Localisation and characterisation of substance P binding to human synovial tissue in rheumatoid arthritis

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Abstract

The neuropeptide substance P is found in perivascular and free unmyelinated nerve fibres in human synovial tissue. Quantitative receptor autoradiography was used to show specific, high affinity ($K_d=0.75$ (0.21), nmol/l (mean (standard error of the mean)), low capacity ($B_{max}=27.8$ (7.9) amol/mm²) binding sites for substance P Bolton Hunter-labelled with iodine-125 localised to vascular endothelial cells in human synovial tissue. The binding could be saturated, was reversible, and was dependent on the magnesium concentration. Unlabelled substance P and neurokinin A competitively inhibited specific binding with 50% inhibition at concentrations of 1.25 (0.21) and 175 (29) nmol/l respectively. Neurokinin B (μ mol/l) and calcitonin gene related peptide (1 μ mol/l) did not inhibit binding. These binding sites show characteristics of the neurokinin 1 tachykinin receptor subtype. This provides further evidence that substance P may play a part in the vascular control of human synovium and may influence inflammatory processes in joints.

The nervous system has been implicated in the aetiology and pathogenesis of human joint disease.¹⁻² Neurological lesions such as strokes and poliomyelitis are protective against rheumatoid arthritis³⁻⁴ and osteoarthritis⁵ in the affected limbs. In contrast, complete sensory denervation can lead to a severe, destructive Charcot's arthropathy.⁶ Similarly, neurological lesions in animals influence the development and severity of experimentally induced arthritis.⁷

The human nervous system has been clearly shown to modulate inflammatory processes in peripheral tissues and to influence each of the classical signs of inflammation: rubor, calor, tumour, and dolor.⁸ The effects of the sympathetic nervous system on vascular tone are well recognised. In addition, unmyelinated sensory fibres contain neuropeptides such as substance P, which may be released centrally to participate in pain pathways, and peripherally where it may modulate vascular function.⁹

There is increasing evidence that substance P can be released into joints from peripheral nerve terminals and may therefore influence the course of joint disease. Nerves containing substance P are associated with blood vessels and occur as free fibres in human synovial tissue.¹⁰⁻¹¹ Antidromic stimulation of the articular nerve in the knee of cats results in the release of substance P-like immunoreactivity into the joint,¹² vasodilatation and plasma extravasa-

tion,¹³ and substance P-like immunoreactivity has been detected in human synovial fluid, suggesting that the local release of the peptide also occurs in human joints.¹⁴⁻¹⁵ Depletion of nerves containing substance P in rats, by neonatal treatment with the sensory neurotoxin capsaicin, reduces the severity of subsequently induced adjuvant arthritis in the adult animal,⁷ whereas intra-articular injection of substance P increases its severity,¹⁶ suggesting a possible part for substance P in the pathogenesis of at least this form of arthritis.

Substance P is a member of the tachykinin family of peptides which also includes the closely related molecules neurokinin A and neurokinin B. Its peripheral actions may result from direct effects on vascular tissue such as the release of endothelium derived relaxing factor (nitric oxide) from endothelial cells,¹⁷ or from the release of mediators from synoviocytes¹⁸ and infiltrating inflammatory cells.¹⁹ Many of its biological effects are believed to be mediated by interactions with specific high affinity receptors on the cell surface.²⁰ Three subclasses of tachykinin receptor have been recognised in non-articular tissues, NK 1, NK 2, and NK 3, each of which shows preferential binding to substance P, neurokinin A, or neurokinin B respectively.²¹

Peptide receptors can be localised and their kinetics studied by quantitative in vitro receptor autoradiography with computerised image analysis.²² We have adapted this technique to study binding sites for substance P in human synovium and have investigated possible sites of action of substance P in human joints.

Patients and methods

Synovial tissue from patients with rheumatoid arthritis, Reiter's syndrome (chronic inflammatory arthritis), or osteoarthritis were collected during operations to replace the knee joint. Normal human synovial tissue was collected during amputation operations. The table gives the details of the patients with arthritis. All tissues were mounted on cork blocks, snap frozen in melting isopentane, and stored at -70°C . Frozen 10 μ m thick sections were cut on a cryostat, mounted on glass slides, air dried, and stored at -20°C with silica gel dessicant.

Each section was preincubated twice at 22°C for 15 minutes in 10 mmol/l HEPES buffer containing 4% enzyme free bovine serum albumin, pH 7.4. Buffer was removed by shaking and the sections were incubated in humidified chambers for 30 minutes at 22°C while exposed to the ligand in incubation buffer

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Accepted for publication
18 June 1991

Details of patients

Patient No	Age (years)	Sex	Diagnosis	Disease duration (years)	Current DMARD	Previous DMARD	Mean specific binding (amol/mm ²)*
1	56	M	RA	20	Nil	P, A, C	2.38
2	58	M	RA	13	Nil	Unk	1.83
3	70	F	RA	22	Nil	P	1.09
4	61	M	RA	24	Unk	Unk	2.67
5	78	M	RA	7	P	Nil	2.24
6	62	M	RA	29	G	Unk	2.32
7	59	F	RA	15	S	Nil	2.78
8	70	F	RA	5	P	G	2.02
9	62	M	RS	30	C	A, M, P	3.27
10	70	M	OA	>5			1.8
11	79	M	OA	>3			3.41
12	19	M	Normal				2.38

*Mean specific binding: density of binding of substance P Bolton Hunter labelled with iodine 125 (¹²⁵I-BH-SP) per mm² of blood vessel at equilibrium using 0.5 nmol/l ¹²⁵I-BH-SP.

Abbreviations: DMARD, disease modifying antirheumatic drug; RA, rheumatoid arthritis; RS, Reiter's syndrome; OA, osteoarthritis; A, azathioprine; C, chlorambucil; G, sodium aurothiomalate; M, methotrexate; P, penicillamine; S, sulphasalazine; Unk, Unknown; F, female; M, male.

(10 mmol/l HEPES, 130 mmol/l sodium chloride, 4.7 mmol/l potassium chloride, 5 mmol/l magnesium chloride, 1 mmol/l EGTA, 1% bovine serum albumin (w/v), pH 7.4). Duplicate consecutive sections were incubated with either 0.5 nmol/l substance P Bolton Hunter labelled with iodine-125 (¹²⁵I-BH-SP) (Amersham, UK) alone (total binding) or 0.5 nmol/l ¹²⁵I-BH-SP plus an excess (1 µmol/l) of unlabelled substance P (Sigma Chemical, Poole, Dorset, UK) (non-specific binding). Each section was then washed twice at 4°C for five minutes in washing buffer (incubation buffer without bovine serum albumin), rinsed in ice cold distilled water and dried in a flow of cold air.

Incubation conditions were optimised by comparing different buffers, pH, temperatures, incubation times, and ligand concentrations. The effect of protease inhibitors on specific binding was also investigated by the addition of chymostatin (0.2%), bacitracin (0.1%) or leupeptin (0.4%) or a combination of the three (all from Sigma) to preincubation or incubation buffers, or both. Competitive inhibition studies were performed by adding to the incubation buffer up to 1 µmol/l of the unlabelled peptides substance P, neurokinin A, neurokinin B, and calcitonin gene related peptide (all from Sigma). To study the time course of dissociation, unlabelled substance P was added to the sections after 30 minutes incubation with 0.5 nmol/l ¹²⁵I-BH-SP, giving a final concentration of 1 µmol/l unlabelled substance P and 0.5 nmol/l ¹²⁵I-BH-SP.

¹²⁵I-BH-SP labelled sections and radiolabelled polymer standards were apposed to autoradiography film (³H Hyperfilm, Amersham, UK) and exposed for seven days at 4°C. Ligand binding to blood vessels was determined on the developed autoradiographs using an IBAS 2000 automatic measuring program (Kontron, Watford, UK). Using images of radiolabelled standards and the specific activity of the ¹²⁵I-BH-SP at the time the film was exposed, a calibration graph was produced for each film relating the grey value to the log of the amount of bound ligand (amol) per square millimetre of section. The mean density of binding for the 10 most densely bound vessels was calculated for each section. Specific binding was defined as the total binding minus the non-specific binding.

Comparison of autoradiograms with counter-stained sections indicated that the measured vessels had a luminal diameter <100 µm.

The dissociation constant (K_d) and maximum binding capacity (B_{max}) for specific binding of substance P to synovial blood vessels from patients with rheumatoid disease were calculated by non-linear regression of competitive inhibition data using 0–32 nmol/l unlabelled substance P. The K_d value for each of six patients with rheumatoid disease was calculated according to the equation

$$K_d = IC_{50} - [L]$$

where IC_{50} is the 50% inhibitory concentration of unlabelled substance P, and $[L] = 0.5$ nmol/l, the concentration of ¹²⁵I-BH-SP. The B_{max} value for each patient was calculated as

$$B_{max} = B_0 \times IC_{50} / [L]$$

where B_0 is the specific binding of ¹²⁵I-BH-SP in the absence of unlabelled substance P.²³

The anatomical localisation of the binding was examined in specimens dipped in emulsion. Sections labelled with ¹²⁵I-BH-SP were fixed in Bouin's fluid, dipped in photographic emulsion at 43°C, dried, exposed for three to seven weeks at 4°C, and then developed and counterstained with haematoxylin and eosin. Endothelium was also identified immunohistochemically in ¹²⁵I-BH-SP labelled sections after exposure to autoradiography film by immunostaining for von Willebrand's factor and by *Ulex europaeus* agglutinin I (UEA 1) lectin binding. Sections were incubated with either antiserum to human von Willebrand's factor (A082; DAKO, UK) or UEA 1 lectin (Vector Laboratories, UK), and the endothelium was visualised using antiserum to UEA 1 (B279; DAKO) and fluorescein isothiocyanate labelled goat antirabbit IgG (Tago, USA).

Specific binding of ¹²⁵I-BH-SP in the presence or absence of magnesium were compared by the paired *t* test on data from three separate experiments each performed in triplicate. Values are expressed as the mean (standard error of the mean). Non-linear regressions, IC_{50} calculations, and curve fitting for graphs were performed using GraphPAD INPLOT version 3.1 (GraphPAD Software, San Diego, A, USA).

Results

Figures 1 and 2 show that ¹²⁵I-BH-SP bound specifically to blood vessels in human synovium with high affinity and low capacity. Using 0.5 nmol/l ¹²⁵I-BH-SP, non-specific binding was less than 10% of the total binding (fig 1B). Specific binding to areas between blood vessels was less than 15% of the density of binding to the blood vessels themselves. Binding reached more than 90% of the maximum within five minutes at 22°C and showed no decline with incubations up to 60 minutes. More than 50% of the bound ¹²⁵I-BH-SP was displaced within 30 minutes of incubation with 1 µmol/l unlabelled substance P.

The addition of the protease inhibitors bacitracin, leupeptin or chymostatin to preincubation or incubation buffers, or both, did not increase

Figure 1 Autoradiographs of substance P Bolton Hunter labelled with iodine-125 (^{125}I -BH-SP) binding to human synovium: (A), (B), patient with rheumatoid arthritis, serial sections; C, patient with osteoarthritis; D, normal subject. (A), (C), (D), total binding; (B) non-specific binding. Arrows indicate synovial surface. Bars=3 mm.

Figure 2 Photomicrograph of a section of rheumatoid synovial tissue dipped in emulsion, in which the binding sites of substance P Bolton Hunter labelled with iodine-125 are shown by silver grains overlying endothelial cells. Bar=50 mm.

the specific binding of ^{125}I -BH-SP to synovial sections (data not shown). The binding of ^{125}I -BH-SP to synovial blood vessels was not affected by pH over the range 5.5–7.5. Specific ^{125}I -BH-SP binding to blood vessels was significantly enhanced in the presence of 5 mmol/l magnesium chloride, with an increase in binding of 343 (56)% ($p < 0.05$) compared with that in the absence of divalent cations. All other experiments were performed in the presence of 5 mmol/l magnesium chloride.

Equilibrium competitive inhibition analysis of binding to synovium from six patients with rheumatoid diseases gave IC_{50} values for substance P and neurokinin A of 1.25 (0.21) and 175 (29) nmol/l respectively (fig 3). Neurokinin B and calcitonin gene related peptide (each 1 $\mu\text{mol/l}$) did not inhibit ^{125}I -BH-SP binding. The calculated K_d and B_{max} values for substance P binding to rheumatoid synovial vessels were 0.75 (0.21) nmol/l and 27–8 (7.9) amol/ mm^2 respectively.

The examination of sections dipped in emulsion, in addition to immunostained sections, confirmed the predominant localisation of specific ^{125}I -BH-SP binding to subsynovial blood vessels (fig 4). In particular, binding was localised to the endothelial cell layer rather than the vessel wall (fig 2). Specific binding was most clearly identified on arterioles in the subsynovial layer, lying between 0.2 and 2 mm from the synovial surface. A thin superficial band of specific binding could also be detected on autoradiographs, corresponding to the synovial lining cell layer 0 to 0.2 mm from the synovial surface (fig 4A). Although this binding was not sufficiently dense to be further characterised, immunostaining for von Willebrand's factor showed that this layer was densely packed with the endothelial cells of small blood vessels (diameter approximately 10 μm) (fig 4B).

Similar specific binding of ^{125}I -BH-SP to synovial vascular endothelium was also shown in a group of patients with diagnoses other than rheumatoid arthritis (table), including osteoarthritis (fig 1C) and synovium from normal control subjects (fig 1D).

Figure 3 Competitive inhibition of the binding of 0.5 nmol/l substance P Bolton Hunter labelled with iodine-125 to blood vessels in synovial tissue from patients with rheumatoid diseases by unlabelled substance P (●) and neurokinin A (▲). Each point represents the mean (standard error of the mean) of six separate patients.

Figure 4 Autoradiograph of vascular binding of substance P Bolton Hunter labelled with iodine-125 in a section of human rheumatoid synovium (A) and an immunofluorescence photomicrograph showing endothelial von Willebrand's factor immunoreactivity in the same section (B). Arrows indicate synovial surface; arrowheads indicate corresponding localisation of binding sites and immunoreactivity. Bar=500 μ m.

Discussion

We have shown specific, high affinity, low capacity binding sites for ^{125} I-BH-SP on endothelial cells of human synovial blood vessels. The ratio of the IC_{50} values of these sites for substance P, neurokinin A, and neurokinin B (1.25:175:>1000) is characteristic of the NK 1 class of tachykinin receptors. We have previously shown substance P-like immunoreactivity in unmyelinated nerves in human synovium.¹¹ Substance P-like immunoreactivity has also been found in inflammatory osteoarthritic, and traumatic synovial effusions, in humans at levels comparable with the K_d concentration for the synovial NK 1 receptor,^{14 15} indicating a possible physiological role.

The interaction of substance P with NK 1 receptors on human synovial endothelial cells may be important in the regulation of local vascular tone. Vasodilatation induced by substance P has previously been shown to be endothelium dependent¹⁷ and is inhibited by N^G -monomethyl-L-arginine, indicating a probable mediation by nitric oxide, the endothelium derived relaxing factor.²⁴ When exposed to substance P, cultured endothelial cells may also release nitric oxide,²⁵ in addition to increased amounts of prostacyclin,²⁶ and the latter response at least appears to be mediated by the NK 1 receptor. Substance P and its receptors are therefore well placed to have a

local vasodilator function in human synovial tissue.

Substance P may also influence plasma extravasation in articular tissues. Plasma extravasation induced in the knees of cats and rats by antidromic nerve stimulation or infusion of exogenous substance P are each inhibited by specific substance P antagonists, suggesting a receptor mediated action.^{13 27} In addition, carrageenan induced inflammation in the knee of rats is inhibited by the specific substance P antagonist d-Pro⁴, d-Trp^{7 9 10}-SP,⁴⁻¹¹ suggesting that a part is played by a specific substance P receptor.²⁷ In addition to its actions on endothelial cells, potentially proinflammatory effects of substance P have been shown in vitro on neutrophils, macrophages, mast cells, lymphocytes, and human synoviocytes.^{18 19} The distinct localisation of ^{125} I-BH-SP binding sites on endothelial cells in human synovium suggests that proinflammatory actions of substance P in joints may be predominantly due to direct vascular actions. However, it was not possible to exclude the presence of low density binding to other cell types owing to the limits of resolution of the technique.

Substance P stimulates the proliferation and migration of cultured bovine adrenal capillary endothelial cells and the proliferation of human umbilical vein endothelial cells.^{28 29} Furthermore, NK 1 dependent stimulation of neo-vascularisation by substance P has been shown in vivo in the corneas of rabbits.²⁸ Rheumatoid synovium is a highly vascular tissue (figs 1 and 4) and it is possible that the actions of substance P on endothelial NK 1 receptors may contribute to this vascular proliferation.

Although nerves containing substance P are depleted in the superficial layers of rheumatoid synovium,¹¹ the detection by some workers of substance P-like immunoreactivity in synovial fluid in rheumatoid arthritis^{14 15} indicates that substance P could still reach receptors located in the synovial lining layer and may therefore continue to affect synovial vasculature in the late stages of rheumatoid disease.

In addition to our previous findings of perivascular nerves containing substance P in human synovium, we have now shown that specific binding sites for ^{125} I-BH-SP with characteristics of NK 1 receptors are localised on human synovial endothelial cells. Nerves containing substance P and substance P binding sites are therefore well placed to play a regulatory part in synovial vasculature.

D A Walsh is an Arthritis and Rheumatism Council Junior Research Fellow; P I Mapp is an Arthritis and Rheumatism Council Research Fellow; and D R Blake is the Arthritis and Rheumatism Council Professor of Rheumatology at the London Hospital Medical School. We are grateful to the Arthritis and Rheumatism Council, Mason Medical Research Foundation and London Hospital Medical College Academic Board for their generous financial support.

- 1 Kidd B L, Mapp P I, Gibson S J, et al. A neurogenic mechanism for symmetrical arthritis. *Lancet* 1989; ii: 1128-30.
- 2 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: 406-11.
- 3 Thompson M, Bywaters E G. Unilateral rheumatoid arthritis following hemiplegia. *Ann Rheum Dis* 1962; 21: 370-7.

- 4 Glick E N. Asymmetrical rheumatoid arthritis after poliomyelitis. *BMJ* 1967; **iii**: 26-9.
- 5 Glynn J H, Sutherland I, Walker G F, Young A C. Low incidence of osteoarthritis in hip and knee after anterior poliomyelitis: a late review. *BMJ* 1966; **ii**: 739-42.
- 6 Slowman-Kovacs S D, Braunstein E M, Brandt K D. Rapidly progressive charcot arthropathy following minor joint trauma in patients with diabetic neuropathy. *Arthritis Rheum* 1990; **33**: 412-7.
- 7 Levine D J, Dardick S J, Roizen M F, Helms C, Basbaum A I. Contribution of sensory afferents and sympathetic efferents to joint injury in experimental arthritis. *J Neurosci* 1986; **6**: 3423-9.
- 8 Holzer P. Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* 1988; **24**: 739-68.
- 9 Pernow B. Substance P. *Pharm Rev* 1983; **35**: 85-141.
- 10 Gronblad M, Konntinen Y T, Korkala O, Liesi P, Hukkanen M, Polak J. Neuropeptides in the synovium of patients with rheumatoid arthritis and osteoarthritis. *J Rheumatol* 1988; **15**: 1807-10.
- 11 Mapp P I, Kidd B L, Gibson S J, *et al*. Substance P-, calcitonin gene-related peptide- and C-flanking peptide of neuropeptide Y-immunoreactive fibres are present in normal synovium but depleted in patients with rheumatoid arthritis. *Neuroscience* 1990; **37**: 143-53.
- 12 Yaksh T L. Substance P release from knee joint afferent terminals: modulation by opioids. *Brain Res* 1988; **458**: 319-24.
- 13 Ferrell W R, Russell N J W. Extravasation in the knee induced by antidromic stimulation of articular C fibre afferents of the anaesthetized cat. *J Physiol* 1986; **379**: 407-16.
- 14 Marshall K W, Chiu B, Inman R D. Substance P and arthritis: analysis of plasma and synovial fluid levels. *Arthritis Rheum* 1990; **33**: 87-90.
- 15 Devillier P, Weill B, Renoux M, Menkes C, Pradelles P. Elevated levels of tachykinin-like immunoreactivity in joint fluids from patients with rheumatic inflammatory diseases. *N Engl J Med* 1986; **314**: 1323.
- 16 Levine D J, Clark R, Devor M, Helms C, Moskowitz M A, Basbaum A I. Intraneuronal substance P contributes to the severity of experimental arthritis. *Science* 1984; **226**: 547-9.
- 17 Furchgott R F. Role of endothelium in the responses of vascular smooth muscle. *Circ Res* 1983; **53**: 557-73.
- 18 Lotz M, Carson D A, Vaughan J H. Substance P activation of rheumatoid synoviocytes: neural pathway in pathogenesis of arthritis. *Science* 1987; **235**: 893-5.
- 19 Stanis A, Scicchitano R, Stead R, *et al*. Neuropeptides and immunity. *Am Rev Respir Dis* 1987; **136**: S48-S51.
- 20 Yokota Y, Sasai Y, Tanaka K, *et al*. Molecular characterization of a functional cDNA for rat substance P receptor. *J Biol Chem* 1989; **264**: 17649-52.
- 21 Lee C-M, Campbell N J, Williams B J, Iversen L L. Multiple tachykinin binding sites in peripheral tissues and in brain. *Eur J Pharmacol* 1986; **130**: 209-17.
- 22 Palacios J M, Dietl M M. Regulatory peptide receptors: visualisation by autoradiography. In: Polak J M, ed. *Regulatory peptides*. 1st ed. Basle: Birkhauser Verlag, 1989.
- 23 DeBlasi A, O'Reilly K, Motulsky H J. Calculating receptor number from binding experiments using same compound as radioligand and competitor. *Trends Pharmacol Sci* 1989; **10**: 227-9.
- 24 Rees D D, Palmer R M J, Moncada S. A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br J Pharmacol* 1989; **96**: 418-24.
- 25 Lang D, Smith J A. Release of endothelium-derived relaxing factor from pig cultured aortic endothelial cells, as assessed by changes in endothelial cell cyclic GMP content, is inhibited by a phorbol ester. *Br J Pharmacol* 1990; **99**: 565-71.
- 26 Marceau F, Tremblay B. Prostacyclin release induced by neurokinins in cultured human endothelial cells. *Can J Physiol Pharmacol* 1989; **67**: 159-62.
- 27 Lam F Y, Ferrell W R. Inhibition of carrageenan induced inflammation in the rat knee joint by substance P antagonist. *Ann Rheum Dis* 1989; **48**: 928-32.
- 28 Ziche M, Morbidelli L, Pacini M, Geppetti P, Alessandri G, Maggi C A. Substance P stimulates neovascularisation in vivo and proliferation of cultured endothelial cells. *Microvasc Res* 1990; **40**: 264-78.
- 29 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**: PL-7-PL-11.