Substance P in intervertebral discs

Binding sites on vascular endothelium of the human annulus fibrosus

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The annulus fibrosus of the human intervertebral disc is sparsely innervated, some of the fibers containing substance P. We could demonstrate, by autoradiography, binding sites for substance P localized on the endothelium of small blood vessels in the annulus fibrosus of human intervertebral discs removed during anterior fusion for back pain. In binding inhibition studies, binding of ^{125}I-Bolton Hunter-substance P was inhibited by unlabeled substance P and the related tachykinins neurokinin A and neurokinin B with a rank order of potency substance P > NKA > NKB. Specific binding was reduced > 75 percent by 5'-guanylylimidodiphosphate, indicating G-protein coupling. These features are characteristic of an NK1 receptor through which vascular effects, i.e., vasodilation, plasma extravasation and angiogenesis of substance P, are mediated. The presence of NK1 receptors on blood vessels in the annulus fibrosus may indicate a role for substance P in tissue repair although acute proinflammatory effects may contribute to discogenic pain.

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The intervertebral disc is innervated by the sinuvertebral nerve and by branches of the sympathetic trunk (Bogduk et al. 1981). Nerve fibers extend into the annulus fibrosus either as free fibers or in association with blood vessels (Malinsky 1959).

Some of these nerve fibers in the outer annulus in man (Coppes et al. 1990, Konttinen et al. 1990, Ashton et al. 1994) and in other species (Weinstein et al. 1988, Ahmed et al. 1991, McCarthy et al. 1993) are immunoreactive for substance P. Substance P is a neuropeptide which, when released from the central terminals of primary afferent neurones in the dorsal horn of the spinal cord, acts as a neurotransmitter or neuromodulator (Lembeck 1981, Henry 1982). In addition, substance P is transported antidromically to peripheral nerve endings where it contributes to inflammatory processes such as mast cell degranulation, vasodilation and increased capillary permeability in skin and joints (Levine 1985, Foreman 1987).

Substance P is a member of the tachykinin group of peptides which includes neurokinin A and neurokinin B (Pernow 1983). The biological effects of the tachykinins are mediated by their interaction with specific high-affinity receptors on cell surfaces. Three subtypes of tachykinin receptor have been identified—i.e., neurokinin (NK)1, NK2 and NK3 which preferentially bind substance P, neurokinin A (NKA) and neurokinin B (NKB), respectively (Burcher 1989). Vascular effects of substance P are largely mediated by the NK1 tachykinin receptor subtype (Scott et al 1992).

Microvascular binding of substance P has previously been demonstrated in human tissues including gut, skin and synovium (Mantyh et al. 1989, Deguchi et al. 1989, Walsh et al. 1992, 1993). Substance P binding sites have not previously been described in the intervertebral disc. As substance P is known to be present in the disc, we tried to identify possible sites of substance P action by localizing substance P binding sites.

Material and methods

Intervertebral discs were obtained from 8 patients aged 29–46 years during anterior fusion for back pain. All patients had previously had discography which revealed either annular tears, or degenerative disc disease with pain reproduction. The disc material, which comprised the anterior and antero-lateral parts of the L4/5 or L5/S1 discs (Table 1) was separated into annulus fibrosus and nucleus pulposus, mounted on cork blocks and frozen immediately in Arcton 12 (dichlorofluoromethane, ICI, Runcorn, Cheshire, UK) precooled in liquid nitrogen. Between

Table 1. Specific binding of ^{125}I-BH-substance P in the annulus fibrosus of the intervertebral disc

Patient	Level	Age	Sex	Blood vessels	Specific binding
1	L4/5	34	F	+	+
2	L4/5	39	F	+	+
3	L4/5	29	M	–	–
4	L4/5	32	F	–	–
5	L5/S1	44	M	+	+
6	L5/S1	30	F	+	+
7	L5/S1	39	M	+	+
8	L5/S1	46	F	–	–

5 and 20 10 µm sections of each specimen of unfixed tissue were cut in a cryostat and thaw-mounted onto microscope slides pretreated with Vectabond (Vector Laboratories, Peterborough, UK).

Sections were preincubated twice for 15 min in buffer A (10 mM HEPES containing 130 mM NaCl, 4.7 mM KCl, 1 mM EGTA and 10 mM $MgCl_2$ at pH 7.4) then further incubated for 45 min at 20 °C with buffer B (Buffer A with the addition of 1% bovine serum albumin) containing 0.5 nM ^{125}I-Bolton Hunter (BH)-labeled substance P (Amersham Radiochemicals, Amersham, UK) either alone (total binding) or together with 1 µM unlabeled substance P (non-specific binding). Specific binding was defined as total binding minus non-specific binding. Sections were subsequently washed twice for 5 min with buffer A at 4 °C, rinsed in distilled water and dried rapidly in a flow of cold air. Binding inhibition studies were performed on consecutive sections by incubation in 0.5 nM ^{125}I-BH-substance P plus either unlabeled substance P (0.1 nM–1 µM), NKA (1 nM–1 µM), NKB (1–10 µM) or 5'-guanylylimidodiphosphate (Gpp(NH)p, 100 µM). Peptides and Gpp(NH)p were obtained from Sigma Chemical Co. (Poole, UK).

^{125}I-BH-substance P labeled sections and 10 µm ^{125}Iodine radiopolymer standards (Amersham) were apposed to autoradiography film (Hyperfilm $^{-3}$H, Amersham) for 7 days at 4 °C and then developed in Kodak D9 at 20 °C for 3 min. Ligand binding was quantified on the developed autoradiographs using image analysis (Sight Systems, Hove, UK). For each film, grey values were related to ^{125}Iodine standards, corrected for the activity date of the ligand. IC_{50} values were derived by iterative nonlinear regression of binding inhibition data, using single-site models with Graph PAD Inplot 4 (San Diego), as previously described (Walsh et al. 1993).

The ^{125}I-BH-substance P labeled sections were then fixed by immersion in Bouin's solution for 60 min at 20 °C, rinsed for 30 min in running tap water and dried under a stream of cold air before being dipped in Ilford K5 emulsion at 43 °C. These dipped sections were dried and exposed at 4 °C for 4 weeks in a light-tight box, developed as for films and then counterstained with hematoxylin and eosin.

Frozen serial sections of the intervertebral disc were immunostained for Von Willebrand factor (VWF) or platelet endothelial cell adhesion molecule (PECAM) by the avidin-biotin complex method (Hsu et al. 1981). Rabbit polyclonal antibodies to VWF (AO82) and mouse monoclonal antibodies to PECAM (JC/70A) were obtained from Dako Ltd (High Wycombe, UK). Biotinylated second-layer antibodies and avidin biotin complex came from Vector Laboratories.

The anatomical localization of substance P binding was examined in emulsion-dipped sections and compared with sections stained for the endothelial markers VWF and PECAM.

Results

Sparse vascularization in the annulus fibrosus sections in 5 of the 8 patients was observed with no demonstrable vessels in the nucleus pulposus. Specific binding of ^{125}I-BH-substance P was confined to blood vessels in the annulus fibrosus and was found in all those specimens in which blood vessels were observed (Table 1). No specific binding was observed in any specimen of nucleus pulposus or non-vascular structures in the annulus fibrosus. Binding was localized to the endothelium of blood vessels (Figure 1).

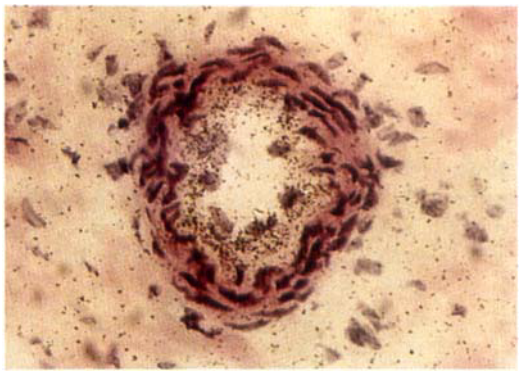

Figure 1. Photomicrograph of emulsion-dipped section of intervertebral disc showing localization of ^{125}I-BH-substance P over vascular endothelium. Counterstained with H&E.

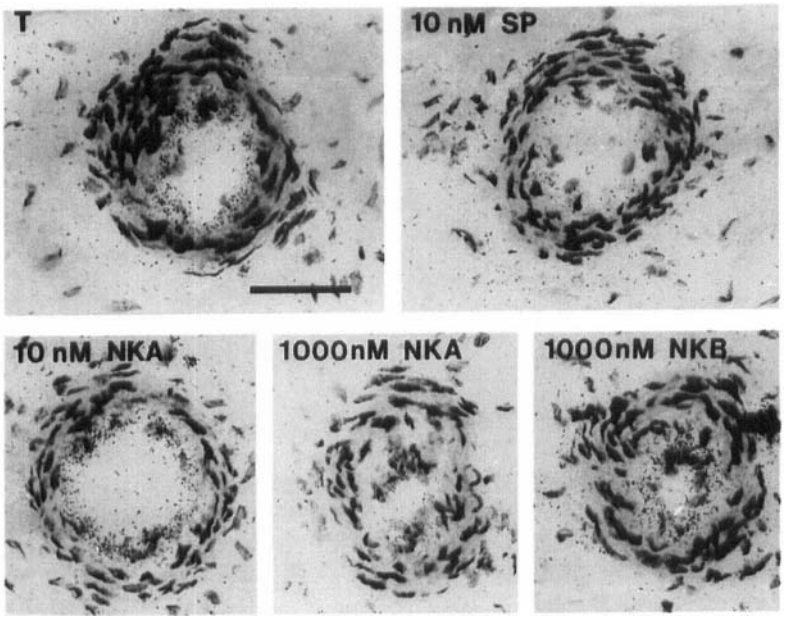

Figure 2. Total binding (T) in the presence of 0.5 nM ^{125}I-BH-substance P was inhibited by coincubation with 10 nM unlabeled substance P (SP), but not by 10 nM NKA, and by 1000 nm NKA but not by 1000 nM NKB, demonstrating the different potencies of SP, NKA and NKB. Bar 20 µm.

In binding inhibition studies, specific binding of ^{125}I-BH-substance P was inhibited by unlabeled tachykinins with a rank order of potency substance P > NKA > NKB (Figure 2), which is characteristic of the NK1 subtype of neurokinin receptor. Few blood vessels were identifiable on film autoradiograms because of the sparse vascularity of disc tissue. Quantification of the one case where vessels were identified throughout serial sections confirmed a rank order of inhibitory potency of substance P > NKA > NKB with IC_{50} values 1.2 nM, 15 nM and > 1000 nM, respectively. Specific binding was > 75 percent inhibited by Gpp(NH)p.

Discussion

The intervertebral disc is normally avascular. However, age or degeneration results in fibrosis and fissuring (Eyre et al. 1989). Vascular ingrowth then commonly occurs, particularly along the margins of clefts and lesions (Vernon-Roberts 1992). In our study, sections of the annulus fibrosus in 5 of the 8 patients were sparsely vascularized. Specific binding sites for substance P were identified only on these blood vessels.

The binding sites for substance P were confined to the endothelium of the vessels and had characteristics of the NK1 type of tachykinin receptor—namely, relative selectivity of substance P as opposed to other tachykinins and inhibition by the non-hydrolyzable GTP analogue Gpp(NH)p, suggesting G-protein coupling. A similar endothelial localization of NK1-like substance P binding is found in human synovium (Walsh et al. 1992, 1993). NK1 receptors mediate several of the vascular effects of substance P including vasodilation, plasma extravasation and angiogenesis (Ziche et al. 1991, Scott et al. 1992, Lam et al. 1993). Prostacyclin release by endothelial cells in response to substance P is also NK1 receptor-mediated (Marceau and Tremblay 1989).

The outer third, approximately, of the annulus fibrosus of the human intervertebral disc is innervated and some of the nerve fibers contain substance P (Coppes et al. 1990, Konttinen et al. 1990, Ashton et al. 1994). The nerve fibers of the annulus fibrosus may be free or perivascular (Konttinen et al. 1990, Ashton et al. 1994). A few of the substance P immunoreactive nerves have recently been found in proximity to blood vessels in the annulus fibrosus (Ashton, unpublished observations). Neuropeptides, including substance P, are released from peripheral terminals of sensory nerves as a result of either chemical or electrical stimulation (Yaksh 1988). We propose that the binding sites which we have identi-

fied represent functional receptors for endogenously released substance P in the human intervertebral disc. In vitro, substance P stimulates endothelial cell proliferation and migration, essential features in the formation of a new vascular network (Ziche et al. 1991). The presence of NK1 receptors suggests that substance P could itself be a contributory factor in neovascularization in human intervertebral disc. Effects of substance P on blood vessels to increase blood flow and angiogenesis may thus promote tissue repair (Walsh et al. 1993), for example, by improved nutrition. On the other hand, sustained effects of substance P could contribute to the inflammatory changes which have been described in the intervertebral disc (Crock 1976, Jaffray and O'Brien, 1986) through the production of inflammatory mediators and cytokines (Lotz et al. 1988) and thus contribute to further degeneration and discogenic pain.

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References

Ahmed M, Bjurholm A, Kreicbergs A, Shultzberg. Substance P and CGRP immunoreactive nerve fibres in the rat lumbar spine. Neuro Orthop 1991; 12: 19–28.

Ashton I K, Roberts S, Jaffray D C, Polak J M, Eisenstein S M. Neuropeptides in the human intervertebral disc. J Orthop Res 1994; 12 (2): 186–92.

Bogduk N, Tynan W, Wilson A S. The nerve supply to the human lumbar intervertebral discs. J Anat 1981; 132 (Pt 1): 39–56.

Burcher E. The study of tachykinin receptors. Clin Exp Pharmacol Physiol 1989; 16 (6): 539–43.

Coppes M H, Marani E, Thomeer R T, Oudega M, Groen G J. Innervation of annulus fibrosis in low back pain (letter) (published erratum appears in Lancet 1990 Aug 4; 336(8710):324). Lancet 1990; 336 (8708): 189–90.

Crock H V. Handbook of clinical neurology (Eds. Vinken, P.J, Bruyn, G.W, Klawans, H.L.) Elsevier, Amsterdam 1976; 25: Injuries of the spine and spinal cord: 487–508.

Deguchi M, Niwa M, Shigematsu K, Fujii T, Namba K, Ozaki M. Specific (125I)Bolton-Hunter substance P binding sites in human and rat skin. Neurosci Lett 1989; 99 (3): 287–92.

Eyre D, Benya P, Buckwalter J, Caterson B, Heinegård D,Oegema T, Pearce R, Pope M, Urban J. Intervertebral disc: Basic science perspectives. New Perspectives in Low Back Pain. (Eds. Frymoyer J W, Gordon S L) Amer Acad Orthop Surg, Park Ridge 1989.

Foreman J C. Peptides and neurogenic inflammation. Br Med Bull 1987; 43 (2): 386–400.

Henry J L. Relation of substance P to pain transmission. Neurophysiological evidence. In: Substance P and the Nervous System. CIBA Foundation. Pitman, London 1982: 206–24.

Hsu S M, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981; 29 (4): 577–80.

Jaffray D, O'Brien J P. Isolated intervertebral disc resorption. A source of mechanical and inflammatory back pain? Spine 1986; 11 (4): 397–401.

Konttinen Y T, Gronblad M, Antti Poika I, Seitsalo S, Santavirta S, Hukkanen M, Polak J M. Neuroimmunohistochemical analysis of peridiscal nociceptive neural elements. Spine 1990; 15 (5): 383–6.

Lam F Y, Ferrell W R, Scott D T. Substance P-induced inflammation in the rat knee joint is mediated by neurokinin 1 (NK1) receptors. Regul Pept 1993; 46 (1–2): 198–201.

Lembeck F, Donnerer J, Colpaert F C. Increase of substance P in primary afferent nerves during chronic pain. Neuropeptides 1981; 1: 175–80.

Levine J D, Collier D H, Basbaum A I, Moskowitz M A, Helms C A. Hypothesis: the nervous system may contribute to the pathophysiology of rheumatoid arthritis. J Rheumatol 1985; 12 (3): 406–11.

Lotz M, Vaughan J H, Carson D A. Effect of neuropeptides on production of inflammatory cytokines by human monocytes. Science 1988; 241 (4870): 1218–21.

Malinsky J. The ontogenetic development of nerve terminations in the intervertebral disc of man. Acta Anat 1959; 38: 96–113.

Mantyh P W, Catton M D, Boehmer C G, Welton M L, Passaro E P Jr, Maggio J E, Vigna S R. Receptors for sensory neuropeptides in human inflammatory diseases: implications for the effector role of sensory neurons. Peptides 1989; 10 (3): 627–45.

Marceau F, Tremblay B, Couture R, Regoli D. Prostacyclin release induced by neurokinins in cultured human endothelial cells. Can J Physiol Pharmacol 1989; 67 (2): 159–62.

McCarthy P W. Sparse substance P-like immunoreactivity in intervertebral discs. Nerve fibers and endings in the rat. Acta Orthop Scand 1993; 64 (6): 664–8.

Pernow B. Substance P. Pharmacol Rev 1983; 35 (2): 85–141.

Scott D T, Lam F Y, Ferrell W R. Acute inflammation enhances substance P-induced plasma protein extravasation in the rat knee joint. Regul Pept 1992; 39 (2–3): 227–35.

Vernon-Roberts B. Age related and degenerative pathology of intervertebral disc and apophyseal joints. In: The Lumbar Spine and Back Pain. (Ed. Jayson M I V) 4th ed. Churchill Livingstone, Edinburgh 1992.

Walsh D A, Mapp P I, Wharton J, Rutherford R A D, Kidd B L, Revell P A, Blake D R, Polak J M. Localization and characterization of substance P binding to human synovial tissue in rheumatoid arthritis. Ann Rheum Dis 1992; 51: 313–7.

Walsh D A, Salmon M, Mapp P I, Wharton J, Garrett N, Blake D R, Polak J M. Microvascular substance P binding to normal and inflamed rat and human synovium. J Pharmacol Exp Ther 1993; 267 (2): 951–60.

Weinstein J, Claverie W, Gibson S. The pain of discography. Spine 1988; 13 (12): 1344–8.

Yaksh T L. Substance P release from knee joint afferent terminals: modulation by opioids. Brain Res 1988; 458 (2): 319–24.

Ziche M, Morbidelli L, Geppetti P, Maggi C A, Dolara P. Substance P induces migration of capillary endothelial cells: a novel NK-1 selective receptor-mediated activity. Life Sci 1991; 48 (2): PL7–11.