Chapter 2

Possible nociceptive structures in the sacroiliac joint cartilage: An immunohistochemical study.

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Abstract

The sacroiliac joint (SI joint) is a known source of low back pain. In the absence of validated physical signs and imaging studies, the diagnosis of SI joint pain can be secured by positive response to SI joint intra-articular infiltration with local anesthetics. The current anatomical and histological knowledge concerning intra-articular structures of the sacroiliac joint is insufficient to explain the efficacy of this infiltration. Consequently, this study was undertaken to detect the intra-articular presence of substance P and Calcitonin Gene-Related Peptide (CGRP) positive nerve fibers, providing indirect evidence of nociceptive innervation of the SI joint. Free-floating sections, obtained from iliac and sacral cartilage and subchondral bone of the SI joint and adjacent ligamentous tissue, of 10 human cadavers were studied immunohistochemically. Tissue of 9 human cadavers showed the presence of substance P and CGRP immunoreactivity in the superficial layer of sacral and iliac cartilage, and the surrounding ligamentous structures. Subchondral bone reacted weakly to the antisera used. These findings support the view that the SI joint may be capable of intra-articular nociception and may explain the positive response to the intra-articular deposition of local anesthetic.

Key words

SI joint; substance P; Calcitonin Gene-Related Peptide; low back pain
Introduction

The sacroiliac joint (SI joint) is a known source of low back pain (LBP) with a reported prevalence of 16 to 30% in patients.\textsuperscript{1-3} In the absence of a specific cause of SI joint pain, such as ankylosing spondylitis, osteoarthritis, malignancy, and/or inflammation, the diagnosis is difficult to confirm without controlled diagnostic blocks.\textsuperscript{2-4} The positive outcome of this infiltration is likely to rely on reaching and subsequent blocking of pain-signaling structures. However, reports in the literature evaluating the (nociceptive) innervation of the SI joint in humans are insufficient. Histological studies of SI joint ligaments showed the presence of sensory nerves in the ventral capsular ligament \textsuperscript{5}, and in the dorsal ligamentous tissue adjacent to the posterior superior iliac spine. \textsuperscript{6,7} These results have been supported by investigations in various animal studies. \textsuperscript{8-11} The above-mentioned findings, however, only concern ligamentous structures surrounding the SI joint cavity, and do not explain pain relief after intra-articular injection. Apparently, the present knowledge of the anatomy of pain stemming from the SI joint is insufficient to explain the effect of intra-articular infiltration with local anesthetics.

Extrapolating from general anatomical knowledge, sensory A-\(\delta\) and C fibers can be found in ligaments, joint capsule, and/or subchondral bone. \textsuperscript{12} Although normal articular cartilage is considered to not be supplied with nerves, recent investigations showed nerve fibers in hyaline cartilage and fibrocartilage immunoreactive for calcitonin gene-related peptide (CGRP) in rat pups. \textsuperscript{13} Another study showed substance P and CGRP immunoreactive nerve fibers in perichondrium and in cartilage canals. \textsuperscript{14} Moreover, substance P together with NK1 receptor reactivity was observed in deep lying chondrocytes of human adult articular cartilage. \textsuperscript{15} Although the presence of CGRP and/or substance P was explained in the above-mentioned studies as related to development \textsuperscript{14}, maintenance, and remodeling of cartilage \textsuperscript{13,15}, these substances are also found in sensory neurons that play a role in pain perception. \textsuperscript{16,17}

Searching for an explanation of SI joint pain, we performed an immunohistochemical study in human cadavers in which we found substance P and CGRP positive nerve fibers in the SI joint anterior capsular ligament and interosseous ligament. \textsuperscript{18} In this study we also found immunoreactive structures in sacral cartilage of a single specimen. To verify our findings we extended our study to examine the distribution of substance P and
CGRP immunopositive structures in iliac and sacral cartilage and subchondral bone of SI joints in humans.

Materials and Methods

Cadavers. Tissue was harvested from 10 human cadavers donated to the Department of Anatomy of the VU University Medical Center Amsterdam between 2004-2006. There were 7 males and 3 females aged between 60 to 90 years (mean 69.8). Nine bodies were embalmed by arterial perfusion within 24 hours post-mortem using 7 liters of perfusion solution containing salicylic acid, alcohol, Sal Carolinum Factitium, thymol, chloral hydrate, and formaldehyde. One body was dissected fresh (12 hours post-mortem) and tissues were fixed by submerging in 4% buffered formaldehyde. A history of arthritis involving the knees, hips, and/or cervical spine was recorded in three cases. No notation of SI joint pain or any disease process affecting the SI joints was recorded for any of the cases.

During the preparation, skin, subcutaneous tissue, muscles, vessels, and the lumbo-sacral plexus were dissected ventrally, enabling a visually guided approach to the ventral part of the SI joint. Tissue blocks (3 cm x 2 cm x 2 cm) of sacral and iliac bone and cartilage were collected from the ventral part of the SI joint at the level of the pelvic brim. All cadaveric tissue was handled in accordance with regulations of the VU University Medical Center in Amsterdam concerning the use of human material.

Tissue preparation. Material obtained from embalmed and unembalmed cadavers was post-fixed in 4% buffered formaldehyde for up to 4 days. After the tissue blocks were rinsed with phosphate buffer (PB), they were decalcified in Kristensen’s solution (13 g sodium formate, 100 ml formic acid 98-100%, 400 ml distilled water). After decalcification, which was accomplished within 22 days (range, 16-28 days), samples were rinsed over 24 hours with PB and immersed in 30% sucrose in PB to cryoprotect the tissue cell structure for another 24 hours. Subsequently, tissue was embedded in 10% gelatin with 30% sucrose in PB. The gelatin tissue blocks were then placed in 4% paraformaldehyde and 30% sucrose in PB for 24 hours until satisfactory cryoprotection was reached (when the tissue no longer floated in the sucrose solution). Blocks were
finally frozen, cut at 40 μm thick on a freezing microtome, placed in vials containing 30% sucrose in PB, and stored in the freezer at -20°C.

**Neuropeptides.** Substance P belongs to the tachykinin group and acts as an NK1 receptor agonist. It is released in response to noxious stimuli, but also plays a role in anxiety, nausea, and vomiting. 20 CGRP is a neuropeptide that acts on different types of CGRP receptors, resulting in nociception, vasodilation, glucose uptake, and glycolysis. 16 Substance P can be co-localized with CGRP in primary sensory neurons.

**Immunohistochemistry protocol for free-floating sections.** Defrosted sections were first rinsed with PB and 50 mM Tris-buffered saline, pH 7.6 (TBS) (Merck, Sigma). Endogenous peroxidase activity was reduced by treating the sections in a 1% solution of hydrogen peroxide (Sigma) in TBS at room temperature for 15 min. Sections were subsequently rinsed 3 times 10 min with TBS and were treated with 5% normal goat serum (DAKO Cytomation code no. X0907) blocking solution in TBS-tx (triton X-100) over 20 min at room temperature. The blocking solution was poured off and every second section was allowed to react for 20 hours at 4°C with the primary antisera against either substance P (Chemicon International Inc., Temecula, catalog no. AB1566) or CGRP (Chemicon International Inc., Temecula, catalog no. AB5920), diluted 1:2000 and 1:1000, respectively. Antibody specificity was previously validated with absorption tests. 21,22 The sections were rinsed with TBS, pH 7.6, and reacted with biotinylated goat anti-rabbit IgG (DAKO Cytomation, Denmark, code no. E0432) diluted at 1:200 for 1 hour at room temperature and washed thoroughly in TBS, pH 7.6. The sites of antibody binding were visualized using the avidin-biotin peroxidase method (ABC Standard kit, Vectastain, Vector Labs) diluted at 1:200 for 1 hour at room temperature. The sections were then rinsed with TRIS-HCl buffer, pH 7.6, and treated with 3,3′-diaminobenzidine (DAB) (Sigma) until the desired color intensity was achieved (10-12 min). Thereafter, the sections were mounted on slides and allowed to dry at room temperature. The sections were counterstained with Nissl-thionin stain and cover-slipped with Entellan. In total, 1047 sections of SI joint bone and cartilage approximately 1 cm x 1.5 cm x 40 μm in dimension were stained: 507 sections with substance P, and 540 with CGRP. The sections were examined with a light microscope (Zeiss) by K.S., verified by P.V.J.M.H.,
and photographed with a Leica DMR microscope. A representative photomicrograph of SI joint cartilage and histologic description of structures is presented in Figure 1.

**Figure 1** Photomicrograph of sacral cartilage with a schematic representation of structures discussed in this study. 1- superficial tangential zone, 2- middle transitional zone, 3- deep radial zone, 4 calcified cartilage, 5- subchondral bone, 6- chondrocytes, 7- ground substance.

*Control tissue.* Control staining was processed along with the study protocol. For positive controls, sections of a cervical spinal cord were used as substance P and CGRP are widespread in laminae I and II of the dorsal horn. Additionally, for negative controls, 3 sections of the series were stained without the primary antiserum.

**Results**

*Technical notes.* Different stages of degenerative changes in the articular cartilage were seen in the sections, varying from small superficial disruptions and fissures to erosion
of the articular surface down to the subchondral bone. The hyaline cartilage often showed superficial fibrillation and damaged parts were filled with irregular fibrous tissue. In spite of the degenerative changes, the articular surfaces were quite easy to separate and no ankylosis was encountered. Occasionally, osteophytes of iliac and/or sacral bone were seen (see Table 1). Sections of one specimen (208) could not be evaluated because of a lack of cartilage tissue in the studied sections and this case was excluded from further evaluation.

**Immunoreactivity for substance P and CGRP.** The results of the immunoreactivity of tissues obtained from the cadavers are presented in Table 1. Forty-five sections showed immunopositivity for CGRP and 32 for substance P. Both the loose and dense connective tissue showed substance P and CGRP positive structures. Solitary immunoreactive structures were found in the peri-articular ligamentous tissue close to blood vessels. The superficial zone of cartilage of two specimens stained positively with both substance P and CGRP antisera (Figs. 1 and 2), and in both cases iliac cartilage reacted better with applied antisera. Furthermore, in another six specimens, either substance P or CGRP positive, beaded fibers were found in the superficial zone. The dimension of the positive fibers varied from 0.8 to 4 μm thick. Immunoreactivity was also observed in chondrocytes of four specimens in the middle and deep cartilage zones. Because the observation of substance P and CGRP positive tissue in superficial cartilage in our samples was quite unique, we also evaluated samples obtained from the knees of two specimens (219 and 279) showing positive immunoreactivity in SI joint cartilage. In these knee samples, no immunoreactivity for substance P or CGRP could be found in the cartilage.

In three sections, beaded fibers approximately 1.5 μm thick and immunoreactive for CGRP were identified in sacral subchondral bone. One substance P immunoreactive fiber was detected in the sacral bone. Spinal cord sections, used as controls for the staining procedure, stained positive. When the first antibody was omitted from the staining procedure, DAB staining was absent.
<table>
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<tr>
<th>Cadaver</th>
<th>Obtained tissue samples</th>
<th>CGRP (N)</th>
<th>Substance P (N)</th>
<th>Cartilage structure*</th>
<th>Cells*</th>
<th>Cartilage-bone integrity*</th>
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</thead>
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<tr>
<td>168</td>
<td>Right SI joint</td>
<td>Negative</td>
<td>Negative</td>
<td>Irregular surface, fissures, superficial zone fibrosis</td>
<td>Hypocellular iliac cartilage, chondrocyte clusters in the middle zone</td>
<td>Crossed by blood vessels</td>
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<td>191</td>
<td>Right SI joint</td>
<td>Negative</td>
<td>Positive (2) Superficial zone</td>
<td>Superficial zone fibrosis</td>
<td>Small chondrocytes, no clusters</td>
<td>Intact</td>
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<tr>
<td>197</td>
<td>Right SI joint</td>
<td>Positive (2) Superficial zone</td>
<td>Negative</td>
<td>Superficial zone fibrosis</td>
<td>Clusters of chondrocytes neighboring the fissures</td>
<td>Locally denuded bone</td>
</tr>
<tr>
<td>199</td>
<td>Right SI joint</td>
<td>Positive (1) Chondrocytes</td>
<td>Positive (4) Superficial zone</td>
<td>Irregularities and superficial zone fibrosis</td>
<td>Hypocellularity</td>
<td>Irregular</td>
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<td>208</td>
<td>Right SI joint</td>
<td>Excluded</td>
<td>Excluded</td>
<td>No cartilage</td>
<td>Hypocellular</td>
<td>Occasionaly denuded bone</td>
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<tr>
<td>219</td>
<td>Left SI joint</td>
<td>Positive (15) Chondrocytes</td>
<td>Positive (4) Superficial zone</td>
<td>Fissures to transitional zone in sacral cartilage</td>
<td>Hypocellular</td>
<td>Occasionaly denuded bone</td>
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<td>Page</td>
<td>Joint</td>
<td>Status</td>
<td>Number</td>
<td>Location</td>
<td>Changes</td>
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<tr>
<td>225</td>
<td>Left SI joint</td>
<td>Negative</td>
<td>1</td>
<td>Superficial zone</td>
<td>Chondrocytes</td>
<td>Irregular and locally disturbed by blood vessels ingrow</td>
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<td>Clusters of chondrocytes in ilium, diffuse</td>
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<td>hypercellularity in sacrum</td>
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<td>229</td>
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<td>Negative</td>
<td>13</td>
<td>Superficial zone</td>
<td>Surface irregularities in sacral cartilage, fissures in the iliac cartilage</td>
<td>Irregular</td>
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<td>Diffuse hypercellularity in sacral cartilage, clusters in iliac cartilage</td>
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<td>232</td>
<td>Right SI joint</td>
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<td>7</td>
<td>Superficial zone</td>
<td>Irregularity and fissures</td>
<td>Intact</td>
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<td>Clusters of chondrocytes</td>
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<td>279</td>
<td>Left and right SI joint</td>
<td>Positive</td>
<td>20</td>
<td>Superficial zone</td>
<td>Fissures and clefts to calcified cartilage</td>
<td>Irregular, crossed by blood vessels</td>
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<td>Clusters in the middle zone</td>
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**Table 1.** Presence of immunoreactivity in tissue samples. *Assessment of cartilage degenerative changes, N-number of immunopositive sections.*
Figure 2. Photomicrograph of the iliac (A and B) and sacral (C and D) cartilage stained for CGRP. The arrowheads point out the positive nerve fiber like structures (A) and the superficial immunoreactive tissue (B-D). Bar = 50μm

Discussion
The present study aimed to demonstrate the distribution of sensory nerves in iliac and sacral bones and cartilage of the SI joint in order to obtain new insights concerning the origin of intra-articular SI joint pain. The microscopic analysis revealed substance P and CGRP positive fiber-like structures in the iliac and sacral cartilage and immunoreactivity in chondrocytes. Immunoreactive fibers were further identified in the fibrous ligamentous tissue adjacent to the cartilage and bone. The bone itself was weakly immunoreactive for the employed antisera. Incubation of adjacent sections without the primary antibody against substance P and CGRP resulted in the absence of positive structures, whereas positive control sections of cervical spinal cord stained strongly.
Although cartilage is said to be non-neural and avascular, a robust growth of neurovascular tissue into knee cartilage was recently reported in humans with advanced osteoarthritic changes. Ingrowth of nerve fibers into damaged fibrous cartilage of intervertebral discs has previously been described by Coppes and coworkers. Although sections used in our study were primarily of hyaline cartilage origin, we saw fibrous degenerative changes related to aging. Also, the expression of substance P and NK1 receptor in human chondrocytes was shown in sections obtained from normal and osteoarthritic adult knee joints. In newborn rats, substance P and CGRP immunoreactive fibers were found in the knee perichondrium in close contact with chondrocytes. Another study in rats demonstrated the presence of nerve fibers in different cartilaginous tissues, predominantly immunoreactive to CGRP. Substance P and CGRP are involved in a variety of biological processes, such as maturation.
maintenance \(^{15}\), or repair of articular cartilage. \(^{13}\) Their immuno-expression may also indicate the presence of sensory nerve fibers which contribute to pain sensations.\(^{23}\) In our studies we found intra-articular substance P and CGRP immunoreactivity on the surfaces of the SI joint cartilage, which has not been reported previously. If the structures that displayed immunoreactivity for substance P and CGRP are indeed nerve fibers, these findings might (in part) explain pain sensations originating from intra-articular structures of the SI joint. Although we did not find sensory nerves in subchondral bone in the present study, we observed occasional positive fibers in the bone marrow, but this does not explain pain originating from the SI joint. We did not use a neural specific stain against nonphosphorylated neurofilament (SMI-32) antibodies in the present study because it does not reveal thin nerve fibers.\(^{18}\)

We observed degenerative changes in our samples, which may be related to degenerative changes caused by normal aging \(^{25}\) or osteoarthritis. While degenerative changes caused by normal aging are not necessarily associated with nociceptive expression, osteoarthritic changes may lead to increased expression of nociceptive structures in cartilage.\(^{23,24}\) The immunoreactivity of our samples with substance P and CGRP could therefore represent an extracellular deposit of these neuropeptides related to degenerative changes (vascularization) of the cartilage. Although SI joint-related pain was not reported in the patients’ records, it may still be possible that osteoarthritic processes occurred in the specimens’ (medical) history. We screened the cellular and architectural changes and tidemark integrity of our sections according to the Histologic/ Histochemical Grading System as introduced by Mankin and colleagues \(^{26}\), but we were not able to score the distribution of safranin-O, since we used Nissl counterstaining. Therefore, it is difficult to establish any relationship between possible SI joint osteoarthritis and the presence of the superficial nerve fibers immunoreactive for substance P and CGRP.

On the other hand, degenerative changes associated with normal aging have been reported to decrease the density of sensory nerve fibers.\(^{27}\) For the present study, we used samples taken from sacroiliac joints of cadavers 60-91 years old. At this age, many structural changes may occur principally in the iliac but also in the sacral articular facet.\(^{25}\) Degenerative changes of cartilage increase with age and include fibrillation, reduced
density of chondrocytes, occurrence of deep fissures in the cartilage, and fibrous
cornections between the auricular facets. 25 Although some of the sections observed in
the present study showed advanced cartilaginous changes, such as deep fissures and
demuded bone, the majority of sections indicated only moderate degeneration.

Taken together, our findings of intra-articular structures immunoreactive for substance
P and CGRP may provide the chemical substrate that verifies the presence of intra-
articular nerve fibers sensitive for pain, and support the theory that nociceptive signals
may originate from the intra-articular structures of the SI joint. Whether these findings
are related to osteoarthritic changes and /or related to normal aging remains to be
established. Consequently, it is necessary to examine younger specimens in future
studies. Furthermore, our results may encourage future studies on the presence of
neuropeptides in intra-articular cartilage of other joints using more advanced
techniques like electron microscopy immunohistochemistry.

Acknowledgments
We would like to extend profound thanks to Evelien Huisman-Timmermans for her
suggestions, comments, and technical assistance in implementation of the
immunohistochemistry staining. The work on this paper was performed in
collaboration with the Pain Management and Research Center, University Hospital
Maastricht, The Netherlands.

The authors have no financial interest in or financial conflict with the subject matter or
materials discussed in the submitted manuscript.
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