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Innervation of the synovium

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This review will describe the normal innervation of the joints, in particular the synovium, and then show how this changes in the rheumatoid arthritis (RA) joint in the human. Substance P will be described in detail, as an example of a particular neuropeptide. The concepts surrounding neuropeptide release and the role of neuropeptides in inflammatory joint disease (neurogenic inflammation) will then be explored. The in vitro and in vivo effects of substance P will be described. The receptors for substance P and the effects of antagonists, peptide and synthetic, for the receptor on inflammatory models will also be discussed. The synovial distribution of the enzyme systems that break down neuropeptides and how this may lead to functional compartmentalisation will be described. Finally, some mechanisms by which pain may arise will be presented.

Normal innervation of the joint

Joints are supplied by both primary and accessory nerves. Primary nerves are branches of peripheral nerves passing near to the joint, whilst accessory nerves are branches of intramuscular nerves crossing the joint capsule. Some joints such as the knee and ankle also receive a nerve supply from cutaneous nerves in the overlying skin. The nerves to any one particular joint always arise from more than one level in the spinal cord. About 50% of the axons that comprise articular nerves are less than 5 μm in diameter; those that are less than 2 μm in diameter are unmyelinated. These fibres carry nociceptive information with a slow conduction velocity. Until relatively recently, the nerve supply to the synovium was thought to be sparse. However, it is now known that the synovium contains a good supply of thinly myelinated or unmyelinated nerve fibres (table). These are of two types: postganglionic sympathetic adrenergic fibres located around the larger blood vessels are responsible for the control of articular blood flow; unmyelinated C fibres are responsible for pain transmission. This latter group of fibres are not normally active and are believed to fire only during tissue damage, either mechanical or chemical. They are therefore sometimes referred to as nociceptive fibres. They are not responsive to normal ranges of movement. However, during an inflammatory response, these fibres may be sensitised by mediators such as prostaglandin E_2, whereupon even movement in the normal range can cause them to discharge, signalling pain.

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Diameters of nerve fibres, their classification and location

Afferent fibre diameter	*Conduction velocity (m/s)*	*Class*	*Location*	*Function*
Myelinated				
10–18 μm	60–100	I (Aα)	Ligaments	Proprioceptive
5–12 μm	20–70	II (Aβ)	Capsule	Proprioceptive
1–5 μm	2·5–20	III (Aδ)	Capsule Synovium	Nociceptive
Unmyelinated				
<1 μm	<1	IV (C)	Capsule Synovium	Nociceptive

INNERVATION OF RHEUMATOID SYNOVIUM

The rheumatoid synovium is a chronically inflamed tissue showing villous hypertrophy of the synovial layer, with an underlying infiltrate of inflammatory cells. Perivascular cuffs of lymphocytes are seen and the tissue may organise itself to resemble a lymphoid follicle.

The innervation of the synovium in rheumatoid arthritis is markedly different from that of the normal synovium. The initial observations in the human synovium, outlined below, were made by Gronblad *et al*,[1] subsequently extended Mapp *et al*,[2] and confirmed independently by Pereria da Silva and Carmo-Fonseca.[3]

Whilst the deeper vessels in the synovium have a nerve population surrounding them, the superficial vessels are apparently not innervated. Also, the number of free fibres is greatly reduced compared with normal synovium. No immunoreactive fibres are seen in intensely inflamed and 'lymphoid' areas of the synovium. Free sensory fibres are not seen in the intimal layer, or in the tissue immediately underlying the intima. Staining for specific peptides shows that the free sensory fibres present in the intimal layer of normal synovium are absent in rheumatoid specimens. The perivascular innervation of the deeper blood vessels is predominantly sympathetic efferent fibres. Similar changes in the innervation of the synovium have been reported in rat models of arthritis.[4 5]

In summary, it would appear that in rheumatoid synovium sensory and sympathetic nerve fibres are absent from the superficial synovium and areas of intense inflammation, whilst the deeper synovium retains its innervation. Staining for specific neuropeptides in the deep synovium is weaker and shows a more varicose distribution than in normal controls, and it is possible that this reflects an increased release of these peptides. The absence of nerve fibres in the superficial synovium may reflect damage to the peripheral terminals of nerve fibres caused by mediators released during the inflammatory response. However, it is possible that the proliferating synovium outstrips the capacity of the nerve supply to innervate it. How these changes in the nerve supply affect the ability of the nervous system to maintain joint homeostasis is a key question, and is the

subject of much debate and research. The sensory and sympathetic fibres exert some of their influences by the release of neuropeptides and this concept is described below.

SENSORY FIBRES CONTAIN NEUROPEPTIDES

Unmyelinated C fibres arise from cell bodies which are located in the dorsal root ganglion, close to the spinal cord. In addition to its peripheral projection, the cell body also has a central projection to the spinal cord. The fine diameter nerve fibres terminate in the superficial layers of the spinal cord, laminae I and II of the dorsal horn, from where they synapse with ascending fibres in the spinal cord. The cell bodies are the site of manufacture of neuropeptides which are transported both centrally and peripherally along the nerve axons. The functions of these peptides are not clearly understood in the central nervous system but some, for example substance P, are thought to be involved in the modulation of pain transmission. Peripherally, where the effects of these peptides are much easier to investigate, they are clearly involved in the inflammatory process—both its induction and its modulation. They have the capacity individually or in synergism with other neuropeptides or mediators to modulate, mediate or prime for an inflammatory response. In addition, they have an effect on vascular tone.

SENSORY AFFERENT FIBRES CAN HAVE AN EFFERENT FUNCTION

In addition to their sensory afferent function, the small unmyelinated fibres present in the synovium, and throughout the body, are able to release the neuropeptides they contain when challenged by a nociceptive stimulus. The production of a nociceptive stimulus within a sensory afferent fibre results in an orthodromic electrical signal (in the direction of the spinal cord) and results in a sensation of a dull pain. However, within the extensive peripheral branches of that same fibre, an antidromic electrical signal (away from the spinal cord) is propagated and this leads to the peripheral release of neuropeptides. This effect is known as the axon reflex. The peripheral release of neuropeptides stimulates an acute reaction—neurogenic inflammation.[6] The principal peptides known to be involved in the induction of neurogenic inflammation are substance P and calcitonin gene related peptide (CGRP). These peptides have a direct effect on the vasculature, causing vasodilatation and oedema, the classical weal and flare reaction. In addition, they may generate indirect effects mediated by stimulation of effector cells, particularly mast cells, with the release of histamine and other inflammatory mediators.

Substance P

In 1931, von Euler and Gaddum extracted from equine brain and intestine a material which was hypotensive and smooth muscle stimulating.[7] They designated this activity preparation P (for powderable) and in later publications referred to it as substance P. It was not until 1971 that the peptide was purified,[8] sequenced and later synthesised.[9]

Substance P is a small polypeptide of 11 amino acids and is one of a family of peptides, the tachykinins, which share four common amino acids at the carboxyl terminal. There appear to be two preprotachykinin (PPT) genes, the SP/NKA PPT I gene and the NKB or PPT II gene. Substance P is encoded by mRNAs from the PPT I gene, alternative RNA splicing resulting in the production of three (α, β and γ) mRNAs, all of which may produce substance P.[10] Substance P is distributed widely throughout the central and peripheral nervous system. In the central nervous system, it is believed to play a role in pain transmission, whilst in the periphery, vascular and numerous other effects have been reported.

IN VITRO INVESTIGATIONS

In vitro, the effects of substance P on a wide variety of cell types have been investigated. These data show release of inflammatory mediators from various cell types, including the degranulation of mast cells,[11] the simulation of prostaglandin E_2 and collagenase from synoviocytes,[12] (Lotz *et al* 1987), secretion of prostaglandins[13] and thromboxane[14] from macrophages, and the modulation of immunoglobulin production from lymphoid tissue.[15] Substance P is also reported to induce the release of interleukin-1 from cultured mouse macrophages.[16] Thus tackykinins may contribute to the maintenance of chronic arthritis. These activities of substance P required doses of the peptide in the nanomolar range, which is consistent with published figures for the dissociation constant of substance P with its receptor (0·5–2·0 nmol/l). Other cells, such as neutrophils, appear to be activated directly only by much larger concentrations of the peptide (>10 μmol/l), suggesting a lack of physiological relevance. However, smaller concentrations of substance P, in the nanomolar range, appear to be able to prime neutrophils to respond to other mediators. In particular, neutrophils have been shown to react, synergistically with substance P, to other chemotactic peptides such as complement-derived C5a or bacterial *N*-formyl-methionyl-leucyl-phenylalanine.[17] Collectively these results suggest that, in addition to a direct effect of substance P on several cell types, a second modulatory mechanism is also operative. The nervous system may therefore be capable of priming cells to respond to smaller doses of certain agents than might otherwise be the case, leading to an apparent hypersensitivity. This mechanism may be particularly important in allergic responses, such as that seen in asthma.

IN VIVO INVESTIGATIONS

As neuropeptides are synthesised in the dorsal root ganglion cell bodies, an alternative approach to determine the involvement of neuropeptides in arthritis would be to induce

an inflammatory response in a single joint and then monitor changes in neuropeptide levels in the dorsal root ganglia that supply that joint. In the case of the knee joint, nerve tracing studies indicated that L4 to L6 are the appropriate ganglia to examine. Using this method, Smith *et al*[18] found a 70% increase in immunoreactive substance P in the ipsilateral dorsal root ganglia. In a Freunds adjuvant model of hind paw inflammation, substance P concentration was found to be increased by 30–40% in the L4 to L6 dorsal root ganglia, five days after the induction of the inflammation.[19] In the same study the substance P content of the sciatic nerve supplying the inflamed paw was increased by 60–75%. The increased biosynthesis of substance P in the dorsal root ganglia of the monoarthritic rat is reflected in the finding of increases in the mRNA levels of preprotachykinin A, the precursor of substance P.[20] These data suggest that, in the early stages of the inflammatory response, the amounts of substance P both synthesised and transported to the inflammatory site are increased.

The involvement of substance P in arthritis has also been studied directly in animal models. Levine *et al*[21] found that infusion of substance P into rat knees increased the severity of adjuvant induced arthritis. This work was extended to show that the substance P receptor was directly involved in this effect. Further experiments will be described in the section on receptors.

EVIDENCE FOR RELEASE OF SUBSTANCE P IN HUMAN JOINT INFLAMMATION

One of the most obvious ways to implicate substance P, or any other neuropeptide, in arthritis is to demonstrate that the peptide is present in the inflammatory joint fluid. Many groups have reported the presence of substance P in synovial fluid, but agreement between the values obtained is poor. Of concern is the reliance on antisera prepared from rabbit plasma for the radioimmunoassay; because different studies have used a different antiserum, the results are not strictly comparable. Of even greater concern must be the handling of the specimens after withdrawal from the joint, as there are numerous enzymes within the joint which are capable of the degradation of substance P (see later). These enzymes are often unaffected by broad spectrum enzyme inhibitors, such as aprotinin, and may therefore continue to be active ex vivo. A further contribution to the confusion surrounding such measurements may be that the substance P is bound to serum albumin[22] in the joint fluid, and therefore the nature of the extraction procedure for the peptide is critical to the final amount observed. These reservations are substantiated by data demonstrating that concentrations of substance P in joint fluids are less than 4·7 pg/ml, which was the detection limit of the assay.[23] The study showed that the inhibition of degrading enzymes and correct extraction procedures were all-important in obtaining reliable results.

RECEPTORS FOR SUBSTANCE P

There are currently three pharmacologically distinct receptors for tachykinins, termed NK1, NK2, and NK3. They are usually defined by the rank order of potency of tachykinins in binding studies. Substance P has the greatest affinity for the NK1 receptor,[24] although it may interact with NK2 and NK3 receptors at higher concentrations. Substance P has effects on many different cell types, from which it may be concluded that each cell type possess a receptor, either constitutive or inducible.

Peptide receptors can be localised and their kinetics studied by quantitative in vitro receptor autoradiography with computerised image analysis.[25] Specific high affinity, low capacity binding sites are present on the endothelial cells of human synovial blood vessel. The ratio of the IC_{50} values for these sites for substance P, neurokinin A, and neurokinin B is 1·25:175:>1000, respectively, and is characteristic of the NK1 class of tachykinin receptor.[26] The interaction of substance P with NK1 receptors on endothelial cells may be important in the regulation of vascular tone. Vasodilatation induced by substance P has been shown to be endothelium dependent and is inhibited by *N*-monomethyl-L-arginine, indicating a probable mediation by nitric oxide, the endothelium derived relaxing factor.[27]

The distinct localisation of substance P receptors on endothelial cells in human synovium suggests that proinflammatory actions of substance P may be predominantly via direct vascular action.

PEPTIDE ANTAGONISTS

The involvement of substance P and its receptors in inflammation and arthritis has been studied using several animal models. Levine *et al*[21] found that infusion of substance P into rat knees increased the severity of adjuvant induced arthritis, but administration of the substance P analogue (D-Pro^2, D-Trp^{7,9})-substance P, a putative substance P receptor antagonist,[28] only produced moderate soft tissue swelling and osteoporosis. Lam and Ferrell[29] showed that the carrageenan model of acute joint inflammation could be virtually abolished by prior administration of the substance P antagonist D-Pro^4, D-Trp^{7,9,10}-substance P (4–11). It has also been shown that injection of capsaicin, the hot component of the chilli pepper, into the synovial cavity of the rat knee inhibited the inflammation seen after injection of substance P. Capsaicin causes the depolarisation of afferent C fibres, leading to the release of substance P and the degranulation of mast cells, but in this case may have been acting by causing a depletion of substance P receptors in the target tissue.[30]

NON-PEPTIDE ANTAGONISTS

Until recently, antagonists of the NK1 receptor were limited to peptide analogues, such as those described in the previous section. However, the commercial pressure to develop non-

metabolisable synthetic antagonists has led to the development of a number of compounds. The first to be described was CP-96,345 (from the Pfizer Company), arising from a high throughput chemical file screening strategy using a ^{3}H-substance P binding assay as the primary screen.[31] This compound is a potent, reversible and competitive antagonist of the NK1 receptor. In vivo studies show that the compound lacks agonist activity and is highly selective. Investigations using CP-96,345 have shown that NK1 receptors display heterogeneity: CP-96,345 has a greater affinity for human and guinea pig NK1 receptors than for those in the rat.[32 33] FK888 (Fujisawa Pharmaceuticals, Osaka, Japan) has similarly been found to have a greater affinity for guinea pig receptors than for rat receptors.[34] There is also evidence for differences in NK1 receptor specificities between different tissues of a single species,[35] and suggestions that receptor expression or affinity may be influenced by inflammation.[36] Results from studies on different tissues in different species or in different pathological states should therefore be interpreted with caution.

Degradation of peptides

The local activities of regulatory peptides depend on a combination of release and clearance from the vicinity of their receptors. Many peptides have a short half lives, rapid clearance being attributable to membrane bound peptidases. These enzymes have characteristic regional distributions, and their activities may vary during inflammation. Understanding the local topography of membrane bound peptidases and their relation to the sites of release and action of those peptide that may act as substrates is therefore essential to understanding peptidergic regulatory systems in the normal and diseased synovium.

Several peptide degrading enzymes have been localised to the synovium, including neutral endopeptidase (NEP), angiotensin converting enzyme (ACE), dipeptidyl peptidase IV (DPPIV), and aminopeptidase M (APM).

NEUTRAL ENDOPEPTIDASE

NEP (EC 3.4.24.11) is capable of the hydrolysis of many peptides, including substance P[37] and CGRP.[38] It is responsible for the majority of the degradation of substance P in the human synovium. This enzyme has been shown to be identical with the common acute lymphoblastic leukaemia antigen (CALLA)[39] and is the CD10 determinant.

NEP is an integral membrane metalloproteinase with a characteristic zinc binding motif in the extracellular carboxyterminal domain.[40] The enzyme hydrolyses peptide bonds at the amino side of hydrophobic amino acids,[41] and is believed to inactivate regulatory peptides at the cell surface. NEP has been hypothesised to modulate neurogenic inflammation, for example in the lung.[42] Its activity has been demonstrated in the synovium of patients with chronic arthritis,[43] and was found to be greater in all patients with rheumatoid arthritis and some patients with degenerative joint disease, compared with traumatic arthritis controls. Since mature B and T lymphocytes and macrophages, which constitute the major inflammatory cell populations in rheumatoid arthritis, contain no detectable NEP activity,[44] it has been speculated that synovial fibroblasts may be the source of this enzyme. Our own data show the localisation of this enzyme in human synovium, as determined by immunocytochemistry, and support the hypothesis that fibroblasts are its source, a restricted population of cells surrounding blood vessels being responsible for the majority of the activity.[45] These may represent a specialist subpopulation of fibroblasts.

As the function of NEP is probably the degradation of locally released regulatory peptides, its presence around the blood vessels makes it ideally located to inactivate vasoactive peptides, such as substance P, which are released from perivascular nerve fibres. There is good evidence from in vivo investigations to support this hypothesis. Substance P in induced oedema in guinea pig skin is potentiated by the NEP inhibitors phosphoramidon and thiorphan,[46] and attenuated by coadministration of NEP. Furthermore, NEP may also limit the activity of endogenously released regulatory peptides. The bronchial contraction induced by capsaicin, which acts by releasing neuropeptides, including substance P, from peripheral nerve endings, is also enhanced by phosphoramidon.[47 48]

ANGIOTENSIN CONVERTING ENZYME

ACE acts as a peptidyl peptidase, inactivating bradykinin and activating angiotensin I by removal of C-terminal dipeptides. In addition, ACE can act as an endopeptidase, cleaving substance P to liberate the C-terminal di- or tripeptide, and subsequently as a peptidyl peptidase, cleaving successive dipeptides from the unprotected C-terminus.[49] The localisation of ACE to the endothelia of all vessels, with the most intense staining on capillaries and arterioles, is similar to that seen in other tissues.

DIPEPTIDYL PEPTIDASE IV

The serine protease DPPIV is identical to the leucocyte antigen CD26[50] and cleaves unprotected N-terminal dipeptides where the penultimate residue is proline. In the case of substance P, this may not abolish biological activity, which resides at its C-terminus, but renders it susceptible to further inactivation by successive cleavage of further N-terminal peptides by APM.

AMINOPEPTIDASE M

APM has been identified as the leucocyte antigen CD13 and also participates in the inactivation of enkephalins.[51] In the inflamed synovium, APM is found in a distribution similar to that of NEP—that is, in spindle

shaped fibroblast like cells surrounding the blood vessels, and also in similarly shaped cells in a layer underlying the hypertrophic intimal cell layer.

FUNCTIONAL COMPARTMENTALISATION

The localisation of membrane peptidases in the human synovium suggests a role, not only in limiting the duration of action of regulatory peptides, but also in localising their activities to the vicinity of their release. This functional compartmentalisation[52] of vascular and stromal regions in synovium is essential to the local regulatory function of peptides and is likely to influence responses to exogenous peptide administered into non-physiological compartments in experimental investigations. Depletion of neuropeptide immunoreactive nerves in the chronically inflamed synovium implies a loss of normal neurovascular regulation, and the abundance of membrane peptidases in inflamed synovial tissue would be expected to exacerbate this situation further. Inhibition of specific peptidases may have a therapeutic role in restoring the protective effects of vasoactive regulatory peptides. Inhibitors of aminopeptidases have anti-inflammatory activity in vivo and the specific ACE inhibitor, captopril, may have slow anti-rheumatic activity.[53]

Pain

Inflammatory mediators may directly stimulate or sensitise the normally silent nociceptive fibres to perceive and react to pain. Such mediators include bradykinin, 5-hydroxytryptamine (serotonin), histamine, and protons (reviewed recently[54]). Undoubtedly, pain in arthritis may arise by such a mechanism. However, if peripheral nerve terminals are damaged by the inflammatory response, other mechanisms may be operative. One such possibility is described below.

As previously mentioned, the nociceptive C fibres have their input into laminae I and II of the dorsal horn of the spinal cord, giving rise to the notion that this anatomical area of the spinal cord is responsible for pain sensation. In rheumatoid arthritis the peripheral terminals of nociceptive fibres are damaged. The central terminals may also be affected; in animal models of axotomy, peripheral nerve damage leads to the withdrawal of central terminals of nociceptive fibres.[55] Furthermore, the laminae I and II become occupied by nerve fibres which sprout in from the adjacent laminae III and IV; such nerve fibres are Aβ fibres and, in the joint, are responsible for proprioception. Thus the possibility arises that proprioceptive nerve fibres have an input into a nociceptive area of the spinal cord. The result of this may be pain on normal range of movement.

Conclusion

This review has considered selected aspects of neuropeptides as they relate to inflammation within the joint. Arguments persist as to the relevance of acute effects of neuropeptides, as opposed to their contribution to chronic disease such as rheumatoid arthritis. Furthermore, it is not clear if some aspects of their action are protective or deleterious. For instance, there is evidence to suggest that oedema formation in adjuvant arthritis of the rat is protective, as increasing oedema correlates with a decrease in severity of joint disease, as judged by radiographic scores. The overall view is that the acute effects of neuropeptide release are protective. The chronic aspects of the involvement of the nervous system in joint disease remain poorly understood, but may well be characterised by the loss of the protective features of acute inflammation.

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