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Effects of platelet activating factor (PAF) and other vasoconstrictors on a model of angiogenesis in the mouse

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Summary. The combination of sponge implant and ^{133}Xe washout technique described in this paper provides a model to study neovascularization in mice which can be observed over several days in the same animal. The local blood flow within the ingrowing granulation tissue has been determined by measuring the washout rate of ^{133}Xe injected into the implants. Tissue infiltration of the sponges was assessed by histological examination and by measurement of sponge wet weight, protein and glycosaminoglycans (GAG) content. The newly formed blood vessels, despite having abnormal configuration, responded to platelet activating factor (PAF) and to endothelin-1 (ET-1) similarly to the normal mature vessels in adjacent skin. However, the sponge blood vessels were more sensitive to angiotensin II than the skin blood vessels. Using this model we have also demonstrated an angiogenic activity of PAF substantiated by increased blood flow and biochemical variables in the implanted sponges.

Keywords: angiogenesis, blood flow, sponge implants

In face of physiological demands such as endometrial repair and wound healing or pathological conditions such as tumour development, the microvascular system is capable of responding with rapid capillary growth (Folkman & Klagsbrun 1987). The process of capillary growth, angiogenesis, involves infiltration of cells, accumulation of fluid, production of mediators, release of hydrolytic enzymes and ingrowth of fibrovascular tissue in a sequential and well ordered series of events. Angiogenesis can also be induced experimentally in sponges implanted *in vivo*. Such implants are particularly useful because they offer scope for modulating the environment within which the angiogenesis occurs, allowing identification of factors that may enhance or inhibit wound repair. Grindlay and Waugh (1951) and later Edwards *et al.* (1960) used polyvinyl alcohol sponges, in dogs and rabbits, respectively, as a framework for the ingrowth of vascularized connective tissue, providing valuable qualitative information on neovascularization and wound healing. The sponge matrix model has also been used to quantitate biochemical parameters of angiogenesis including collagen metabolism (Paulini *et al.* 1974), proteoglycan turnover (Bollet *et al.* 1958), fibronectin deposition

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(Holund *et al.* 1982), the kinetics of cellular proliferation (Davidson *et al.* 1985). This model has also been used for studying responses to pharmacological agents (Ford-Hutchinson *et al.* 1978).

Another measure of wound healing is the amount of local blood flow and methods for estimating blood flow in healing wounds, based on the washout of radioactive isotope, offer a relatively straightforward and well established technique (Weiber 1959; Lim *et al.* 1969; Nyman *et al.* 1972). This method is based on the principle (Kety 1949) that the amount of a locally deposited radioactive tracer decreases at a rate proportional to the blood flow at the site of the injection. The decrease of radioactivity is exponential and the $t_{1/2}$ for the washout is inversely related to the local blood flow (Kety 1949). A combination of the sponge implant and the washout of ^{133}Xe has been used to study the process of angiogenesis in rats (Andrade *et al.* 1987). We report here the successful adaptation of this combination of techniques to mice, measuring the development of the vasculature and biochemical variables and the pharmacological reactivity of the new blood vessels. We have also demonstrated an angiogenic effect of platelet activating factor (PAF) in this model. Some of these results have been presented in a preliminary form (Andrade 1991).

Methods

Animals

Male Balb/c mice weighing 20–28 g were used for all experiments.

Sponge implants

Polyether polyurethane sponge discs, 4 mm height × 8 mm diameter (Vitafoam Ltd, Manchester, UK) were used as the matrix for vessel growth. One end of a polythene tube 1·2 cm long × 1.2 mm internal diameter (Portex Ltd, Hythe, Kent, UK) was secured with two 5.0 silk sutures (Ethicon Ltd, UK) to the centre of each disc in such a way that the tube was perpendicular to the disc face. The sponge discs with cannula attached were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 min.

Some implants were thicker (6 mm × 8 mm) and had no cannula attached.

Implantation of sponges

Discs were implanted using aseptic techniques, in mice anaesthetized by intramuscular (i.m.) injection of Hypnorm and Hypnovel (0.5 ml/kg of each). The dorsal hair was shaved and the skin wiped with 70% ethanol. A 1-cm mid-line incision was made and through it one subcutaneous pocket was prepared by blunt dissection. A sterilized sponge implant was then inserted in the pocket, its cannula being pushed through a small incision which had been previously made on top of the pocket. The base of the cannula was sutured to the skin. The cannula was then plugged with a smaller sealed polythene tube. The mid-line incision was closed and the animals kept singly with free access to food and water after recovery from the anaesthetic.

Blood flow measurement

For blood flow measurement the animals were immobilized with Hypnorm/Hypnovel as before and sterile saline (10 μl) containing 1×10^6 c.p.s. ^{133}Xe was injected into the implant via the cannula, using an Alga micrometer fitted with an all-glass syringe (Wellcome Reagents Ltd, Beckenham, UK). The cannula was quickly plugged to prevent evaporation of ^{133}Xe and the radioactivity in the implant measured using a collimated gamma-scintillation detector (sodium iodide–thallium activated crystal; 1 inch by 1 inch (2.5 cm × 2.5 cm)) positioned 1 cm directly above the site of the injection. The radioactivity was accumulated for 40 s over at least 6 min after injection and the 40-s counts printed automatically on an SR7

scaler ratemeter (Nuclear Enterprises Ltd, London, UK). The rate of washout of ^{133}Xe was expressed in terms of its half-time ($t_{1/2}$; time taken for the radioactivity to fall to 50% of its original value).

Histology of sponge implants

At fixed times during the period of vascularization, mice were anaesthetized and killed by cervical dislocation and the sponge implants excised carefully, dissected free of adherent tissue, and fixed in formalin (10% w/v in isotonic saline). Sections (5 μm) were stained with haematoxylin and eosin (H & E) and processed for light microscopy studies.

Biochemical variables

Estimates of the cellular infiltration of the implant were derived from the wet weight of the sponge discs and its content of protein and glycosaminoglycans (GAG). For this the implants were removed on day 4, 7, 14 post-implantation. Immediately after being removed the implants were weighed, finely chopped with scissors in 1 ml of PBS, centrifuged, and the supernatant retained for analysis. The protein concentration in the sponge supernatant (10 μl) was measured by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard. The glycosaminoglycan content was measured as described by Farndale *et al.* (1982) with chondroitin sulphate as standard, using a 200-μl sample of supernatant.

Pharmacological reactivity of newly formed blood vessels

This was measured at 14 days post-implantation. Injections (50 μl) of vasoconstrictors PAF (0.1, 0.5 and 1 μg), endothelin-1 (0.125 and 1.25 ng) or angiotensin II (0.05 and 0.5 μg) or sterile saline were made into the sponge immediately before the ^{133}Xe solution was given. The reactivity of normal dorsal skin blood vessels was assessed in exactly the same manner, giving the injections (10 μl) intradermally in sites adjacent to the implant.

Angiogenic effect of PAF

On day 4 after implantation of the sponge, PAF or lyso-PAF (2 μg in sterile saline 50 μl) was injected into the sponge and this treatment repeated daily for another 4 days (five injections in total). A control group of mice received 50 μl of phosphate-buffered saline (PBS) in the same schedule. These injections were made immediately after the assay of ^{133}Xe washout.

Chemicals

Xenon injection, ^{133}Xe (10 mCi in 3 ml), was obtained from Amersham International, UK. Platelet-activating factor (1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine), Lyso-PAF, angiotensin II, Folin-Ciocalteu Phenol Reagent, Type A chondroitin-4-sulphate from whale cartilage, Type III papain, *N*-acetyl cysteine and EDTA (ethylene diaminetetraacetic acid) all from Sigma Chemical Co Ltd; endothelin-1 porcine (Peninsula Laboratories, Inc., Belmont, Calif, USA); Hypnorm (0·315 mg/ml fentanyl citrate and 10 mg/ml fluanisone acetate) from Janssen Pharmaceuticals, Oxford, UK; and Hypnovel (5 mg/ml of midazolam hydrochloride) from Roche Pharmaceuticals, Welwyn Garden City, UK.

Statistical analysis

Results are given as mean (±s.e.m.) values from n animals. Comparisons between groups were made with Student's t-test for unpaired data and a P-value less than 0.05 was considered significant.

Results

Effects of sponge size on angiogenesis in implants

The intensity and speed of the tissue response elicited by the sponge depend on several

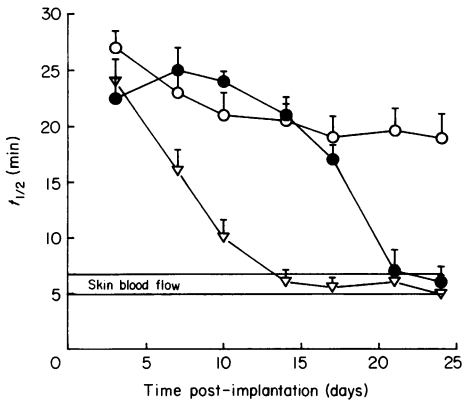


Fig. 1. Effects of sponge size on the development of blood flow measured by $t_{1/2}$ of ^{133}Xe washout over several days post-implantation. Sponge implant of ○, 6 mm × 8 mm; ●, 6 mm × 8 mm with cannula; ▽, 4 mm × 8 mm with cannula. Data represent mean ± s.e.m. from seven or eight animals.

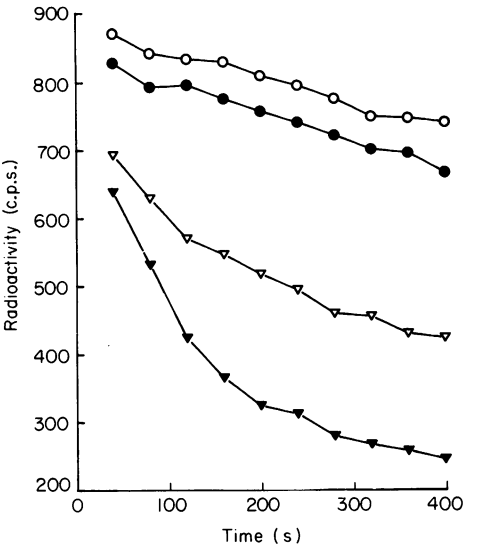


Fig. 2. ^{133}Xe washout curves from an implant of 4 mm × 8 mm with cannula at different days post-implantation reflecting the development of blood flow. The curves were obtained from the same animal at days ○, 4; ●, 7; ▽, 10 and ▼, 14 post-implantation.

factors including the type of sponge material and its size (Salvatore *et al.* 1961; Holund *et al.* 1979). To optimize angiogenesis in the mouse model three different types of implants were tested. Angiogenesis was assessed by the progressive fall in ^{133}Xe $t_{1/2}$, that is, progressive increase in local blood flow, and the results of these initial experiments are shown in Fig. 1. The 6 mm × 8 mm sponge implant with no cannula showed no sign of vascularization with $t_{1/2}$ remaining high for up to 24 days post-implantation. The same size of implant with a cannula did show neovascularization but only after 17 days post-implantation with $t_{1/2}$ reaching the normal skin value on day 21. In smaller implants (4 mm × 8 mm) with a cannula, there was a steady increase in blood flow over 14 days reaching a value which remained stable over the subsequent 10 days. This type of implant was adopted for all subsequent experiments. As illustrated in Fig. 1, the mean value of $t_{1/2}$ fell to about 15 min on day 7 from its starting value of about 24 min, with progressive falls to 10 min (10 days) and to 5 min (14 days). This last value was within the range for normal untreated skin. One of the features of this model is the non-destructive nature of the ^{133}Xe washout assay enabling each implant to be monitored over the whole period of neovascularization. In Fig. 2, the washout of ^{133}Xe is shown for one implant on four successive assays over 14 days, illustrating the progressive increase in the rate of washout with consequent falls in $t_{1/2}$.

Correlation between ^{133}Xe washout and cellular changes in implanted sponges

Soon after implantation (5–7 days) sponge implants became extensively infiltrated with cells. The early cellular infiltrate consisted mainly of polymorphonuclear leucocytes with the interstitial space occupied by fibrinous material, free red blood cells, spindle-shaped cells and macrophages (Fig. 3a).

By day 10 post-implantation, the sponge centre was completely occupied by an amorphous fibrinous matrix with numerous spindle-shaped cells. Also present in the matrix

were macrophages, endothelial cells, fibroblasts and capillary lumina containing red blood cells. On day 14 the implant sections had a more organized appearance with reduced cellularity and increased collagen matrix deposition. A phagocytic response was also apparent from the presence of giant cells. There was a mature capillary network with erythrocytes in the lumina of these new vessels (Fig. 3b).

Biochemical variables in implant

The three variables measured, wet weight, protein and GAG content, in implants over 14 days are summarized in Table 1. Although the wet weight showed a small increase in mean value, this change was not significant over 14 days. There were significant increases in protein and GAG content between 4 and 14 days, with proportionately greater increases in GAG (about 45%) than in protein (35%).

Responses of skin and sponge blood vessels to vasoconstrictors

Vasomotor responses of the newly formed blood vessels in implants were studied at 14 days using endogenous vasoconstrictors and compared with the responses of vessels in normal skin. As shown in Fig. 4 there was a progressive increase in $t_{1/2}$ when increasing doses of PAF, ET-1 and angiotensin II were injected into the implants or in normal skin. The responses of the blood vessels in the implants to PAF and ET-1 did not differ from those of the skin vessels. By contrast, responses to angiotensin II were relatively weak in normal skin with the $t_{1/2}$ up to about 9 and 15 min with 50 and 500 ng respectively, but very clearly enhanced in the implant vessels at both doses of this vasoconstrictor.

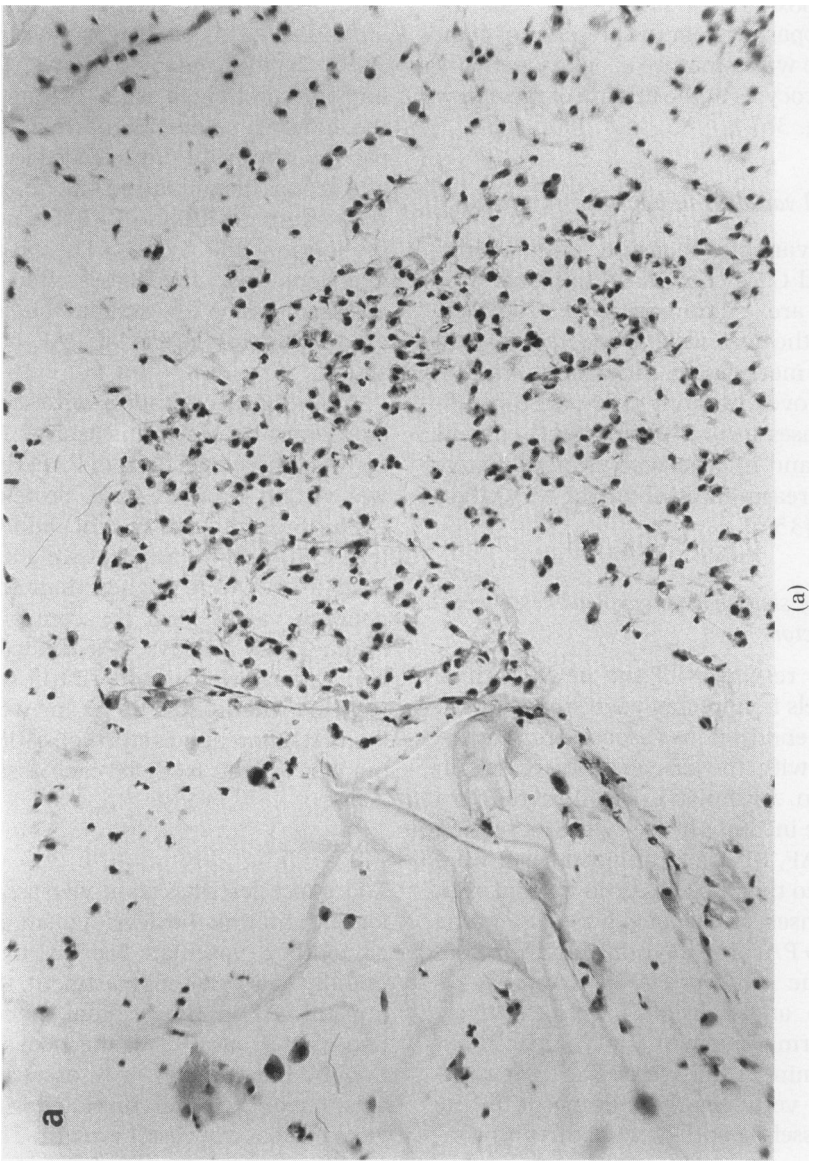
Angiogenic effect of PAF

This was assessed by the ^{133}Xe washout assay and by measuring the three biochemical variables in sponges implanted in mice receiving a single daily injection of PAF for 5 days (days 4–8). As shown in Fig. 5 pretreatment with PAF accelerated the fall in $t_{1/2}$ relative to the animals treated with saline or with lyso-PAF, the inactive natural metabolite of PAF. This effect was most marked at about 7 days and by 10 days, the $t_{1/2}$ in implants pretreated with PAF had reached the limiting value (for normal skin) compared with the 14 days needed by the other two groups of implants. At all times of assay, pretreatment with lyso-PAF had no effect on the normal fall in $t_{1/2}$. The pretreatment injections were made just after the ^{133}Xe washout assay to exclude the immediate vasoconstrictor effects of PAF on the $t_{1/2}$ assay.

A similar acceleration was seen for the biochemical variables measured. At 7 days, i.e. after three injections of PAF, the implant wet weight (263 ± 5 mg), protein content (216 ± 5 μg/mg wet weight) and GAG content (370 ± 14 ng/mg wet weight; $n=7$ for each assay) were higher than the corresponding values from the control implants (Table 1). By 14 days, PAF treated implants had normal wet weight (230 ± 6 mg) and protein content (208 ± 9 μg/mg wet weight), but GAG content was still high (408 ± 10 ng/mg wet weight; $n=7$ for each assay).

Discussion

This paper describes an in-vivo assay in mice for quantitating the development of a microcirculatory vascular bed in a localized, initially avascular compartment, the sponge implant. As in the original model in rats (Andrade *et al.* 1987), the neovascularization of the implant was assessed by the washout of ^{133}Xe and correlated with histological evidence of vessel growth. Thus, being readily adaptable to other types of rodent this technique offers further advantages to the study of angiogenesis. The smaller size of the mouse and the implant also means that lesser amounts of angiogenic or anti-angiogenic factors, vasoactive agents and other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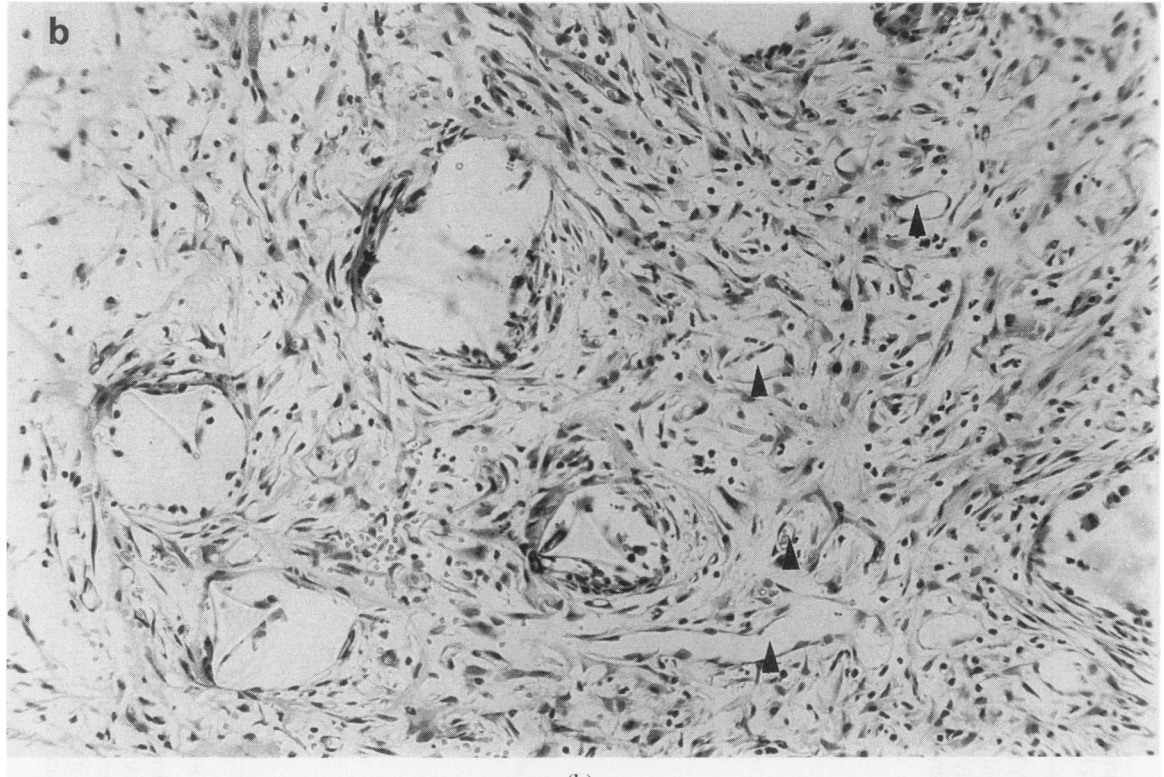

(b)

Fig. 3. Histological sections of implants. Sponge implants were removed on day a, 7 and b, 14 post-implantation, fixed in formalin, embedded in paraffin and 5-μm sections stained. The sponge matrix is seen as triangular shapes. In a, the pores of the implants are filled with a fibrinous network with numerous neutrophils granulocytes. In b, the granulation tissue is characterized by the presence of fibroblasts, lymphocytes, macrophages, foreign body giant cells and new blood vessels (arrows). H & E. $\times 100$.

Table 1. Biochemical changes during angiogenesis in sponges implanted in mice

	Days after implantation		
	4	7	14
Wet weight (mg)	227±11	217±12	247±5
Protein content (μg/mg wet weight)	139±7	128±5	187±25*
GAG (μg GAG/mg wet weight)	192±7	241±10*	279±7*

The values in the Table are the mean (±s.e. mean) values from five animals at day 4, 10 animals at day 7 and seven animals at day 14. Although the wet weight did not change, both protein and GAG contents increased over 14 days as the angiogenesis progressed.

* Significantly different from 4-day value.

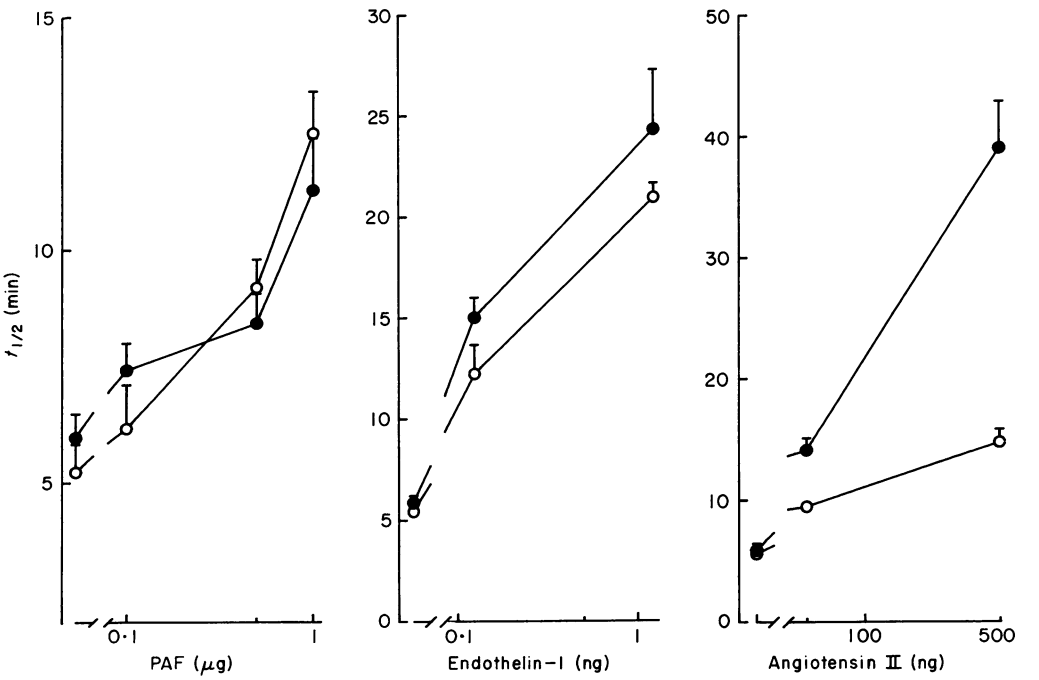

Fig. 4. Vasoconstrictor effects of PAF, endothelin-1 and angiotensin II in ○, skin and ●, sponge blood vessels (day 14). The values to the left of the break represent ^{133}Xe washout from untreated skin or sponge vessels. Agonists were injected into skin or sponge immediately before the radioactive tracer. The progressive increase in $t_{1/2}$ demonstrates vasoconstriction of blood vessels by the vasoactive agents. The values shown represent mean±s.e.m. from five to eight animals at each dose.

agonists are needed to produce their effects. Further, for the study of tumour-induced angiogenesis the range of syngeneic tumours is far greater in mice, with many inbred strains available.

The progressive fall in $t_{1/2}$, reflecting an increase in implant blood flow, was correlated with histological evidence for angiogenesis. Sponges excised from the animals at different times following implantation

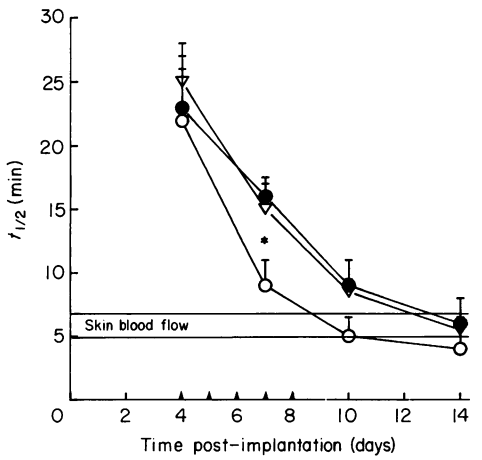

Fig. 5. Enhanced angiogenesis elicited by ○, PAF indicated by lower $t_{1/2}$ values compared to ●, lyso-PAF or ▽, PBS. Data represent mean ± s.e.m. from six to eight animals per group. $^*P < 0{\cdot}05$, different from control. Arrows, injections.

showed a reproducible and temporally-ordered set of events. The early appearance of neutrophils and deposition of fibrin was followed by the appearance of macrophages, fibroblasts and endothelial cells. Subsequently, new capillaries developed and the extra-cellular matrix became organized (Fig. 3). These changes started at the periphery of the implanted sponge disc and then progressed in a centripetal fashion to fill the sponge by day 14 post-implantation.

Further support for the process of neovascularization was provided by three biochemical measurements, wet weight, protein and GAG content. Increase of protein content and wet weight of sponge implants in rats have been used as positive evidence of fibrovascular infiltration following stimulation by growth factors (Davidson *et al.* 1985; Buckley *et al.* 1985). In our implants, protein increased over the 14 days as did GAG content. The latter molecules in their form of protein complexes, proteoglycans, are the major structural component of the colloid-rich ground substance of the extra-cellular matrix, produced extensively during angiogenesis by the endothelial cells (Ausprunk 1982). Hence the increase in GAGs that was observed reflects the deposition of extracellular matrix in the implanted sponges, perhaps as a corollary to the formation of the capillary network.

We consider the correlation between ^{133}Xe $t_{1/2}$, histology and biochemical assays to support strongly the validity of $t_{1/2}$ as a true representation of the neovascularization process here in the mouse as already shown in the rat (Andrade *et al.* 1987).

Results from our experiments also agree with the findings of others using implants to stimulate angiogenesis in that the physical nature of the implant crucially affects the extent and progression of the angiogenesis (Salvatore *et al.* 1961; Holund *et al.* 1979). In our assays, angiogenesis in the larger disc occurred after a considerable delay and was complete in 23 days. The smaller disc yielded a more gradual process and was clearly better suited to a study of angiogenesis. It is clear that the presence of the cannula is an important feature for successful neovascularization, perhaps by providing a cut surface in the skin and thus a 'wound' to promote acceleration of the healing process.

A particular advantage of the ^{133}Xe $t_{1/2}$ assay is that it allows non-destructive and thus repeated measurements of blood flow in the same animal over the period of neovascularization of the sponge. Some recently developed in-vivo models of angiogenesis (Fajardo *et al.* 1988; Plunkett & Halley 1990), although giving quantitative results, do not allow sequential observations as each animal provides information for only one time point. The assay we used clearly requires fewer animals over the period of angiogenesis and would also allow an estimate of variability in individual animals.

There are well established morphological and functional differences between newly formed vessels in granulation tissue and mature vessels (Abell 1946; Williams 1959; Cliff 1963; Warren 1979) and some of these differences, such as tortuosity and dilatation, we have observed in our implants. However, the pharmacological reactivity of the

implant blood vessels to two of the vasoconstrictors was identical to that of normal mature skin blood vessels, suggesting for these two agonists at least, pharmacological normality in the presence of morphological difference. The responses to angiotensin II showed a hyper-reactivity in the implant vessels, a result which needs further analysis before a mechanism could be proposed. Nevertheless, these results contrast sharply with the recent preliminary finding that vessels in implants bearing tumour cells were resistant to these vasoconstrictors relative to those in normal implants (Andrade *et al.* 1991).

The angiogenic effect of PAF given during vessel growth, rather than its acute vasoconstrictor effect, was investigated because angiogenic effects have been attributed to vasoconstrictor agents such as angiotensin II in other models (Fernandez *et al.* 1985). Several vasoactive agents causing venoconstriction, and thus vasocongestion at the capillary level, have been postulated to synergize to cause angiogenesis (Chiarugi *et al.* 1986). If this were so, treatments with exogenous vasoconstrictors could add to the endogenous stimuli and accelerate neovascularization. This may be the explanation of the results of the pretreatment with PAF. However, there are other possibilities. For instance, PAF is an inflammatory mediator and could contribute to local oedema and attract leucocytes by a mechanism separate from its vasoconstrictive properties. It is clear that diffusible angiogenic stimuli can be released by activated lymphocytes (Sidky & Auerbach 1975) and activated macrophages (Polverini *et al.* 1977). Furthermore, the vasoconstriction caused by PAF is relatively short lasting; in the 14-day implant, retesting the ^{133}Xe washout after an injection of PAF showed that by one hour after 1 μg PAF, the $t_{1/2}$ value had returned to the normal value (4–5 min; data not shown). This would suggest that for most of the 24 h between PAF treatments, vasoconstriction was not present. From our present results, the relevant component of PAF's biological activity cannot be determined. The importance of vasoconstriction *per se* could be tested by using other vasoconstrictors without the pro-inflammatory properties of PAF. Other chemoattractant molecules without vasoactivity, such as leukotriene B_4, might be used to assess the importance of inflammatory cell infiltration. However, our results are quite clear and the paradox of the same mediator decreasing blood flow acutely and increasing it by chronic treatment could be explained by the combination of both properties of the mediator.

In conclusion, this present modification of a previous model of angiogenesis (Andrade *et al.* 1987) still retains the essential correlation between histological and biochemical evidence of neovascularization and increase in blood flow detected by ^{133}Xe washout. With it we have shown that the new blood vessels exhibit pharmacological reactivity comparable to that of normal vasculature and that the model can be used to demonstrate stimulation of the angiogenesis by PAF. We consider this model in mice will contribute significantly to the further qualitative analysis of angiogenesis in normal and pathological conditions.

Acknowledgements

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