Enhancement of Angiogenesis by Endogenous Substance P Release and Neurokinin-1 Receptors During Neurogenic Inflammation

HÉLÈNE C. SEEGER, VIVIENNE C. HOOD, BRUCE L. KIDD, SIMON C. CRUWYS, and DAVID A. WALSH

ABSTRACT

Early angiogenesis is a key step in the transition from acute to persistent inflammation. The nervous system has long been known to play a role in inflammation, in part through the release of substance P from peripheral nerve terminals (neurogenic inflammation). Application of substance P can stimulate vessel growth in a variety of angiogenesis assays, although it was previously not known whether endogenous substance P released from sensory nerves could modulate angiogenesis. We hypothesized that endogenous substance P could initiate angiogenesis during acute neurogenic inflammation. Here we show that 10 nmol of substance P can stimulate angiogenesis within the rat knee synovium, as shown by increased endothelial cell proliferation index (PCNA index 95% confidence interval (CI), 17 to 20%) compared with saline injected knees [6% (95% CI, 4% to 8%), p < 0.05]. Moreover, this was prevented by coadministration of an antagonist of the neurokinin-1 (NK1) subtype of neurokinin receptor SR140333 (nolpitantium), 1 μmol [8% (95% CI, 5% to 11%)]. Capsaicin 0.5%, which stimulates release of endogenous substance P from sensory nerves, was also found to enhance synovial angiogenesis, [PCNA index 17% (95% CI, 14% to 19%)] compared with saline injected control knees [2% (95% CI, 1% to 3%), p < 0.05], and this was also inhibited by 1 μmol of SR140333 [11% (95% CI, 8 to 16%)]. Inhibition of capsaicin-enhanced angiogenesis was incomplete, and this may indicate a contribution of other neuropeptides, in addition to substance P-NK1 receptor interactions, in capsaicin-enhanced angiogenesis. NK1 receptor antagonists could have therapeutic potential in conditions where neurogenic angiogenesis contributes to disease.

Angiogenesis, the growth of new vessels from a pre-existing vasculature, is a common feature of chronic inflammation (for review, see Hladky and Wall, 2000) and synovitis (Walsh et al., 1998a). Using the rat model of carrageenan synovitis, we have previously shown that angiogenesis during the acute phase can predict persistent inflammation (Walsh et al., 1998a). Understanding and targeting early angiogenesis may then be of potential benefit for the treatment of chronic inflammation.

Sensory nerves and nerve-derived neuropeptides have been implicated in peripheral inflammation (for review, see Holzer, 1988) and involvement of substance P in joint inflammation has been extensively studied (for review, see Garrett et al., 1992). Substance P is released from the peripheral terminals of fine unmyelinated sensory nerves in vivo, where it mediates acute neurogenic inflammation (for review, see Richardson and Vasko, 2002). Its release can also be stimulated by application of capsaicin, the pungent component of chili pepper (Szallasi and Blumberg, 1999). Through its primary action on tachykinin NK1 receptors on the surface of vascular endothelial cells, substance P induces vascular permeability, plasma extravasation, and edema, i.e., neurogenic inflammation (Eglezos et al., 1991; Xu et al., 1992). Substance P released from endogenous sources has not previously been reported to enhance angiogenesis, however. Substance P-enhanced endothelial cell proliferation and in vivo angiogenesis are mimicked by selective NK1 receptor antagonists.
agonists and inhibited by antagonists of neurokinin receptors (Ziche et al., 1990; Fan et al., 1993; Ziche et al., 1994). Substance P may also stimulate neurokinin-2 receptors (NK2) in some peripheral tissues, although it binds NK2 receptors with lower affinity than NK1 receptors. Specific, nonpeptide antagonists of neurokinin receptors have been developed, of which SR140333 and SR144190 display high affinity, stereoselectivity, and receptor subtype specificity at rat NK1 and NK2 receptors, respectively (Emonds-Alt et al., 1993; Oury-Donat et al., 1994; Emonds-Alt et al., 1997). The effects of these have not been tested on neurogenic angiogenesis, however.

We have investigated the effects of exogenous and endogenous substance P on angiogenesis in the rat knee, the receptor subtypes that mediate these effects, and the ability of antagonists of these receptors to inhibit angiogenesis during a model of synovitis. We hypothesized that endogenous substance P initiates angiogenesis during the early phase of synovitis through an action on NK1 receptors.

### Materials and Methods

Experiments, licensed under the UK Home Office regulations, were performed on male Wistar rats (180 g).

**Animal Models.** On day 0, rats were injected in the right knee with substance P in 0.9% normal saline or 0.5% capsaicin in 10% ethanol, 10% Tween 80, and 80% normal saline (v/v/v) (Mapp et al., 1996), each either alone, or mixed and injected together with pharmacological agents. Neurokinin receptor antagonists and inactive enantiomers were dissolved in ethanol to a stock concentration of 100 mM and then diluted in normal saline to give a final ethanol concentration ≤10% (v/v). Preliminary controls with the capsaicin vehicle demonstrated no increase in angiogenesis (data not shown). Left knees were injected with normal saline alone, which does not increase indices of angiogenesis, macrophage infiltration, or knee diameter at 24 h compared with naive animal knees (Walsh et al., 1998a). Volumes for all intra-articular injections were 100 μl, performed under neuroleptanalgesia (0.15 ml kg⁻¹ Hypnorm (0.315 mg ml⁻¹ fentanyl citrate, 10 mg ml⁻¹ fluanisone); Janssen-Cilag Ltd., High Wycombe, UK). Knee diameters (millimeters) were measured with a digital electronic caliper (Mitutoyo UK Ltd., Andover, UK).

**Tissue Collection and Preparation.** Twenty four hours after intra-articular injection, rats were killed by asphyxiation in carbon dioxide, and the right and left knee synovia with patellae were immediately harvested. Synovia were embedded in Tissue Tek (Miles, Inc., Elkhart, IN), frozen in melting isopentane onto cork block mounts, and stored at −80°C until use.

**Staining Procedures.** Multiple sequential staining was used to identify endothelial proliferation. Sections (4 μm) were first immunostained for proliferating cell nuclear antigen (PCNA) using monoclonal antibody clone PC10 and then for endothelium using a monoclonal antibody directed against CD31 (Waseem and Lane, 1990; Male et al., 1995). Nuclei were counterstained by immersion of Tissue Tek (Miles, Inc., Elkhart, IN), frozen in melting isopentane onto cork block mounts, and stored at −80°C until use.

**Quantification.** Quantification was performed by an observer blinded to experimental details using a Zeiss microscope (Carl Zeiss GmbH, Jena, Germany) with a 16× objective lens. Transmitted light and fluorescence images of the same field were each captured using a 3-CCD camera and analyzed using a KS300 image analysis system (Imaging Associates Ltd., Thame, UK).

### Results

**PCNA Index.** Synovia from saline-injected knees displayed low levels of endothelial cell proliferation (Fig. 1). There was a dose-dependent increase in endothelial cell PCNA index in synovia 24 h after injection of substance P (Fig. 1 and 2). For all the experiments with administration of 10 nmol of substance P, the mean endothelial cell PCNA index was 19% (95% CI, 17 to 20%) and was higher than in the saline injected knee [6% (95% CI, 4 to 8%), p < 0.05].

Intra-articular injection of 0.5% capsaicin was also followed by an increase in endothelial cell PCNA index [17% (95% CI, 14 to 19%)] compared with saline injected control knees [2% (95% CI, 1 to 3%), p < 0.05].

Coinjection of the NK1 receptor antagonist SR140333 (0.01 to 1 μmol) together with 10 nmol of substance P or 0.5% capsaicin dose dependently attenuated the increases in endothelial cell PCNA indices (Figs. 3 and 4). At the highest dose of SR140333 (1 μmol), endothelial cell PCNA indices were lower than in synovia from knees that had been injected with either substance P or capsaicin alone. The inactive enantiomer of SR140333, SR140603 (1 μmol), did not significantly attenuate the increased endothelial cell PCNA index observed 24 h after injection of substance P or capsaicin (Figs. 3 and 4).

Coinjection of the neurokinin-2 receptor antagonist SR140190 (1 μmol) did not significantly attenuate the increased endothelial cell PCNA index observed 24 h after injection of substance P [18% (95% CI, 13 to 23%)]. In the
absence of substance P or capsaicin, intra-articular injection of either NK1 or NK2 receptor antagonist did not affect endothelial cell PCNA indices when compared with saline-injected control knees [7% (95% CI, 4 to 10%) and 10% (95% CI, 6 to 14%) respectively.]

Vascular Densities (Endothelial Percentage Areas). Synovia from saline-injected knees were highly vascularized. The mean vascular densities in saline injected knees for each experiment was in the range 3.8 to 5.8%. Vascular densities were higher 24 h after intra-articular injection of substance P (10 nmol) [6.2% (95% CI, 5.0 to 7.6%)] or 0.5% capsaicin [6.5% (95% CI, 5.9 to 7.2%)] than after saline injection [4.5% (95% CI, 3.1 to 5.7%), \( p < 0.05 \)] or [5.2% (95% CI, 4.5 to 6.0%), \( p = 0.01 \)], respectively.

Inflammation (Macrophage Infiltration and Knee Diameter). Intra-articular injections of substance P (10 nmol) or 0.5% capsaicin induced significant increases in knee joint diameter and macrophage infiltration (ED1 percentage area) at 24 h compared with saline-injected knees (Table 1).

Co-injection of either NK1 or NK2 antagonists (SR140333 or SR140190, respectively) or the inactive enantiomer SR140603 (1 \( \mu \)mol each), did not significantly attenuate the increased diameter or macrophage infiltration observed 24 h after injection of substance P or capsaicin (Table 1).

**Discussion**

In this study, we have shown that exogenous application of substance P (10 nmol) and capsaicin (0.5%) each enhanced endothelial cell proliferation and increased vascular density, which could be inhibited by the coadministration of specific NK1 receptor antagonist. Collectively our results strongly suggest that the effect of endogenous substance P on NK1...
receptors is an important component of the neurogenic enhancement of angiogenesis in rat knee synovitis.

Normal synovium has evolved to be one of the most highly vascular tissues in the body to meet the metabolic requirements of the avascular articular cartilage. In inflamed synovia, edema, cellular hyperplasia, and vascular regression blunt the increase in vascular density that would otherwise accompany synovial angiogenesis (Stevens et al., 1991; Walsh et al., 1998a,b). We have found that endothelial cell PCNA index is a more sensitive measure of synovial angiogenesis than is vascular density, and its use increases statistical power for demonstrating effects of interventions (Walsh et al., 1998a,b).

Enhancement of angiogenesis by substance P was dose dependently and, at the highest dose, completely inhibited by the nonpeptide NK1 receptor antagonist SR140333 but not by its inactive enantiomer SR140603 (1 μmol). Arithmetic means (±S.E.M.) of five or six rats. *, p < 0.05 compared with knees injected with capsaicin alone.

Inhibition of capsaicin-enhanced endothelial cell proliferation by NK1 receptor antagonist. Dose-dependent inhibition of 0.5% capsaicin-enhanced endothelial cell proliferation by the NK1 receptor antagonist SR140333 (0.01 to 1 μmol) but not by its inactive enantiomer SR140603 (1 μmol). Arterial means (±S.E.M.) of five or six rats. *, p < 0.05 compared with knees injected with capsaicin alone.

Fig. 4. Inhibition of capsaicin-enhanced endothelial cell proliferation by NK1 receptor antagonist.
infiltration in these models is mediated, at least in part, by pathways that do not require the NK1 receptor.

In conclusion, our findings indicate that substance P, either exogenously applied or released from endogenous sources, can stimulate syngangliogenic angiogenesis through an action on NK1 receptors. Selective NK1 receptor antagonists can inhibit angiogenesis under these circumstances. Further work is required to determine the contributions of substance P and NK1 receptors to angiogenesis in specific pathologies.

References


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Address correspondence to: David A. Walsh, Academic Rheumatology, University of Nottingham Clinical Sciences Building, City Hospital, Hucknall Road, Nottingham, NG5 1PB, UK. E-mail: david.walsh@nottingham.ac.uk