[Leu⁸]des-Arg⁹-bradykinin inhibits the angiogenic effect of bradykinin and interleukin-1 in rats

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1 Subcutaneous implantation of sterile polyether sponges in rats elicited a reproducible neovascular response over 14 days, as determined by measurements of relative sponge blood flow by a ¹³³Xe clearance technique. The angiogenic response was verified by quantitation of haemoglobin contents and histological evaluation of vascularized sponges.

2 Daily administration of 1 nmol of bradykinin (BK) into the implants significantly enhanced the basal sponge-induced neovascularization, leading to higher ¹³³Xe clearance values, increased haemoglobin contents, cellularity and vascularity.

3 When given alone, lower doses of BK (10 pmol) or recombinant human interleukin-1 α (IL-1 α , 0.3 pmol) produced no apparent effects on the basal sponge-induced angiogenesis. However, co-administration of these two peptides produced an angiogenic response similar to that elicited by 1 nmol of BK.

4 The BK/IL-1 α -induced neovascularization was abolished by the bradykinin B₁ receptor antagonist, [Leu⁸]des-Arg⁹-BK (1 nmol day⁻¹), but not by the B₂ receptor antagonist Ac-D-Arg-[Hyp³,D-Phe⁷,Leu⁸]-BK (1 nmol day⁻¹).

5 Thus, if such interaction between BK and IL-1 α contributes to the excessive neovascularization in chronic inflammatory diseases, the blockade of B₁ receptors may provide an effective treatment.

Keywords: Angiogenesis; bradykinin; interleukin-1; angiosuppression; B₁ and B₂ receptor antagonists

Introduction

Angiogenesis is an important physiological process closely involved with normal embryonic development, endometrial regeneration and wound healing. Angiogenic diseases such as rheumatoid arthritis, diabetic retinopathy and cancer are thought to be due to the imbalance between stimulators and inhibitors of angiogenesis (Folkman & Klagsbrun, 1987). One potentially attractive approach to achieve angiosuppression is to block the activity of angiogenic factors at the receptor level. However, little work in this area has been undertaken.

Bradykinin (BK) is an endogenous peptide which causes vasodilatation, oedema and pain. It is detected in large quantities in rheumatoid synovial fluid (reviewed in Regoli & Barabe, 1980; Proud & Kaplan, 1988). More recent *in vitro* studies showed that BK also interacts with the inflammatory cytokine interleukin-1 (IL-1) to stimulate proliferation of BALB/3T3 fibroblasts (Kimball & Fisher, 1988) and prostaglandin E₂ (PGE₂) release by human synovial cells (O'Neill & Lewis, 1989) and gingival fibroblasts (Lerner & Modeer, 1991). These observations have implicated BK in the pathogenesis of chronic inflammatory diseases.

The aim of the present study was to investigate whether BK, alone or in combination with IL-1, would stimulate angiogenesis *in vivo*. Furthermore, we attempted to characterize the BK receptors associated with such a response using B₁ and B₂ receptor antagonists.

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 flunitrazepam; 0.1 ml kg⁻¹, i.m.). Test substances (50 μ l) were injected daily into the sponges through the cannulae and the neovascular response was assessed as a function of blood flow through the implants by use of a ¹³³Xe clearance technique described by Andrade *et al.* (1987).

Two additional approaches were taken to verify the angiogenic response. First, the sponges were homogenized in phosphate buffered saline (PBS) and their haemoglobin contents measured colorimetrically with a commercial kit. Second, sponge sections (10 μ m) were stained with haematoxylin and eosin (H&E) or an endothelial cell marker *Bandeirea simplicifolia* lectin I, isolectin B₄ (BSL-B₄) for the assessment of cellularity and vascularity, respectively. The specimens were analysed 'blind' and recorded on Ektachrome ASA 64T film.

Materials

Recombinant human IL-1 α was kindly supplied to Dr Jerry Saklatvala of Strangeways Research Laboratory, Cambridge. Other materials were purchased from the following companies: BK, [Leu⁸]des-Arg⁹-BK (Peninsula Laboratories, UK); Ac-D-Arg-[Hyp³,D-Phe⁷,Leu⁸]-BK (Novobiochem Co., UK); ¹³³Xenon injection (10 mCi in 3 ml saline, Amersham International, UK); polyether foam sheet (R.E. Carpenter & Co., Suffolk, UK); polythene tubings (Portex Ltd., UK); Hypnorm (Janssen Pharmaceuticals, UK); *Bandeirea simplicifolia* lectin I, isolectin B₄ (BSL-B₄, Vector Laboratories Ltd., Peterborough, UK); phosphate buffered saline (PBS) and haemoglobin kit (Sigma Chemical Co. Ltd., UK). All solutions were prepared daily in PBS and sterilized by membrane filtration (0.22 μ m) before use.

Statistical analysis

Statistical analysis was performed by two-tailed Student's *t* test for unpaired observations.

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Figure 1 Effect of bradykinin (BK) on sponge-induced angiogenesis. Symbols represent sponges treated daily with phosphate buffered saline (□), 10 pmol BK (▨), 100 pmol BK (■) and 1000 pmol BK (▩). Data represent the mean $\pm$ s.e. $n = 4-10$. ** $P < 0.01$ (1000 pmol BK vs PBS).

Results

Basal sponge-induced angiogenesis

Subcutaneous implantation of sterile polyether sponges in rats elicited a reproducible neovascular response over 14 days, as demonstrated by (i) a gradual increase in the ability of the vascularized sponges to clear ^{133}Xe -saline (Figure 1) and (ii) a corresponding elevation of haemoglobin contents in sponges recovered at various intervals (Figure 2).

Enhanced angiogenic response induced by bradykinin and interleukin-1

Figure 1 also shows daily administration of BK into the implants caused a dose-dependent enhancement of ^{133}Xe clearance, suggesting that 1 nmol doses BK augmented the basal sponge-induced neovascular response. This enhanced angiogenic response was verified histologically. Figure 3a shows the increased cellularity in the test sponges as com-

Figure 2 Effect of bradykinin (BK) and interleukin-1 α (IL-1 α) on the haemoglobin contents in sponge implants. Symbols represent sponges treated daily with phosphate buffered saline (□) or a combination of BK and IL-1 α (■). Data represent the mean $\pm$ s.e. $n = 4-10$. * $P < 0.05$.

pared to controls (Figure 3b). Furthermore, the BK-treated sponges showed a distinct increase in vascularity as identified by the endothelial cell marker *Bandeirea simplicifolia* lection I, isolectin B₄ (Figure 3d and 3e).

Lower doses of BK (10 pmol or 100 pmol) did not influence the basal sponge-induced neovascular response (Figure 1). However, subthreshold doses of BK (10 pmol) interacted with subthreshold doses of IL-1 α (0.3 pmol) to produce an enhancement of angiogenesis (Figure 4) which was similar in magnitude to that elicited by 1 nmol BK (Figure 1). Consistently higher levels of haemoglobin were detected in sponges treated with 10 pmol BK plus 0.3 pmol of IL-1 α (Figure 2). The angiogenic effect of BK/IL-1 α was confirmed histologically (Figure 3c and 3f).

Blockade of bradykinin-induced angiogenesis by B₁ receptor antagonist

Since kinins act through at least two different receptors (B₁ and B₂), we attempted to characterize the receptor subtype associated with the angiogenic activity of BK. Figure 5a shows that the effect of BK was abolished by the B₁ receptor antagonist [Leu⁸]des-Arg⁹-BK (1 nmol), but not by the same doses of B₂ receptor antagonist Ac-D-Arg-[Hyp³,D-Phe⁷,Leu⁸]-BK (Figure 5b).

Discussion

The two most significant findings of this paper are that subthreshold doses of BK and IL-1 α can interact to produce an intense neovascular response in rats, and that this angiogenic response can be blocked by the B₁ receptor antagonist [Leu⁸]des-Arg⁹-BK. These results not only provide further evidence for an important role of BK in the pathogenesis of chronic inflammatory diseases, but also suggest the possible involvement of B₁ receptors in angiogenesis.

Angiogenesis is a multi-step process that involves degradation of basement membrane by metalloproteases, migration and proliferation of endothelial cells and capillary tube formation (Folkman & Klagsbrun, 1987). BK and IL-1 can elicit new blood vessel formation by multiple pathways. IL-1 may have a direct action since it is potent inducer of collagenase (Dinarello, 1991) and a mitogen for microvascular endothelial cells (Detmar *et al.*, 1992). In contrast, it is not known if BK has a direct action in angiogenesis. The effect of BK may result in part from the generation of active metabolites. For example, cleavage of the C-terminal arginine residue leads to the formation of the biologically active metabolite des-Arg⁹-BK, which is a B₁ agonist. Although the physiological role of B₁ receptors is not well established, they appear to be related to the regulation of tissue repair and cell growth. Indeed, B₁ receptors are synthesized *de novo* following tissue injury (Regoli & Barabé, 1980) and the responsiveness of these receptors is up-regulated by IL-1 (Deblois *et al.*, 1988). Furthermore, des-Arg⁹-BK has been shown to stimulate DNA synthesis by cultured lung fibroblasts. This action of des-Arg⁹-BK is inhibited by the B₁ antagonist [Leu⁸]des-Arg⁹-BK (Goldstein & Wall, 1984). It is also possible that BK causes the release of substance P from capsaicin-sensitive sensory nerves (Geppetti *et al.*, 1991). Substance P has already been shown to stimulate endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (Ziche *et al.*, 1990).

BK has been shown to stimulate phosphatidylinositol turnover, calcium mobilization, arachidonate release, and production of prostaglandins, platelet-activating factor and endothelium-derived relaxing factor in endothelial cells, via the activation of B₂ receptors (see Proud & Kaplan, 1988). Lerner & Modeer (1991) demonstrated that both B₁ (des Arg⁹-BK) and B₂ (BK, Lys-BK) receptor agonists synergize with IL-1 β to stimulate PGE₂ production by human gingival fibroblasts. Our data suggest that B₁ receptors play a prominent role in angiogenesis.

Figure 3 Histological sections of 10-day old sponges illustrating the intense neovascular response induced by bradykinin (BK, 1 nmol day⁻¹) or bradykinin/interleukin-1 α (BK/IL-1 α , 10 pmol/0.3 pmol day⁻¹). Bar = 100 μ m. (a) Sponge treated with BK (1 nmol day⁻¹; haematoxylin and eosin (H&E) stained). (b) Sponge treated with phosphate buffered saline (PBS, 50 μ l; H&E stained). (c) Sponge treated with BK/IL-1 α (10 pmol/0.3 pmol day⁻¹; H&E stained). (d) Sponge treated with BK (1 nmol day⁻¹; BSL-B₄-stained, see Methods). (e) Sponge treated with PBS (50 μ l; BSL-B₄-stained). (f) Sponge treated with BK/IL-1 α (10 pmol/0.3 pmol day⁻¹; BSL-B₄-stained).

It might be argued that vascular reactivity to BK might interfere with the assessment of angiogenesis by ¹³³Xe measurement. However, the new vessels will have to be developed before being influenced by BK. To exclude their possible acute effects on the microvasculature (dilatation or constriction), the test substances were always injected via the cannulae into the sponges 24 h before ¹³³Xe measurements. As we have previously reported, laser Doppler flowmetry studies showed that BK (1 nmol) produced only a transient

Figure 4 Effect of bradykinin (BK) and interleukin-1 α (IL-1 α) on sponge-induced angiogenesis. Symbols represent sponges treated daily with phosphate buffered saline (PBS) (□), 10 pmol BK (○), 0.3 pmol IL-1 α (■) and 10 pmol BK plus 0.3 pmol IL-1 α (●). Data represent the mean $\pm$ s.e. $n = 4-10$. ** $P < 0.01$ (BK plus IL-1 α vs PBS).

Figure 5 (a) Effect of [Leu⁸]des-Arg⁹-BK on angiogenesis induced by bradykinin (BK) and interleukin-1α (IL-1α) (10 pmol and 0.3 pmol). Symbols represent sponges treated daily with BK and IL-1α plus phosphate buffered saline (PBS) (●) or BK and IL-1α plus 100 pmol (■) or 1000 pmol (□) of the antagonist. Data represent the mean ± s.e. $n = 4-10$. ** $P < 0.01$ (BK and IL-1α plus 1000 pmol vs BK and IL-1α plus PBS). (b) Effect of Ac-D-Arg-[Hyp³,D-Phe⁷,Leu⁸]-BK on angiogenesis induced by BK and IL-1α. Symbols represent sponges treated daily with PBS (□), BK and IL-1α plus PBS (●), BK-IL-1α plus 1000 pmol (Δ) or 100 pmol (▲) of the antagonist. Data represent the mean ± s.e. $n = 4-10$. The effect of the B₂ antagonist + BK + IL-1α was not statistically different from PBS + BK + IL-1α. This was not due to any agonist effect of the B₂ antagonist since the results with the antagonist were not significantly different from the PBS control.

(<10 min) increase in rat cutaneous blood flow (Fan *et al.*, 1992). On the other hand, intradermal injection of 10 pmol BK, 0.3 pmol IL-1α or co-injection of these two peptides failed to show any local blood changes. Parallel studies with the ¹³³Xe clearance technique to follow the effects of BK and other peptides at 6 h, 12 h and 24 h after injection also failed to show any protracted vascular responses (unpublished data). Furthermore, these peptides did not show any vasoactivity in 14-day old sponges.

In conclusion, we have shown that [Leu⁸]des-Arg⁹-BK can

inhibit the angiogenic response elicited by a combination of subthreshold doses of BK and IL-1. These data illustrate the potentially attractive approach to achieve angiosuppression by blocking the activity of angiogenic factors at the receptor level.

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