Original Full Length Article

Behavioral signs of pain and functional impairment in a mouse model of osteogenesis imperfecta

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A B S T R A C T

Osteogenesis imperfecta (OI) is a congenital disorder caused most often by dominant mutations in the COL1A1 or COL1A2 genes that encode the alpha chains of type I collagen. Severe forms of OI are associated with skeletal deformities and frequent fractures. Skeletal pain can occur acutely after fracture, but also arises chronically without preceding fractures. In this study we assessed OI-associated pain in the Col1a1Jrt/+ mouse, a recently developed model of severe dominant OI. Similar to severe OI in humans, this mouse has significant skeletal abnormalities and develops spontaneous fractures, joint dislocations and vertebral deformities. In this model, we investigated behavioral measures of pain and functional impairment. Significant hypersensitivity to mechanical, heat and cold stimuli, assessed by von Frey filaments, radiant heat paw withdrawal and the acetone tests, respectively, were observed in OI compared to control wildtype littermates. OI mice also displayed reduced motor activity in the running wheel and open field assays. Immunocytochemical analysis revealed no changes between OI and WT mice in innervation of the glabrous skin of the hindpaw or in expression of the pain-related neuropeptide calcitonin gene-related protein in sensory neurons. In contrast, increased sensitivity to mechanical and cold stimulation strongly correlated with the extent of skeletal deformities in OI mice. Thus, we demonstrated that the Col1a1Jrt/+ mouse model of severe OI has hypersensitivity to mechanical and thermal stimuli, consistent with a state of chronic pain.

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1. Introduction

Osteogenesis imperfecta (OI) or brittle bone disease is associated with bone fragility, bone deformities, short stature and many other skeletal manifestations of widely varying severity. Extra-skeletal abnormalities associated with OI include joint hyperlaxity, muscle weakness, brittle teeth, bluish-gray sclera and hearing defects. The prevalence of OI at birth is about 1 in 10,000 [1]. In the large majority of individuals with OI, the disorder is caused by mutations in genes encoding collagen type I, COL1A1 and COL1A2.

Skeletal pain is a major issue in OI patients [2]. Even mild forms of OI pain can be associated with decreased health-related quality of life [3]. However, scientific data on the topic remain scarce. The most extensive study on pain in OI obtained a 1-week pain diary in 35 children and concluded that both acute and chronic pain were common and interfered with activities of daily life [2]. Thus, even though the importance of pain in OI is universally acknowledged, little is known about the underlying mechanisms and no mechanistic studies on pain in OI animal models have been reported.

Here we used the recently developed Col1a1P1/+ mouse model of OI to study OI-related pain. Col1a1P1/+ (+/OI) mice exhibit small stature, low bone mineral density and fragile bones similar to type IV OI patients [4]. In mice, evoked responses to sensory noxious stimuli (nociception) of different modalities (mechanical, heat and cold) serve as proxy behavioral indices suggestive of increased pain sensitivity [5]. Other behavioral measures of functional impairment that may be secondary to pain include decreased motor activity and limping.

OI and WT mice were tested for behavioral signs of sensory hypersensitivity and functional impairment. The OI mice displayed significant hypersensitivity and reduced motor ability compared to WT mice. There was a significant correlation between some evoked behavioral responses and skeletal deformities in OI mice. In contrast, no differences
were observed in measures of either cutaneous innervation density or sensory neuron plasticity. This study provides insights into the relationship between bone health and behavioral indices of pain and functional impairment in WT and OI mice.

2. Materials and methods

2.1. Animals

All experiments were approved by the Animal Care Committee at McGill University and conformed to the ethical guidelines of the Canadian Council on Animal Care and the guidelines of the Committee for Research and Ethical Issues of IASP [6]. The Col1a1Jrt/+ mice were developed by screening of N-ethyl-N-nitrosourea-induced mutagenesis resulting in T to C transition in the COL1A1 gene leading to an 18 amino acid deletion in the main triple helical domain of Col1a1, as described in [4]. The Col1a1Jrt/+ mice were bred on a FVB background. Mice were a gift from Dr. Jane Aubin's laboratory, University of Toronto. The breeding colony was maintained at the Animal Care Facility of the Shriners Hospitals for Children®—Canada. Animals had unrestricted access to food and water, and were on a 12-hour alternating light and dark cycle. A total of 29 (3–6 month old males); 15 WT and 14 Col1a1Jrt/+ mice were used in the study.

2.2. Behavioral assessment

Animals were transferred to the Alan Edwards Centre for Research on Pain and habituated for one week prior to testing. Behavioral measurements were taken weekly for 7 consecutive weeks and at week 10. Animals were acclimatized for one hour prior to each testing session.

2.2.1. Behavioral measures of sensitivity to mechanical, heat and cold stimuli

2.2.1.1. Mechanical hypersensitivity. Mechanical hypersensitivity was performed using von Frey filaments (Stoelting Co., Wood Dale, IL). Animals were placed on an elevated wire mesh grid and covered individually with glass compartments. Filaments were applied to the plantar surface of the hind paw to the point of bending. A positive response was defined as a withdrawal from the filament within two seconds. The fifty percent withdrawal threshold in grams was calculated according to the up-and-down method [7]. A decrease in withdrawal threshold corresponds to an increase in mechanical sensitivity.

2.2.1.2. Heat hypersensitivity. Heat hypersensitivity was measured using the IITC Life Science Inc. plantar analgesia meter as previously described [8]. A radiant heat beam was directed to the planter surface of the hind paw and latency to withdrawal was recorded in seconds. A cut off was set to 22.7 s to avoid tissue damage. Decreases in withdrawal latency correspond to an increase in thermal sensitivity.

2.2.1.3. Cold hypersensitivity. Cold hypersensitivity was adapted from Choi et al. [9]. Cold hypersensitivity test was performed by placing a drop of acetone (~25 μL) on the planter surface of the hind paw using a syringe loaded needle. Total time spent engaging in acetone-evoked behaviors such as lifting and shaking of the paw was recorded for one minute. Increases in acetone-evoked behaviors are suggestive of increased sensitivity to cold.

2.2.2. Behavioral measures of functional impairment

2.2.2.1. Open field assay. The open field assay. Was performed by placing mice individually in a transparent Plexiglas chamber with 40 cm high walls and a square base of 27 × 27 cm. A video camera was placed on the top of the chamber to track the animals’ motor activity with ANY-maze software (Stoelting, USA) for 5 min. Total distance traveled was determined.

2.2.2.2. Rearing. Rearing was determined by an observer blind to genotype as the time spent standing on the animals hind limbs in an upright position during the open field assay. Rearing time was recorded in seconds.

2.2.2.3. Limping score. A limping score modified from [10], was given to each mouse by an observer blind to genotype after 5 min of non-forced ambulation in the open field setting. The assigned scale was as follows: 0 = complete lack of limb use, 1 = partial non-use of the limb in locomotor activity, 2 = limping and guarding behavior, 3 = substantial limping, 4 = normal use. A score was given to each limb according to the scale. An average score of all four limbs was given to each animal.

2.2.2.4. Voluntary running wheel test. The voluntary running wheel test was performed by placing an individual mouse in a home cage containing a rotating wheel with sensor, wireless transmitter and receiving software (Med Associates, Inc.). Voluntary running behavior was measured as the number of wheel rotations detected during a one hour test period.

2.3. Radiography

Live digital radiographs (Faxitron® MX-20) were taken at weeks 3, 8 & 11. Mice were anesthetized with an intra-peritoneal (i.p.) injection of 0.01 mL/kg of ketamine (100 mg/mL), xylazine (20 mg/mL) and acepromazine (10 mg/mL). Animals were scanned in a prone position after taping to the exact location for each scan. Radiographs were analyzed by two independent observers blind to genotype. Skeletal abnor- malities including atlanto-occipital joint dislocation, scoliosis-like spine deformity, reduced cervical intervertebral disc space, reduced length of long bones, deformation and fracture of olecranon processes, deform- ation of coxae, arthritic knees, hindpaw and forepaw bone deformities, loss of transverse processes of caudal vertebrae, hyperelastic callus formation, and osteophytes were recorded for each mouse and a skeletal deformity score was calculated as a total number of all observed abnormalities.

2.4. Tissue processing for micro-computed tomography and immunohistochemistry

Mice were deeply anesthetised and perfused transcardially with vascular rinse (5% 0.2 M phosphate buffer, 0.95% NaCl, 0.026% KCl, 0.05% NaHCO3 in distilled water, 0.1% NaNO2 added at day of perfusion) followed by 50 mL of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Femurs were cleaned thoroughly, stored in 70% ethanol at 4°C then scanned by Micro Computed Tomography. Glabrous hindpaw skin was cryoprotected in 30% sucrose in phosphate-buffered saline (PBS) overnight at 4°C, embedded in optimum cutting temperature medium (OCT; Tissue Tek®) and sectioned (40 μm). Upper lumbar dorsal root ganglia (DRG) were dissected, post-fixed in 4% paraformaldehyde in phosphate buffer for one day at 4°C, cryoprotected in 30% sucrose in PBS for 4 days at 4°C, embedded in OCT, sectioned at a thickness of 10 μm and thaw-mounted onto gelatin-coated slides.

2.5. Micro-computed tomography

Right femurs were scanned in PBS using cone beam computed tomography (CT) (Skyscan 1172) at a voxel size of 6 μm. Scan parameters included a 0.45-degree increment angle, 3 frames averaged, an 84-kVp and 118-mA X-ray source with a 0.5-mm Al filter to reduce beam hardening artifacts. Trabecular bone was analyzed in a region starting at 0.5 mm proximal to the distal femoral growth plate (to avoid primary
spongiosa) and scanning a 1 mm section of bone in a proximal direction. Trabecular bone was manually selected along the inner cortical surface. Scans were quantified using the system’s analysis software (SkyScan CT Analyser, Version 1.11.8.0). To analyze cortical bone scanning was performed starting at a distance 44% of the total femur length from the distal end and scanned for 1 mm proximally. Average outer bone diameter and average diameter of the bone marrow cavity were determined from cross-sectional areas assuming a circular bone cross-section. Cortical thickness was calculated as the difference of these two diameters divided by 2.

2.7. Data analyses

All data are expressed as means ± S.E.M., with n indicating number of animals, p < 0.05 was considered statistically significant. Two-way repeated-measures ANOVA was used for comparing groups over time (Figs. 1,2,3). Non-parametric Spearman correlation analyses were used to assess correlations between evoked pain behavior modalities and skeletal deformities scores in Col1a1Jrt/+ mice (Fig. 4). Student t-test was used to compare between two groups at a single time point (Figs. 1,5). Statistical analyses were performed using GraphPad Prism software version 6.00 for Windows, California, USA.

3. Results

3.1. Characterization of bone deformities in OI mice

OI mice weighed less than their WT littermates. The average weight of WT mice was 32.9 ± 1.8 g, while the average weight of OI mice was 23.3 ± 1.5 g (Fig. 1A). WT animals gained 2.0 ± 1.6 g over 11 weeks while OI mice gained 1.1 ± 0.9 g. Radiographic assessment of OI mice revealed a smaller skeleton and widespread skeletal deformities. The number of skeletal deformities per animal (i.e. skeletal deformity score) was significantly and persistently increased in OI compared to WT mice (Fig. 1B). The skeletal deformities included atlanto-occipital joint dislocation (Fig. 1C), deformity and fracture of olecranon processes and osteophytes (Fig. 1D), hyperplastic callus formation (Fig. 1E), scoliosis-like spine deformity (Fig. 1F), improper healing of the femur at the hip joint (Fig. 1G), reduced length of long bones (not shown), deformity of coxae (Fig. 1H), arthritic knees (Fig. 1I), hindpaw bone deformities and tarsal-metatarsal fracture (Fig. 1J). Micro-CT scans revealed a significant decrease in OI femur length (Fig. 1K), bone volume/tissue volume (Fig. 1L), cortical thickness (Fig. 1M), and trabecular number (Fig. 1N) compared to WT femurs. The bone deformities reported here are consistent with previous reports [4,11].

3.2. Increased sensitivity to mechanical, heat and cold stimuli in OI compared to WT mice

We tested sensitivity to mechanical, heat and cold stimulation in OI and WT mice. OI mice have decreased paw withdrawal thresholds to mechanical stimulation in comparison to WT (Fig. 2A). Testing OI mice for heat sensitivity revealed decreased withdrawal latency compared to WT mice (Fig. 2B). OI mice showed significantly increased behavioral responses to acetone compared to WT (Fig. 2C). Overall, these results indicate that OI mice are hypersensitive to sensory stimuli.

3.3. Increased functional impairment in OI compared to WT

We tested the mice for signs of physical functional impairment that may be secondary to OI-induced pain and skeletal deformities. OI mice traveled less distance in the open field glass chamber compared to WT mice (Fig. 3A). In the same experimental setting, rearing time (time spent standing on their hind limbs) (Fig. 3B) and limping score (4 = normal walking, 0 = no limp use) (Fig. 3C) were recorded during a 5 minute observation. OI mice exhibited substantial limping as well as decreased rearing attempts compared to WT mice. Voluntarily running was measured with a home cage running wheel for one hour. While both OI and WT mice improved their performance following their initial exposure to the running wheels, likely due to the presence of a training component in the test, the overall counts of rotations were significantly reduced in OI compared to WT mice (Fig. 3D).
Fig. 1. Skeletal characteristics and bone histomorphometric parameters in OI and WT mice. (A) Weight in grams over the 11 week experiment period in WT and OI animals. (B) The total number of skeletal deformities per animal was quantified by giving a score of 1 to each deformed, fractured or arthritic bone/joint at weeks 3, 8 and 11. Data are mean ± S.E.M., n = 14–15/group. ****p < 0.0001 indicates significant group differences between OI and WT mice assessed by two-way repeated-measures ANOVA. (C–J) Radiographs demonstrating examples of skeletal deformities observed in OI mice: (C) atlanto-occipital joint dislocation (arrow) and reduced cervical intervertebral disc space, (D) ulnar fracture (lower arrow) and osteophyte in humerus (upper arrow), (E) hyperplastic callus formation of olecranon process, (F) scoliosis-like spine, (G) improper healing of femur at hip joint, (H) severe hip bone deformation, (I) arthritic knee and, (J) tarsal–metatarsal fracture. (K–N) Bone histomorphometric parameters quantified by micro-CT scans: (K) Femur length; (L) bone volume/tissue volume; (M) cortical thickness; (N) trabecular number. Data are means ± S.E.M., n = 14–15/group. **p < 0.01 and ****p < 0.0001 indicate significant differences between OI and WT mice assessed by unpaired t-test. These results are consistent with previous studies characterizing the skeletal and histomorphometric phenotype of the Col1a1Jrt/+ mice [4,11].

Fig. 2. Hypersensitivity to sensory stimuli in the hind paw of OI mice compared to WT littermates. (A) Changes in mechanical sensitivity assessed as 50% withdrawal threshold in grams (g) using von Frey filaments. (B) Changes in heat sensitivity assessed as withdrawal time (s) in response to radiant heat stimulation. (C) Changes in cold sensitivity assessed as time (s) spent responding to acetone application. Direction of arrow indicates increased hypersensitivity (i.e. more pain). Data are means ± S.E.M., n = 14–15/group. **p < 0.01 and ****p < 0.0001 indicate significant differences between OI and WT groups assessed by two-way repeated-measures ANOVA.
3.4. Hypersensitivity to mechanical and cold stimuli correlates with skeletal deformities in OI mice

To assess if behavioral measures in OI mice are related to bone health, we performed correlation analyses between the number of skeletal deformities, sensory thresholds and functional impairment. We found significant correlation between the total number of skeletal deformities and sensitivity to mechanical stimulation (Fig. 4A) and to acetone-evoked behaviors (Fig. 4C) at week 3 (Fig. 5). No correlations were observed with heat sensitivity (Fig. 4B) or with any functional impairment measures (data not shown).

3.5. Peripheral innervation in glabrous hindpaw skin and sensory neuronal plasticity in OI and WT mice

To assess if changes in peripheral innervation in OI mice might contribute to increased sensory sensitivity in the hindpaw, immunohistochemistry using the pan-neuronal marker PGP 9.5 was performed and nerve fiber density was quantified in the upper dermis of the hindpaw. PGP 9.5-immunoreactive nerve fibers were not different between WT and OI mice (Fig. 5A,B). In order to detect potential pain-related changes in sensory neuron cell bodies, we stained the upper lumbar dorsal root ganglia (DRG) for CGRP, a neuropeptide involved in pain transmission. The percent of CGRP-immunoreactive sensory neurons was not different between WT and OI mice (Fig. 5C,D).

4. Discussion

Our data demonstrate increased sensitivity to mechanical, heat and cold stimuli and significant functional impairment in the Col1a1Jrt/+ mouse model of OI compared to their WT littermates. Consistent with previous studies, Col1a1Jrt/+ mice exhibited skeletal deformities including fracture-prone bones, kyphosis and small stature, similar to the clinical features of severe OI [4,11]. The changes in sensory thresholds in Col1a1Jrt/+ mice were not associated with changes in nerve fiber density in the skin or with changes in CGRP-expression in dorsal root ganglia. Mechanical and cold hypersensitivity significantly correlated with bone deformities in Col1a1Jrt/+ mice. These data suggest that the...
Col1a1Prt/+ mice represent a clinically-relevant animal model for understanding the mechanisms and exploring therapeutic interventions in OI-related pain.

4.1. Col1a1Prt/+ mice as a model for osteogenesis imperfecta

Col1a1Prt/+ mice were recently established as a model of severe dominant OI [4]. These mice present multiple skeletal abnormalities including fractures, hyperplastic callus formation at previously fractured sites, bone hypertrophy, severely deformed bones and arthritic joints, kyphosis and scoliosis, which are consistent with skeletal findings in severely affected patients [12]. In addition, a 3-fold increase in the marker of osteoclast activity CTX was reported in the Col1a1Prt/+ mice compared to WT [11]. These data are consistent with studies in humans with OI [13], as well as with other OI mouse models, such as the BrtlIV mice [14] or the oim/oim mouse [15]. Thus, the Col1a1Prt/+ mice mimic the features of inheritance and skeletal phenotype observed in patients with severe OI.

4.2. Possible causes of pain in Col1a1Prt/+ mice

We report significant and persistent increases in sensory sensitivity to mechanical, heat and cold stimuli in OI mice compared to control. Multiple mechanisms can contribute to the pain phenotype observed in this model. Pain in the Col1a1Prt/+ model may be related to multiple fractures [16], Bone abnormalities and fracture disrupt the periosteum rich in sensory innervation, and allow stimulation of the peripheral nerve endings supplying bone tissues [17]. Another source of pain could be condensation of vertebral bodies and kyphosis, which might compress the sensory nerve roots innervating the hind limbs, leading to hindpaw hypersensitivity [18]. In addition, the knee arthritis observed in Col1a1Prt/+ mice might contribute to both the hindpaw sensory hypersensitivity and motor impairment [19]. It is also well established that osteoclast activity contributes to pain in various animal models of bone-related pain [20–22], and may be relevant to this model. In addition, muscle weakness might contribute to functional impairment in the OI mice [23]. It is unlikely, however, that functional/motor impairment is responsible for the observed hypersensitivity in the sensory assays because faster and/or more vigorous motor responses indicate hypersensitivity to sensory stimuli; motor impairment would be expected to have the opposite impact. Finally, inflammation due to recurrent fractures or abnormal healing processes might contribute to the pain phenotype [24].

4.3. Pain and collagen mutations

Diseases related to collagen disturbances in quality or quantity such as OI and Ehlers–Danlos syndrome are often associated with musculoskeletal pain. Collagen is one of the main components of tendons, ligaments and myofibrils and is important for tissue healing and scar formation. In our study, total nerve fiber density in the skin was unaltered in Col1a1Prt/+ mice, suggesting that this collagen mutation does not alter dermal innervation. In addition, the mutation did not affect the dorsal root ganglia expression of CGRP, a neuropeptide associated with nociceptive neuroplasticity. Overexpression of CGRP in DRG neurons is detected in several animal models of experimental musculoskeletal pain including degenerative disc disease [25], tibial fracture [26], osteoarthritis [27] and osteoporosis [18]. The absence of detectable changes in sensory neurons supports the hypothesis that skeletal deformities and not changes in the nervous system are the primary drivers of hypersensitivity and functional impairment in this model.

4.4. Limitations and future directions

The combination of OI-like skeletal, sensory and functional changes in the Col1a1Prt/+ mice suggest that this is a suitable model for understanding the mechanisms behind OI-related pain. For example, future experiments could use this model to examine the efficacy of bisphosphonates or other disease-modifying treatments to understand the relationships between bone- and pain-related outcomes, to explore the relative contributions of inflammatory vs. neuropathic pain mechanisms or to identify the most effective analgesic agents. The current study has several limitations: First, only male mice were used; future
studies will need to examine OI-related pain in both male and female mice. Second, investigations into the role of neuronal plasticity were limited to total innervation in the skin and to one pain-related neuropeptide in the sensory neuron cell bodies. Studies in other tissues such as muscles, synovial membranes and bones will be of great interest, as would be studies examining signs of plasticity in the spinal cord or in supraspinal regions.

4.5 Conclusion

In spite of the multidisciplinary approaches to OI therapy and the use of analgesics, pain is one of the main complaints that drive patients to seek treatment. The pain in OI is both acute and chronic in nature and is not necessarily associated with recurrent fractures [2]. The mobility limitations secondary to pain and physical and emotional distress further decrease quality of life [3,28]. Therefore, molecular mechanisms underlying chronic non-fracture pain in OI patients need to be investigated. The Col1a1+/− mutation results in OI-like changes in skeletal, sensory and functional parameters. We propose the Col1a1+/− as a translational tool to investigate targeted therapeutic options for OI-related pain and to lay the groundwork for the establishment of pain management protocols for OI patients.

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