate damaging OH· radicals or restore damaged DNA. Ethiophos (WR 2721) is a phosphorothiol which was developed at the Walter Reid Hospital as a whole body radioprotective agent for military purposes. In the late 1960s and 1970s it was investigated as an adjunct to radiotherapy because it was shown to give much more of a protective effect for bone marrow (a factor of 3) than for a rodent tumour (Yuhas and Storer 1969). Further studies showed that it was capable of giving considerable protection to a range of normal tissues, particularly at short intervals after injection (Yuhas 1980). Studies using labelled Ethiophos showed greater and earlier uptake into normal tissue, but the cause of this difference was ill understood. Ethiophos is a phosphorylated compound, with the thiol group blocked by a phosphate. For activation, and indeed for transport into cells, the compound must be dephosphorylated. Yuhas suggested that the phosphatase activity might differ in tumour and normal cells. What now seems more likely is that the reduced alkaline phosphatase activity in tumour endothelium may effectively prevent passage of ethiophos across the vessel wall, until a pool of the dephosphorylated compound created in normal vessels has saturated the normal tissues and provided a systemic reservoir of the diffusible product (Denekamp and Rojas 1988; Murray *et al.* 1989a). Differential activation of prodrugs based on differences in surface enzymes in mature and immature endothelium may be a productive path to investigate. This could in theory lead to differential activation of cytotoxic drugs, or to the activation of DNA repair enzymes in normal tissues after irradiation (Ward 1986).

Photodynamic therapy

Photodynamic therapy depends upon the activation of a compound, e.g., haematoporphyrin, by visible light to release toxic activated oxygen species which kill the cell in which it has been formed. This extremely reactive radical would not diffuse to adjacent cells. It is evident under gross illumination that the compound is retained preferentially in the tumour some 72–96% hours after administration. At first, this was attributed to leaky tumour blood vessels, i.e., altered permeability (Bugelski *et al.* 1981). However, recently it has been suggested that the retention is within the endothelial cells of the newly formed tumour blood vessels rather than extravascularly. Reduced blood flow and vascular destruction have both been demonstrated (Selman *et al.* 1984; Star *et al.* 1986) and delayed cell death

has also been measured after photodynamic therapy (Henderson *et al.* 1985). Indeed the damage to normal vessels exposed to light and haematoporphyrin has also been shown to be vascular mediated (Moore *et al.* 1990). The reason for the endothelial localisation is totally unknown, but would indicate that differential endothelial retention might be an important parameter when screening for alternative compounds instead of the existing haematoporphyrin derivatives. At present the emphasis is entirely on development of more active compounds, or those responding to more penetrating wavelengths and the vascular uptake is not monitored in the screening of new agents.

Cytokines

The biological response modifiers or cytokines have evoked a great deal of interest, and many clinical trials are in progress using recombinant DNA technology to provide pure tumour necrosis factor, interferons and interleukins. Attention has been focused on their relationship to growth factors and their immune-mediated reactivity. A host response is clearly involved. It has been assumed that the eventual target is the malignant cell. Evidence is accumulating that vascular effects are also involved. Bloksma *et al.* (1982) showed regional necrosis, one of the features of vascular-mediated injury, after endotoxin. MacPherson and North (1986) showed reduced blood flow to tumours after endotoxin which was correlated with ultrastructural damage to endothelial cells. Haemorrhagic necrosis after endotoxin, interferons, interleukins and tumour necrosis factor have all been conspicuous features of the response. Injury to the microvasculature has been identified after interferon (Dvorak and Gresser 1989) and changes in blood flow or in the process of angiogenesis after TNF (Kalinowski *et al.* 1989), interferon (Sidky and Borden 1987) and interleukins (Belvilacqua *et al.* 1984; Kotasek *et al.* 1988). These effects may be mediated by changes in endothelial cell function rather than by endothelial cell killing. Changes in surface markers after TNF (Pober *et al.* 1986) and alterations of blood coagulation parameters (Bevilacqua *et al.* 1986; Nawroth *et al.* 1988) may be indicators of this. Figure 7 shows the many complex pathways by which alterations in blood flow to a tumour may be achieved. It is clear that several overlapping and complex fields of physiology, pharmacology and endothelial cell biology may be needed to unravel these effects.

Chemotherapeutic agents

Most cytotoxic drugs kill cells by causing damage to DNA, or by interfering with the normal processes of DNA replication and division. Considerable effort has been put into developing drugs which are freely diffusible through tissue to reach the tumour cells most distant from the capillaries. Drug delivery is however a major problem because of the poor circulation. Flow in some vessels is sluggish, or may be static for minutes or hours at a stretch. The transvascular and interstitial movement of cytotoxic agents may be suboptimal because of the sinusoidal nature of many vessels and the high interstitial pressures which result from poor lymphatic drainage (Jain 1989). If the drug is only active on cycling cells a further problem is encountered because active proliferation is limited to the first few cell layers around each vessel because of nutrient limitations. Thus the poor tumour vasculature creates problems in the effective use of cytotoxic drugs. Nevertheless significant tumour regression is seen in many solid tumours and it is pertinent to ask whether this is ever mediated by the drugs causing vascular damage instead of direct tumour cell kill. Very few studies of blood flow changes after chemotherapy have been performed. In the study of melphalan and misonidazole (Murray *et al.* 1987) only a small effect was seen when melphalan was used alone. Indirect evidence of a rapid host-mediated cytotoxicity is available from the studies of *in vitro* survival after excising tumours treated *in vivo* (Stephens and Peacock 1978). For most of the drugs tested, the yield of cells from tumours harvested shortly after drug administration or 24 hours later was reduced to 50 to 75%. After vinblastine and vincristine an even greater loss in cell yield was seen in the tumours excised at 1 day. This is com-

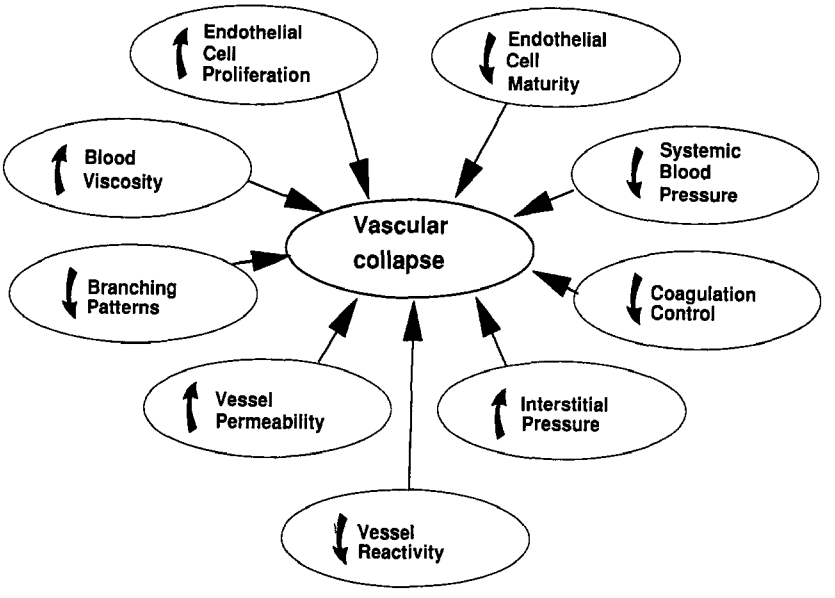

Fig. 7. Schematic illustration of the aspects of the vascular network that could lead to collapse or occlusion of tumour blood vessels after various agents.

patible with the rapid necrosis that is normally associated with vascular collapse. Further studies looking for vascular reactivity would be desirable with other chemotherapy regimes. It is quite possible that altered schedules would be needed to maximise endothelial cell killing, e.g. reduced solubility or dosage regimes that would limit drug diffusibility.

Flavone acetic acid has been the subject of intensive study in animal tumour models over the last 5 years. This agent was not developed originally as a cytotoxic drug, but rather as an anti-inflammatory agent. It has no antimetabolite or DNA binding properties. In NCI screens, it was found to be remarkably effective on all solid tumour models, but had no effect on lymphomas or leukaemias. It was ineffective when given immediately after tumour cell injection, but was effective when given in repeated doses or to large established tumours. FAA is tolerated in high doses *in vitro* and the effects *in vivo* are incompatible with the low *in vitro* toxicity (Finlay *et al.* 1988), indicating a host response must be involved. A rapid haemorrhagic necrosis is visible within a few hours of administering FAA (Smith *et al.* 1988) and this is accompanied by, or preceded by a fall in tumour perfusion (Bibby *et al.* 1989; Evelhoch *et al.* 1988; Hill *et al.* 1989b). Alterations of tumour perfusion have been detected within 15 minutes by some techniques and these persist for at least 24 hours. Hill *et al.* (1989b) developed a panel of tumour showing a range of sensitivity to FAA, two of which are shown in Fig. 8. The FAA-sensitive tumours show extensive growth delay, or even local control, after single doses of the agent, whereas the unresponsive tumours show little delay after double the drug dose. The extent of reduction in blood flow is shown below each response panel. Although some reduction is seen for both tumours, it is clearly more marked in the responsive tumour. It is interesting that this slow growing Ca Rh was one of the least responsive tumours to hyperthermia.

Figure 9 summarizes such FAA studies from a range of different tumours, with the gross tumour effect expressed as specific growth delay (delay divided by pre-treatment doubling time) in an attempt to normalize for differences in growth rates. It is only when extensive and prolonged vascular occlusion is seen that the extensive growth delay, accompanied by histological evidence of massive and rapid necrosis, occurs.

Recent studies by Zwi *et al.* (1990) have demon-

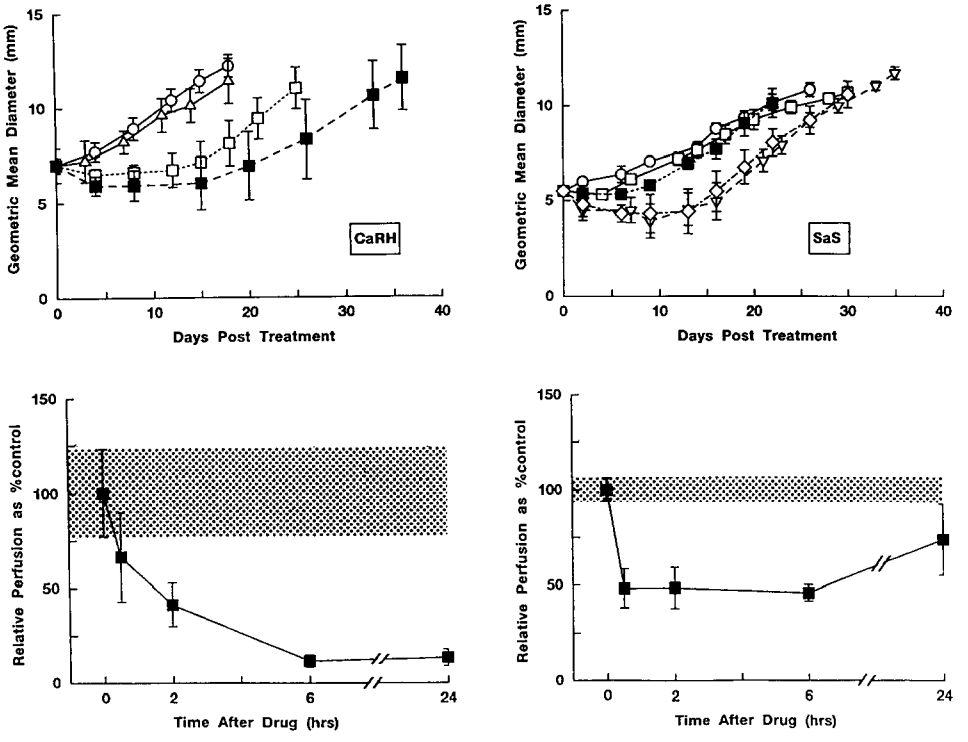

Fig. 8. Tumour response to a single dose of flavone acetic acid, in terms of growth retardation after various FAA doses (upper panels) or of blood perfusion after 200 mg/kg (lower panels). FAA causes a reduced perfusion in both tumours but it is more marked and more prolonged in the tumour which shows the greatest growth delay.

○ = no FAA
△ = 100 mg/kg
□ = 150 mg/kg
■ = 200 mg/kg
◇ = 250 mg/kg
▽ = 300 mg/kg

strated very convincingly that FAA action is greatly enhanced in vascularised tumours. They have developed an elegant model of intraperitoneal tumour spheroids. These dense aggregates of tumour cells are grown *in vitro* to a size of 0.5–1.2 mm diameter. When injected intraperitoneally, these attach to peritoneal surfaces and develop a vascular supply, or may exist as free floating non-vascularised spheroids. Thus in the same animal the effect on similar sized tumours can be studied with and without a vascular network. The damage in the spheroids was assessed histologically 18 hours after FAA and was much more marked in the vascularised tumours. There was even a differential effect in the vascularised and non-vascularised regions of the same spheroid. A comparison of the response in non-adherent spheroids *in vivo* or *in vitro* showed an enhanced response in the mouse, with leucocyte involvement, but this increase in effect was minor compared with the vascular mediated necrosis.

An interesting feature of FAA is that it is much more toxic when it is administered to tumour-bearing mice in a manner that is related to tumour load and also to the tumour sensitivity to FAA (Hill *et al.* 1990b). The antitumour action of the drug appears to be related to the whole body toxicity, even if different tumours are grown in the same strain of mice and treated at the same small size (~ 150 mg). This is illustrated in Fig. 10 where a large specific growth delay after 200 mg/kg is seen to be associated with low LD_{50} values. The increased toxicity can be induced, though to a lesser extent, by other angiogenic stimuli, e.g. by surgery, or by the implantation of a surgical sponge. Sloughing of the implanted surgical sponge occurs at 10–14 days in mice given FAA 2–4 days after implant, showing an antivascular effect even in this non-malignant mod-

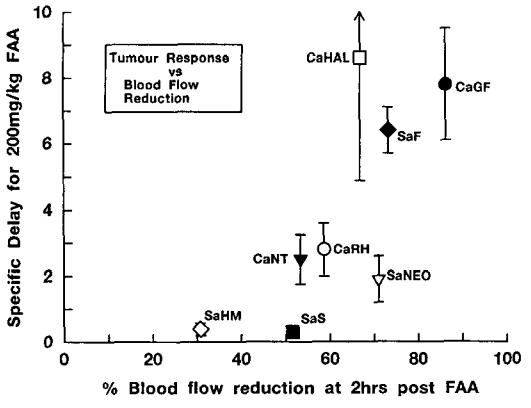

Fig. 9. Correlation between FAA induced growth delay (a measure of tumour damage) and the reduced perfusion measured at 2 hours after FAA administration. A similar correlation is seen with ^{86}Rb extraction data obtained at 6 and 24 hour intervals (from Hill *et al.* 1989b).

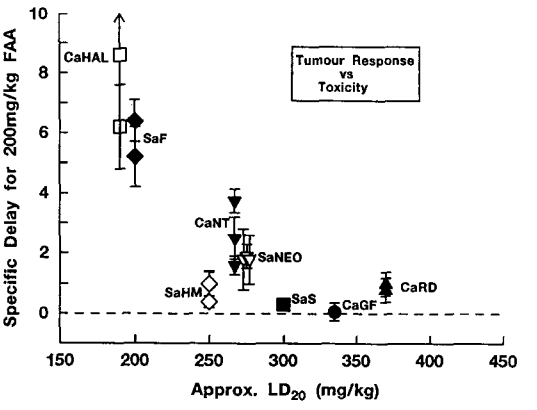

Fig. 10. Tumour bearing mice show an increased sensitivity to the toxic effects of the agent FAA. The more responsive the tumour to 200 mg/kg, the less well tolerated is the drug (from Hill *et al.* 1990b).

el (Williams *et al.* 1990). The time course of this sloughing is similar if a repeat experiment is done at a late time in the same mice, indicating that it is not likely to be immune mediated.

It therefore appears that a systemic effect is being induced by the presence of a tumour, or by a network of immature blood vessels. Changes in coagulation parameters have been demonstrated within a short time after drug injection. Initially the blood has a shorter clotting time, but after 24 hours this is reversed, perhaps indicating depletion of the clotting precursors (Murray *et al.* 1989b; Thurston *et al.* 1990). Detailed studies have indicated that the coagulation cascade is modulated by endothelial-derived factors, and that the presence of a tumour can tip the balance towards a pro-coagulant state (Falanga *et al.* 1987; Murray *et al.* 1989; Zacharski *et al.* 1987; Stern *et al.* 1985; Murray *et al.* 1990) by modifying endothelial activity. Evidence is accumulating that this balance can be further tipped by the addition of TNF or FAA to the system, either *in vitro* or *in vivo* (Murray *et al.* 1990; Clauss *et al.* 1990). This mechanism might explain the increased toxicity to the whole mouse, since the blood in a tumour-bearing mouse will have been primed by the products of such tumour cell: endothelial cell interactions. Indeed this influence of the tumour on coagulation parameters after FAA can be ameliorated by isolating the blood supply to the tumour from the main circulation for a period of 12–18 hours before administering FAA (Smith *et al.* 1990). A correlation between tumour responsiveness and platelet depletion has been demonstrated in the range of tumours illustrated in Fig. 9. Platelet depletion is much greater in mice bearing FAA sensitive tumours (Hill *et al.* 1990a).

Flavone Acetic Acid should perhaps be regarded as a lead compound for future anti-vascular therapies. It has certainly indicated some potential new modes of vascular attack. However, it has been universally disappointing in the early clinical trials. There has been little evidence for even partial regressions (Kerr *et al.* 1987; Weiss *et al.* 1988), in marked contrast to the massive effects seen in most rodent models. The early clinical studies showed that the main limiting side effect was severe hypotension, and also that decreased platelet aggregability was observed after 1–2 days. In order to minimise the troublesome hypotension, to maximise the chance of tumour cytotoxicity, and to avoid possible crystallization of FAA in the kidney tubules, a schedule was developed for the Cancer Research Campaign clinical trial using slow intravenous infusion over 6–8 hours in patients previously alkalinised with sodium bicarbonate. This schedule may seem to differ in minor ways relative to the acute bolus administration in mice but it may be the key to the disasspointing clinical results. If continuous infusion, or repeated small doses are

administered over a period of 6–8 hours to simulate the clinical infusion used in the CRC study the effect of 200 mg/kg, which gives 12 days' delay with a bolus injection is reduced to 4 days. An equivalent 12-day growth delay can be obtained by giving 300 mg/kg in the 6 injection regime (Denekamp and Hill 1990). Thus the protracted exposure which gave the greatest effect *in vitro* is not matched by the *in vivo* response. There is also a marked effect of prior alkalinization of the mice. In one tumour the induced growth delay is completely abolished, and in two others it is reduced to half or less. These effects cannot be explained by changes in the pharmocokinetics of the parent compound (Hill *et al.* 1990a).

Future directions

The appeal of vascular mediated cancer therapy is that it will be more effective on large tumours than small, but it will have the potential of being effective on any tumour with a newly evoked vascular network, i.e., of ~0.5–1 mm in diameter. These studies with many different anticancer agents have illustrated the potential complexity of responses induced by an agent that appears to cause tumour cell death mainly by collapse or occlusion of the blood supply. They have also focused attention on features of disparate agents, e.g., TNF, FAA which may share similar pathways. No single feature of neovasculature can be highlighted as the route by which such antivascular therapy should be targeted (Fig. 7). Rapid proliferation of the endothelial cells may prove to be a target, or may influence differentiation characteristics, so that the immature cells will function abnormally. The permeability of these poorly formed vessels may lead to extravasation of proteins (or erythrocytes), leading to increased interstitial pressures and hence to vascular collapse. Changes in systemic blood pressure, cardiac output, viscosity or coagulation would all have differential effects in tumours and normal vessels. Whether these effects are mediated through endogenous lymphokines or through the arachidonic acid and prostaglandin synthetic pathways needs further investigation. Clearly both vascular patho-physiology and the complexity of endothelial cell function and its imbalance in neovasculature will be important in understanding the mechanism of action of anti-vascular strategies (Folkman 1985, 1986; Jain 1989; Fajardo 1989).

The body of evidence that vascular-mediated damage occurs after many existing forms of antitumour therapy is rapidly accumulating. Conventional screens will not detect that this is the mode of action. Scheduling of existing treatments and the search for novel agents may be inappropriate if this component of injury is ignored or overlooked. The hallmarks of vascular-mediated damage are that cell death occurs in large patches, rather than in individual scattered cells. Histologically viable cords may persist around single capillaries whilst immediately adjacent cords are totally destroyed. This is an all or none phenomenon, with the surviving cords being similar in size to those observed prior to therapy. If a much greater effect is observed *in vivo* than predicted from *in vitro* studies, a host effect is clearly indicated. Whilst this could be immune-mediated, the rapid timescale and the histological pattern can sometimes distinguish that from ischaemic cell death. Similarly if solid tumours show more effect than disseminated (non-vascularized) tumours and if the effect is even greater in large than in small tumours then a vascular component of damage should be suspected. If an excision assay shows lower survival when tumours are removed 1 or 2 days after treatment instead of within an hour, an indirect mode of cell kill (or prolonged pharmacokinetics of a drug) may be implicated.

Finally the model of intraperitoneal spheroids is a powerful tool for comparing the response of vascularised and non-vascularised masses. Clearly for all these studies experimental animals will be needed, and *in vitro* substitutes will not yield relevant answers.

Different approaches to experimental cancer therapy are needed to ensure that this important new avenue is fully exploited. The vascular patterns of tumours will become important and these may differ in different tumour sites, in transplanted versus spontaneous tumours and in xenografts versus established rodent models. The tumour cells'

influence on endothelial cell function may be tumour specific. The branching frequency of the vascular network will be critical in determining how many cells die as a result of a single occlusion. Absolute tumour size rather than micro-environmental parameters may be important. We have much to learn and it promises to be an exciting and challenging multidisciplinary frontier calling for cooperation between tumour biologists, vascular and endothelial biologists and physiologists, immunologists and medicinal chemists.

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