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ORIGINAL ARTICLE

Presence of NGF and its receptor TrkA in degenerative lumbar facet joint specimens

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Abstract In a preliminary study, the recurrent presence of nervous terminations was demonstrated with optical microscopy in several slides of degenerative lumbar facet joints and surrounding soft tissues. The purpose of this study was to prove the presence of NGF (nerve growth factor) and its receptor TrkA (tyrosine kinase receptor) with immunofluorescence. The peri/articular tissues were harvested from the lumbar facet joints of ten patients surgically treated for degenerative diseases. There were seven females (one bilateral) and two males whose mean age at surgery was 72 years (range, 67–80 years). The affected levels were L3–L4 in two cases and L4–L5 in seven cases (one bilateral). All specimens were fixed in formalin, dehydrated and enclosed in paraffin. From each specimen, four slides were obtained. Two slides were employed for the search of NGF: one was treated with specific antibodies and marked with FITC (fluorescein isothiocyanate conjugated), and the second slide was for control purposes. It was exposed to FITC, but without prior exposure to the specific antibody. The same procedure was repeated to obtain on two more slides, to repeat the search for Trka with specific antibodies. All the slides were finally studied on a fluoromicroscope. The analysis of these specimens revealed the presence of the neurotrophin (NGF) and its own receptor (TrkA) in all cases: the immunohistochemical reaction between the specimens and the specific antibodies marked with FITC was seen under fluoromicroscopy, but in none of the control cases treated with FITC only. NGF is released by mastocytes, fibroblasts and other cell types involved in the inflammatory processes. The level of peripheral NGF is increased in inflammatory processes, while the administration of exogenous NGF has a hyperalgesic effect on rats and produces muscular pain in humans. Furthermore, NGF produces hypersensitization to heat stimulation in humans and mammals in general. There is considerable evidence showing that the system constituted by the NGF and its high-affinity receptor TrkA plays a fundamental role in the molecular processes underlying the main forms of "persistent" pain. This indicates a possible therapeutic area for the antibodies that could block the NGF/TrkA system, in order to modulate the frequency and the duration of the action potential of nociceptive neurons during chronic inflammation. This study demonstrated the presence of NGF and TrkA in specimens collected from degenerative facet joints, suggesting that specific molecules could be used in order to modulate chronic pain in patients with degenerative lumbar spine.

Keywords Nerve growth factor · Receptor trkA · Spine · Low back pain · Osteoarthritis

Introduction

Nerve growth factors (NGF) were characterized more than four decades ago and belong to the neurotrophins family. They are small homodimeric polypeptides with a varied repertoire of biological activities influencing the generation, differentiation, survival and regeneration of vertebrate neurons [1, 2]. They are closely structurally related: NGF, BDNF, NT-3 and NT-4 share approximately 50% of the sequence identity. The regions of sequence similarity and variation are clustered, indicating probable regions of structural and functional importance.

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Neurotrophins bind to two different classes of transmembrane receptor proteins, the Trks and the neurotrophin receptor p75. This dual system allows the transduction of very different signals following binding, such as signaling cell death through p75 or cell survival through the Trk receptors.

The Trk receptors belong to the family of receptor tyrosine kinases, and three *trk* genes have been identified in mammals. The TrkA protooncogene was first identified as an NGF receptor [3, 4], followed by TrkB and TrkC [5]. NGF is the preferred ligand for TrkA; BDNF and NT4/5 are preferred for TrkB; and NT3 for TrkC [12]. These specificities are not absolute, and NT3 is also a ligand for TrkA and TrkB.

P75 was the first member to be molecularly cloned [6] of a large family of receptors, which includes both TNF receptors, Fas (Apo-1/CD95) and CD40, and 15 other members [7]. The defining motifs of this receptor family are cysteine repeats in the extracellular domain, which form the ligand-binding domain.

Binding of the neurotrophins to the Trk receptors leads to receptor tyrosine phosphorylation [8]. This triggers the activation of pathways leading to the prevention of programmed cell death and neuronal differentiation. Ligand-induced dimerization [9] results in the phosphorylation of specific tyrosine residues that leads to an open conformation of the receptor, resulting in *trans*-phosphorylation and allowing the access of substrates to the kinase. Phosphotyrosine residues on Trk receptors then act as docking sites for adapter molecules.

Tissue inflammation is typically accompanied by hyperalgesia and pain. It increases the sensitivity of high-threshold receptors, so that stimuli of lower intensity than normal can activate them, and NGF is implicated in the regulation of hyperalgesia. In particular, systemic injections of large quantities of NGF into rats cause hypersensitivity to noxious heat and mechanical stimuli that persists for days [10]. Similar observations also were made with humans with lower doses of NGF [11]. The role of NGF in inflammation and pain has been documented by the following observations:

- the levels of NGF in damaged or inflamed tissue are markedly increased [12, 13];
- cytokines typically involved in tissue damage and inflammatory processes, such as interleukin-1b (IL-1b), increase NGF levels, both in vitro [14] and in vivo [15];
- blockade of endogenous NGF with antibodies or similar reagents prevents both heat and mechanical hyperalgesia, which normally follows tissue inflammation, without affecting the inflammation itself 16, 17].

The basis for the hyperalgesic action of NGF appears to include an up-regulation of peptide neurotransmitters expressed by nociceptors, such as calcitonin gene-related peptide (CGRP) and substance P. Both are clearly regulated by the availability of NGF and contained in a subset of NGF-responsive DRG neurons expressing NGF-specific receptors [18]. Also, NGF applied to peripheral targets increases the excitability of spinal cord neurons to activation of the treated afferents. Finally, mast cells express trkA receptors, and NGF triggers the release of their granules, which contain substances, such as histamine and serotonin, capable of sensitizing nociceptors [14, 19].

A preliminary study with optical microscopy conducted on several specimens taken from degenerative lumbar facet and surrounding soft tissue proved the presence of abundant nervous terminations in patients undergoing surgery for chronic low back pain (CLBP). From a review of literature, the role of NGF in inflammation and pain and also its presence in inflammatory chronic diseases such as asthma, BID and others has emerged.

The purpose of this descriptive study was to prove the presence of "in vivo" NGF and its receptor TrkA with immunofluorescence in peri/articular tissues harvested from lumbar facet joints of patients affected by CLBP.

Materials and methods

Several biopsy samples of peri/articular tissues were obtained from the lumbar facet joints and surrounding tissue of ten patients surgically treated for degenerative diseases. There were seven females (one bilateral) and two males whose mean age at surgery was 72 years (range, 67–80 years). The affected levels were L3–L4 in two cases and L4–L5 in seven cases (one bilateral).

From each specimen, four slides were obtained. Two slides were employed for the search of NGF: one was treated with specific antibodies and marked with FITC, nd the second slide was for control purposes and was exposed to FITC, but not to the specific antibody. The same procedure was repeated to obtain two more slides in order to repeat the search for Trka.

All specimens were fixed in formalin and embedded in paraffin; then, 4–6μm of tissue sections were cut and applied to the slides. They were deparaffinized in xylene using three changes for 5 min each; the sections were hydrated gradually through graded alcohol: washed in 100% ethanol twice for 10 min each, then 95% ethanol twice for 10 min each. Then, they were washed with deionized H_2O.

All slides were incubated with 10% normal blocking serum (normal goat serum) in PBS (phosphate-buffered saline) for 20 min to suppress non-specific binding of IgG. Blocking serum ideally should be derived from the same

species in which the secondary antibody was raised and then washed with PBS.

The group of specimens used to reveal the presence of NGF have been incubated with antibody anti NFG (NGF (H-20): sc-548 Santa Cruz Biotechnology®, Inc) for 60 minutes. Optimal antibody concentration should be determined by titration; then they have been washed with three changes of PBS for 5 minutes each. The control group was not treated with the antibody anti NFG.

All these specimens were incubated for 45 min with fluorochrome-conjugated secondary antibody (goat anti-rabbit IgG-FITC: sc-2012; Santa Cruz Biotechnology®, Inc) diluted to 1:750 in PBS in a dark chamber, washed extensively with PBS and finally all the slides were observed with a fluoromicroscope.

The same procure was performed to reveal the presence of Trka: in this case antibodies anti TrkA (TrkA (763): sc-118 Santa Cruz Biotechnology®, Inc) were used first. Mouse anti-rabbit IgG (Santa Cruz Biotechnology®, Inc), conjugated with fluorochrome was then employed.

In order to confirm that the presence of both NGF and Trka in facet joints was associated with CLBP, a sample harvested from a 45-year-old patient, treated surgically for a disk herniation and not suffering from CLBP, was processed according to the method described. All the slides were finally studied under a fluoromicroscope (Microscope Leitz, Aritoplan) for the presence of a positive immunoistochemical reaction.

Results

The analysis of these specimens revealed the presence of the neurotrophin (NGF) and its receptor (TrkA) in all the cases. The immunohistochemical reaction between the specimens and the specific antibodies marked with FITC was seen under fluoromicroscopy, but in none of the control cases treated with FITC only. In addition, in the case of the younger patient, neither the presence of NGF nor of its receptor TrkA could be demonstrated.

Discussion

Inflammatory pain is a multifaceted syndrome that comprises distinct components: spontaneous pain referred to the site of the inflammation, an amplification of the response to noxious stimuli, hyperalgesia, and finally allodynia, the generation of pain by normal entity stimuli. These last two components are manifest both in the inflamed tissue and in the surrounding non-inflamed tissue. A number of different mechanisms operating at different times and sites contribute to inflammatory pain: direct activation of chemosensitive nociceptors by irritants or inflammatory mediators will elicit spontaneous pain[20]; an alteration in transduction sensitivity of nociceptors by sensitizing mediators like bradykinin or prostaglandin will contribute to primary hyperalgesia[21]; NGF may contribute to inflammatory hypersensitivity by producing both local and central changes in sensitivity.

Several pathological conditions, particularly those caused by tissue injury or inflammation, are associated with the tonic activation and sensitization of these sensory neurons, with lowered thresholds for activation and vigorous responds to noxious stimuli.

Prostaglandins, bradykinin, serotonin, histamine, hydrogen ions and NGF can lead to this sensitization of nociceptors. Many of these agents are released into the damaged tissue. The rapid onset of hyperalgesia after subcutaneous injections of NGF strongly suggests a peripheral action. The ability of NGF to induce neurogenic extravasation [22, 23] also strongly suggests such a role. Finally, the direct electrophysiological observations of sensitization of primary sensory neurons clearly demonstrates this effect [24].

It has been demonstrated that many nociceptors express the trkA receptor [25]. It is possible that sensitization occurs following the direct binding and activation of this receptor by NGF. However, there are other cellular elements in peripheral tissues, which express the trkA receptor and it is therefore possible that sensitization of nociceptors arises indirectly. TrkA receptors are known to be expressed by both sympathetic postganglionic neurons and by some mast cells [26], and NGF is known to be a potent degranulator of mast cells [26].

The demonstration of the presence of NGF and its specific receptor Trka, in peri/articular tissues from lumbar facet joint, according to the most recent literature[27], is an original finding.

A control group, considering the difficulty of collecting samples from unaffected patients, was judged not necessary and consequently no statistical analysis was performed in this descriptive study.

Moreover, the only control case of a younger patient, which was possible to observe was negative for the presence of both NGF and Trka. This suggests that the simultaneous presence of the neurotrophin and its specific receptor play a role in the tissues where chronic inflammation and hyperalgesia processes are generated and self-maintained.

This study describes the unvarying presence of NGF and TrkA in specimens collected from degenerative facet joints and surrounding tissues in patients undergoing surgery and affected by chronic low back pain, thus suggesting that specific molecules could be used in order to modulate CLBP in patients with degenerative affections of the lumbar spine.

Conflict of interest statement None of the authors has any potential conflict of interest.

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