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Comparative studies of the angiogenic activity of vasoactive intestinal peptide, endothelins-1 and -3 and angiotensin II in a rat sponge model

De-En Hu, C. Robin Hiley & [1]Tai-Ping D. Fan

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

1 The angiogenic activity of four vasoactive peptides with a range of vasodilator and vasoconstrictor properties, i.e. vasoactive intestinal peptide (VIP), endothelin-1, endothelin-3 and angiotensin II, were investigated in a rat sponge model. Neovascularization was assessed by the ^{133}Xe clearance technique and confirmed by histological studies.

2 Daily doses of the vasodilator peptide, VIP (1000 pmol), caused intense neovascularization, but a lower dose (10 pmol) produced no apparent effect. However, the lower dose of VIP, when given with a subthreshold dose of interleukin-1α (0.3 pmol), produced an angiogenic response similar to that seen with the higher dose of VIP. The neovascular response induced by co-administration of VIP and interleukin-1α was inhibited by simultaneous administration of 100 pmol VIP (10–28), a specific VIP receptor antagonist.

3 In contrast, daily doses of 10, 100 or 1000 pmol endothelin-3 (a mixed vasoconstrictor and vasodilator with more marked vasodilator activity) or of 100 or 1000 pmol endothelin-1 (also with mixed activity but with much more pronounced vasoconstrictor response) produced no apparent effect on sponge-induced angiogenesis.

4 The vasoconstrictor peptide, angiotensin II, in daily doses of 1000 pmol, caused an intense neovascularization like VIP but lower doses of angiotensin II (10 or 100 pmol) produced no apparent effect. The lowest dose of angiotensin II (10 pmol) when administered with the subthreshold dose of interleukin-1α (0.3 pmol) had no effect on the basal neovascular response in the sponges. The angiotensin II-induced neovascular response was inhibited by co-administration of 100 nmol of the specific AT_1 receptor antagonist, losartan, but not by the AT_2 receptor antagonist, PD 123319.

5 These data show that VIP and angiotensin II possess angiogenic activity. However, endothelin-1 and endothelin-3 had no activity at the doses used. Thus the angiogenic response is not related to local vasoconstriction or vasodilatation in the sponges. The blockade of VIP- and angiotensin II-induced angiogenesis at the receptor level suggests that receptor modulation could provide a strategy for the management of angiogenic diseases.

Keywords: Angiogenesis; vasoactive intestinal peptide (VIP); endothelin-1; endothelin-3; angiotensin II; losartan; interleukin-1α

Introduction

Angiogenesis is normally under stringent control and occurs only during embryonic development, endometrial development and wound repair. However, in many pathological conditions, such as cancer, diabetic retinopathy, psoriasis, rheumatoid arthritis and atherosclerosis, the diseases appear to be driven by, or are dependent upon, persistent unregulated vessel proliferation, suggesting anti-angiogenesis as a new therapeutic approach for the treatment of these diseases (Folkman, 1995; Fan *et al.*, 1995).

The angiogenic process is controlled by the balance of activity of angiogenic inducers and inhibitors. At least twenty angiogenic peptides have been discovered (Folkman & Shing, 1992; Fan & Brem, 1992). Some directly stimulate vascular endothelial cells to migrate, proliferate, form tubes, or a combination of these activities, while others act indirectly by mobilising host cells (macrophages, mast cells and occasionally lymphocytes) to release endothelial cell growth factors. The directly-acting factors include acidic and basic fibroblast growth factor (aFGF and bFGF), interleukin-8 and vascular endothelial growth factor (VEGF). Examples of the indirectly-acting factors are tumour necrosis factor (TNF-α), transforming growth factor-β and platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP). The activities of these angiogenic factors are counter-balanced by at least ten endogenous inhibitors of angiogenesis (Fan *et al.*, 1995). Furthermore, it is becoming clear, that some of the activities of vasoactive peptides such as substance P, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide and endothelins may be likened to those of cytokines or growth factors (Woll, 1991; Kramer *et al.*, 1992; Waschek, 1995). However, their role in angiogenesis has not been well characterized.

Using the rat sponge model for the quantitative assay of angiogenesis (Andrade *et al.*, 1987; Hu *et al.*, 1995), we have established roles of cytokines and growth factors (e.g. interleukin-8, TNF-α, bFGF, VEGF and PD-ECGF/TP) in angiogenesis and shown that the angiogenic responses they elicit can be neutralised by their antibodies or blocked by either selective receptor antagonists or selective signalling pathway inhibitors (Hu *et al.*, 1993; 1994; 1995; Hu & Fan, 1995a, b; Moghaddam *et al.*, 1995). Additionally, we have shown that the inflammatory polypeptides, substance P and bradykinin, might be involved in angiogenesis and use of receptor antagonists has shown that the neovascular responses to these polypeptides are mediated by the NK_1 and B_1 receptor subtypes, respectively (Fan *et al.*, 1993; Hu & Fan, 1993).

These findings raise the possibility that other vasoactive peptides may also contribute to the aberrant neovascularization often associated with chronic inflammatory diseases. However, the possibility remains that, in many models of

[1] Author for correspondence.

angiogenesis, including the sponge model that we have used, the changes in angiogenesis evoked by vasoactive peptides might be the consequence of local changes in blood flow. Therefore, in the present study, we have used the rat sponge model to examine if VIP (a vasodilator), endothelin-1 and endothelin-3 (both vasoconstrictors and vasodilators), and angiotensin II (a potent vasoconstrictor) possess angiogenic activity and, if so, whether their actions may be blocked by selective antagonists.

Methods

The sponge implant model

Sterile circular polyether sponge discs (6 mm thick, 1.2 cm diameter; R. E. Carpenter & Co., Haverhill, Suffolk) with a central cannula (1.3 cm long, 1.4 mm internal diameter; Portex Ltd, Hythe, Kent) 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.; Janssen Pharmaceuticals, Oxford). Test substances (in 50 μl volume) were injected daily into the sponges through the cannula. In order to exclude possible acute effects (dilatation or constriction) of the test substances on the sponge microvasculature, the doses were given 18–24 h prior to the ^{133}Xe measurements. The neovascular response was assessed as a function of blood flow through the implants using a ^{133}Xe clearance technique which has been validated by comparison with four other methods, namely, (i) tracer microspheres to measure absolute blood flow in the sponges; (ii) the carmine red dye method for measurement of neovasculature; (iii) colourimetric measurement of haemoglobin levels in the implants and (iv) histological and morphometric studies using a computer-assisted image analysis system (Hu *et al.*, 1995).

Histology

The animals were killed by asphyxiation in CO_2 and the sponges were then 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, Masson Trichrome or an endothelial cell marker *Bandeiraea simplicifolia* lectin I, isolectin B_4 ($BSL-B_4$ from Vector Laboratories, Peterborough, Cambridgeshire).

Drugs

VIP, VIP (10–28) and angiotensin II were from Peninsula Laboratories (Europe) Ltd (St Helens, Merseyside). Endothelin-1 and endothelin-3 were from The Peptide Institute (Osaka, Japan). Losartan and PD 123319 ([S]-1-{[4-(dimethylamino)-3-methylphenyl]methyl}-5-(diphenylacetyl)-4,5,6,7, tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid, ditrifluoroactetate, monohydrate) were gifts from Dr Ronald Smith (Du Pont Merck Pharmaceutical Co., Wilmington, DE, U.S.A.) and Dr Marc de Gasparo (Ciba-Geigy Pharmaceuticals, Basel, Switzerland), respectively. ^{133}Xe injection was from Medgenix plc (High Wycombe, Buckinghamshire) and phosphate buffered saline (PBS) from the Sigma Chemical Co. Ltd (Poole, Dorset). Solutions were prepared daily in PBS and sterilised by membrane filtration (0.22 μm) before use.

Statistics

Statistical analysis was carried out with 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%. Data are presented as the mean ± s.e.mean.

Results

Effect of VIP on sponge-induced angiogenesis

Daily doses of 1000 pmol VIP caused a marked intensification of neovascularization but a lower dose (10 pmol) produced no apparent effect. However, this lower dose of VIP interacted with a dose of interleukin-1α (0.3 pmol), previously shown to be subthreshold (Fan *et al.*, 1993), to produce an angiogenic response similar to that seen with the higher dose of VIP (Figure 1). Histological studies of sponge sections showed that both cellular infiltration and neovascularisation in the sponges treated with either VIP or co-administration of VIP and interleukin-1α were greatly increased as compared to controls (Figure 2). The neovascular response induced by co-administration of VIP and interleukin-1α was inhibited by simultaneous administration of 100 pmol VIP (10–28), a specific VIP receptor antagonist (Turner *et al.*, 1986; Figure 3). The effect of VIP (10–28) on VIP-induced angiogenesis was confirmed histologically (Figure 2).

Effect of endothelins on sponge-induced angiogenesis

Daily doses of 10, 100 or 1000 pmol endothelin-3 or of 100 or 1000 pmol endothelin-1 produced no apparent effect on the rate of neovascularization of the sponges (Figure 4). Histological studies of sponge sections showed that there were no significant differences in the degree of cellular infiltration, neovascularization or connective tissue formation between the endothelin-3 or endothelin-1-treated sponges and the control group (results not shown).

Effect of angiotensin II on sponge-induced angiogenesis

Daily doses of 1000 pmol angiotensin II caused an intense neovascularization but lower doses of angiotensin II (10 and 100 pmol) produced no apparent effects. Also, the lowest dose of angiotensin II (10 pmol) did not interact with the subthreshold dose of interleukin-1α (0.3 pmol) to produce an enhanced neovascular response (Figure 5). Histological studies of sponge sections showed that both the cellular infiltration and neovascularization in the sponges treated with 1000 pmol angiotensin II were profoundly increased relative to control sponges (Figure 2). This angiotensin II-induced neovascular

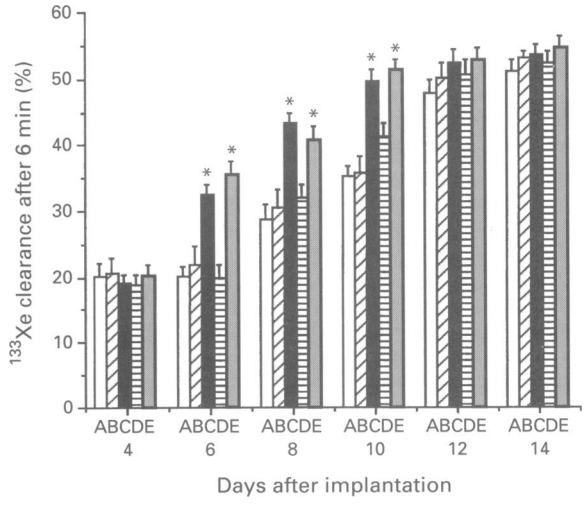

Figure 1 Effects of VIP on sponge-induced angiogenesis as shown by changes in ^{133}Xe clearance. Columns represent sponges treated daily with PBS (A), 10 pmol VIP (B), 1000 pmol VIP (C), 0.3 pmol interleukin-1α (D) or 10 pmol VIP plus 0.3 pmol interleukin-1α (E). Data are shown as the mean ± s.e.mean of 10 experiments. * $P<0.01$, VIP or VIP plus interleukin-1α versus PBS.

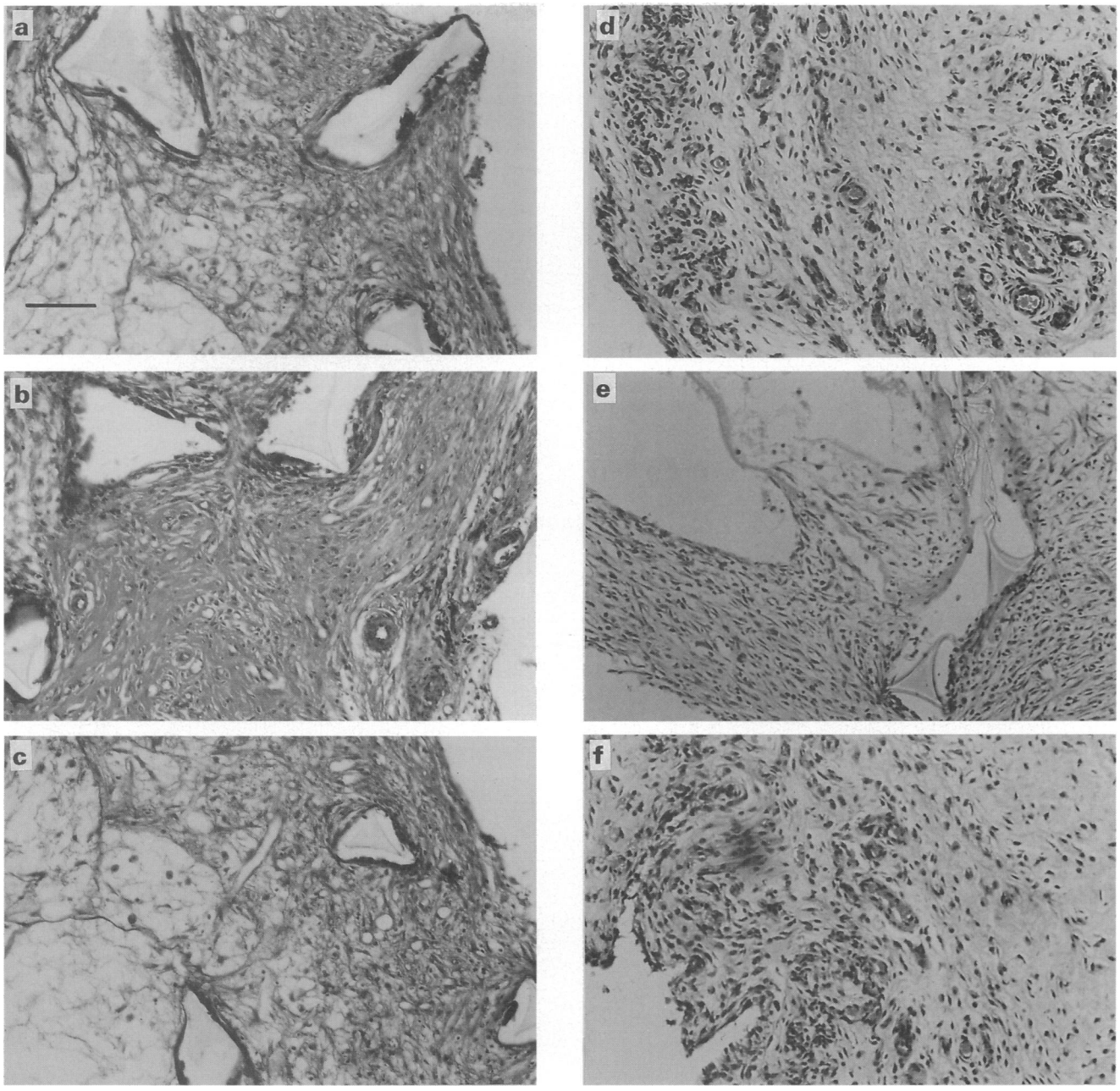

Figure 2 Histological sections of 8-day old sponges illustrating (i) sponge treated with PBS (section a, stained with Masson Trichrome); (ii) the intense neovascular response induced by 10 pmol VIP plus 0.3 pmol interleukin-1α (section b, stained with Masson Trichrome); (iii) the inhibition of VIP/interleukin-1-induced angiogenesis by VIP (10–28) (100 pmol day^{-1}), (section c, stained with Masson Trichrome); (iv) the inhibition of angiotensin II-induced angiogenesis (1000 pmol day^{-1}, section d, stained with haematoxylin and eosin) by the AT_1 receptor antagonist, losartan (100 nmol day^{-1}, section e, stained with haematoxylin and eosin), but not by the AT_2 antagonist, PD 123319 (100 nmol day^{-1}, section f, stained with haematoxylin and eosin). All sections photographed at $\times$ 200 magnification. Bar = 100 μm.

response was inhibited by co-administration of 100 nmol of the specific AT_1 receptor antagonist, losartan but not by the AT_2 antagonist, PD 123319 (Figure 6). Histological studies of sponge sections showed that cellular infiltration and neovascularization in the sponges given 1000 pmol angiotensin II were suppressed by the specific AT_1 receptor antagonist losartan (Figure 2). In the absence of exogenous peptides, the sponge-induced angiogenesis was not modified by losartan or PD 123319 (data not shown).

Discussion

Angiogenesis is a fundamental process in which new blood vessels are formed. It is presumed that four main steps are involved: degradation of basement membranes, migration of endothelial cells, proliferation of endothelial cells and formation of capillary tubes. These steps involve extensive interplay between cells, soluble factors, blood flow and extracellular matrix components (Folkman, 1995). In addition to the cytokines and polypeptide growth factors (e.g. interleukin-1, bFGF and VEGF), increasing number of neuropeptides (e.g. bombesin, substance P and VIP) have been shown to act as growth factors (Zachary *et al.*, 1987; Woll, 1991). The present study has been designed to compare the angiogenic actions of four vasoactive peptides in order to see if angiogenic activity might be correlated with their acute vascular effects. The peptides were also chosen because they have been reported to have growth stimulating activity on cells which would be consistent with them having angiogenic activity.

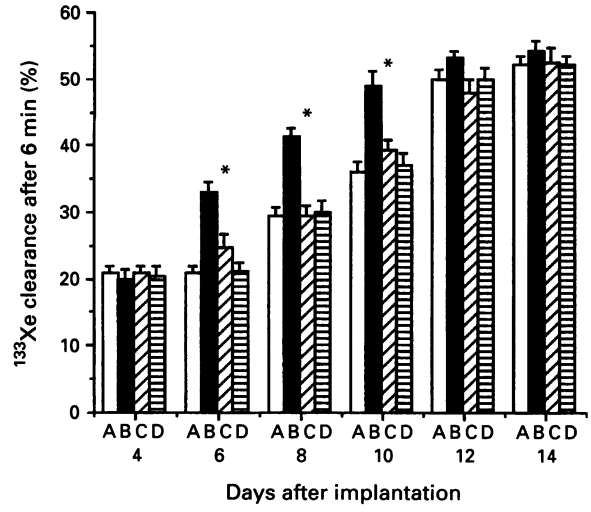

Figure 3 Effects of VIP on sponge-induced angiogenesis and its inhibition by the VIP receptor antagonist VIP (10–28) as shown by changes in ^{133}Xe clearance. Columns represent sponges treated daily with PBS (A), 10 pmol VIP plus 0.3 pmol interleukin-1α (B), 10 pmol VIP and 0.3 pmol interleukin-1α plus 100 pmol VIP (10–28) (C) or 100 pmol VIP (10–28) (D). Data are shown as the mean ± s.e.mean of 10 experiments. *$P<0.01$, VIP/interleukin-1α plus VIP (10–28) versus VIP/interleukin-1α.

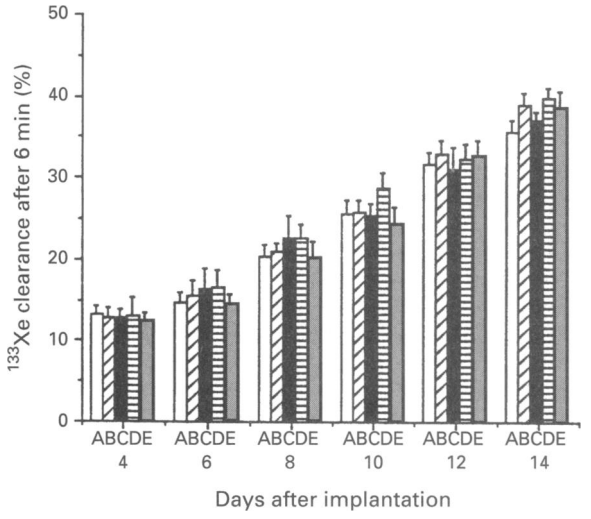

Figure 4 Effects of endothelin-3 and endothelin-1 on sponge-induced angiogenesis as shown by changes in ^{133}Xe clearance. Columns represent sponges treated daily with PBS (A), 10 pmol endothelin-3 (B), 100 pmol endothelin-3 (C), 1000 pmol endothelin-3 (D) or 1000 pmol endothelin-1 (E). Data are shown as the mean ± s.e.mean of 10 experiments.

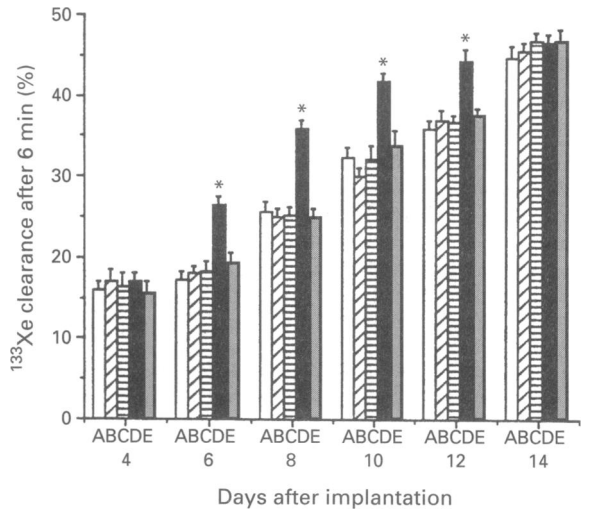

Figure 5 Effects of angiotensin II on sponge-induced angiogenesis as shown by changes in ^{133}Xe clearance. Columns represent sponges treated daily with PBS (A), 10 pmol angiotensin II (B), 100 pmol angiotensin II (C), 1000 pmol angiotensin II (D), or 10 pmol angiotensin II plus 0.3 pmol interleukin-1α (E). Data are shown as the mean ± s.e.mean of 10 experiments. *$P<0.01$, 1000 pmol angiotensin II versus PBS.

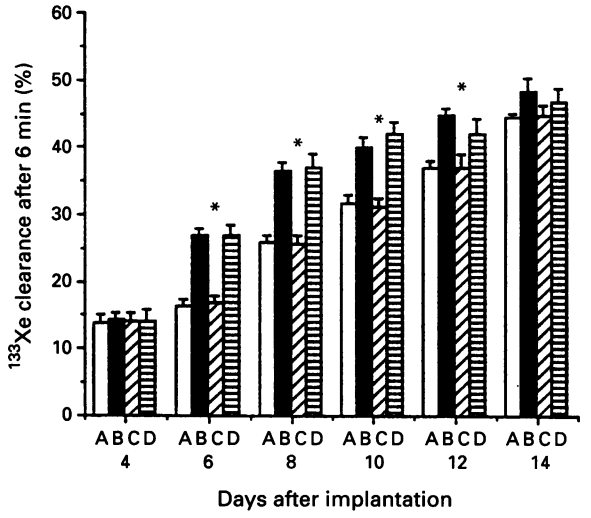

Figure 6 Effects of AT_1 and AT_2 receptor antagonists on angiotensin II-induced changes in sponge-induced angiogenesis as shown by ^{133}Xe clearance. Columns represent sponges treated daily with PBS (A), 1000 pmol angiotensin II (B), angiotensin II plus 100 nmol losartan (C), or angiotensin II plus 100 nmol PD 123319 (D). Data are shown as the mean ± s.e.mean of 6 experiments. *$P<0.01$, angiotensin II plus losartan versus angiotensin II.

Thus, one reason for choosing VIP for this study is that it is not only a potent vasodilator (Said & Mutt, 1970; Hattori *et al.*, 1992) but regulates various immune cells (O'Dorisio *et al.*, 1985) and stimulates the proliferation of many cell types *in vitro* (Bepler *et al.*, 1988; Zurier *et al.*, 1988; Haegerstrand *et al.*, 1989; Pincus *et al.*, 1990; Takahashi *et al.*, 1993). However, its effects on endothelial cells and angiogenesis have been unclear until now, and this is the first account of the angiogenic activity of VIP *in vivo*. Although daily injection of 1000 pmol VIP enhanced angiogenesis in the rat sponge model, a hundred fold lower dose of VIP was inactive on its own. This lower dose (10 pmol) did, however, interact with a subthreshold dose of interleukin-1α (0.3 pmol) to produce an angiogenic response similar to that seen with the higher dose of VIP. The neovascular response induced by combination of VIP with interleukin-1α was inhibited by co-administration of a VIP receptor antagonist, VIP (10–28) (Turner *et al.*, 1986). These data suggest that the angiogenic effect of VIP in the rat is mediated by a specific VIP receptor but determination of whether or not VIP is an endothelial mitogen awaits further investigation.

VIP has widespread distribution, including central and peripheral neurones (Said, 1984). Since it stimulates the proliferation of keratinocytes, it has been speculated that VIP, released from sensory nerve endings in the skin, may be involved in wound healing (Haegerstrand *et al.*, 1989). Another rich source of VIP is mast cells (Cutz *et al.*, 1978) which are frequently localized around blood vessels in tissues and VIP released from these cells is likely to promote wound healing. Also, angiogenesis is a hallmark of inflammation, it is especially important in both the healing process which follows an

acute inflammatory response and in chronic inflammatory diseases. Thus, VIP may lead to aberrant neovascularization in rheumatoid arthritis. In support of this hypothesis is the earlier report of elevated plasma and tissue levels of VIP in many types of tumours (Said & Faloona, 1975). Furthermore, VIP receptors are present in a human pancreatic adenocarcinoma cell line (Estival *et al.*, 1983) and localized in patients with gastrointestinal tumours (Virgolini *et al.*, 1994). Our findings suggest that, in these clinical situations, VIP might contribute to tumour angiogenesis and subsequent metastasis.

Unlike VIP, the endothelins are capable of both vasoconstriction and vasodilatation. Endothelin-1, the most potent vasoconstrictor yet described (Yanagisawa *et al.*, 1988), is also capable of reducing regional vascular resistance (Wright & Fozard, 1988) and causes endothelium-dependent relaxation (Warner *et al.*, 1989; Douglas & Hiley, 1990). Endothelin-3 is more potent than endothelin-1 as a vasodilator (Douglas & Hiley, 1990) and pressor responses in anaesthetized rats require much higher doses of endothelin-3 than of endothelin-1 (Douglas & Hiley, 1991). Therefore, in view of their mixed actions on vasculature, it was of interest to study both of these members of the endothelin family. In addition to mediating short-term changes in cellular activity, such as causing contraction of all investigated types of smooth muscle, endothelins have many long-term effects, including stimulation of mitogenesis in vascular smooth muscle cells (Komuro *et al.*, 1988) and rat brain capillary endothelial cells (Vigne *et al.*, 1990). It is noteworthy that preproendothelin-1 mRNA has been detected in several tumours, including an established endometrial adenocarcinoma cell line (Economos *et al.*, 1992) which secretes immunoreactive ET-1 into their growth medium. Since angiogenesis is essential for tumour growth, these data collectively suggest that ET-1 may be involved in angiogenesis.

To our knowledge, there have been no previous direct studies on the angiogenic effects of exogenous endothelins. It has recently been reported that recombinant human erythropoietin (rHuEPO) stimulates endothelial cell migration and proliferation and this might be mediated by endothelin-1 since rHuEPO can increase endothelin-1 release by the endothelial cell (Carlini *et al.*, 1993). Furthermore, stimulation of angiogenesis by rHuEPO was blunted by an antibody to endothelin-1 (Carlini *et al.*, 1995). Thus, it seems that rHuEPO stimulates angiogenesis *in vitro,* and that this is due at least in part to enhanced autocrine release of endothelin-1 by rHuEPO. In an *in vitro* vascular repair model, it has been shown that endothelin-3, but not endothelin-1 accelerated proliferation in wounded monolayers of human umbilical vein endothelial cells (Wren *et al.*, 1993). Furthermore, Dashwood *et al.* (1994) found [^{125}I]-endothelin-1 binding sites in regions of neovascularization in atherosclerotic human coronary artery (although these did not appear to be ET_A receptors like those in the tunica media). In contrast, the present study found no enhancement of angiogenesis, over the dose-ranges used, as a result of treatment of the sponges with either endothelin-1 or endothelin-3. It is possible that the absence of an effect on angiogenesis was due to insufficiently high doses being used even though the same maximal dose (1000 pmol) was used for both endothelins and angiotensin II. However, Weber *et al.* (1994b) reported that, although angiotensin II treatment of rat aortic smooth muscle resulted in a greater maximal mitogenic effect (4 to 7 fold) than that observed for endothelin-1 (3 fold), the response to endothelin-1 was maximal at a 10 fold lower concentration than that at which angiotensin II reached its maximal response. Thus it seems unlikely that the absence of response with the endothelins is simply due to insufficient doses being used.

Although there have been several reports that endothelin-1 is mitogenic for vascular smooth muscle cells (Komuro *et al.*, 1988; Hirata *et al.*, 1989; Yu *et al.*, 1991), this effect may be dependent on cell phenotype (Serradeil-Le Gal *et al.*, 1991). In itself, mitogenic action on vascular cells does not necessarily imply a role for a substance in angiogenesis and Hahn *et al.* (1993) have argued that the main role of endothelin-1 is in differentiation of vessel constituents rather than in mitogenesis. In contrast, they proposed that the mitogenic effects of angiotensin II, a potent vasoconstrictor, involve modulation of autocrine activity of vascular smooth muscle cells, modulation of the extracellular matrix synthesis, and induction of DNA synthesis in endothelial cells and medial cell layers. Angiotensin II induces proliferation of fibroblasts, smooth muscle cells and endothelial cells *in vitro* (Sadoshima & Izumo, 1993; Weber *et al.*, 1994a; Stoll *et al.*, 1995) and it appears to have angiogenic effects in the rabbit cornea (Fernandez, *et al.*, 1985) and the chorioallantoic membrane of the chick embryo (Le Noble *et al.*, 1991). It has also been reported that angiotensin II is important in the development of collateral vessels to kidney after aortic stenosis (Fernandez *et al.*, 1982). In the present study, we found that daily injection of 1000 pmol angiotensin II caused intense neovascularisation although a 10 fold lower dose had no effect.

Angiotensin II acts via specific cell surface receptors and two major subtypes have been identified: AT_1 and AT_2. Losartan is a selective AT_1 receptor antagonist, whereas PD 123319 selectively blocks the AT_2 receptor (Timmermans *et al.*, 1993). Here we show that angiotensin II-induced angiogenesis was inhibited by co-administration of losartan with angiotensin II, but not by PD 123319. Thus, the angiogenic response in the sponge is mediated by the same receptors, AT_1, that predominate in adult rat skin while, in foetal rat skin, 97% of the receptors are of the AT_2 subtype (Kimura *et al.*, 1992). A role for AT_1 receptors in promoting cellular proliferation is further supported by a recent account of the growth-promoting effect of angiotensin II on rat coronary endothelial cells (Stoll *et al.*, 1995). In contrast, angiotensin II-induced angiogenesis in the chick embryo chorioallantoic membrane cannot be inhibited by either AT_1 or AT_2 antagonists (Le Noble *et al.*, 1993). Taken together, these results indicate different specificities of angiotensin II receptors between fowl and mammals.

In order to extend the study, it was decided to investigate the interactions of the two active angiogenic agents, VIP and angiotensin II, with interleukin-1α. The latter is an inflammatory mediator with a wide spectrum of activities including activation of fibroblasts to proliferate and to secrete prostaglandins, collagenase, and plasminogen activator (Dinarello, 1991). There is a potentially important synergistic interaction between interleukin-1α and neuropeptides in inflammation (Arai *et al.*, 1990; Buckley *et al.*, 1991) and we have previously shown that daily administration of 3 pmol interleukin-1α caused intense neovascularization. A lower dose of interleukin-1α (0.3 pmol) was ineffective when given alone but, when combined with a subthreshold dose of either substance P (10 pmol) or bradykinin (10 pmol), it produced an angiogenic response similar to that seen with the higher dose of interleukin-1α. Furthermore, angiogenesis induced by combined administration of interleukin-1α with substance P or interleukin-1α with bradykinin can be blocked by either NK_1, B_1 or interleukin-1 receptor antagonists, as appropriate (Fan *et al.*, 1993; Hu & Fan, 1993).

In the present study, we found that the lowest dose of VIP used, but not the lowest dose of angiotensin II (both 10 pmol), when given with a subthreshold dose of interleukin-1α (0.3 pmol) interacted to produce an enhanced neovascular response. These results suggest that there are differences between vasoactive peptides in their capacity to interact with interleukin-1α in angiogenesis. This could be explained by proposing that the synergistic interaction between agonists occurs when they activate different signalling pathways. The effects of VIP are mediated by elevation of cyclic AMP without protein kinase C activation or Ca^{2+} mobilization (Zurier *et al.*, 1988). Thus, its positive interaction with interleukin-1α could be due to VIP activating adenylyl cyclase whilst interleukin-1α activates protein kinase C and induces mitogen activated protein kinase in its target cells (Muegge & Durum, 1990; Saklatvala *et al.*, 1993). In contrast, because angiotensin II also activates protein kinase C and induces Ca^{2+} mobilization (Tsuda *et al.*, 1991), it may be less likely to interact with interleukin-1α.

In summary, we have shown that vasoactive peptides, such as VIP and angiotensin II can accelerate angiogenesis and this is not simply related to their acute vasoactivity, *i.e.* constriction or dilatation. However, the results with endothelin-1 and endothelin-3 indicate that not all vasoactive peptides accelerate angiogenesis in the sponge model. Like substance P (Fan *et al.*, 1993) and bradykinin (Hu & Fan, 1993), the angiogenic responses induced by VIP and angiotensin II were blocked at the receptor level. Taken together, these data clearly demonstrate that receptor modulation of angiogenic factors could provide a strategy for the management of angiogenic diseases.

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References

ANDRADE, S.P., FAN, T.-P.D. & LEWIS, G.P. (1987). Quantitative in-vivo studies on angiogenesis in a rat sponge model. *Br. J. Exp. Pathol.*, **68,** 755–766.

ARAI, K., LEE, F., MIYAJIMA, A., MIYATAKE, S., ARAI, N. & YOKOTA, T. (1990). Cytokines: coordinators of immune and inflammatory responses. *Ann. Rev. Biochem.*, **59,** 783–836.

BEPLER, G., ROTSCH, M., JAQUES, G., HAEDER, M., HEYMANNS, J., HARTOGH, G., KIEFER, P. & HAVEMANN, K. (1988). Peptides and growth factors in small cell lung cancer: production, binding sites and growth effects. *J. Cancer Res. Clin. Oncol.*, **114,** 235–244.

BUCKLEY, T.L., BRAIN, S.D., COLLINS, P.D. & WILLIAMS, T.J. (1991). Inflammatory edema induced by interactions between IL-1 and the neuropeptide calcitonin gene-related peptide. *J. Immunol.*, **146,** 3424–3430.

CARLINI, R.G., DUSSO, A.S., OBIALO, C.I., ALVAREZ, U.M. & ROTHSTEIN, M. (1993). Recombinant human erythropoietin (rHuEPO) increases endothelin-1 release by endothelial cells. *Kidney Int.*, **43,** 1010–1014.

CARLINI, R.G., REYES, A.A. & ROTHSTEIN, M. (1995). Recombinant human erythropoietin stimulates angiogenesis in vitro. *Kidney Int.*, **47,** 740–745.

CUTZ, E., CHAN, W., TRACK, N.S., GOTH, A. & SAID, S.I. (1978). Release of vasoactive intestinal polypeptide in mast cells by histamine liberators. *Nature*, **275,** 661–662.

DASHWOOD, M.R., ALLEN, S.P., LUU, T.N. & MUDDLE, J.R. (1994). The effect of the ET_A receptor antagonist, FR 139317, on [^{125}I]-ET-1 binding to the atherosclerotic human coronary artery. *Br. J. Pharmacol.*, **112,** 386–389.

DINARELLO, C.A. (1991). Interleukin-1 and interleukin-1 antagonism. *Blood*, **77,** 1627–1652.

DOUGLAS, S.A. & HILEY, C.R. (1990). Endothelium-dependent vascular activities of endothelin-like peptides in the isolated superior mesenteric arterial bed of the rat. *Br. J. Pharmacol.*, **101,** 81–88.

DOUGLAS, S.A. & HILEY, C.R. (1991). Endothelium-dependent mesenteric vasorelaxant effects and systemic actions of endothelin (16–21) and other endothelin-related peptide in the rat. *Br. J. Pharmacol.*, **104,** 311–320.

ECONOMOS, K., MACDONALD, P.C. & CASEY, M.L. (1992). Endothelin-1 gene expression and biosynthesis in human endometrial HEC-1A cancer cells. *Cancer Res.*, **52,** 554–557.

ESTIVAL, A., MOUNIELOU, P. & TROCHERIS, V. (1983). Presence of VIP receptors in a human pancreatic adenocarcinoma cell line. Modulation of the cAMP response during cell proliferation. *Biochem. Biophys. Res. Commun.*, **111,** 958–963.

FAN, T.-P.D. & BREM, S. (1992). Angiosuppression. In *The Search for New Anti-Cancer Drugs*. Cancer Biology & Medicine Series Vol. 3 ed. Waring, M.J. & Ponder, B.A.J., pp. 185–229. Dordrecht/Boston/London: Kluwer Academic Publishers.

FAN, T.-P.D., HU, D.-E., GUARD, S., GRESHAM, G.A. & WATLING, K.J. (1993). Stimulation of angiogenesis by substance P and interleukin-1 in the rat and its inhibition by NK_1 or interleukin-1 receptor antagonists. *Br. J. Pharmacol.*, **110,** 43–49.

FAN, T.-P.D., JAGGAR, R. & BICKNELL, R. (1995). Controlling the vasculature: angiogenesis, anti-angiogenesis and vascular targeting of gene therapy. *Trends Pharmacol. Sci.*, **16,** 57–66.

FERNANDEZ, L.A., CARIDE, V.J., TWICKLER, J. & GALARDY, R.E. (1982). Renin-angiotensin and development of collateral circulation after renal ischemia. *Am. J. Physiol.*, **243,** H869–H875.

FERNANDEZ, L.A., TWICKLER, J. & MEAD, A. (1985). Neovascularization produced by angiotensin II. *J. Lab. Clin. Med.*, **105,** 141–145.

FOLKMAN, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Med.*, **1,** 27–31.

FOLKMAN, J. & SHING, Y. (1992). Angiogenesis. *J. Biol. Chem.*, **267,** 10931–10934.

HAEGERSTRAND, A., JONZON, B., DALSGAARD, C.J. & NILSSON, J. (1989). Vasoactive intestinal polypeptide stimulates cell proliferation and adenylate cyclase activity of cultured human keratinocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 5993–5996.

HAHN, A.W., RESINK, T.J., KERN, F. & BÜHLER, F.R. (1993). Peptide vasoconstrictors, vessel structure, and vascular smooth muscle proliferation. *J. Cardiovasc. Pharmacol.*, **22** (Suppl. 5), S37–S43.

HATTORI, Y., NAGASHIMA, M., ENDO, Y. & KANNO, M. (1992). Glibenclamide does not block arterial relaxation caused by vasoactive intestinal polypeptide. *Eur. J. Pharmacol.*, **213,** 147–150.

HIRATA, Y., TAKAGI, Y., FUKUDA, Y. & MARUMO, F. (1989). Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Atherosclerosis*, **78,** 225–228.

HU, D.-E. & FAN, T.-P.D. (1993). [Leu^8]des-Arg^9-bradykinin inhibits the synergistic interaction between bradykinin and interleukin-1 in angiogenesis. *Br. J. Pharmacol.*, **109,** 14–17.

HU, D.-E. & FAN, T.-P.D. (1995a). Suppression of VEGF-induced angiogenesis by the protein tyrosine kinase inhibitor lavendustin A. *Br. J. Pharmacol.*, **114,** 262–268.

HU, D.-E. & FAN, T.-P.D. (1995b). The protein kinase C inhibitor calphostin C prevents cytokine-induced angiogenesis in the rat. *Inflammation*, **19,** 39–54.

HU, D.-E., HILEY, C.R., SMITHER, R.L., GRESHAM, G.A. & FAN, T.-P.D. (1995). Correlation of ^{133}Xe clearance, blood flow and histology in the rat sponge model for angiogenesis: further studies with angiogenic modifiers. *Lab. Invest.*, **72,** 601–610.

HU, D.-E., HORI, Y. & FAN, T.-P.D. (1993). Interleukin 8 stimulates angiogenesis in rats. *Inflammation*, **17,** 135–143.

HU, D.-E., HORI, Y., PRESTA, M., GRESHAM, G.A. & FAN, T.-P.D. (1994). Inhibition of angiogenesis by IL-1 receptor antagonist and selected cytokine antibodies. *Inflammation*, **18,** 45–58.

KIMURA, B., SUMNERS, C. & PHILLIPS, M.I. (1992). Changes in skin angiotensin II receptors in rats during wound healing. *Biochem. Biophys. Res. Commun.*, **187,** 1083–1090.

KOMURO, I., KURIHARA, H., SUGIYAMA, T., TAKAKU, F. & YAZAKI, Y. (1988). Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. *FEBS Lett.*, **238,** 249–252.

KRAMER, B.K. NISHIDA, M., KELLY, R.A. & SMITH, T.W. (1992). Endothelins: myocardial actions of a new class of cytokines. *Circulation*, **85,** 350–356.

LE NOBLE, F.A.C., HEKKING, J.W.M., VAN STRAATEN, H.W.M., SLAAF, D.W. & STRUIJKER BOUDIER, H.A.J. (1991). Angiotensin II stimulates angiogenesis in the chorioallantoic membrane of the chick embryo. *Eur. J. Pharmacol.*, **195,** 305–306.

LE NOBLE, F.A.C., SCHREURS, N.H.J.S., VAN STRAATEN, H.W.M., SLAFF, D.W., SMITS, J.F.M., ROGG, H. & STRUIJKER BOUDIER, H.A.J. (1993). Evidence for a novel angiotensin II receptor involved in angiogenesis in chick embryo chorioallantoic membrane. *Am. J. Physiol.*, **264,** R460–R465.

MOGHADDAM, A., ZHANG, H.T., FAN, T.-P.D., HU, D.-E., LEES, V.C., TURLEY, H., FOX, S.B., GATTER, K.V., HARRIS, A.L. & BICKNELL, R. (1995). Thymidine phosphorylase is angiogenic and promotes tumor growth. *Proc. Natl. Acad. Sci. U.S.A.*, **92,** 998–1002.

MUEGGE, K. & DURUM, S.K. (1990). Cytokines and transcription factors. *Cytokine*, **2,** 1–8.

O'DORISIO, M.S., WOOD, C.L. & O'DORISIO, T.M. (1985). Vasoactive intestinal peptide and neuropeptide modulation of the immune response. *J. Immunol.*, **135,** (Suppl.), 792S–796S.

PINCUS, D.W., DICICCO-BLOOM, E.M. & BLACK, I.B. (1990). Vasoactive intestinal peptide regulates mitosis, differentiation and survival of cultured sympathetic neuroblasts. *Nature*, **343**, 564–567.

SADOSHIMA, J.I. & IZUMO, S. (1993). Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts; critical role of the AT_1 receptor subtype. *Circ. Res.*, **73**, 413–423.

SAID, S.I. (1984). Vasoactive intestinal polypeptide (VIP): current status. *Peptides*, **5**, 143–150.

SAID, S.I. & FALOONA, G.R. (1975). Elevated plasma and tissue levels of vasoactive intestinal polypeptide in the watery-diarrhea syndrome due to pancreatic, bronchogenic and other tumors. *N. Engl. J. Med.*, **293**, 155–160.

SAID, S.I. & MUTT, V. (1970). Potent peripheral and splanchnic vasodilator from normal gut. *Nature*, **225**, 863–864.

SAKLATVALA, J., RAWLINSON, L.M., MARSHALL, C.J. & KRACHT, M. (1993). Interleukin-1 and tumour necrosis factor activate the mitogen activated protein (MAP) kinase kinase in cultured cells. *FEBS Lett.*, **334**, 189–192.

SERRADEIL-LE GAL, C., HERBERT, J.M., GARCIA, C., BOUTIN, M. & MAFFRAND, J.P. (1991). Importance of the phenotypic state of vascular smooth muscle cells on the binding and the mitogenic activity of endothelin. *Peptides*, **12**, 575–579.

STOLL, M., STECKELINGS, U.M., PAUL, M., BOTTARI, S.P., METZGER, R. & UNGER, T. (1995). The angiotensin AT_2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J. Clin. Invest.*, **95**, 651–657.

TAKAHASHI, K., NAKANISHI, S. & IMAMURA, S. (1993). Direct effects of cutaneous neuropeptides on adenylyl cyclase activity and proliferation in a keratinocyte cell line: Stimulation of cyclic AMP formation by CGRP and VIP/PHM, and inhibition by NPY through G protein-coupled receptors. *J. Invest. Dermatol.*, **101**, 646–651.

TIMMERMANS, P.B.M.W.M., WONG, P.C., CHIU, A.T., HERBLIN, W.F., BENFIELD, P., CARINI, D.J., LEE, R.J., WEXLER, R.R., SAYE, J.A.M. & SMITH, R.D. (1993). Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol. Rev.*, **45**, 205–251.

TSUDA, T., KAWAHARA, Y., SHII, K., KOIDE, M., ISHIDA, Y. & YOKOYAMA, M. (1991). Vasoconstrictor-induced protein-tyrosine phosphorylation in cultured vascular smooth muscle cells. *FEBS Lett.*, **285**, 44–48.

TURNER, J.T., JONES, S.B. & BYLUND, D.B. (1986). A fragment of vasoactive intestinal peptide, VIP (10–28), is an antagonist of VIP in the colon carcinoma cell line, HT29. *Peptides*, **7**, 849–854.

VIGNE, P., MARSAULT, R., BREITTMAYER, J.P. & FRELIN, C. (1990). Endothelin stimulates phosphatidylinositol hydrolysis and DNA synthesis in brain capillary endothelial cells. *Biochem. J.*, **266**, 415–420.

VIRGOLINI, I., RADERER, M., KURTARAN, A., ANGELBERGER, P., BANYAI, S., YANG, Q., LI, S., BAYYAI, M., PIDLICH, J., NIEDERLE, B., SCHEITHAUER, W. & VALENT, P. (1994). Vasoactive intestinal peptide-receptor imaging for the localization of intestinal adenocarcinomas and endocrine tumors. *N. Engl. J. Med.*, **331**, 1116–1121.

WARNER, T.D., DE NUCCI, G. & VANE, J.R. (1989). Rat endothelin is a vasodilator in the isolated perfused mesentery of the rat. *Eur. J. Pharmacol.*, **159**, 325–326.

WASCHEK, J.A. (1995). Vasoactive intestinal peptide: an important trophic factor and developmental regulator? *Dev. Neurosci.*, **17**, 1–7.

WEBER, H., TAYLOR, D.S. & MOLLOY, C.J. (1994a). Angiotensin II induces delayed mitogenesis and cellular proliferation in rat aortic smooth muscle cells: correlation with the expression of specific endogenous growth factors and reversal by suramin. *J. Clin. Invest.*, **93**, 788–798.

WEBER, H., WEBB, M.L., SERAFINO, R., TAYLOR, D.S., MORELAND, S., NORMAN, J. & MOLLOY, C.J. (1994b). Endothelin-1 and angiotensin II stimulate delayed mitogenesis in cultured rat aortic smooth muscle cells: evidence for common signaling mechanisms. *Mol. Endocrinol.*, **8**, 148–158.

WOLL, P.J. (1991). Neuropeptide growth factors and cancer. *Br. J. Cancer*, **63**, 469–475.

WREN, A.D., HILEY, C.R. & FAN, T.-P.D. (1993). Endothelin-3 mediated proliferation in wounded human umbilical vein endothelial cells. *Biochem. Biophys. Res. Commun.*, **196**, 369–375.

WRIGHT, C.E. & FOZARD, J.R. (1988). Regional vasodilation is a prominent feature of the haemodynamic response to endothelin in anaesthetized, spontaneously hypertensive rats. *Eur. J. Pharmacol.*, **155**, 201–203.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

YU, S., YU, C., WONG, H. & YUAN, H. (1991). Effect of endothelin-1 on the vascular smooth muscle cell cycle. *J. Cardiovasc. Pharmacol.*, **17**, (Suppl. 7), S239–S241.

ZACHARY, I., WOLL, P.J. & ROZENGURT, E. (1987). A role for neuropeptides in the control of cell proliferation. *Dev. Biol.*, **124**, 295–308.

ZURIER, R.B., KOZMA, M., SINNETT-SMITH, J. & ROZENGURT, E. (1988). Vasoactive intestinal peptide synergistically stimulates DNA synthesis in mouse 3T3 cells: role of cAMP, Ca^{2+} and protein kinase C. *Exp. Cell Res.*, **176**, 155–161.

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