Suppression of VEGF-induced angiogenesis by the protein tyrosine kinase inhibitor, lavendustin A

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1 Vascular endothelial growth factor (VEGF) is a heparin-binding angiogenic factor which specifically acts on endothelial cells via distinct membrane-spanning tyrosine kinase receptors. Here we used the rat sponge implant model to test the hypothesis that the angiogenic activity of VEGF can be suppressed by protein tyrosine kinase (PTK) inhibitors.

2 Neovascular responses in subcutaneous sponge implants were determined by measurements of relative sponge blood flow by use of a ¹³³Xe clearance technique, and confirmed by histological studies and morphometric analysis.

3 Daily local administration of 250 ng VEGF₁₆₅ accelerated the rate of ¹³³Xe clearance from the sponges and induced an intense neovascularisation. This VEGF₁₆₅-induced angiogenesis was inhibited by daily co-administration of the selective PTK inhibitor, lavendustin A (10 µg), but not its negative control, lavendustin B (10 µg). Blood flow measurements and morphometric analysis of 8-day-old sponges showed that lavendustin A reduced the ¹³³Xe clearance of VEGF₁₆₅-treated sponges from 32.9 ± 1.5% to 20.9 ± 1.6% and the total fibrovascular growth area from 62.4 ± 6.1% to 21.6 ± 6.8% (*n* = 12, *P* < 0.05).

4 Co-injection of suramin (3 mg), an inhibitor of heparin-binding growth factors, also suppressed the VEGF₁₆₅-elicited neovascular response. In contrast, neither lavendustin A nor suramin produced any effect on the basal sponge-induced angiogenesis.

5 When given alone, low doses of VEGF₁₆₅ (25 ng) or basic fibroblast growth factor (bFGF; 10 ng) did not modify the basal sponge-induced neovascularisation. However, co-administration of these two peptides to a single sponge together caused a significant increase in the rate of ¹³³Xe clearance and angiogenesis similar to that seen with the high dose of VEGF₁₆₅ (250 ng) acting alone. This VEGF/bFGF neovascular response was also blocked by daily co-administration of lavendustin A (10 µg), suramin (3 mg) or a monoclonal anti-bFGF antibody (DG2, 1 µg), but not lavendustin B (10 µg).

6 These results suggest that selective inhibition of PTK could have therapeutic potential in angiogenic diseases where VEGF plays a dominant role. Furthermore, blockade of the angiogenic activity of VEGF and VEGF/bFGF by suramin reveals an alternative strategy in angiosuppression.

Keywords: Angiogenesis; vascular endothelial cell growth factor (VEGF); protein tyrosine kinase (PTK); lavendustin; suramin

Introduction

Angiogenesis is normally under stringent control and occurs only during embryonic development, the female reproductive cycle and wound repair. In many pathological conditions (e.g., solid tumour, rheumatoid arthritis, diabetic retinopathy and atherosclerosis), the disease itself appears to be driven by persistent unregulated vessel proliferation. Folkman hypothesized in the early 1970s that tumour growth is angiogenesis-dependent and pioneered the idea that an anti-angiogenesis strategy might represent a new therapeutic approach for the treatment of solid tumours (Folkman, 1972). Since the first successful clinical treatment of an angiogenic disease using interferon alpha-2a (White *et al.*, 1989), several natural and synthetic compounds have shown promising therapeutic potentials as angiosuppressants. Some of them are currently undergoing clinical trials for the treatment of cancer (Fan, 1994).

It is now clear that the angiogenic process is tightly controlled by the balance between many positive and negative regulatory signals (Folkman & Klagsbrun, 1987; Folkman & Shing, 1992; Fan & Brem, 1992). However, vascular endothelial growth factor (VEGF) has attracted much attention recently because it is the only angiogenic factor that specifically acts on endothelial cells (Connolly, 1991; Ferrara *et al.*, 1992; Senger *et al.*, 1993). The isolation of VEGF from several tumour cell lines and macrophages has implicated it as a major tumour angiogenesis factor in some human gliomas

(Plate *et al.*, 1992) and chronic inflammatory diseases (Senger *et al.*, 1993). Another unique feature of VEGF is that its cell-surface receptors are exclusively expressed in endothelial

Figure 1 Angiogenesis induced by vascular endothelial growth factor (VEGF). Columns represent sponges treated daily with phosphate-buffered saline (PBS, open columns), 25 ng VEGF (hatched columns) or 250 ng VEGF (solid columns). Data represent the mean ± s.e. *n* = 6. **P* < 0.05, (VEGF vs PBS).

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cells (Millauer *et al.*, 1993; Plate *et al.*, 1993). Thus, VEGF is a promising target for the development of specific angiopressive drugs.

Using a rat sponge implant model, we have previously demonstrated that it is possible to block the neovascular responses induced by growth factors/cytokines *in vivo* by their respective neutralising antibodies, receptor antagonists (Hu & Fan, 1993; Fan *et al.*, 1993; Hu *et al.*, 1994), or selective inhibitors of the signal-transduction pathways (Hu & Fan, 1994a). Since the mitogenic and angiogenic activities of VEGF are thought to be mediated by distinct membrane-spanning tyrosine kinase receptors, we predicted that specific inhibitors of protein tyrosine kinase (PTK) should block the biological functions of VEGF *in vivo*. To test this hypothesis, we used a highly potent and specific PTK inhibitor lavendustin A (Onoda *et al.*, 1989; Odell *et al.*, 1991) and showed that it is an effective inhibitor of the angiogenic activity of VEGF.

Suramin is an effective analogue of heparin/heparan sulphate which inhibits the activities of several heparin-binding growth factors such as fibroblast growth factors (FGFs) and platelet-derived growth factor (Myers *et al.*, 1990). Although suramin blocks the growth-stimulating activity of VEGF *in vitro* (Olander *et al.*, 1991), its action against VEGF *in vivo* is unknown. Here we also show that suramin blocks the angiogenic activity of VEGF in the rat sponge model.

Methods

The sponge implant model

Sterile circular polyether sponge discs (5 mm thick, 1.2 cm diameter) with central cannulae (1.3 cm long, 1.4 mm internal diameter) were implanted subcutaneously in male Wistar rats (180–200 g) after induction of neuroleptanalgesia by Hypnorm (0.315 mg ml⁻¹ fentanyl citrate and 10 mg ml⁻¹ fluanisone; 0.1 ml kg⁻¹, i.m.). Test substances (50 µl) were injected daily into the sponges through the cannulae. To exclude the possible acute effects of the test substances (dilatation or constriction) on the microvasculature, they were given 18–24 h prior to the ¹³³Xe measurements. The neovascular response was assessed as a function of blood flow through the implants by use of a ¹³³Xe clearance technique (Andrade *et al.*, 1987). Briefly, animals were anaesthetized with Hypnorm as before and 10 µl ¹³³Xe in sterile PBS was injected into the sponges through the cannulae. The washout of radioactivity from the implants was monitored with a gamma scintillation detector over a period of 6 min. The clearance value was calculated by the formula % ¹³³Xe clearance = [(initial radioactivity – residual radioactivity at 6 min)/initial radioactivity] × 100%. The use of this technique to assess angiogenesis has been validated by comparison with four other methods, namely, (i) ¹¹³Sn microsphere wash-in technique to measure absolute blood flow in the sponges; (ii) the carmine red dye method for measurement of neovascularity; (iii) colorimetric measurement of haemoglobin levels in the implants and (iv) histological and morphometric studies using a computer-assisted image analysis system.

Histology and morphometric analysis of fibrovascular growth areas

The animals were killed by cervical dislocation and sponges dissected out, carefully removing the cannulae and any adherent fat. The samples were then bisected and fixed in formal saline at 4°C for 1 h. Sections (10 µm) were prepared and stained with haematoxylin and eosin (H & E), Masson Trichrome or a specific endothelial cell marker *Bandeiraea simplicifolia* lectin I, isolectin B₄ (BSL-B₄). The specimens were analysed in a 'blind' manner and recorded on Kodak Gold II ASA 200 film. For simplicity, we use the term fibrovascular growth area to describe the tissue growth into a sponge. This is composed of blood vessels and surrounding stroma containing fibroblasts, macrophages, neutrophils and

endothelial cells. As reported by Kowalski *et al.* (1992), the area occupied by blood vessels is a consistent percentage of the fibrovascular growth area. Thus, the measurement of fibrovascular growth area provides an accurate index of angiogenesis. To analyse morphometrically fibrovascular growth areas of the sponges, we adopted the axial strip sampling technique as described by Mayhew & Sharma (1984). The H & E-stained sections were observed with a 4 × objective and images relayed to a computer-assisted image analysis system (Seescan, Cambridge, U.K.). By a point counting procedure, using a 96-point square lattice with a field area of 0.66 mm², the volume fraction occupied by fibrovascular growth was estimated.

Materials

Recombinant human VEGF₁₆₅ was purchased from R & D Systems, Minneapolis, U.S.A. Lavendustin A and B were purchased from Calbiochem-Novabiochem (UK) Ltd., Nottingham, U.K. Recombinant human basic FGF and the monoclonal antibody to basic FGF, DG2 (Reilly *et al.*, 1989), were gifts from Professor M. Presta, University of Brescia, Brescia, Italy and Dr T.M. Reilly, Du Pont Merck Pharmaceuticals, Wilmington, Delaware, U.S.A., respectively. Other materials were purchased from the following companies: suramin (Imperial Chemical Industries Ltd., Macclesfield, U.K.); polyether foam sheet (R.E. Carpenter & Co., High Wycombe, U.K.); polythene tubings (Portex Ltd., Hythe, U.K.); Hypnorm (Janssen Pharmaceuticals, Oxford, U.K.); ¹³³Xe injection (Medgenix plc, High Wycombe, U.K.) and phosphate buffered saline (PBS) (Sigma Chemical Co. Ltd., Poole, U.K.). Lavendustin A and B were dissolved in dimethyl sulphoxide (DMSO) and then diluted with PBS. The final concentration of DMSO was less than 1.25%. Other solutions were prepared daily in PBS and sterilized by membrane filtration (0.22 µm) before use.

Statistics

Statistical analysis was done using StatView II (Abacus Concepts, Berkeley, CA, U.S.A.) on a Macintosh computer. Statistical significance was tested by analysis of variance (ANOVA) using a multi-comparison significance level for one factor ANOVA of 95% shown in the figures, or by Student's *t* test shown in the table.

Results

Effect of VEGF₁₆₅ on sponge-induced angiogenesis

Daily doses of 250 ng VEGF₁₆₅, the most abundant form of VEGF found *in vivo* (Ferrara *et al.*, 1992), caused a significant enhancement of ¹³³Xe clearance from the sponges as compared to the controls, while lower doses (25 ng) had no apparent effect (Figure 1). Histological studies of sponge sections showed that the cellular infiltration, neovascularisation and connective tissue in the VEGF₁₆₅-treated sponges were profoundly increased (Figure 2d–2f), as confirmed by a large increase of the fibrovascular growth area (Table 1).

Effect of lavendustin A and B on VEGF-induced angiogenesis

Figure 3 clearly shows that the enhanced ¹³³Xe clearance in VEGF₁₆₅-treated sponges was significantly inhibited by daily co-administration of a selective PTK inhibitor, lavendustin A (10 µg), but not its negative control, lavendustin B (10 µg). Lower doses of lavendustin A and lavendustin B (1 µg) were ineffective. Histological studies of sponge sections showed that the cellular infiltration, neovascularisation and connective tissue in the VEGF-treated sponges were markedly suppressed by lavendustin A (Figure 2g and 2h), as confirmed by

Figure 2 Histological sections of 8-day old sponges illustrating (i) sponge treated with PBS (sections a–c); (ii) the intense neovascular response induced by vascular endothelial growth factor (VEGF) (250 ng day⁻¹, sections d–f); (iii) the inhibition of VEGF-induced angiogenesis by lavendustin A (10 μg day⁻¹, section g), but not by lavendustin B (10 μg day⁻¹, section h); (iv) the inhibition of VEGF-induced angiogenesis by suramin (3 mg day⁻¹, section i), (v) the intense neovascular response induced by a combination of VEGF (25 ng day⁻¹) and bFGF (10 ng day⁻¹, section j); (vi) the inhibition of VEGF/bFGF-induced angiogenesis

a large reduction of the fibrovascular growth area (Table 1). By contrast, neither lavendustin A nor lavendustin B (both at 10 μ g) was able to inhibit the basal sponge-induced neovascularisation (data not shown).

Effect of suramin on VEGF-induced angiogenesis

The VEGF₁₆₅-induced neovascular response was inhibited by co-administration of 3 mg suramin, while 1 mg suramin was

ineffective (Figure 4). Histological studies of sponge sections showed that the cellular infiltration, neovascularisation and connective tissue in the VEGF-treated sponges were effectively suppressed by suramin (Figure 2i), again confirmed by a large reduction of the fibrovascular growth area (Table 1). In the absence of exogenous peptides, suramin (1 or 3 mg daily) produced no apparent effect on the ¹³³Xe clearance and angiogenesis in control sponges, as confirmed histologically (data not shown).

by lavendustin A (10 μ g day⁻¹, section k), suramin (3 mg day⁻¹, section l) or by a monoclonal anti-bFGF antibody (1000 ng day⁻¹, section m), but not by lavendustin B (10 μ g day⁻¹, section l). All sections photographed at $\times 200$ magnification. Bar = 100 μ m. (a) Sponge treated with PBS (H & E stained); (b) sponge treated with PBS (BSL-B₄ stained); (c) sponge treated with PBS (Masson Trichrome stained); (d) sponge treated with VEGF (H & E stained); (e) sponge treated with VEGF (BSL-B₄ stained); (f) sponge treated with VEGF (Masson Trichrome stained); (g) sponge treated with VEGF plus lavendustin A (H & E stained); (h) sponge treated with VEGF plus lavendustin B (H & E stained); (i) sponge treated with VEGF plus suramin (H & E stained); (j) sponge treated with VEGF plus bFGF (Masson Trichrome stained); (k) sponge treated with VEGF and bFGF plus lavendustin A (Masson Trichrome stained); (l) sponge treated with VEGF and bFGF plus lavendustin B (Masson Trichrome stained); (m) sponge treated with VEGF and bFGF plus suramin (Masson Trichrome stained); (n) sponge treated with VEGF and bFGF plus DG2 (Masson Trichrome stained).

Table 1 Effect of vascular endothelial growth factor on the total fibrovascular growth area (%) of sponge implants

Treatment groups	Fibrovascular growth area (%)
Control	19.2 ± 5.3
VEGF (250 ng day ⁻¹)	67.4 ± 6.0*
VEGF (250 ng) + lavendustin A (10 µg)	21.6 ± 6.8***
VEGF (250 ng) + lavendustin B (10 µg)	62.4 ± 6.1
VEGF (250 ng) + suramin (3 mg)	23.3 ± 6.7**
VEGF (25 ng) + bFGF (10 ng)	61.1 ± 5.9*
VEGF (25 ng) + bFGF (10 ng) + lavendustin A (10 µg)	23.7 ± 6.5****
VEGF (25 ng) + bFGF (10 ng) + lavendustin B (10 µg)	62.9 ± 6.6****
VEGF (25 ng) + bFGF (10 ng) + suramin (3 mg)	22.2 ± 6.3****
VEGF (25 ng) + bFGF (10 ng) + DG2 (1 µg)	24.2 ± 6.9****

Data derived from histological sections of 8-days old sponges, $n = 12$ for each group. * $P < 0.01$ (vs Control); ** $P < 0.01$ (vs VEGF); *** $P < 0.05$ (VEGF + lavendustin A vs VEGF); **** $P < 0.01$ (vs VEGF + bFGF).

Figure 3 Inhibition of vascular endothelial growth factor (VEGF)-induced angiogenesis by lavendustin A. Columns represent sponges treated daily with phosphate buffered saline (A), 250 ng VEGF (B), 250 ng VEGF plus 1 µg lavendustin A (C), 250 ng VEGF plus 10 µg lavendustin A (D) or 250 ng VEGF plus 10 µg lavendustin B (E). Data represent the mean ± s.e. $n = 6$. * $P < 0.05$, (VEGF plus lavendustin A vs VEGF).

Figure 4 Inhibition of vascular endothelial growth factor (VEGF)-induced angiogenesis by suramin. Columns represent sponges treated daily with phosphate buffered saline (open columns), 250 ng VEGF (solid columns), 250 ng VEGF plus 1 mg suramin (hatched columns) or 250 ng VEGF plus 3 mg suramin (horizontally lined columns). Data represent the mean ± s.e. $n = 6$. * $P < 0.05$ (VEGF plus suramin vs VEGF).

Synergism between VEGF and bFGF

When given alone, low doses of VEGF (25 ng) or bFGF (10 ng) did not modify the basal sponge-induced neovascularisation (data not shown). However, co-administration of these two peptides to a single sponge together caused a significant increase in angiogenesis and ¹³³Xe clearance (Figures 2j and 5) similar to that seen with the high dose of VEGF (250 ng) acting alone. This VEGF/bFGF neovascular response was also blocked by daily co-administration of lavendustin A (10 µg), suramin (3 mg) or a monoclonal anti-bFGF antibody (DG2, 1 µg), but not lavendustin B (10 µg). Histological studies of sponge sections showed that the cellular infiltration, neovascularisation and connective tissue in the VEGF/bFGF-treated sponges were suppressed by lavendustin A (Figure 2k), suramin (Figure 2m) or DG2 (Figure 2n), as confirmed by a large reduction of the fibrovascular growth area (Table 1).

Discussion

VEGF is a highly conserved, multifunctional cytokine expressed and secreted at high levels by activated macrophages and many tumour cells of animal and human origin (Senger *et al.*, 1993). Among the various angiogenic factors identified

to date, VEGF is unique because it specifically acts on endothelial cells (Connolly, 1991; Ferrara *et al.*, 1992) and its cell-surface receptors are exclusively expressed in endothelial cells (Millauer *et al.*, 1993; Plate *et al.*, 1993). The over-expression of VEGF and its receptors are associated with chronic inflammation, tumour growth and other angiogenic diseases. Thus, VEGF is a prime target for the development of specific angiostatic drugs.

Here we show for the first time that the angiogenic activity of VEGF *in vivo* can be prevented by lavendustin A. Lavendustin A is a potent and selective PTK inhibitor *in vitro*, with an IC₅₀ of 11 nM. In contrast, the IC₅₀ of its negative control, lavendustin B, is 1.3 µM (Onoda *et al.*, 1989). Thus, the ability of lavendustin A and B to suppress VEGF-induced angiogenesis is entirely consistent with their relative potency as PTK blockers. Recently, lavendustin A has been shown to inhibit antigen-induced activation of PTK and histamine release from mast cells with IC₅₀ of 80 µM and 112 µM, respectively (Kawakami *et al.*, 1992). This large discrepancy in the ability of lavendustin A to inhibit PTK enzyme activity in membrane fractions and in intact cells is possibly due to its poor penetration into the cells (Onoda *et al.*, 1990). Two other tyrosine kinase inhibitors, herbimycin A (Oikawa *et al.*, 1989) and genistein (Fotsis *et al.*, 1993), have also been shown to inhibit angiogenesis in the chick embryo chorioallantoic membrane and endothelial cell proliferation *in vitro*. Experiments are in progress to assess the inhibitory activities

Figure 5 Effects of lavendustin A, suramin and a monoclonal anti-bFGF antibody on the enhanced neovascular response elicited by a combination of vascular endothelial growth factor (VEGF) and bFGF. Columns represent sponges treated daily with phosphate buffered saline (A), 25 ng VEGF and 10 ng bFGF (B), 25 ng VEGF and 10 ng bFGF plus 10 µg lavendustin A (C), 25 ng VEGF and 10 ng bFGF plus 3 mg suramin (D) and 25 ng VEGF and 10 ng bFGF plus 1 µg DG2 (E). Data represent the mean $\pm$ s.e. $n = 4-8$. * $P < 0.05$, (all drug treatment groups vs VEGF and bFGF).

of these compounds on VEGF-induced angiogenesis in the sponge model.

It is important to note that in the absence of exogenous VEGF, lavendustin A did not modify the basal sponge-induced angiogenesis. In a previous report, we showed that the basal sponge-induced angiogenesis was almost completely inhibited by the combination of IL-1 receptor antagonist and neutralising antibodies to bFGF, TNF- α and IL-8 (Hu *et al.*, 1994), suggesting that the interactions between endogenous bFGF, TNF- α , IL-1 α and IL-8 are mainly responsible for the neovascularisation elicited by sponges alone. Taken together, these findings strongly suggest that the action of lavendustin A in the present study is directed against VEGF-elicited responses.

Suramin, a polysulphonated naphthylurea, is the prototype of anti-cancer drugs that work by binding growth factors. It inhibits the activities of several heparin-binding growth factors such as transforming growth factor- β , fibroblast growth factor and platelet-derived growth factor (Coffey *et al.*, 1987; Kopp & Pfeiffer, 1990; Myers *et al.*, 1990). In a rat brain tumour model, suramin inhibits endothelial and tumour cell proliferation (Takano *et al.*, 1993). Our data show that suramin is equally effective against angiogenesis induced by VEGF without any sign of toxicity. Previously, we have shown that the neovascular response induced by bFGF was markedly suppressed by co-administration of 3 mg suramin (Hu & Fan, 1992). In contrast, the angiogenic activity of non-heparin-binding growth factors such as platelet-derived endothelial cell growth factor was resistant to suramin (Fan *et al.*, 1993). In view of the narrow therapeutic window for suramin (La Rocca *et al.*, 1990), we are currently evaluating the anti-angiogenic activity of suramin analogues

which are 5 to 10 fold less toxic than suramin (Braddock *et al.*, 1994).

Several other approaches have been used to inhibit VEGF activity. For example, pretreatment with a monoclonal antibody specific for VEGF has been shown to reduce the vascular density and suppress the growth of several human tumours in nude mice, without affecting the growth rate of tumour cells *in vitro* (Kim *et al.*, 1993). Similarly, we have shown that the VEGF-induced neovascular response was neutralised by co-administration of a polyclonal antibody to VEGF (Hu & Fan, 1994a). Two types of VEGF receptors have been identified: Flt and KDR (de Vries *et al.*, 1992; Terman *et al.*, 1992). In a recent study, recombinant human soluble Flt (sFlt) has been shown to bind VEGF with high affinity and inhibit its mitogenic activity for vascular endothelial cells (Kendall & Thomas, 1993). Future work may generate sFlt derivatives as specific high-affinity antagonists of VEGF.

VEGF has been reported to act synergistically with bFGF to stimulate both endothelial cell growth and endothelial cell invasion through collagen gels *in vitro* (Pepper *et al.*, 1992), and on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels (Goto *et al.*, 1993). Here we showed that VEGF acts synergistically with bFGF to enhance neovascularisation *in vivo*. The synergistic effect of VEGF and bFGF was blocked by co-administration of lavendustin A, suramin or a monoclonal anti-bFGF antibody, DG2. These data suggest that in situations where an angiogenic response is elicited by VEGF and bFGF, strategies based on the antagonism of either angiogenic factor could prove feasible.

When given intravenously, bFGF has been reported to dilate blood vessels and decrease arterial blood pressure (Cuevas *et al.*, 1992). However, we did not detect any significant increase in ¹³³Xe clearance when VEGF and/or bFGF were injected intradermally or into the sponges (data not shown). Furthermore, all the test substances were given 18–24 h before the ¹³³Xe measurements to exclude the possible acute effects (dilatation or constriction) on the microvasculature. Thus, the enhanced angiogenesis in sponges treated with VEGF and/or bFGF is not likely to be due to vasodilatation.

We have recently shown that cytokine-induced angiogenesis *in vivo* can be partially prevented by the potent and specific PKC inhibitor calphostin C (Hu & Fan, 1994b; 1995). Here we report that the PTK inhibitor, lavendustin A, can suppress VEGF-induced neovascularisation. Taken together, these data clearly demonstrate that it is possible to down-regulate angiogenesis by inhibitors of signalling pathways. In conclusion, further elucidation of the functions of PKC, PTK and other signalling pathways in angiogenesis will no doubt facilitate the development of enzyme-specific inhibitors that could prove useful in formulating new strategies for the prevention and treatment of angiogenic diseases. Specifically, in angiogenic diseases where VEGF plays a dominant role, we anticipate more potent and permeable derivatives of lavendustin A in combination with VEGF-specific antibodies or soluble VEGF receptors to provide an effective treatment.

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