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Editorial

Purinergic Signaling in Bone

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In recent years, it has become apparent that extracellular nucleotides, signalling via P2 receptors, play an important role in the regulation of bone turnover. Furthermore, purinergic signalling has been associated in the pathophysiology of several bone and cartilage diseases, including osteoarthritis, rheumatoid arthritis, and osteoporosis and might also be implicated in the deleterious skeletal effects of cancer and on bone pain. Widespread expression of multiple P2 receptor subtypes by bone and cartilage cells has now been reported, and the functional effects of receptor activation are being determined. Of all of the P2 receptors expressed, it is the P2X7 receptor which has emerged as being central in the pathogenesis of several skeletal conditions, though also a number of other P2Y and P2X receptors have important roles in regulation of bone turnover.

There are many studies, using human and animal models, which have described the pivotal role of the P2X7 receptor in rheumatoid arthritis, a complex, multifactorial inflammatory disease with no current successful treatment. The review entitled “Modulating P2X7 receptor signaling during rheumatoid arthritis: new therapeutic approaches for bisphosphonates” by A. Baroja-Mazo and P. Pelegrin included in this special issue summarises some of this research. In particular, it focuses on the therapeutic potential of P2X7 receptor antagonists, both alone and in combination with bisphosphonates, as a treatment for rheumatoid arthritis.

Recent work has shown that the P2X7 receptor is also important in bone-related conditions, particularly osteoporosis. A number of clinical studies have associated single-nucleotide polymorphisms (SNPs) in the P2X7 receptor gene with increased fracture risk, low bone mineral density, and increased bone loss in humans. The importance of genetic variation within the P2X7 receptor in relation to bone mass and strength is addressed by two original research articles by S. Syberg et al. in this special issue. Firstly, the paper entitled “Association between P2X7 receptor polymorphisms and bone status in mice” investigates the effects of one SNP (P451L) using an in vivo animal model. The second article entitled “Genetic background strongly influences the phenotype of P2X7 receptor knockout mice” provides a detailed analysis of the differences of bone phenotype between two different strains of P2X7 receptor knockout. Combinedly these research papers highlight the importance of genetic background when looking at the functional effects of the P2X7 receptor and suggest that when mouse models are used to test the efficacy of P2X7 receptor agonists and antagonists it is taken into account. Also they demonstrate the role of the P2X7 receptor in regulation of bone mass.

Bone is both a site of primary tumor formation and metastatic spread of high incidence neoplasias such as breast and prostate cancers. To date, the efficacy of therapies intended to reduce bone alterations and related pain in cancer is limited. In this special issue, two papers point out to P2X receptors as possible targets for the treatment of bone cancer and associated pain. E. Adinolfi et al. cover recent findings linking P2X7 receptor and bone biology with a focus on P2X7-mediated osteoblast proliferation and osteoclast differentiation. The authors report evidence on the role of the P2X7 receptor as an oncogene implicated in cancer growth, neovascularization, and metastatic dissemination.
Their paper relies on data reported by the recent literature and examination of Affymetrix-based expression databases. Examined tumors include osteosarcoma, neuroblastoma, multiple myeloma, and breast and prostate cancers.

The review from S. Falk et al. is centered on the role of purinergic nociception in cancer-induced bone pain. In particular, the authors report evidence in favor of P2X3 involvement in bone-related pain both at the peripheral and central levels concentrating on studies conducted in different rodent models of cancer-induced pain states. The authors also report on the role of other P2X and P2Y receptors in the complex network of cells involved in bone pain development, with a critical perspective taking into account all the possible problems linked to model representative potential. “P2X7 receptor function in bone-related cancer” and “The role of purinergic receptors in cancer-induced bone pain” both take into consideration the role of the natural agonist of P2X receptors, extracellular ATP, in cancer. Indeed, several publications reported the presence of high levels of ATP in cancer microenvironment, and a role for this nucleotide in increasing the host immune response was suggested. Data summarized in the reviews by E. Adinolfi et al. and S. Falk et al. suggest a mechanism by which extracellular ATP released from tumor cells, through the P2X7 receptor, might affect osteoblast growth and osteoclast activity, while it might induce pain through the P2X3 receptor.

Increasing evidence supports the role of purinergic signaling through P2 purinergic receptors in regulating normal bone turnover, but also seems to play a role in the pathophysiology of a range of bone diseases including postmenopausal osteoporosis, immune-mediated bone loss, and cancer-induced bone disease as well as in bone-pain.

Thus, accumulating evidence provide us with a range of new therapeutic targets to treat the above-mentioned diseases, for some of which efficacious treatment options are not currently available. In terms of therapeutic strategy for cancer-induced bone disease, one could speculate that treatment with a cocktail of drugs contemporarily targeting multiple P2X receptor could prove efficacious both in reducing cancer growth, dissemination and pain sensation, while the widespread expression of P2 receptors on the different types of bone cells could prove to be a novel target for the regulation of bone formation and resorption in both postmenopausal osteoporosis and osteoporosis related to other diseases.

It will certainly be interesting to follow the continued progress in the field of purinergic signaling in bone in the future, and the growing acceptance of this concept is supported by the recent funding by the European Commission’s 7th Framework Programme of the ATPBone project and a dedicated session at the European Calcified Tissue Society 2012 conference to “ATP and bone,” putting this area of research concept on the scientific map.

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Cancer-induced bone pain significantly compromises the quality of life of many cancer patients. A large proportion of patients with bone metastasis experience severe pain and bone pain is often the first sign of metastatic spread in patients suffering from breast, lung, or prostate cancer. Current treatment options are radiotherapy, anti-inflammatory agents, opioids, and bisphosphonates, but still up to 45% of patients are left with inadequate pain control [1–4]. The poor management of cancer-induced bone pain is a consequence of the complexity of the pain state, involving a combination of both background pain and breakthrough pain. The background pain is described as a constant pain with increasing intensity as the disease progresses and can usually be treated with opioids with a satisfying result. Breakthrough pain can be divided into movement-evoked pain and spontaneous pain. These pain states, which have a rapid onset and a short duration of 15–30 minutes [1], are generally difficult to treat. Opioids given orally have a slow onset of analgesia of approximately 30 minutes, that is, an analgesic effect only at the end of the average breakthrough pain episode, if at all. Additionally, oral opioids have a long duration of action, typically 4–6 hours, which is much longer than required to treat breakthrough pain episodes [5] and often causes dose-limiting side effects [6]. A rapid onset and short acting opioid such as sublingual or nasal spray fentanyl is a more promising approach for the treatment of breakthrough pain [3].

In order to accommodate the clinical need for new and improved therapies of cancer-induced bone pain, a better understanding of the mechanisms underlying the pain state is needed. Cancer-induced bone pain exhibits components of both inflammatory and neuropathic pain, but the complete mechanism is not yet fully characterized [7]. When tumor cells invade the bone tissue, multiple mechanisms are initiated. Osteoclasts are stimulated resulting in increased bone degradation with release of growth factors from the bone matrix, and as the tumor cells invade the bone, they compress and damage the sensory fibers present in the bone. Also inflammatory cells infiltrate the tissue and release various cytokines and growth factors [8] that may contribute to the development and maintenance of the pain state. Furthermore, cancer-induced bone pain causes cellular and neurochemical changes in the spinal cord which appear to be mechanistically distinct compared to neuropathic or inflammatory pain states [7].

The advanced understanding of the underlying mechanisms of cancer-induced bone pain is mainly due to the recent development of in vivo bone cancer models displaying...
pain-related behavior mimicking the clinical condition. Since the first murine model of cancer-induced bone pain was developed in 1999 [9], various syngeneic animal models of cancer-induced pain states have been developed in rodents. The rodent models are based on direct injection of cancer cells into the intramedullary space of femur, humerus, or tibia or in and around calcaneus [10]. This allows correlation of tumor growth, tumor microenvironment, bone destruction, and neurochemistry with site-specific pain-related behaviors and has given new insight into the different cellular and molecular mechanisms driving cancer-induced bone pain and thereby provided opportunities to develop targeted therapies [10]. A number of cytokines, growth factors, and other molecules such as tumor necrosis factor-alpha (TNF-α), nerve growth factor (NGF), bradykinins, and prostaglandins have thus been identified to play a role in cancer-induced bone pain [11–14]. Adenosine 5-triphosphate (ATP), which plays a key role in nociception, both as a neurotransmitter and as a modulator of glial activity, has also been identified to be involved in cancer-induced bone pain. This paper provides an overview of current knowledge regarding the role of purinergic receptors in cancer-induced bone pain.

2. ATP and Purinergic Receptors

The involvement of ATP and purinergic signaling in nociception has long been recognized. Already back in 1966 Collier et al. reported that ATP could initiate pain when applied to human skin [15]. However, extracellular ATP was not accepted as a functional neurotransmitter until the 1990s. Today, the role of ATP in pain transmission is well established [15–18].

ATP is an agonist for two classes of purinergic receptors, the ligand gated ion channels, P2X receptors (P2X1–7) and the G protein-coupled P2Y receptors (P2Y1, 2, 4, 6, 11–14). The P2X receptors consist of two transmembrane domains with intracellular N- and C-terminals and a long extracellular loop between the transmembrane regions. The extracellular domain contains binding sites for ATP, competitive antagonists and modulatory metal ions [19–22]. The N-terminal has similar length in all subtypes, whereas the C-terminal varies considerably from 30 residues in the P2X6 receptor to 240 residues in the P2X7 receptor [18, 23], indicating that the different functional properties of the subtypes are linked to the C-terminal. A characteristic of the P2X7 receptor, but also some of the other P2X receptors, is the ability to induce pore formation allowing the permeation of large molecules [24–26]. The P2X receptors form homo- or heterotrigeminal ATP-gated nonselective cation channels [23]. The P2Y receptors contain seven transmembrane regions similar to other G protein-coupled receptors [18]. Unlike the P2X receptors, which are all stimulated by native ATP and synthetic ATP analogues, most of the P2Y receptors have greater affinity for ADP, UTP, or UDP [27].

The P2 receptors are found on almost every cell in the body [27]. When it comes to their involvement in chronic pain states, some purinergic receptors have been much more intensely studied than others. In the 1990s the first antagonists for the P2X receptors became available [28, 29] and this, together with recent development of various knockout models, has facilitated the investigation of the purinergic system in chronic pain states. A solid amount of data have demonstrated a key role of especially the P2X3 receptor, but also the P2X4 and P2X7 receptors in the development of both neuropathic and inflammatory pain [16, 30], and recently the number of studies on other P2X and also P2Y receptor subtypes are starting to rapidly expand the field. In relation to cancer-induced bone pain the involvement of the purinergic receptors is still poorly understood, but purinergic receptors are speculated to be important for the nociceptive transmission in cancer-induced bone pain for multiple reasons. First of all, the involvement of ATP in other chronic pain states, such as neuropathic and inflammatory pain, has been firmly established [16]. Secondly, nociceptors have been found to project not only to the periosteum, but also deeply into the bone and bone marrow and are therefore in close proximity to both tumor cells and tumor-associated immune cells and stromal cells. Thirdly, growing tumor cells are thought to release ATP, thus possibly producing a microenvironment of extracellular ATP stimulating the P2 receptors directly at the peripheral terminals of the nociceptors.

Although, an understanding of the role of purinergic signaling in the pathogenesis of cancer-induced bone pain is slowly emerging, the studies are complicated by molecular and cellular variation among the different in vivo models. One of the complicating factors is the variation in expression pattern of various purinergic receptors on the nociceptors in different models, as described in the following sections.

3. Purinergic Receptor Expression on Nociceptive Neurons

The nociceptive neurons are specialized pseudounipolar primary afferent neurons having their cell bodies in the dorsal root ganglion (DRG) or trigeminal ganglion and projecting to both the peripheral sites and the dorsal horn of the spinal cord [31]. Sensory afferent fibers can be divided into two major populations, the myelinated A-fibers and the nonmyelinated C-fibers, with the C-fibers often being further classified into peptide-rich and a peptide-poor groups according to the neuropeptides, receptors and channels they express, and the A-fibers being classified into thick Aα- and β-fibers and thin Aδ-fibers. Aδ- and C-fibers, and possibly Aβ-fibers are considered nociceptors [31, 32], and it is generally accepted, at least for skin, that the myelinated Aδ-fibers conduct the fast signal, perceived as the sharp “first pain,” whereas the slower nonmyelinated C-fibers conduct the more dull “secondary pain” [31]. From the DRG the nociceptive neurons project to different laminae in the dorsal horn, thereby grouping them into anatomical subpopulations. The myelinated Aδ-fibers and the peptidergic population of the C-fibers send input to lamina I and the outer part of lamina II. The nonpeptidergic
population of the C-fibers project to the inner lamina II, whereas the deeper layers, such as lamina V, only receives few input from Aδ-fibers [33].

Even though the expression of the P2X and P2Y receptors on the nociceptive neurons has been intensively studied, the expression pattern is still not clear, as conflicting results are reported. The characterization of the expression pattern is complicated by species differences [34, 35], and variation is observed in different compartments of the neurons, as some receptors are expressed both at the peripheral and central projections and in the cell body, whereas others might only be expressed at the central or peripheral terminals.

When studying the expression of P2X receptors on peripheral terminals, most information is currently found on the P2X3 receptor. In contrast to many of the other P2X receptors, which are found on various cell types throughout the body, the P2X3 receptors are predominantly expressed in small- and medium-sized sensory neurons (C- and Aδ-fibers), and presumably in both the cell body and the peripheral and central terminals [35, 36]. P2X3 receptors are mainly expressed on an IB4-expressing subpopulation of the nociceptive neurons, with up to 94% of the P2X3 receptor-positive neurons also expressing IB4 [37, 38]. However, variation in expression has been demonstrated for the P2X3 receptor at both the peripheral and central terminals of the afferent neurons, and this is likely similar for the other P2 receptors. For instance, different models of neuropathic pain have demonstrated opposite responses in the expression of the P2X3 receptor depending on which method was used to induce the peripheral injury. Whereas one model showed downregulation of P2X3-receptors in the spinal cord, another displayed an increase in the number of P2X3-positive neurons in both the DRGs and the spinal cord [37, 39]. Furthermore, the expression level can change at both the peripheral and central terminals according to different peripheral stimuli, such as nerve damage, inflammation or tumor growth [40–42].

When moving from the peripheral terminal to the cell body of the nociceptors in DRG, more information about the expression pattern is starting to accumulate. In the dorsal root and trigeminal ganglia up to 90% of the neurons express various subtypes of P2X receptors. With the exception of the P2X7 receptor all of the subtypes are present, however, at different expression levels and with the P2X3 receptors more highly expressed than the remaining P2 receptors [43, 44]. In rats up to 40% of the DRG neurons express P2X3 receptors [38, 43]. Of these 73–84% also express P2Y1, while 25–35% are expressing P2Y4 [45]. Using immunohistochemistry, P2X2 and P2X3 receptors have been demonstrated to display a high degree of colocalization in rat DRG, although single-labeled neurons are also present, and P2X2 receptors are also observed in satellite cells [34]. Whereas the expression of P2X3 receptors in DRG is fairly well established, the reports on the expression of the remaining subtypes are still conflicting.

At the level of the dorsal horn the same confusion seems to exist when it comes to the distribution of the different P2X subtypes. Attempting to clarify the matter, Aoyama et al. recently reported a systematic analysis of the distribution of all seven P2X receptors in the dorsal horn and compared their findings to earlier reports [46]. From this they concluded that P2X1 and P2X3 receptor subunits are densely distributed mainly in laminae I and II of the dorsal horn and are presumably expressed at the afferent nerve terminals [38]. This is in agreement with earlier studies reporting P2X3-expressing projections from nonpeptidergic nociceptive DRG neurons to lamina II [37, 38]. For the P2X2 receptor Aoyama et al. concluded that it was only weakly expressed in the fibers of the dorsal roots, and almost completely absent in the gray matter, whereas earlier reports have demonstrated dense P2X2 receptor immunostaining in the spinal cord, especially in the dorsal horn [47]. In addition, while Aoyama et al. reported P2X4 receptor expression in small cells in the entire dorsal horn and in dorsal root fibers and neuropils surrounding neurons, others have demonstrated P2X4 receptor expression in microglia, but not in astrocytes and neurons in the dorsal horn [48, 49]. Also, P2X7 receptors were strongly detected in dorsal root fibers, in neuropils in the entire dorsal horn, and also in astrocytes. The expression on astrocytes is supported by functional studies in astrocyte cultures isolated from both the spinal cord, cerebral cortex, and hippocampus [50–53]. However, this is inconsistent with other studies reporting expression in microglia, but not in astrocytes and neurons [54, 55]. As this summary illustrates, the expression profile of the P2X receptors is unclear, and both the spatial and temporal distributions are likely affected by species variation, method of nerve injury and peripheral stimuli.

4. P2X Receptors at the Peripheral Site in Relation to Cancer-Induced Bone Pain

The bone is innervated by a tight network of both sympathetic and sensory neurons, and although the periosteum seems to be the most densely innervated part, when the total volume of each tissue is considered, the bone marrow is receiving the greatest number of nerve fibers followed by the mineralized part of the bone and lastly the periosteum [56, 57]. In rodents, both myelinated A-fibers and unmyelinated peptidergic C-fibers are found throughout the periosteum, in the compact and trabecular part of the mineralized bone and in the bone marrow [56, 58]. The innervation of the bone by nociceptors has, in addition to traditional immunohistochemical analysis, been established using retrograde labeling demonstrating that the size, neurochemistry, and segmental distribution of the neuronal projection from the bone to the DRG and dorsal horn are consistent with a functional role in nociception [59].

The expression of P2X and P2Y receptors on both nerves and bone cells is interesting in several aspects in relation to cancer-induced bone pain. During the last decade a number of groups have reported expression of both P2X and P2Y receptors in osteoblasts, including P2X1–7 and P2Y1, 2, 4, 6 and 12–14, and in osteoclasts, including P2X1–5 and 7 and P2Y1-2, 4–6 and 11–14 [60]. The action of P2 receptors on osteoclasts includes P2X2-induced bone resorption, increased osteoclast formation and bone resorption through P2Y1 receptor activation, increased survival by P2Y6.
receptor activation, and P2X7-mediated precursor cell fusion and decreased apoptosis (although there have been reports on increased apoptosis) [60]. The presence of P2X and P2Y receptors on osteoblasts, and especially on osteoclasts, could be essential for understanding the progression of cancer-induced bone pain. For instance, it has been demonstrated that low concentrations of extracellular ATP stimulates resorption pit formation by mammalian osteoclast [61]. This is speculated to be controlled by the action of the P2X2 receptors, which are the only of the P2 receptors that require extracellular acidification to be fully activated by ATP [62, 63]. Moreover, P2X2 receptor knockout mice display a phenotype with increased bone mass, further pointing to the involvement of P2X2 receptors in bone turnover [64]. As cancer-induced bone pain is correlated with degradation of the bone [65], and tumor cells probably release ATP, it is likely that these events are linked together through the action of purinergic receptors on the osteoclasts, a likely candidate being the P2X2 receptors.

Tumor cells have also been shown to have a direct effect on the organization of the nerves innervating the bone, as it has recently been reported that bone cancer induces sprouting and disorganization of both sensory and sympathetic fibers in the periosteum through the action of NGF in a murine model of cancer-induced bone pain [66]. How this interruption of the normal innervation is effecting the expression of the purinergic and other nociceptive receptors, and thereby the transmission of the nociceptive signal to the spinal cord is clearly target for further investigation.

So far, the P2X3 receptor is the most studied purinergic receptor in relation to cancer-induced bone pain (Table 1). Several studies have investigated the neuronal expression of the P2X3 receptor and the effect of P2X3 receptor inhibitors in various models of cancer-induced bone pain [41, 42, 67–70]. An upregulation of P2X3 receptor expression on epidermal nerve fibers overlaying a cancer-induced area was demonstrated by Gilchrist et al. in a murine model of cancer-induced bone pain [66]. How this interruption of the normal innervation is effecting the expression of the purinergic and other nociceptive receptors, and thereby the transmission of the nociceptive signal to the spinal cord is clearly target for further investigation.

The recent development of selective P2X3 receptor antagonists has provided the tools for further investigating the role of P2X3 receptor-mediated purinergic signaling in cancer-induced bone pain [72]. The competitive antagonist A-317491 blocks both P2X3 and P2X2/3 receptors [73] and was shown to attenuate pain-related behaviors in two different murine models and a rat model of cancer-induced bone pain [68–70]. González-Rodríguez et al. showed that A-317491 injected subcutaneously over a tibial tumor mass dose-dependently inhibited tumor-induced thermal hyperalgesia in the affected limb but not in the nontumor bearing limb. Interestingly, coadministration of an anti-met-enkephalin antibody abolished the antihyperalgesic effect of A-317491 suggesting that in this model the effect of A-317491 occurs through stimulation of peripheral opioid receptors [68]. The involvement of endogenous opioid mechanisms in P2X3 and P2X2/3 receptor-related antinoception has also been described in rats models of inflammatory pain [74].

An antinoceptive effect of A-317491 in cancer-induced bone pain was further demonstrated in a study by Hansen et al. where A-317491 was administered systemically in a murine model with mammary carcinoma cells injected into the femoral medullar cavity. Chronic administration of A-317491 resulted in an attenuation of pain-related behaviors in the early stage of the bone cancer model; however, no effect of neither chronic nor acute treatment with A-317491 was observed in the late and progressed stages of the pain model [69]. The limited effect of A-317491 in the cancer model might be explained by its pharmacokinetic properties. A-317491 has a high plasma protein binding and a limited CNS penetration which makes it less suitable for in vivo testing and indicates that the effect of A-317491 is predominantly in the periphery [72]. An additional explanation for the lack of effect of A-317491 in the progressed stage of cancer-induced bone pain could be that the expression or activity of the P2X3 and P2X2/3 receptors changes with the development of the cancer and/or that other nociceptive mechanisms dominate at this stage. A recent rat study by Kaan et al. also showed less analgesic effect of P2X3 receptor inhibition in the late stage of cancer-induced bone pain [67]. Kaan et al. used AF-353 (RO-4) in a rat model of tibial cancer-induced bone pain. AF-353 potently blocks P2X3 and P2X2/3 receptors and in contrast to A-317491 it has low plasma protein binding and good CNS penetration. Oral administration of AF-
| Receptor Species | Inoculation site | Cell type | Antagonist | Effect of cancer at peripheral sites | Effect of cancer at dorsal root or trigeminal ganglia level | Effect of cancer at spinal cord level | Behavioral Tests | Effect of receptor inhibition on bone cancer pain-related behavior | References |
|------------------|------------------|-----------|------------|-------------------------------------|------------------------------------------------------------|--------------------------------------|----------------|---------------------------------------------------------------|------------|
| C3H mice         | Into and around the calcaneus bone | NCTC 2472 fibrosarcoma cells | A-317491 s.c. | Increased expression (epidermis) (protein) | Increased expression (protein) | Mechanical | | [41] |
| Fisher rats      | Subperiosteal tissue of the lower gingiva | SCC-158 squamous cell carcinoma | AF-353 (RO-4) i.t. or p.o. | Increased expression | | Mechanical | | [42] |
| C3H/HeJ mice    | Tibia            | NCTC 2472 fibrosarcoma cells | A-317491 i.t. or s.c. with α,β-me ATP | Increased expression (mRNA and protein) | | Thermal | Attenuation | [68] |
| P2X3, (P2X2/3) Sprague-Dawley rats | Tibia | NCTC 2472 fibrosarcoma cells | A-317491 i.t. or s.c. | Increased expression (mRNA and protein) | | Weight-bearing | Attenuation | [67] |
| Wistar rats     | Tibia            | Walker 256 cell breast carcinoma cell | A-317491 i.t. or s.c. | Increased expression (mRNA and protein) | | Mechanical | Limb use Flinching | [70] |
| C3H/HeN mice    | Femur            | NCTC 2472 fibrosarcoma cells | A-317491 s.c. | Mechanical | | Weight-bearing | Limb use Attenuation (early phase) | [69] |
| Balb/c mice     | Femur            | 4T1 mammary carcinoma | A-317491 s.c. | Mechanical | | Weight-bearing | Limb use Attenuation (early phase) | [69] |
| P2X7            | C3H/HeN mice     | NCTC 2472 fibrosarcoma cells | A-438079 s.c. | Weight-bearing | | No effect | | [113] |
| Balb/c mice, WT and KO | Femur | 4T1 mammary carcinoma | A-438079 s.c | Weight-bearing | | No effect | | [113] |
| P2Y1            | Sprague-Dawley rats | Walker 256 cell breast carcinoma cell line | MRS2179 i.t. | Increased expression (mRNA) | Increased expression (mRNA) | Mechanical | Limb use Attenuation | [120] |

WT: wild-type mice, KO: knockout mice, s.c.: subcutaneous, i.t.: intrathecal, and p.o.: oral administration.
353 in a prophylactic treatment regimen reduced cancer-induced pain-related behaviors to the level of control rats, and some effect of AF-353 was also observed when it was given at a more progressed disease stage [67]. Both peripheral and central effects of P2X3 receptor inhibition were suggested. The argument for a peripheral effect was based on experiments showing that MRMT-1 carcinoma cells release ATP in vitro and that a P2X3 receptor-mediated increase in phosphorylated ERK immunoexpression was found in dorsal root ganglion neurons cocultured with MRMT-1 cell, indicating an activation of the ERK-signaling pathway. Although this is in agreement with other studies demonstrating enhanced phosphorylated ERK expression in models of chronic pain [75], the results remain to be confirmed in vivo.

A central effect of AF-353 was investigated by administering AF-353 directly into the spinal cord of cancer bearing rats. AF-353 dose-dependently reduced the electrically evoked responses observed in the Aδ- and C-fibers of the dorsal horn, and additional a significant reduction in the postdischarge was found at the high doses of AF-353, together suggesting that the cancer-induced hyperexcitability of the dorsal horn neurons can be modulated by P2X3 and P2X2/3 receptor antagonism [67]. Taken together, the results described above provide evidence that inhibition of P2X3 and P2X2/3 receptor activity at the periphery and at the level of the spinal cord could have therapeutic potential in the treatment of cancer-induced bone pain. It should be noted that although many of the purinergic receptors are expressed in bone cells, it is unlikely that the effect of P2X3 and P2X2/3 receptor antagonism is mediated through decreased bone destruction. None of the P2X3 and P2X2/3 receptor antagonist studies found any effect on bone destruction and only very few osteoclasts express functional P2X3 receptors [76].

5. The Role P2X Receptors at the Central Level of Nociception

A state of hypersensitivity is introduced in the spinal cord as a response to peripheral nociceptive stimuli in chronic pain states. The hypersensitivity is a consequence of the highly complex processing and modulation of the peripheral nociceptive input through excitatory and inhibitory mechanisms in the dorsal horn of the spinal cord causing a general state of hyperexcitability in the neurons [77]. The output from the dorsal horn will under normal conditions be balanced by excitatory and inhibitory control mechanisms, but in pathological pain states the output is greatly increased, caused by increased excitatory synaptic transmission and/or suppressed inhibitory transmission. Increasing evidence points to the interaction of neurons and nonneuronal cells to be the underlying cause of this hypersensitivity, and various molecular and cellular changes in the spinal cord have been observed in different chronic pain states. Interestingly, inflammatory, neuropathic, and cancer-induced pain states can be distinguished by these neurochemical changes in the spinal cord. In models of inflammatory pain an increased level of substance P and calcitonin gene-related peptide includes some of the changes, whereas neuropathic models display an opposite decreased expression of the same molecules [7]. For the cancer-induced pain state the changes in the spinal cord include increased expression of c-Fos in laminae I and II, increase in the number of dynorphin-expressing neurons, and often massive astrocyte hypertrophy without neuronal loss [7, 9, 78, 79].

Purinergic signaling is a key element in the modulation of the hypersensitivity at the level of the spinal cord. ATP mediates neuron-neuron, neuron-glia, and glia-glia communication through activation of purinergic receptors expressed in the presynaptic terminals of the afferent sensory neurons in the spinal cord, in postsynaptic neurons, and in spinal microglia and astrocytes [35, 72, 80, 81]. The importance of the purinergic receptors, especially in microglia, is well established in animal models of both neuropathic and inflammatory pain [81], but much is still quite unexplored when it comes to their role in cancer-induced bone pain.

The P2X4 receptor is a good example of a purinergic receptor recognized to be important for the microglia-mediated contribution to neuropathic pain [48]. In response to peripheral nerve injury a de novo expression of P2X4 receptors occurs in microglia in the dorsal horn, which, upon stimulation by ATP released from the afferent neurons, results in activation of p38-mitogen-activated protein kinase (MAPK), leading to synthesis and exocytotic release of brain-derived neurotrophic factor (BDNF) [49, 82, 83]. The released BDNF induces, through the activation of the TrkB receptor, a depolarizing shift in the anion reversal potential in lamina I neurons, causing a general neuronal hyperexcitability in lamina I by reducing GABA_A-ergic and glycineric inhibition [84]. The P2X4 receptors have not been directly linked to cancer-induced bone pain, but might be indirectly involved, as the pain state displays some elements of neuropathic pain caused by compression of the peripheral nociceptive terminals as the tumor grows.

The specific role of microglial P2X7 receptors is still not fully understood, but is speculated to contribute to the hyperexcitability through the action of both TNF-α and interleukin-1β (IL-1β) [54, 85–88]. ATP, for example, released from astrocytes and acting through microglial P2X7 receptors has been reported to be an endogenous factor causing microvesicle shedding and IL-1β release in microglia [89]. ATP is released from astrocytes both through vesicular release [90] and via various membrane channels, such as connexin [91] and connexin [92], and possible through P2X7 receptor pore formation [53, 93]. The function of the P2X7 receptors in astrocytes is recognized to be tightly linked to the hyperexcitation of the dorsal horn neurons, not only through release of IL-1β and ATP, but also through glutamate signaling [50]. It has been reported that peripheral nerve injury results in an initially increased, but later persistent decreased expression of glutamate transporter-1 and glutamate-aspartate transporter in astrocytes [94–97] and that activation of the P2X7 receptors could decrease glutamate uptake in both astrocytes and microglia [98–100]. This means that stimulation of astrocytes is not only causing ATP-induced ATP release, and thereby activation of
neighboring neurons, microglia, and astrocytes through their purinergic receptors, but also that the glutamate released upon stimulation is inadequately removed from the synapse, additional contributing to a general hyperexcitation of the postsynaptic neurons and further adding to the stimulation of the neighboring astrocytes.

The ability of the P2X7 receptor as well as the P2X2 and P2X4 receptors to induce membrane permeabilization to large molecules has long been recognized and the mechanisms underlying this have been widely studied. Contrary to the P2X2 and P2X4 receptors that do not seem to rely on pannexin hemichannel association for pore formation [26, 101], the P2X7 receptor can associate with pannexin-1 to form large pores [102–104]. However, recent studies have shown that Pannexin-1 is not always involved in P2X7-receptor-mediated pore formation and other interactions as well as intrinsic ion channel dilation are possible mechanisms [105–108].

The P2RX7 gene is highly polymorphic and several nonsynonymous single nucleotide polymorphisms (SNPs) have been shown to affect either receptor channel function or pore function or both [109]. Multiple studies have connected specific P2X7 receptor SNPs to diseases such as bipolar and major depression disorders, infections, cancer, and some bone diseases [109, 110]; however, it is only recently that variations in the P2X7 receptor gene have been associated with pain [111]. Sorge et al. demonstrated that interstrain variation in mice specifically affecting the pore forming function of the P2X7 receptor influences the hypersensitivity developed in neuropathic and inflammatory pain states. Mice carrying a mutation causing impaired pore formation demonstrated less pain-related behavior than mice with normal pore formation properties of the P2X7 receptor. In addition, administration of a peptide, which blocked pore formation, also reduced pain-related behavior. Together these data indicate that it might be the properties related to pore formation and not to channel function which affect chronic pain states. The effect was found to translate to humans, as a genetic association between lower pain intensity and a hypofunctional allele of the P2X7 receptor was found in a cohort study of patients with chronic pain [111].

The importance of the P2X7 receptor in both neuropathic and inflammatory pain has additionally been demonstrated using P2X7 receptor knockout mice [112, 113]. The development of both thermal and mechanical hypersensitivity following nerve ligation was completely absent in the P2X7 receptor knockout animals, and, in addition, the animals did not develop hypersensitivity following intraplantar injections of a proinflammatory agent. The baseline nociceptive behavior was, however, unaffected, as their response to noxious heat was similar to the wild-type animals in the absence of any neuropathic or inflammatory insult [112]. Unexpectedly, it was found that the P2X7-receptor-deficient mice were susceptible to cancer-induced bone pain and even had an earlier onset of pain-related behavior compared to cancer-bearing wild-type mice. Furthermore, no effect of P2X7 receptor inhibition was found when the P2X7 receptor antagonist A-438079 was tested in two different murine models of cancer-induced bone pain.

A-438079 was applied in a model with no cancer-induced microglial or astrocyte activation (BALB/c) and in one with strong astrogliosis, but no microglial activation (C3H/HeN). Inhibiting the P2X7 receptors did not alleviate pain-related behaviors in either of the models, suggesting that the P2X7-receptor-mediated contribution to the progression of the cancer-induced pain state in these murine models is limited. Neither of the models had microglial activation, which might explain the lack of effect of P2X7 receptor inhibition. This could reflect a species difference, since microglia activation seems to be important for the progression of cancer-induced bone pain in rat models [114, 115]. Wang et al. reported an upregulation of the microglia/macrophage marker OX-42 and of BDNF in the spinal cord 6 days after tibial cancer cell inoculation in a rat model of cancer-induced bone pain. Intrathecal injection of minocycline, an inhibitor of microglial activation, resulted in a decrease in pain-related behaviours and decreased OX-42 and BDNF mRNA expression in the dorsal horn. The antinociceptive effect of minocycline was only present, when the compound was injected at the early stage of disease.

Complicating the interpretation of data from P2X7 receptor knockout mice is the fact that the mice used in the pain experiments express a splice variant of the P2X7 receptor that escaped deletion [116]. This rodent splice variant, P2X7k, uses an alternative exon 1 compared to the P2X7a variant. The P2X7k and P2X7a variants have different, but overlapping, expression, and the P2X7k variant is activated by lower ATP and bzATP concentrations than the P2X7a variant [108, 116]. Recently it was shown that the splice variants are differentially affected by a P451L SNP. Only the P2X7a variant was sensitive to the P451L SNP, which affects pore formation [108].

6. P2Y Receptors in relation to Cancer-Induced Bone Pain

Compared to the P2X receptors, the P2Y receptors are less studied in relation to chronic pain states. P2Y12 is expressed on microglia cells, and is, like P2X4, upregulated in response to peripheral nerve injury [117, 118]. Activation of the P2Y12 receptors results in phosphorylation of MAPK [117], thereby possibly linking the action of the P2Y12 receptor to the P2X4-mediated BDNF release. A role of P2Y12 in pain-related behavior has been demonstrated in models of neuropathic pain in rats. In these models intrathecal administration of different P2Y12 antagonists and antisense knockdown of P2Y12 expression suppressed the development of pain-related behaviors, and additionally intrathecal infusion of a P2Y12 agonist was found to elevate pain-related behavior in naïve rats [117, 118]. Several other types of P2Y receptors, such as the P2Y2 and P2Y1 receptors, have begun to be recognized to contribute to the hypersensitivity that occurs in chronic pain states. The P2Y1 receptors are localized on small diameter neurons in DRG and are like many of the other purinergic receptors upregulated in response to peripheral nerve injury [119]. So far, the P2Y1 receptor is the only one of the P2Y receptors studied in...
relation to cancer-induced bone pain (Table 1). In a recent study it was demonstrated that the level of P2Y1 receptor mRNA is increased in the DRGs and spinal cord of rats with bone cancer and additionally that inhibiting the receptor with intrathecal injections of a P2Y1 receptor antagonist temporarily attenuated the nociceptive behavior in the early stage of tumor growth [120]. This is in contrast to a previous report, suggesting that the P2Y1 receptor might constitute an inhibiting role in release of nociceptive transmitters in the spinal cord by inhibiting the activity of the P2X3 receptor [121, 122].

7. Conclusion and Perspectives

Even though the development of animal models has provided clues to the mechanisms underlying cancer-induced bone pain, much is still unknown. The role of purinergic signaling has begun to be elucidated and points to an involvement of the P2X3 and P2X2/3 receptors in the early development of cancer-induced bone pain. Other purinergic receptors such as P2X7 and P2Y1 have also been investigated; however, for the P2X7 receptor the interpretation of the data is complicated by variation in the activation of glial cells in different in vivo models of cancer-induced bone pain. All data so far have been obtained in rat or mouse models and it remains to be established which model is the best representation of the human disease.

Purinergic signaling is complicated not only by involving numerous receptors that can combine both as homo- and heterotrimers, but also by the fact that released ATP can rapidly be degraded by ectonucleotidases thus producing ADP, AMP, and adenosine. Investigating these complex interactions in vivo is almost impossible, and we might have to wait for new research tools before getting a fuller picture of the role of purinergic signaling in chronic pain including cancer pain. Nevertheless, based on the knowledge we already have of the importance of the purinergic receptors in nociceptive signaling in other chronic pain states, it is reasonable to hypothesize that the purinergic receptors are important for the development of cancer-induced bone pain.

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Review Article

P2X7 Receptor Function in Bone-Related Cancer

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Modulation of tumor microenvironment by different mediators is central in determining neoplastic formation and progression. Among these molecules extracellular ATP is emerging as a good candidate in promoting cell growth, neovascularization, tumor-host interactions, and metastatization. This paper summarizes recent findings on expression and function of P2X7 receptor for extracellular ATP in primary and metastatic bone cancers. Search of mRNA expression microchip databases and literature analysis demonstrate a high expression of P2X7 in primary bone tumors as well as in other malignancies such as multiple myeloma, neuroblastoma, breast, and prostate cancer. Evidence that P2X7 triggers NFATc1, PI3K/Akt, ROCK, and VEGF pathways in osteoblasts promoting either primary tumor development or osteoblastic lesions is also reported. Moreover, P2X7 receptor is involved in osteoclast differentiation, RANKL expression, matrix metalloproteases and cathepsin secretion thus promoting bone resorption and osteolytic lesions. Taken together these data point to a pivotal role for the P2X7 receptor in bone cancer biology.

1. Introduction

Primary bone cancers, malignancies that originate directly from bone cells, are quite rare diseases. According to USA National Cancer Institute about 2810 people were diagnosed, and 1500 died of bone and joint cancer in 2011. A similar incidence, one affected individual every 100,000, was reported in the same year by the Italian Association for Cancer Research (AIRC). Primary bone cancers generally originate in long bones of limbs and affect children or young adults accounting for 6% of all new pediatric cancer per year [1]. Among primary bone cancers osteosarcoma is the most frequent [2]. Two different osteosarcoma variants are known: a conventional high-grade form intramedullary located at the metaphysis of long tubular bones, frequent in adolescents, and a rarer low-grade variant arising at the surface of long bones, showing a better prognosis, more frequent among adults than young people. Frequency of low-grade is 20 times lower than high-grade ones [3].

A peculiar primary tumor, causing bone damage without need of spreading from the original site, is multiple myeloma. Multiple myeloma is a clonal B-cell malignancy characterized by an accumulation of mature plasma cells in the bone marrow, leading to bone destruction and failure of normal hematopoiesis [4, 5]. The incidence is 3–9 cases/100,000/year; it is more frequent among elderly with a slight male prevalence. Multiple myeloma remains an incurable disease even with the use of proteosome inhibitor bortezomib, immuno-modulatory drugs (thalidomide or lenalidomide), and high-dose chemotherapy associated to autologous stem cell transplantation, as part of first-line therapy [6]. In multiple myeloma, tumor and stromal cells interact via adhesion molecules and cytokine networks to simultaneously promote tumor cell survival, drug resistance, angiogenesis, and disordered bone metabolism.

Bone metastasis of extra-bone high-grade solid tumors is more frequent than primary bone cancers. The rate of bone metastasis is about 70% in breast, melanoma, lung and prostate cancer and about 15–30% in colon, stomach, bladder, uterus, rectum, thyroid, and kidney carcinomas [7]. Symptoms related to bone metastasis include pain, fractures, spinal cord compression, and hypercalcemia leading to poor quality of life and reduced life expectancy. It is estimated that the appearance of bone metastasis can reduce the five-years
survival rate of breast cancer patients from 98% to 26% and that of prostate cancer patients from 100% to 33% [8]. Both primary and secondary cancers involving the skeleton can cause osteoblastic (sclerotic) or osteolytic lesions, although generally the final histological picture is a mixture of the two. Osteoblastic lesions originate from proliferation of osteoblasts, while osteolytic lesions are generally due to osteoclast activation caused by factors secreted by cancer cells. Common sites of metastasis in the skeleton are the spine, rib cage, limbs and skull. Once settled into the bone, tumor cells release factors that activate matrix resorption leading to bone destruction; this facilitates cancer spread and proliferation [8, 9]. Since bone resorption activity is followed by an increase in bone formation, as it occurs in normal remodeling, these two processes are intimately linked and typically present at sites of bone metastasis. Osteolytic metastases are common in breast cancer, mainly due to stimulation by tumor cells of osteoclast differentiation and activity [10]. An associated local bone formation usually occurs, presumably in an attempt to activate repair, but this response is often inefficient, thus leading to final bone loss. In multiple myeloma tumor cells in the bone marrow cause exclusively osteolytic lesions, with almost complete absence of bone formation [4]. This seems to be due to suppression of osteoblast activity. On the contrary, prostate cancer metastasis are primarily osteoblastic, with possible presence of osteolytic components [11]. Most of the factors implicated in osteolytic metastatization are also involved in the pathogenesis of osteoporosis to the point that pharmacological treatment of osteolytic metastasis and osteoporosis is similar.

PTHRP (parathormone-related protein) and TGF-β (transforming growth factor beta) are among the most important osteolytic mediators [9]. PTHrP secreted by breast cancer and other tumors, is a well-known potent stimulator of osteoclast activity in bone metastasis. PTHrP acts through the release of RANKL (receptor activator of nuclear factor κB ligand) which binds to RANK receptor on osteoclasts, the system RANK–RANKL being the main pathway for osteoclast differentiation and activation. Factors released by metastatic cells or by the primary tumor activate bone resorption, this in turn triggers release of TGF-β from bone matrix. TGF-β and tumor-derived PTHrP are believed to act in a vicious cycle of local bone destruction in osteolytic metastasis: TGF-β released in active form during osteoclastic resorption of bone matrix stimulates PTHrP production by tumor cells. In turn, PTHrP mediates bone destruction by stimulating osteoclasts [4, 12]. Furthermore, TGF-β released from bone exerts suppressive effects on T lymphocytes and NK cells, thus reducing immune cell response to tumor [13].

Growth of primary bone tumors and development of bone metastasis are complex processes involving bone-tumor cells crosstalk mediated by several different cytokines and growth factors. In this context the study of molecules moulding tumor micro-environment is of great importance for a better understanding of cancer biology and to find new and efficacious therapies. Here we suggest a possible role of extracellular ATP and its receptor P2X7 in the development of both primary bone cancers and skeletal metastasis.

2. Role of ATP and P2X7 Receptors in Cell Growth and Carcinogenesis

In recent years investigation of tumor microenvironment has gained great importance for the understanding of tumor formation and progression [14]. Several molecules are released by tumor cells, acting as promoters of proliferation, allowing for immune system escape, helping cell matrix infiltration, neovascularization and distant site invasion. Interestingly, mediators of inflammatory-immune response can also influence cancer progression [15]. A good candidate molecule for many of these functions is extracellular ATP. This nucleotide has been recently shown to accumulate both at inflammatory sites [16, 17] and into the interstitium of solid tumors of different origin [18]. ATP release by dying tumor cells, following chemotherapy, is also associated to immunogenic cell death [19]. Immunogenic cell death implies that the host immune system is essential for antitumor effect of certain chemotherapeutic agents such as anthracyclines, and that molecules released from dying tumor cells act as danger signals to activate the host immune response. In this context ATP released from neoplastic cells drives recruitment of dendritic cells and T lymphocytes into the tumor site [20, 21]. Effects of ATP are mediated by two families of plasma membrane purinergic receptors: P2Y, metabotropic G coupled receptors, and P2X, ligand gated ion channels [22]. The P2X7 receptor is involved in many of the tumor-promoting and immune-modulatory effects of extracellular ATP. Like other members of the P2X family, P2X7 mediates cation fluxes across the plasma membrane but thanks to its peculiar C terminal tail it also gates a large nonselective pore [23]. Opening of this pore is coupled to the well-known P2X7 cytotoxic activity usually triggered by high (i.e. mM) pharmacological ATP concentrations. On the contrary, basal tonic P2X7 activation mediated by endogenous ATP release causes cell proliferation [24, 25]. Proliferation and other tumor transformation hallmarks seem to be dependent on the channel activity since they are retained also by cells expressing a C-terminal truncated P2X7 splice variant, which lacks pore-forming activity [26]. Several reports suggest an association between P2X7 and cancer [27]. In different cell models P2X7 expression supports organellar calcium increase, NFATc1 activation, matrix infiltration and cell growth [24–26, 28, 29]. There is good evidence that P2X7 receptor can influence energy production by increasing mitochondrial potential and intracellular ATP levels [25], a condition linked to the PI3 K/Akt pathway that positively influences cell proliferation [30]. Moreover, P2X7 activation alters the biochemical composition of tumor by causing release of microvesicles [31, 32] and secretion of cytokines [33], tissue factor [34], matrix metalloproteases (MMPs) [35] and prostaglandins [36]. Furthermore, P2X7 itself can be a conduit for ATP release [37]. Direct participation of P2X7 in tumor progression was demonstrated in a recent in vivo study by our laboratory [38]. We showed that P2X7 inhibition by either pharmacological tools or RNA interference caused a dramatic reduction of tumor masses, and vice-versa that P2X7 overexpression accelerated tumor growth. Interestingly P2X7 expressing tumors showed a
thicker vascular network and higher secretion of vascular endothelial growth factor (VEGF). Accordingly, tumor growth was inhibited by administration of the anti-VEGF antibody bevacizumab [38].

3. Extracellular ATP and P2X7 as Modulators of Osteoblasts and Osteoclasts Responses

The role of P2X7 receptor in bone cells has been extensively studied and was recently appraised in several reviews [39–42]. P2X7 receptor is expressed by both osteoblasts [43] and osteoclasts [44, 45] of different species and plays a central role in mediating osteoblast-osteoclasts crosstalk via calcium oscillations [46] and other signaling pathways [39]. One of the main roles attributed to P2X7 receptor in osteoblasts is to promote cell growth and osteodeposition [36] through a series of different pathways including c-fos [47], ERK [48], PI3 K [49], and COX [36]. Moreover, P2X7 likely mediates osteoblast ATP release as shown by the inhibitory effect on nucleotide release of P2X7 blockers [50, 51].

Skeletal disorders, such as osteoporosis and tumor-induced bone resorption, are caused by increased activity of bone-resorbing osteoclasts. The role of P2X7 in osteoclast biology is still poorly understood. It has been suggested that P2X7 participates in cell fusion, a central step in osteoclastogenesis [52, 53], but osteoclasts from P2X7 KO mice are normal in number and size [54, 55]. P2X7 receptor might, however exert its activity on osteoclast fusion indirectly by extracellular adenosine generation [53] or, simply, by increasing survival via RANKL [44] and NF-xB [56] pathways. The effect of P2X7 knock-down on bone phenotype probably depends on the different mice models considered [57]. Whatever is the role of P2X7 in osteoclast fusion and activation, a reduced activity of the receptor has been associated to increased susceptibility to osteoporosis [58]. All the known polymorphisms of human P2X7 have now been studied in different postmenopausal women cohorts [59–61]. These studies revealed an association between different complications of osteoporosis and loss of function of P2X7; a lower incidence of vertebral fractures in women expressing a gain of function receptor polymorphism was also evident [60]. These data suggest that, depending on the P2X7 polymorphism carried, one could be more or less exposed to osteolytic bone cancer complications.

4. P2X7 and Cell Metabolism in Cancer: Warburg Effect and Signaling

Ability to adapt to unfavorable conditions is a key feature of cancer cells, making them more and more aggressive. Tumor-cell survival runs through a reorganization of metabolic pathways to balance energy generation and production of biosynthetic intermediates. Aerobic glycolysis (also known as “Warburg effect”) is known to be the preferred metabolic path adopted by cancer cells, in presence of oxygen. Lactate release, as a consequence of glucose degradation, is observed in many solid tumors and leukemias. Detection of increased glucose uptake in tissues is commonly used for diagnosis of cancer by positron emission tomography (PET) [62]. In a recent paper, Grol and colleagues showed that in the osteoblast like MC3T3-E1 cell line, P2X7 activation triggers, via PI3 K, release of lactate and increased glucose metabolism [49]. PI3 K activates the serine thereonine kinase Akt, one of the most studied paths involved in tumor progression and aggressiveness [63, 64]. Indeed, activation of PI3 K/Akt pathway has been correlated with many cellular critical events such as proliferation, apoptosis, metabolism, adhesion, cytoskeleton modifications, tumorigenesis, metastatization, and drugs resistance [65, 66]. A direct effect of P2X7 activation on Akt has been shown in several cell lines [30, 67–72]. In some models such as neuroblastoma [70] and non-small-cell lung cancer [72], P2X7 was reported to reduce Akt phosphorylation while in others such as astrocytes [68], neurons [69, 73], and osteoblasts [49], the P2X7-Akt axis promoted proliferation and survival. Another Akt-mediated effect during tumor development is induction of HIF-1α that in turn leads to VEGF production and neovascularization [74, 75]. One might speculate that P2X7-mediated VEGF secretion from tumoral masses [38] could be dependent on PI3 K/Akt also in tumor proliferating osteoblasts; VEGF being a known positive regulator of osteoblastic lesions [76].

5. P2X7 Receptor in Primary Bone Tumors

A search of EMBL-EBI Atlas database (http://www.ebi.ac.uk/) revealed an association between P2X7 overexpression and different malignancies including blood and bone tumors [77]. In particular, P2X7 expression was increased in osteosarcoma, Ewing’s sarcoma, chordromyxoid fibroma, and multiple myeloma. Moreover, P2X7 receptor was found to be expressed and active in multiple myeloma cell lines where it mediates MMPs activation [78].

Osteoblasts and osteosarcoma were among the first cell models in which a proliferative activity of P2X7 and ATP was suggested [36]. Although direct in vivo proof of an oncogenic role of the receptor in osteosarcoma is missing, several experimental findings point to such an involvement. Osteoblast like (MC3T3-E1) and osteosarcoma cells lines (SaOs-2, HOS) generally show high expression of P2X7 at mRNA, protein, and functional level [43, 79, 80]. P2X7 is expressed in MSC osteoblastic precursors [81] and is highly and constantly detected during osteoblast differentiation [82]. P2X7 activity has been associated to proliferation and osteodeposition [36] as well as to upmodulation of the osteosclerotic factor FosB [83] in osteoblasts [84]. In a recent study, Liu and Chen demonstrated a trophic effect of ATP on HOS cells that was abolished by suramin, a P2 purinoceptors antagonist. Suramin also inhibited ATP-dependent cytosolic calcium increases. The ATP growth promoting effect was likely mediated via both P2X4 and P2X7 [80]. A role for P2X7 receptor in osteoblasts proliferation and osteogenesis was also indicated by Panupinthu et al. who reported reduced cell growth and osteodeposition by calvarial cell cultures from P2X7 KO mice [36]. P2X7 activated pathways in osteoblasts include cyclooxygenase (COX), lysosphatidic acid, and prostaglandin E2 [36]. A further study by Gavala et
al. showed that P2X7 dependent AP-1/Fos-B activation was responsible for COX-2 expression [84]. Moreover, mechanical stimulation triggers ATP release and P2X7-dependent activation of several kinases, including ERK [48] and PI3 K [49]. It is tempting to speculate that a condition, such as cancer, in which extracellular ATP levels are known to be upregulated [18], might mimic mechanical loading causing and stimulating osteoblast proliferation. Furthermore, mouse osteoblasts and osteoclasts constitutively release ATP into extracellular microenvironment via P2X7-dependent pathway [51].

NFAT is one of the main pathways activated through Ca2+ and calcineurin following P2X7 stimulation [29, 85–88]. We have shown that NFATc1 activation is central for P2X7 trophic activity as treatment with the NFATc1 inhibitors cyclosporine and VIVIT obliterates P2X7-dependent cell growth [29]. Moreover, P2X7-positive tumors overexpress NFATc1 [38]. On the other hand NFAT has a central role also in osteoblast biology. Mice expressing in osteoblasts a constitutively nuclear NFATc1 variant, NFATc1(nuc), develop bone masses characterized by osteoblast overgrowth [89]. Accordingly, viable NFATc1-deficient mice have defects in bone formation, in addition to impaired osteoclast development. Calcineurin/NFAT-signaling in Osteoblasts controls the expression of chemoattractants for monocytic osteoclast precursors, thereby coupling bone formation and bone resorption, and regulating bone mass [89]. Elevated levels of NFAT are among factors necessary for in vitro invasiveness of mice metastatic osteosarcoma cell lines [90]. Finally, calcineurin/NFAT pathway is implicated in prostate cancer bone metastasis. Prostate tumor cells that engraft in the bone stimulate osteoblasts by secreting growth-promoting factors among which endothelin 1 (ET-1). In osteoblasts ET-1 activates calcineurin, causes nuclear translocation of NFAT and, thus, osteoblasts stimulation [91]. On the other hand, a negative role for NFAT in osteoblasts has been proposed by Choo et al. who demonstrated that constitutively active NFAT inhibits alkaline phosphatase activity and mineralization [92]. These observations might suggest that P2X7 could cause NFAT activation and osteoblast proliferation both in primary and metastatic osteoblastic lesions.

Moreover, Ca2+-NFAT signaling is essential for osteoclast differentiation [93]. Intriguingly, NFAT activation in osteoclasts has also been related to malignant progression of multiple myeloma. Several studies reported osteoblast NFAT reduction associated with decreased osteoclastogenesis, following myeloma treatment [94–97].

P2X7 was shown to induce ATP secretion from both osteoclasts and osteoblasts [51]. Increase in extracellular ATP is followed by extracellular adenosine accumulation viaCD39 and CD73 ectonucleotidases [98]. Adenosine was demonstrated to stimulate proliferation of MC3T3-E1 osteoblastic-like cell line [99]. Furthermore, HCC1 cells release increased amounts of IL-6 and osteoprotegerin following adenosine receptor stimulation [100], likely modulating osteoclastogenesis and bone resorption.

VEGF production was found increased in different experimental tumors expressing P2X7 receptor [38]. The P2X7-VEGF connection in tumors is also supported by the finding that P2X7 activation in rat C6 glioma cells is linked to increased release of proinflammatory factors (MCP-1, IL-8 and VEGF) and to tumor-cell migration [101]. Accordingly, patients with osteosarcoma showed increased VEGF plasma levels, and this was reduced following tumor removal by surgery [102]. Moreover, VEGF secretion is known to be central in malignant progression of multiple myeloma; the first lymphohaemopoietic tumor in which increased angiogenesis was detected, and which greatly benefits of the treatment with VEGF-targeted agents [103].

6. P2X7 Receptor Activity in Bone Metastasizing Cancers

The complications of bone metastasis are thought to be due to the perturbation of the interaction between osteoblasts and osteoclasts. This disruption is thought to be caused by tumor-derived humoral mediators produced by the metastasized cancer cells within the bone marrow. Among factors regulating bone cancer metastasis RANKL plays a central role, as demonstrated by the efficacy of an anti-RANKL antibody in the therapy of such secondary tumors [104]. During physiological bone remodeling RANKL, produced by proliferating osteoblasts, causes activation of osteoclasts ensuring a balance between osteodeposition and bone resorption. However, the presence of cancer cells can alter bone microenvironment causing an increased production of RANKL, favoring osteoclasts activation and osteolysis. Several factors, such as IL-1, IL-6, COX2/prostaglandins, and VEGF, can cause positive shifts in RANKL production [10, 105]. All these mediators are released upon P2X7 stimulation from different cell types, including osteoblasts [36], immune [106, 107], and cancer cells [38, 108, 109]. Moreover, P2X7-mediated ATP secretion [26, 37] from tumor cells [18] could itself upregulate osteoblasts RANKL expression [44]. Extracellular ATP is rapidly degraded to adenosine, which can directly modulate osteoclasts formation through A2 receptors [53]. P2X7-dependent Rho-kinase1 (ROCK) activation has been demonstrated in several cell types [31, 110–112], including osteoblasts [113]. ROCK is known to activate changes in cell morphology, adhesion, and motility, and is associated to P2X7-dependent cell blebbing, a response that might be related to invasive phenotype of P2X7-expressing cells [26, 38, 114]. Interestingly, an increased ROCK signaling has been shown to contribute to breast cancer invasiveness [115] as, if overexpressed, the kinase conferred a bone metastatic phenotype to a human breast cancer cell line in an in vivo model. ROCK is also known to mediate activation of the proresorptive factor PTHR1 through activation of TGF-β signaling [116].

Several studies reported an association between primary tumors causing bone metastatization and P2X7 overexpression and function (for a recent review see [27]). Upregulation of P2X7 in breast cancer was shown for the first time by Slater et al. who demonstrated an association between receptor expression and tumor invasiveness [117]. Recently, Jelassi et al. also showed an involvement of P2X7 in breast cancer metastasis formation [114]. P2X7 activation in a
highly aggressive breast cancer cell line (MDA-MB-435s) caused increased in vitro cell motility and extracellular matrix infiltration [114]. Accordingly, P2X7 inhibition significantly reduced in vivo cell migration in a zebrafish embryo metastatic model [114]. As previously reported in macrophages [118], P2X7 receptor activation caused release of a broad range of cathepsins, including cathepsin k, also from breast cancer cells [114]. Another bone-degrading enzyme which is secreted in a P2X7-dependent fashion is MMP9 [35]. Cathepsin k and MPPs release in resorption pits during bone degradation make these enzymes attractive therapeutic targets to block osteolysis [11]. Since cathepsin k inhibitors showed adverse effects during clinical trials [11], pharmacological inhibition of upstream pathways, such as P2X7, could prove useful in therapy.

A peculiar case of tumor causing osteolytic metastasis is neuroblastoma, which is the second most common pediatric malignancy worldwide and is responsible for 15% of childhood cancer deaths [119]. Patients with high-risk metastatic disease (stages III-IV) show a mere 42% survival rate, despite treatment [120]. Few years ago Raffaghello et al. showed that P2X7 receptor is expressed in specimens from neuroblastoma patients and in all neuroblastoma cell lines examined [109]. Interestingly, in these cells P2X7 receptor lacks its well-known cytotoxic activity but rather supports proliferation [109]. Wu et al. have recently reported expression of P2X7 also in murine neuroblastoma cell lines [121]. In these cells P2X7 inhibits differentiation [121], promotes proliferation [70], and microvesicles release [122]. P2X7 implication in neuroblastoma progression is further supported from in vivo experiments from our laboratory showing a 2-3 fold reduction in neuroblastoma tumor masses upon P2X7 silencing [38]. Bone involvement is observed in 55–68% of neuroblastoma patients who present metastatic disease at diagnosis [123], particularly at the bony orbit. Bone metastasis is generally associated with osteolytic lesions due to either direct action of cancer cells on osteoclasts or to an indirect involvement of osteoclasts finally causing bone resorption [123]. Although direct evidence of P2X7 involvement in neuroblastoma bone metastatization is still missing all experimental evidence points to such an association [38, 53, 109].

Patients with advanced prostate carcinoma generally develop osteoblastic metastasis due to deregulation of physiological bone remodeling. In these patients prostate cancer cells are adjacent to large numbers of osteoblasts, which are responsible for woven bone deposition [76]. P2X7 expression by prostate cancer cells has been reported long time ago [124, 125]. Furthermore, ATP treatment increases prostate cancer cell invasion [126]. The cross-talk between prostate cancer cells and osteoblasts contributes to metastatic development. If on one hand cancer cells secrete TGFβ, VEGF and other factors promoting osteoblast proliferation and differentiation, on the other osteoblasts can prompt cancer cells to secrete MMP9 and stimulate tumor cell growth [76]. P2X7 activation has been involved in secretion of almost all the above factors either from osteoblasts [44] or from cancer cells [35, 38].

7. Conclusions and Future Perspectives

Over-expression of a potentially cytotoxic receptor such as P2X7 by cancer cells is puzzling. However, the discovery that this receptor has oncogene-like properties may provide a logical explanation for this finding [38, 114]. Due to altered skeletal phenotype of P2X7 KO mice, bone has been one of the tissues in which P2X7 activity has been best characterized. Nevertheless, there is only sporadic evidence linking bone related cancers and P2X7 so far. Here we propose that P2X7 might have a central role in bone cancer development and progression by causing NFATc1, PI3K/Akt, ROCK, VEGF activation, thus driving osteoblast proliferation in primary bone tumors and osteoblastic metastasis. P2X7 might also stimulate bone resorption by causing osteoclast activation [53] as well as secretion of cathepsins and MMPs [35, 114] thus contributing to osteolytic metastasis formation.

The processes of bone resorption and bone formation are tightly coupled and treatments that primarily target the osteoclasts generally exert secondary inhibitory effects on bone formation. The discovery of new pathways occurring in tumorigenic transformation and aggressiveness of bone-related cancers made clinicians pay attention to new targeting drugs: blocking bone lesions acting on osteoblasts/osteoclasts regulation, like bisphosphonates do, is not the only chance anymore. Clinical trials with RANKL blocking antibody (denosumab) produced very encouraging results showing that denosumab was tolerated better than bisphosphonates (zolendronic acid) and even increased patient survival [104]. Inhibitors of mTOR, an Akt downstream effector are in clinical trials for the treatment of multiple bone related cancers [127]. In particular, rapamycin is known to arrest cell growth in osteosarcoma [128, 129] and breast cancer [130].

Since P2X7 receptor is an upstream regulator of all the paths inhibited by the RANKL and mTOR blockers, it is an attractive therapeutic target for bone-related diseases too. Several P2X7 antagonists are currently in phase I and II clinical trials for the treatment of chronic inflammatory diseases, showing so far excellent safety profiles [131]. These drugs are, in principle, available to be used at patient’s bed and could be a good therapeutic opportunity for those cancers, such as neuroblastoma and multiple myeloma, which still lack an efficacious cure.

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Research Article

Genetic Background Strongly Influences the Bone Phenotype of P2X7 Receptor Knockout Mice

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The purinergic P2X7 receptor is expressed by bone cells and has been shown to be important in both bone formation and bone resorption. In this study we investigated the importance of the genetic background of the mouse strains on which the P2X7 knockout models were based by comparing bone status of a new BALB/cJ P2X7\(^{-/-}\) strain with a previous one based on the C57BL/6 strain. Female four-month-old mice from both strains were DXA scanned on a PIXImus densitometer; femurs were collected for bone strength measurements and serum for bone marker analysis. Bone-related parameters that were altered only slightly in the B6 P2X7\(^{-/-}\) became significantly altered in the BALB/cJ P2X7\(^{-/-}\) when compared to their wild type littermates. The BALB/cJ P2X7\(^{-/-}\) showed reduced levels of serum C-telopeptide fragment (s-CTX), higher bone mineral density, and increased bone strength compared to the wild type littermates. In conclusion, we have shown that the genetic background of P2X7\(^{-/-}\) mice strongly influences the bone phenotype of the P2X7\(^{-/-}\) mice and that P2X7 has a more significant regulatory role in bone remodeling than found in previous studies.

1. Introduction

In bone, endogenous nucleotides, such as ATP and UTP, are released from many types of cells, including bone cells [1, 2]. Nucleotides are released in response to a number of stimuli including inflammation [3, 4] and mechanical stimulation [5–7]. They act as autocrine and paracrine signaling molecules by activating purinergic (P2) receptors [8]. Bone cells express several types of P2 receptors [9], allowing them to respond differently to nucleotides, depending on the types of nucleotide present, their concentration, and the duration of exposure [8, 9]. The P2X7 receptor subtype is probably one of the most important P2 receptors in the regulation of bone turnover. It is most abundant in cells of haematopoetic origin [10], including osteoclasts [8, 11], but also in osteoblasts that are of mesenchymal origin [12–14].

Several roles of P2X7 have been suggested including ATP-induced apoptosis [12, 15, 16], maturation of interleukin-1β, and its subsequent release [17]. Low agonist concentrations lead to low-level activation of P2X7 receptors and might initiate osteoclast formation through activation of pathways initiating production of transcription factors [18, 19].
Prolonged exposure to high agonist concentrations induces the formation of large pores in the membrane, permeable to hydrophilic molecules as large as 900 Da [20, 21]. P2X7 receptors are expressed in both osteoclast precursors and active osteoclasts [8, 11, 22]. Therefore, in addition to activating the apoptotic pathway, the P2X7 receptor could play a role in osteoclast development [23, 24] and activation [25].

To further explore the role of the P2X7 receptor in the regulation of the skeletal system, the bone phenotype of two mouse models with ablation of the P2X7 receptor was examined [13, 22, 26]. Both of these exhibited a skeletal phenotype different from the genetic background strain. The P2X7 knock-out model first published by Solle et al. [27] showed reduced total bone mineral content (BMC), decreased periosteal bone formation, and increased bone resorption [13], which resembles the effects of disuse on the skeleton. In line with this it was shown that P2X7−/− mice have impaired response to mechanical loading [26]. The effects are partially due to increased osteoclast numbers which were further supported by in vitro studies showing that the P2X7 receptor is not necessary for murine osteoclast formation [13]. The second murine P2X7 knock-out model generated by Chessell et al. [28] shows a different bone phenotype with increased cortical thickness of the tibial shaft, but surprisingly no changes in total BMD [22].

The contradicting observations have been attributed to the dissimilar sample sizes, methods of the gene knockout, and different genetic backgrounds of the inbred strains used to generate the mice. Solle’s P2X7−/− mice were generated on 129/Ola, C57Bl/6 (B6), and DBA/2 mixed genetic background but afterwards maintained on the B6×DBA/2 background [13, 27], hereafter called DBAxB6 P2X7−/−. The P2X7−/− mice generated by Chessell et al. were maintained on B6 background but originate from a B6/129 hybrid [28], hereafter called B6 P2X7−/−.

Interestingly, mice of the DBA and B6 background have a naturally occurring mutation in the P2X7 receptor as shown by Adriouch et al. [29]. Presence of the mutation impairs the normal function of the receptor; HEK cells transfected with constructs of both genotypes showed that ATP-induced pore formation was reduced by 50% in cells carrying the mutated allele (P451L) [29]. In osteoclasts from mouse strains carrying the 451L allele we found a reduction in pore-forming activity compared to osteoclasts from strains carrying the P451 allele [30]. In murine thymocytes the P451L mutation affects the mechanism of apoptosis acting through the pore formation induced by ATP [31]. As the response of the P2X7 receptor to ATP is lower in cells harbouring the 451L allele of the P2X7 gene, consequently the observed effects of the P2X7−/− in the two models may have been underestimated in the published studies. By introducing a different genetic background to a P2X7−/− model a more pronounced bone phenotype could maybe be found.

In support of this, we recently showed that DBA/2 and B6 mice have low BMD along with impaired bone strength [30], which could make it difficult to detect ovariectomy-induced bone loss in mouse models of postmenopausal osteoporosis. Therefore, both the B6 and DBA are less suitable as mouse models of osteoporosis. Of the four inbred strains of mice that have been reported to have the P451 allelic genotype [29], only 129X1/SvJ and BALB/cJ proved ideal for a new P2X7−/− strain. Both strains had high baseline BMD, relatively strong bones and high trabecular bone volume [30]. Since the 129X1/SvJ strain is resistant to cancellous bone loss induced by ovariectomy [32] only the BALB/cJ strain is suitable as a candidate strain for the generation of a new P2X7 knock-out model. The BALB/cJ mice are characterized by having high BMD, high Tb.Th, and trabecular numbers along with strong bones. Since the BALB/cJ mice clearly respond to ovariectomy with bone loss [33], it is well suited for studying the bone specific effect of P2X7 knock-out.

Therefore, the overall aim of this study was to generate a new P2X7−/− strain with a genetic background not harbouring the P451L mutation subsequent to characterizing the BALB/cJ P2X7−/− mice and comparing the bone phenotypes of this model with that of the B6 P2X7−/−.

2. Materials and Methods

2.1. Animals. Female mice of the B6 P2X7−/− strain [28] were kindly provided by GlaxoSmithKline and crossed into the BALB/cJ inbred strain from Jackson Laboratories (Bar Harbor, ME) for five generations. Founders were selected by PCR for the knockout of exon of the P2X7 gene. Since the mice generated were only insipient congenic with the BALB/cJ genome (~97%), wild type littermates from heterozygote breeding couples were used as control groups in both strains, instead of inbred B6 or BALB/cJ.

Requests for BALB/cJ P2X7 animals should be addressed to Niklas Rye Jørgensen (Niklas@dadlnet.dk). The authors do not currently have access to B6 P2X7−/− animals, so requests for these should be directed to GlaxoSmithKline.

2.2. Study Protocol. All animal procedures were approved by The Danish Animal Welfare Council prior to initiating the experiments (protocol: 2002/561-634 and 2006/561-1209). Female mice (n = 15 mice per strain) were kept at a 12:12 hour light/dark cycle at 20 ± 0.7°C, fed the Purina mouse diet formula 5K52 (Purina, St. Louis, MO), and had access to tap water ad libitum. Ten and two days before sacrifice, animals were injected with 25 mg/kg tetracycline i.p. or 20 mg/kg calcine i.p., respectively, in order to label formative surfaces of bone for dynamic bone histomorphometry. At the age of 120 days, they were starved overnight (to minimize biological variation in bone markers) and euthanized by CO2. Blood was collected into 2 mL syringes by cardiac puncture, and serum was collected for later measurements of bone markers. The animals were scanned on the PIXIms (Lunar Corp.) densitometer shortly after blood was collected.

2.3. P2X7−/− Genotyping. P2X7−/− animals of both strains (B6 and the BALBc/J) were genotyped by PCR using the protocol outlined in the following. Earpieces or tail parts were used for DNA isolation with the QIAamp DNA Blood Mini Kit (Qiagen), which was used as template in PCR
2.4. Bone Formation and Resorption Markers. To investigate possible differences in bone formation markers between the genotypes, osteocalcin was measured in serum samples in duplicate using the Mouse Osteocalcin RIA Reagents from Biomedical Technologies, Inc. (Stoughton, MA), following the protocol supplied with the reagents. Interassay CV was 12% and intra-assay CV was 6%.

Bone resorption as expressed by fragments of type I collagen (CTX) in mouse serum was measured in duplicate using the RatLaps Elisa Assay (C-telopeptide collagen type I fragment Assay) developed by Nordic Bioscience Diagnostics (Herlev, Denmark) and following the procedure supplied with the kit. Interassay CV was 14.8% and intra-assay CV was 9.2%.

Alkaline phosphatase activity was measured in duplicate in mouse serum using the Alkaline Phosphatase Reagent Kit (Sigma). The kit measures total alkaline phosphatase activity and does not distinguish the different organ-specific subtypes. Alkaline Phosphatase activity was measured directly on the serum in the multiwell plates, using a slight modification of the standard clinical chemistry procedure. Serum replicates were diluted with alkaline buffer solution, and substrate solution was added to each well, and the plate was incubated at 37°C for 30 min. Finally, 2.0 M NaOH was added to each well to stop the reaction. Absorbance was measured on a plate reader at 405 nm. Interassay CV was 5.9% and intra-assay CV was 2.4%.

2.5. Bone Strength Measurements. On the day of sacrifice the mouse femurs were collected, cleaned for soft tissue, wrapped in saline moistened gauze in a tube, and frozen at −20°C for later ex vivo biomechanical measurements, as described earlier [34]. The strength of the femoral diaphysis was determined by a 3-point bending test on a Lloyd Instruments compression device (LR50K, Lloyd Instruments, Fareham, UK), after rehydration in a saline solution at room temperature. Load-deformation curves were generated, and maximal load was recorded at a speed of 2 mm per minute with a 100 N load cell.

2.6. Bone Histomorphometry. To investigate histologic and morphometric changes in the P2X7−/− models histomorphometric analyses were performed. In short the total spines and tibias were collected and fixed in 70% ethanol at 5°C. After methyl-methacrylate embedding, bones were sectioned into 7 μm thick slices on a Polysciences (heavy-duty microtome) and mounted on slides. Five subsequent slides were stained with Goldner-Trichrome for determination of bone volume in percentage of total volume (BV/TV, %), cortical thickness (C.Th, μm), trabecular thickness (Tb.Th, μm), and eroded surface as percentage of bone surface (ES/BS). Five slides from each bone were left unstained for quantification of mineralizing surfaces as percentage of bone surface (MS/BS, %) under fluorescent light. Further, mineral appositional rate (MAR, μm/day) was calculated. The previously mentioned indices were determined using an Olympus BX51 microscope equipped with a C.A.S.T.-Grid system and reported according to standard bone histomorphometry nomenclature.

2.7. Statistics. Statistical analyses were performed using the SPSS software, v.11.5. Standard parametric tests were used. Differences were considered statistically significant when P ≤ 0.05. Simple descriptives were presented as means ± standard error of the mean (SEM). The two types of knockout animals were compared to the respective wild type animals by Student’s t-test.

3. Results

3.1. Comparison of the Two P2X7+/− Strains. The new P2X7−/− strain was made by backcrossing the B6 P2X7−/− mice generated by Chessel et al. [28] into the BALB/cJ inbred strain for five generations, hereafter called BALB/cJ P2X7−/−. As a point of reference there were no significant differences between the wild type (P2X7+/+) mice of the two strains used for the knockout in the majority of assessed bone parameters (Table 1), but the BMD, femoral strength, and concentration of bone markers were significantly lower in the B6 strain (Table 1, Figures 1(b) and 1(c)) than in the BALB/cJ. Furthermore, bone resorption (levels of s-CTX and ES/BS%) were lower in the wild type B6 compared to the wild type BALB/cJ strain (Tables 1 and 2 and Figure 1(d)), and the bone formation markers alkaline phosphatase and osteocalcin were decreased in the B6 (Table 1).

3.2. B6 P2X7−/− Mice. There were no significant differences in the basic characteristics such as weight, length, and body composition between the two genotypes of the B6 P2X7 animals (data not shown). The markers of bone resorption and bone formation did not alter significantly when P2X7 was ablated in the B6 mice (Table 2). The increase in whole body BMD (4.5%, P = 0.011) in B6 P2X7−/− compared to the P2X7+/+ animals (Figure 1(a)) was accompanied by significant increased bone strength in B6 P2X7−/−, 18.22 N compared to 16.29 N in B6 P2X7+/+ (P = 0.018). The histomorphometric analysis of bone indices in the vertebrae showed only significant increase in Tb.Th in B6 P2X7−/−.
Figure 1: Bone mineral density, bone strength, and serum concentration of the resorption marker in the P2X7−/− in the strains BALB/cJ and B6 compared to their wild type littermates, displayed as means ± SEM. (a) Bone mineral density in BALB/cJ and B6 measured by DXA scanning on the PIXIImus. The mice homozygote for the P2X7 deletion in both strains had significant higher BMD than the wild type. There was also significant difference in BMD between the two wild type groups. (b) Bone mineral density in the femoral region of BALB/cJ and B6 measured by DXA scanning on the PIXIImus. The mice homozygote for the P2X7 deletion in both strains had higher BMD than the wild type; however it was only significant in the BALB/cJ strain. There were also significant differences in BMD between the two wild type groups. (c) Strength of femurs in BALB/cJ and B6 measured by a three-point bending test. The mice homozygous for the P2X7 deletion in BALB/cJ had significant stronger bones than the wild type BALB/cJ. B6 wild type bones were significantly weaker in the test and only a small significant difference when compared to the knockout group. (d) Concentration of s-CTX (ng/mL) in BALB/cJ and B6. There were no significant differences between the wild type and homozygote in the B6 strains. In BALB/cJ the serum concentration was significantly 46% lower in the KO animals compared to the wild type.

mice (P = 0.011) compared to B6 P2X7+/+ (Table 2). Histomorphometric analysis of the proximal tibia revealed that in resemblance with the vertebrae Tb.Th was increased (32.3 μm to 35.5 μm) however not significant in this region (P = 0.119). As seen on Figure 2, the thickness of the tibial cortex was increased to 162.6 μm in B6 P2X7−/− compared to 127.4 μm in B6 P2X7+/+ (P < 0.001). When the histomorphometric indices of B6 P2X7−/− were compared to B6 P2X7+/+, the changes were of different amplitude and direction in the two regions, tibia and vertebrae, as presented in Table 2.

3.3. BALB/cJ P2X7−/− Mice. First, we analyzed the data concerning the basic characteristics such as weight, length, and body composition (lean versus fat tissue). No significant difference between BALB/cJ P2X7−/− mice and wild type littermates was found (Table 1). The total BMD in BALB/cJ P2X7−/− assessed by DXA, was higher compared to wild type animals (Figure 1(a), Table 1); however here the difference between genotypes was higher in the BALB/cJ strain than in the B6 strain. Also differences in BMC were detected, with the higher value in the knock-outs (0.470 g versus 0.558 g, P < 0.001), as well as in bone area, again assessed by DXA.
|                  | N  | Mean   | SEM  | N  | Mean   | SEM  | P  |
|------------------|----|--------|------|----|--------|------|----|
| **N**            |    |        |      |    |        |      |    |
| B6               |    |        |      |    |        |      |    |
| Body weight (g)  | 15 | 20.12  | 0.45 | 14 | 21.65  | 1.63 | 0.380 |
| Lean tissue (g)  | 15 | 15.6   | 0.3  | 14 | 15.7   | 0.4  | 0.786 |
| Fat tissue (g)   | 15 | 4.2    | 0.3  | 14 | 5.5    | 1.5  | 0.427 |
| Fat percentage   | 15 | 20.8   | 1.3  | 14 | 22.7   | 3.1  | 0.587 |
| Bone area (cm²)  | 15 | 9.10   | 0.10 | 14 | 9.07   | 0.14 | 0.847 |
| Whole body BMC (g) | 15 | 0.454  | 0.007| 14 | 0.473  | 0.012| 0.179 |
| Whole body BMD (g/cm²) | 15 | 0.0499 | 0.000| 14 | 0.0521 | 0.0006| 0.005 |
| Femur length (mm) | 15 | 15.50  | 0.05 | 13 | 15.66  | 0.21 | 0.500 |
| Femoral BMC (g)  | 15 | 0.036  | 0.001| 14 | 0.037  | 0.001| 0.321 |
| Femoral BMD (g/cm²) | 15 | 0.0650 | 0.0007| 14 | 0.0682 | 0.0012| 0.038 |
| Maximal load (N) | 15 | 16.29  | 0.29 | 14 | 18.22  | 0.68 | 0.018 |
| RatLabs (ng/mL)  | 15 | 9.7    | 0.5  | 14 | 9.1    | 0.6  | 0.441 |
| ALP concentration (nmol/mL) | 12 | 247.6  | 13.5 | 13 | 241.0  | 12.7 | 0.727 |
| Osteocalcin (ng/mL) | 15 | 39.2   | 2.9  | 14 | 44.5   | 2.4  | 0.161 |
| **BALB/cJ**      |    |        |      |    |        |      |    |
| Body weight (g)  | 14 | 20.68  | 0.28 | 15 | 20.42  | 0.25 | 0.499 |
| Lean tissue (g)  | 14 | 16.3   | 0.2  | 15 | 16.1   | 0.2  | 0.555 |
| Fat tissue (g)   | 14 | 3.6    | 0.1  | 15 | 3.3    | 0.1  | 0.069 |
| Fat percentage   | 14 | 18.0   | 0.4  | 15 | 17.0   | 0.4  | 0.086 |
| Bone area (cm²)  | 14 | 9.19   | 0.08 | 15 | 9.90   | 0.13 | <0.001 |
| Whole body BMC (g) | 14 | 0.471  | 0.008| 15 | 0.558  | 0.009| <0.001 |
| Whole body BMD (g/cm²) | 14 | 0.0513 | 0.0005| 15 | 0.0563 | 0.0005| <0.001 |
| Femur length (mm) | 14 | 15.06  | 0.17 | 15 | 15.61  | 0.16 | 0.029 |
| Femoral BMC (g)  | 14 | 0.040  | 0.001| 15 | 0.044  | 0.001| 0.004 |
| Femoral BMD (g/cm²) | 14 | 0.0764 | 0.0006| 15 | 0.0826 | 0.0010| <0.001 |
| Maximal load (N) | 14 | 24.62  | 0.44 | 15 | 33.95  | 0.59 | <0.001 |
| RatLabs (ng/mL)  | 14 | 19.1   | 1.2  | 15 | 270.6  | 15.0 | <0.001 |
| ALP concentration (nmol/mL) | 14 | 314.2  | 11.0 | 14 | 270.6  | 15.0 | <0.001 |
| Osteocalcin (ng/mL) | 14 | 55.9   | 3.1  | 15 | 63.2   | 2.9  | 0.101 |

4. Discussion

The role of P2X7 in the regulation of bone turnover has already been established over the last decade. Extracellular nucleotides have been shown to be involved in calcium signalling and thus intercellular communication among osteoblasts [8]. Nevertheless, the reported effects of the relatively specific P2X7 agonist BzATP on the activity of bone cells in vitro have been conflicting [12, 18, 19].

In the current study, we have shown that the P2X7 receptor has a distinct role in the regulation of bone mass and turnover, as BMD was increased in P2X7−/− mice from two strains with different genetic backgrounds. The mechanisms underlying the rise in BMD are presumably multiple but could be related to the observed changes in bone formation and resorption markers, even though no difference was seen in the histomorphometric analyses.
Table 2: Histomorphometric analysis for B6 and BALB P2X7+/+ and P2X7−/− mice.

|                | B6+/+ | B6−/− | t-Test |
|----------------|-------|-------|--------|
|                | Mean  | SEM   | Mean   | SEM   | P      |
| Vertebral      |       |       |        |       |        |
| N              | 12    | 8     |        |       |        |
| MS/BS%         | 43.8  | 2.9   | 42.5   | 3.9   | 0.796  |
| MAR (μm/day)   | 1.07  | 0.03  | 1.00   | 0.04  | 0.077  |
| ES/BS (%)      | 7.9   | 0.4   | 9.1    | 0.7   | 0.180  |
| BV/TV (%)      | 17.7  | 1.5   | 20.4   | 0.9   | 0.122  |
| Tb.Th. (μm)    | 32.8  | 1.1   | 36.5   | 0.7   | 0.011  |
| C.Th. (μm)     | 93.0  | 3.6   | 93.7   | 4.8   | 0.917  |
| Tibial         |       |       |        |       |        |
| N              | 12    | 8     |        |       |        |
| MS/BS%         | 46.1  | 2.1   | 44     | 2.4   | 0.517  |
| MAR (μm/day)   | 1.16  | 0.03  | 1.2    | 0.04  | 0.378  |
| ES/BS (%)      | 8.3   | 0.6   | 8.0    | 0.3   | 0.676  |
| BV/TV (%)      | 10.0  | 1.7   | 13.2   | 1.7   | 0.197  |
| Tb.Th. (μm)    | 32.3  | 1.1   | 35.5   | 1.7   | 0.138  |
| C.Th. (μm)     | 127.4 | 3.8   | 162.6  | 6.1   | <0.001 |

Changes in histomorphometric parameters in P2X7−/− compared to P2X7+/+ in the tibia and vertebral (only B6). Displayed as mean ± SE for the group. The direction of changes is equal in the different regions of the B6 mice, besides MAR where it is decreased in the vertebral region of P2X7−/− and decreased in the tibia. The ES/BS (%) has the opposite directions in vertebral and tibia. Besides the cortical thickness and MS/BS (%) the directions of changes are the opposite in the BALB mice compared to the B6 mice. The size of the changes in the histomorphometric parameters P2X7−/− mice is different in relation to region and strain analyzed. Significant differences from wild type animals at the P < 0.05 levels, determined by Student t-test, are displayed.

Figure 2: Representative photomicrographs of Goldner-Trichrome stained slices of proximal tibia from (a) P2X7−/− and (b) wild type animals of B6 strain.
The new P2X7−/− on BALB/cJ background (BALB P2X7−/−) showed significantly increased BMD, BMC, and bone area determined by DXA compared to the wild type genotype. BMD/BMC was also increased in the femoral region, which was accompanied by an increase in bone strength of the femoral diaphysis. The difference could be caused by reduced resorption compared to bone formation, since we found big reduction in s-CTX and a significant decrease in the bone formation marker alkaline phosphatase. Morphological changes in other compartments could also explain the observed results, but these have not been investigated in this study. Almost the same picture was present when the B6 P2X7−/− mice were examined though the effect sizes were smaller. Thus, the increases in BMDs and in femoral strength were smaller. When histomorphometric analysis of the vertebrae was compared to the tibia, site-specific differences were detected in the resorption index ES/B5%, but even the effect was in the opposite direction; it was insignificant at both sites. The measured Tb.Th was significantly higher in the vertebrae but not in the tibia. We found increased C.Th in the proximal tibia, but not in the vertebrae. The fact that we observe the same effect of P2X7 ablation, but with a smaller effect size in the strain carrying the 451L allele, confirms the role of the P2X7 receptor in regulation of bone mass.

Serum CTX levels express the total bone resorbing activity of the organism, and interestingly we found striking differences in s-CTX between the two strains. In BALB/cJ, the wild type animals have a significantly higher s-CTX level than both the corresponding knock-out and the B6 wild type (Figure 1(d)). In contrast, the B6 wild type and the B6 P2X7−/− s-CTX levels are not different, suggesting a distinct role of the cytoplasmic tail of the P2X7 receptor in osteoclastic bone resorption. In a former study we found a reduction in pore formation in osteoclasts from the B6 strains compared to osteoclasts from the BALB/cJ, but no difference in resorative activity of cultures not stimulated with nucleotides [30]. Even though there are multiple genetic differences between the two strains, the major finding in this study is that the genetic background is of significant importance when determining the effect of the P2X7 ablation.

As seen in Figure 2, our study confirms the results of the previous study where Garland et al. showed increased cortical thickness in tibia of P2X7−/− [22]. Since they were not able to detect any difference in the formation and number of osteoclasts, they suggested that the apoptosis of the osteoblasts was affected. They also observed an increased number of osteocytes in cortex, suggesting that instead of undergoing apoptosis the osteoblasts were incorporated in matrix. That could be a plausible explanation for the differences found in B6 P2X7−/−, but it cannot explain the decrease in s-CTX in the BALB P2X7−/−. In the study by Ke et al., they found an increased number of osteoclasts in Pfizer DBA/B6 P2X7−/− tibiae but could not detect any difference in osteoclastogenesis in vitro [13]. Garland et al. also reported that there was no significant difference in the development of osteoclasts between B6 P2X7−/− and B6 WT in vitro [22]. The question is if the unnatural culture conditions, for example, the lack of systemic hormones, accessory cells, and mechanical stimulation, could explain the lack of effect of P2X7 ablation upon osteoclasts when assessed in vitro compared to the differences seen in vivo/ex vivo.

Finally, the recently described P2X7-k splice variant suggested to escape knockout in the Glaxo mice [35] could be a contributing factor to the differences between the Glaxo and Pfizer models. However, even though normal osteoclasts express P2X7, none could be detected in osteoclasts derived from BALB/cJ-P2X7−/− mice, indicating that the P2X7-k expression splice variant was undetectable on the protein level [36].

However, even if there are some conflicting results from the two different models of P2X7 knock-out in mice, they both point towards an important role of the receptor in regulation of bone formation. It emphasizes that even though mice are widely used as models to study the regulation of bone mass and turnover, there are important differences between murine and human bone physiology. Human studies are highly warranted to investigate the role of the P2X7 receptor in human bone turnover and in conditions of altered bone metabolism.

The two background strains on which the B6xDBA P2X7−/− from Pfizer and B6 P2X7−/− from Glaxo mice were based carry the 451L allele of the naturally occurring mutation in P2X7. The contradictory effects on bone status between the two strains could be due to a number of factors, including differences in experimental parameters like sex, age, or diet [13, 22], which makes it difficult to compare the experiments with different background. Ke et al. found a larger effect of the knockout in DBA/B6 P2X7−/− males compared to the females. Garland et al. do not inform about the sex of the B6 P2X7 animals in their study [22]. In our study, only females were used. Of critical importance is the choice of wild type animals, whether it is wild type siblings from heterozygote breeding couples or purchased from the inbred background strain. Another important parameter to consider is the diet, as it is known to influence bone mass and structure. Our earlier experiments with the B6 P2X7 mice showed only insignificant differences in s-CTX. By changing the diet from Altromin 1430 or 1319 to LabDiet 5K52, which contains more calcium (0.8% to 1.15%), more D-vitamin (1 IU to 4 IU), and less fat (7.5% to 4%) it showed significant effects on the BMD, also in female mice. In this study the BALB and B6 P2X7−/− mice were fed the same diet and had similar age and sex, so the only difference is the genetic background. Bouxsein et al. have shown that the genetic background is extremely important for the bone status in the different strains [33]. In this study BALB/cJ mice had higher bone turnover (indicated by levels of s-CTX and alkaline phosphatase) than B6. Therefore it is possible that it is easier to detect the decrease in resorption in the P2X7−/− on the BALB/cJ background. Similar strain-specific differences are seen in other knockout mice, like diverse effects of treatment are seen in different strains [37].

In conclusion, we have shown that the P2X7 receptor plays an important role in the control of bone remodelling. Furthermore, absence of P2X7 receptor expression seems to be associated with increased bone mass and strength in two
mouse models. The effect of P2X7 ablation was underestimated in the model based on the B6 strain carrying the naturally occurring mutation, since the difference between the knockouts and their wild type littermates is higher in the BALB/c model than in the B6 model. Further studies are warranted in order to understand the complex roles of P2X7 in bone turnover, where especially human studies are important in order to fully understand the role of the receptor in human bone physiology.

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Association between P2X7 Receptor Polymorphisms and Bone Status in Mice

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Macrophages from mouse strains with the naturally occurring mutation P451L in the purinergic receptor P2X7 have impaired responses to agonists (1). Because P2X7 receptors are expressed in bone cells and are implicated in bone physiology, we asked whether strains with the P451L mutation have a different bone phenotype. By sequencing the most common strains of inbred mice, we found that only a few strains (BALB, NOD, NZW, and 129) were harboring the wild allelic version of the mutation (P451) in the gene for the purinergic receptor P2X7. The strains were compared by means of dual energy X-ray absorptiometry (DXA), bone markers, and three-point bending. Cultured osteoclasts were used in the ATP-induced pore formation assay. We found that strains with the P451 allele (BALB/cJ and 129X1/SvJ) had stronger femurs and higher levels of the bone resorption marker C-telopeptide collagen (CTX) compared to C57Bl/6 (B6) and DBA/2J mice. In strains with the 451L allele, pore-formation activity in osteoclasts in vitro was lower after application of ATP. In conclusion, two strains with the 451L allele of the naturally occurring mutation P451L, have weaker bones and lower levels of CTX, suggesting lower resorption levels in these animals, which could be related to the decreased ATP-induced pore formation observed in vitro. The importance of these findings for the interpretation of the earlier reported effects of P2X7 in mice is discussed, along with strategies in developing a murine model for testing the therapeutic effects of P2X7 agonists and antagonists upon postmenopausal osteoporosis.

1. Introduction

In the past decade several reports have shown that P2-receptor signalling plays a central role in bone physiology [1–5]. Bone cells express several types of P2 receptors [6], allowing them to respond differently to nucleotides, depending on the types of nucleotides present, their concentration, and the duration of exposure [7, 8]. Prolonged exposure to high agonist concentrations initiates the formation of large pores in the membrane mediated by the P2 subtype P2X7, a feature often assessed by ATP-induced dye uptake [7, 9]. Activation of the P2X7 receptor results in changed cellular morphology and membrane blebbing [10], which initiates necrotic and apoptotic mechanisms in macrophages [11]. There are several studies directing a role to the P2X7 receptor in mediating ATP-induced apoptosis in other cell types [7] and accordingly increased osteoclast numbers have been found in mice with ablation of the P2X7 receptor (P2X7−/− mice) [3]. In humans the single nucleotide polymorphism Glu496Ala is associated with abolished pore formation activity of the P2X7 receptor [12] and with decreased ATP-induced apoptosis of osteoclasts in vitro [13]. The P2X7 receptors are expressed in both osteoclast precursors and resorbing osteoclasts [8, 14, 15], and therefore, in addition...
to activating the apoptotic pathway, the P2X7 receptor could play a role in osteoclast development [16–18] and activation [19].

In calvarial cells in vitro activation of P2X7 receptors increases expression of osteoblast markers, enhances mineralization, and induces membrane blebbing [20, 21]. The effects of P2X7 activation could also be mediated through the activation of the cytokine interleukin β (IL-1β) on bone cells or on cells adjacent to the bone compartment, resulting in a systemic effect on bone [22]. In macrophages from people carrying two C alleles of the Glu496Ala (A1513C) polymorphism, the processing of pro-IL-1β is impaired, leading to decreased levels of mature serum IL-1β.

The ablation of the P2X7 receptor generated by Solle (Pfizer P2X7−/−) in mice, led to reduced total bone mineral content (BMC), as a result of increased trabecular bone formation [20, 21]. The effects of P2X7 activation could also be mediated through the ATP-induced pore formation [25]. The reduced function of P2X7 receptors to ATP in mice with the P451L mutation could have led to underestimation of the responsiveness of the P2X7 receptors to ATP in mice with the P451L mutation a eff fthymocytes the P451L mutation acts in cells carrying the mutated 451L allele [24]. In murine strains, effects apoptosis acting through the ATP-induced pore formation [25]. The reduced responsiveness of the P2X7 receptors to ATP in mice with P451L could have led to underestimation of the effects of the P2X7−/− upon bone and other parameters. Pfizer P2X7−/− mice were generated on 129/Ola × C57Bl/6(B6) × DBA/2 genetic backgrounds, and maintained on the B6 × DBA/2 background [3, 26]. The GSK P2X7−/− mice were maintained on B6 background, but originate from a B6/129 hybrid [15, 23].

Two papers have described the expression of a novel splice variant (called P2X7-k) for the rodent P2X7 [27, 28]. The splice variant was first found in lymphocytes of the GSK P2X7−/− mice by Taylor et al. [27]. Nicke et al. reported shortly after that P2X7-k shows higher agonist sensitivity and slower deactivation than the normal P2X7 receptor complexes [28]. The alternative exon of the P2X7-k splice variant includes the intracellular N-terminus and ~80% of the first transmembrane domain, and thereby escapes gene inactivation in the GSK P2X7−/− mice. However, the expression of the splice variant P2X7-k is tissue-specific, and not expressed on the plasma membrane of osteoclasts from BALB/cj P2X7−/− [29].

None of the current P2X7−/− models are quite suitable for studying the therapeutic potential of the agonist acting through P2X7 in ovariectomized mice. First, the impaired ATP response in P451L mutated cells could mask the “true” effect of agonists and antagonists. Second, some of the founder strains included in the genetic background of these animals lacks response upon ovariectomy. The inbred mouse strains traditionally used for bone research represent different skeletal phenotypes during development and aging [30], which could be of importance in the interpretation of the precise role of P2X7 receptors in bone remodelling. In the present study, the major goal was to investigate the distribution of the P451L alleles in inbred strains of mice. Furthermore, we wanted to investigate the bone status of these animals in order to select a suitable strain with the preferred genetic background (i.e., carrying the P451L allele) for crossing the P2X7−/− genotype into.

2. Materials and Methods

2.1. Sequencing of P2X7. A single-coding mutation (T1352C) in the murine P2X7 gene resulting in a change from proline to leucine at position 451 (P451L) has earlier been reported in four major strains of inbred mice [24]. To examine the distribution of the two alleles of T1352C (P451L) among laboratory mice genomic DNA was analyzed by sequencing exon 13 in the murine P2X7 gene. DNA from 20 different inbred strains (C3H/HeJ, C3H/HeJ/Crl, NZB/B1NI, NZW/J, SJL/J, AKR/J, BALB/cJ, BALB/cByJ, BALB/cAnCrl, 129J, 129X1/SvJ, SWR/J, C57L/J, C57BL/10J, DBA/1J, DBA/2J, SM/J, NOD, DDD/cJ, and CALB/Rk) and from 2 strains of outbred (Mus caroli/Ei and M. spreitus) was obtained from The Jackson Laboratories (Bar Harbor, ME). The B6 originates from Charles River (Germany), The Danish Pest Infestation Laboratory (Lyngby, Denmark) donated ten wild Mus musculus domestic, from different locations. Earpieces and tail parts were used for DNA isolation with the QIAamp DNA Blood Mini Kit (Qiagen), which was used as template in PCR reactions with the following primers recognizing exon 13: F-ACT TGA GGG GTT GTC ATT GC and R-TCC AAG GGA AGC TGT ATT GTG giving an amplicon of 595 bp. For each PCR product two sequencing reactions were prepared with the specific sequencing primers (F-TGC TGA TGG GTC ATT GC and R-TCC AAG GGA AGC TGT ATT GTG) in the sequencing reaction with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as described by Ohlendorf et al. 2007 [13], and loaded on the ABI PRISM 3100 Genetic Analyzer. Duplicate amplification and sequencing reactions were run per strain to confirm the sequences.

2.2. Animals. Female mice of nine inbred strains (C3H/HeJ, NZB/B1NI, SJL/J, AKR/J, BALB/cJ, 129X1/SvJ, SWR/J, C57L/J, and DBA/2J) were obtained at eight weeks of
age from Jackson Laboratories (Bar Harbor, ME). The B6 originated from Charles River (Germany).

2.3. Study Protocol. The Danish Animal Welfare Council approved all animal procedures in advance (protocol: 2002/561–634). Female mice at the age of 120 days were starved overnight before euthanized by CO₂. Blood was collected into 5 mL syringes by cardiac puncture. Serum was collected and stored at −80°C for later measurements of bone markers. The animals were scanned on a PIXImus (Lunar Corporation, Madison, WI) densitometer.

2.4. Bone Mineral Measurements and Body Composition. Bone mineral measurements and body composition of the animals were determined on the PIXImus densitometer. Animals were fixed in a standard position, and measurements were performed sequentially, with duplicate determinations. Intra assay CV was 0.47% and inter assay CV 0.73%. Due to the large mineral content in the skull, it was excluded from the calculations.

2.5. Bone Formation and Resorption Markers. To investigate possible differences in bone formation markers between the genotypes, osteocalcin was measured in serum samples in duplicate using the Mouse Osteocalcin RIA Reagents from Biomedical Technologies, Inc. (Stoughton, MA), following the protocol supplied with the reagents. Intraassay CV was 12% and intraassay CV was 6%.

Bone resorption as expressed by fragments of type I collagen in mouse serum (s-CTX) was measured in duplicate using the RatLaps Elisa Assay (C-telopeptide collagen type I fragment Assay) developed by Nordic Bioscience Diagnostics (Herlev, Denmark) and following the procedure supplied with the kit. Intraassay CV was 14.8% and intraassay CV was 9.2%.

Alkaline phosphatase activity was measured in duplicate mouse serum using the Alkaline Phosphatase Reagent Kit (Sigma). Alkaline phosphatase activity was measured directly on the serum in multiwell plates, using a slight modification of the standard clinical chemistry procedure. Serum replicas were diluted with alkaline buffer solution and substrate solution was added to each well, and the plate was incubated at 37°C for 30 min. Finally 2.0N NaOH was added to each well to stop the reaction, before the absorbance was measured on a plate reader at 405 nm. Intraassay CV was 5.9% and intraassay CV was 2.4%.

2.6. Bone Strength Measurements. On the day of sacrifice the mouse femurs were collected, cleaned for tissue, wrapped in saline gauze, and frozen at −20°C until biomechanical testing. The strength of the femur was measured by the 3-point-bending test on a Lloyd Instruments compression device (Lloyd Instruments, Fareham, UK), performed after rehydrating the femur in a saline solution at room temperature, as described earlier [31].

2.7. Bone Histomorphometry. To investigate histologic and morphometric changes in four different inbred strains histomorphometric analyses were performed, as described earlier [31]. In short, the total spines and tibia were collected and fixed in 70% ethanol at 5°C. The following indices were determined; bone volume in percentage of total volume (BV/TV%), cortical thickness (C.Th, μm), trabecular thickness (Tb.Th, μm), and eroded surface as percentage of bone surface (ES/BS%). Under fluorescent light the mineralizing surfaces were determined as percentage of bone surface (MS/BS%). Further, mineral appositional rate (MAR, μm/day) was calculated.

2.8. In Vitro Dye Uptake Assay. Osteoclasts were isolated from bone marrow from the long bones of 3–4 weeks old female mice of the strains; BALB/cJ, 129X1/SvJ, DBA, and B6 (10 mice per strain) as described by Wu et al. [32]. Bone marrow monocyte/macrophage lineage precursors were seeded in α-MEM (10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 50 ng/mL M-CSF, and pH = 7.35–7.4) and plated with a density of 2 × 10⁶ cells/cm². More than 91% of both mononuclear and multinucleated cells in these cultures stained positively for TRAP (tartrate resistant acid phosphatase) using the leukocyte acid phosphatase kit (Sigma).

To investigate pore formation in osteoclasts from the four strains, as described for osteoblasts by Ke et al. [3], the medium was changed after 7 days in culture to a HEPES/glucose (20/10 mM) divalent cation-free buffer and the osteoclasts ability to take up the dye YO-PRO (Molecular Probes) upon ATP (200 nM) stimulation was assessed. After 9 minutes Hoechst 33342 (5 μg/mL, BioWhittaker) was added to the culture for another minute to reveal unstained nuclei in the osteoclasts. Only multinucleated osteoclasts (with 3 or more nuclei) were counted on a Leica BZ:03 Microscope and the number of YO-PRO containing cells was compared to the number of total osteoclasts in ten randomly chosen fields of sight. Control cultures were incubated with YO-PRO and Hoechst, but not ATP. Intraassay CV was 9.8% and intraassay CV was 5.4%.

2.9. Resorption Assay. Osteoclasts were isolated and cultured as described above for the dyeuptake assay, but seeded in 96-well plates on bovine bone slices (Nordic Bioscience, Herlev Denmark) or glass-cover slips. After 10 days of culture the osteoclasts on glass-cover slips were stained for TRAP and counted by visible inspection of ten randomly chosen fields of sight. Only TRAP positive cells with more than 3 nuclei were quantified as multinucleated osteoclasts. The cells were removed from the bone slices and the resorption pits were stained with hematoxylin, prior to quantification with the C.A.S.T. (Computer Assisted Sterological Toolbox) Grid system on Olympus Bx51. The results were calculated as area resorbed in percentage of total area. Intraassay CV was 0.67% and intraassay CV was 0.45%.

2.10. Statistics. Statistical analyses were performed using the SPSS software, v.11.5. Standard parametric and nonparametric tests were used. For the comparison of results from the different strains the one-way ANOVA was used, with
Table 1: The distribution of the strains in two groups with different allelic versions of the P451L mutation. Strains written in italic are outbred or mice from wild populations. *Confirming the data also shown by Adriouch et al. [24].

| Strains with P451L | Strains with 451L |
|--------------------|------------------|
| *Mus spretus*      | C57BL/6j (B6)*   |
| *Mus musculus*     | C57BL/10J*       |
| *Mus caroli*       | C57BL/6NCrI      |
| BALB/cByJ*         | DBA/1J*          |
| BALB/cAnNCrI*      | DBA/2J*          |
| NOD*               | AKR/J            |
| NZW*               | C57L/J           |
| 129/J*             | NZB/BINJ         |
| 129X1/SvJ          | SIL/J            |
|                    | SWR/J            |
|                    | DDDY/J           |
|                    | SM/J             |
|                    | CALB/RkJ         |

multiple comparisons and post hoc Bonferroni corrections. Differences were considered statistically insignificant when \( P \leq 0.05 \). Simple descriptives of data were presented as means ± standard error of the mean (SEM).

3. Results

3.1. Distribution of the P451L Mutation in the Inbred Strains. The distribution of the two alleles of the P451L mutation among common strains of laboratory mouse strains was determined in 21 strains and sublines by sequencing genomic DNA. Only mice from wild populations, outbred strains and a few of the inbred strains, which included the four major strains, BALB, NOD, NZW, and 129, had the P451 allele, hereafter called the original genotype of the P451L mutation. The rest of the examined strains and sublines had the mutated 451L allele (Table 1).

3.2. Bone Phenotypes in Relation to P451L Mutation. Ten inbred strains frequently used in bone related studies, were examined by DXA, three-point bending and serum concentration of bone markers to reveal the bone phenotype. As summarized in Table 2 there were significant differences in all measured parameters between groups, but only few associated to the different allelic versions of the P2X7 P451L mutation. With the genetic background of the two existing P2X7−/− mice [23, 26] in mind, the inbred strains of 129X1/SvJ and BALB/c] (P451) were compared with B6 and DBA/2 (451L) with respect to bone status and these background strains were further examined by histomorphometric analysis, resorption activity, and in vitro dyeuptake assay.

3.3. Bone Status of the Background Strains. When focusing on BMD/BMC 129X1/SvJ had significantly (\( P < 0.001 \)) higher whole body bone mineral (BMD and BMC) compared to the other three strains (Figure 1(a)). In the load-bearing region, femoral BMD and BMC were higher in the strains 129X1/SvJ and BALB/c] than B6 and DBA/2 (\( P < 0.001 \). Figure 1(b)).

The ultimate way of determining bone quality is to test the ability of bone to resist mechanical forces. Determined by a three-point bending test, the femoral bone strength was different among the strains of mice (Table 2). Of the four background strains 129X1/SvJ and BALB/c] were more resistant to mechanical forces at the femur than both DBA/2 and B6 strains (\( P < 0.001 \), Table 2, Figure 1(c)).

In vivo bone markers are summarized in Table 2. The serum concentration of the bone formation markers, alkaline phosphatase and osteocalcin, were not associated with the P451L mutation in the P2X7 gene. The BALB/c] strain had significantly (\( P < 0.001 \)) higher s-CTX than all the other strains (Figure 1(d)). 129X1/SvJ mice had significantly higher s-CTX than the B6 mice, but not the DBA/2 (Table 2).

3.4. Histomorphometric Analysis in Relation to the P451L Mutation. Bone volume (%) in the vertebrae was higher in 129X1/SvJ than in the BALB/c] and the B6 (\( P < 0.001 \)), whereas BALB/c] and B6 did not differ significantly (Figure 2(a)). No significant difference was found in the Tb.Th between the different strains. The cortical thickness (C.Th) for BALB/c] (102.1 \( \mu \)m) was not significantly different from B6 (93.0 \( \mu \)m) and DBA/2 (104.4 \( \mu \)m). The mean C.Th was 121.5 \( \mu \)m for the 129X1/SvJ strain, was significantly higher than in the three other strains. Neither MAR nor MS/BS showed any significant differences between the four strains. However ES/BS% showed that the 129X1/SvJ strain had a significantly higher ES/BS% than B6, and BALB/c], B6, and DBA/2 (Figure 2(b)).

3.5. Ex Vivo Bone Resorption Assay. The area of resorption pits per bone slice was calculated as percentage of total area in 10 mice from each strain. BALB/c] and 129X1/SvJ osteoclasts showed 19.1% and 18.7% area resorbed in 10 mice from each strain; BALB/c] and 129X1/SvJ had 79.5 and 83.7% area resorbed in comparison to 17.4% and 16.3% for B6 and DBA/2, with no significant difference (\( P = 0.478 \) between BALB/c] and DBA/2). The number of TRAP-stained osteoclasts did not differ significantly. BALB/c] and 129X1/SvJ had 79.5 and 83.7 TRAP-positive osteoclasts in comparison to 93.6 and 97.4 for B6 and DBA/2, with no significant difference (\( P = 0.108 \) between BALB/c] and DBA/2).

3.6. In Vitro Dye Uptake Assay. To elucidate the cellular bases of these changes, we examined the response to ATP stimulation in a dye uptake assay, comparing the pore formation in 4 cultures from 10 mice in each of the following strains; B6, BALB/c], DBA/2, and 129X1/SvJ. Half of the cell cultures were incubated with dye, but without ATP stimulation. These cultures were used as controls to determine the spontaneous background staining, compared to the total number of cells in the assay. A fraction of the osteoclasts in the un-stimulated control cultures showed spontaneous dye uptake, with no significant difference between the strains. Interestingly,
| Sample size (n) | 129X1/SvJ | BALB/cJ | B6 | DBA/2J | SWR/J | C57L/J | AKR/J | SJL/J | NZB/BlNJ | C3H/HeJ | ANOVA one way |
|----------------|-----------|---------|----|--------|-------|--------|-------|-------|-----------|----------|---------------|
| P451L genotype | P | P | L | L | L | L | L | L | L | L | |
| Bone Parameters | | | | | | | | | | | |
| BMD (g/cm²) | 0.0562* (±0.0020) | 0.0513A,D (±0.0018) | 0.0502A,D (±0.0020) | 0.0468* (±0.0022) | 0.0477 (±0.0013) | 0.0579 (±0.0030) | 0.0576 (±0.0021) | 0.0576 (±0.0015) | 0.0548 (±0.0015) | 0.0525 (±0.0041) | 0.0563 (±0.0018) | <0.001 |
| BMC (g) | 0.535* (±0.045) | 0.471A,D (±0.028) | 0.458D (±0.031) | 0.358* (±0.029) | 0.394 (±0.035) | 0.548 (±0.042) | 0.593 (±0.046) | 0.459 (±0.025) | 0.465 (±0.025) | 0.558 (±0.036) | <0.001 |
| Area (cm²) | 9.53D (±0.53) | 9.19D (±0.30) | 9.13D (±0.40) | 7.65* (±0.41) | 8.26 (±0.54) | 9.46 (±0.35) | 10.29 (±0.60) | 8.38 (±0.40) | 8.83 (±0.60) | 9.90 (±0.50) | <0.001 |
| BMD in femur region (g/cm²) | 0.0798B,D (±0.0039) | 0.0764A,D (±0.0021) | 0.0655A,C (±0.0028) | 0.0639A,C (±0.0027) | 0.0650 (±0.0024) | 0.0786 (±0.0036) | 0.0782 (±0.0036) | 0.0774 (±0.0032) | 0.0786 (±0.0032) | 0.0826 (±0.0037) | <0.001 |
| BMC in femur region (g) | 0.0439B,D (±0.0044) | 0.0402D (±0.0028) | 0.0364A,D (±0.0026) | 0.0281* (±0.0026) | 0.0307 (±0.0021) | 0.0466 (±0.0044) | 0.0466 (±0.0044) | 0.0391 (±0.0029) | 0.0578 (±0.0032) | 0.0439 (±0.0036) | <0.001 |
| Area of femur region (cm²) | 0.552D (±0.035) | 0.529D (±0.031) | 0.558D (±0.027) | 0.443* (±0.035) | 0.476 (±0.031) | 0.595 (±0.046) | 0.599 (±0.026) | 0.507 (±0.032) | 0.511 (±0.035) | 0.533 (±0.028) | <0.001 |
| Femur length (mm) | 15.25D (±0.36) | 15.06D (±0.64) | 15.53D (±0.23) | 14.34* (±0.45) | 14.29 (±0.40) | 15.79 (±0.33) | 16.00 (±0.69) | 14.08 (±0.28) | 15.90 (±0.45) | 16.31 (±0.64) | <0.001 |
| Strength/Max. load femurs (N) | 3.15* (±3.2) | 2.6* (±1.7) | 1.63A,C (±1.1) | 1.87A,C (±1.5) | 1.67 (±0.8) | 2.80 (±2.4) | 3.05 (±2.8) | 2.0 (±3.0) | 23.3 (±2.3) | 33.9 (±2.3) | <0.001 |

**In vivo bone markers**

| Osteocalcin (ng/mL) | 40.0C,D (±9.48) | 55.91* (±1.78) | 38.42C,D (±11.11) | 71.72* (±10.89) | 38.44 (±8.97) | 36.49 (±8.75) | 72.37 (±11.25) | 51.09 (±10.20) | 48.49 (±8.06) | 63.22 (±11.38) | <0.001 |
| ALP (nmol/mL) | 266.8 | 314.2 | 260.2 | 317.6 | 272.5 | 179.7 | 190.9 | 213.7 | 299.4 | 270.6 | <0.001 |
| RatLaps-Telopeptide collagen (ng/mL) | 14.28C,D (±56.1) | 19.09 (±41.2) | 9.81A,C (±63.8) | 12.13C (±60.5) | 10.75 (±39.9) | 9.37 (±48.3) | 7.07 (±62.5) | 7.15 (±59.6) | 8.83 (±36.3) | 8.76 (±56.3) | <0.001 |
the percentage of osteoclasts taking up dye upon ATP-stimulation was significantly higher when isolated from strains carrying the P451 allele (BALB/cJ and 129X1/SvJ) as compared to strains with the 451L-allele (DBA/2 and B6) \((P < 0.05)\) (Figure 3). When corrected for background dye uptake, the number of dye uptaking osteoclasts from P2X7 451L strains was reduced by approximately 30% compared to osteoclasts from the P451 strains, suggesting deleterious effects of the mutation on P2X7-mediated pore formation.

4. Discussion

The P451 allele of the P451L mutation in P2X7 was present in different wild mouse populations, in the outbred strains \textit{Mus caroli/Ei} and \textit{M. spretus} and the four inbred strains, BALB, NOD, NZW, and 129 (Table 1). In the bone phenotypic study, two of the strains we characterized, had the natural version (P451) of the mutation, the other eight strains had the 451L version. We found great diversity in all the examined bone phenotypic parameters, but only a few were
associated to the P451L mutation. The strain 129X1/SvJ had higher BMD, stronger femurs and higher bone resorption in bone histomorphometric analysis, compared to BALB/cJ, DBA/2 and B6. BALB/cJ had stronger femurs with higher BMD and higher levels of the bone resorption marker s-CTX than DBA/2 and B6.

We found strong femurs with high BMD in the P451 mice. Mechanical stress of load-bearing parts of the body could induce release of ATP in the bone compartment.

Mechanical stimulation of bone formation has been shown to be partly mediated through the P2X7 receptor [4]. However, we did not detect any significant differences in bone formation markers associated to the P451L alleles. Perhaps the local effect of ATP stimulation in the femurs cannot be detected on the systemic bone markers in the serum samples. The histomorphometric analysis was only of the vertebrae, where higher BV/TV% was found in the P451 mice.

In the bone phenotypic part of the study we found that strains with the naturally occurring mutation 451L in the gene for P2X7 had lower resorption than strains with the wild type allele (P451). Agonists activating P2X7 in low concentrations can initiate osteoclast formation [33, 34]. Significant differences in the number of osteoclasts after 10 days of culture were not detected, but bear in mind that osteoclast culture was done without agonist stimulation. The absence of an effect of the P451L mutation on osteoclast development in vitro could also be due to the lack of systemic hormones, mechanical stimulation, and lack of accessory cells in the culture system.

The next step was to investigate if there were any differences in resorption activity in vitro though we did not detect a significant difference in resorbed area there appeared to be a tendency to higher resorption in cultures with osteoclasts derived from P451 strains. The duration of resorption was not investigated in this study, but keeping in mind that the P2X7 receptor is involved in apoptosis of osteoclasts [13], there could be differences in the length of the osteoclasts’ resorption phase related to the P451L allelic versions.

In humans, the polymorphism called Glu496Ala abolishes the pore formation activity of the P2X7 receptor [12] and has been associated with decreased ATP-induced apoptosis of osteoclasts in vitro [13]. A tendency towards more
TRAP-positive osteoclasts was seen in the 451L osteoclast cultures. Apoptosis was not directly addressed in this study, but in vitro osteoclasts derived from the examined strains showed differences in the ATP-induced dye uptake assay. The examined mutation P451L is in the C-terminal part of P2X7, which is thought to be involved in the initiation of the pore formation, by activating pannexin-1 [35, 36]. In this manner the 451L mutated P2X7 receptors could blunt the pannexin-1 dependent dye uptake, and explain the reduced dye uptake we found in B6 and DBA/2 osteoclasts.

The physiological importance of the pore formation remains to be elucidated, but besides having a role in apoptosis it could be related to IL-1β processing and its release through pannexin-1 [37, 38]. Interleukin-1β has been reported to affect both bone resorption and bone formation dependent on the duration of the administration [39]. Interleukin-1β has also been shown to induce osteoclast precursor proliferation and differentiation, and increases bone resorption [40]. This suggests that IL-1β induces bone resorption initially, but that the long-term effect increased bone turnover, which corresponds to our data.

In macrophages from people carrying two C alleles of the Glu496Ala (A1513C) polymorphism, the processing of pro-IL-1β is impaired, leading to decreased levels of mature serum IL-1β. Following the theory that the reduced activity of the cytoplasmic tail of P2X7 (measured by pore formation) has significant importance in determining the bone cells’ response to ATP, the mechanical stimulation of bone cells in strains with the mutation should be impaired, and thereby the release of mature IL-1β reduced. Then the level of IL-1β induced resorption would be decreased in strains with the mutation and bone remodelling would favour bone formation, and that correlates with the decreased levels of resorption measured in vivo in B6 and DBA/2.

Background strains of both the previously described P2X7−/− mice harbor the 451L allele of the examined mutation in P2X7. The observed differences in the two P2X7−/− murine models cannot be addressed to the P451L mutation in P2X7 found in the background strains, since both models are maintained on genetic background harboring the 451L allele. However, the different bone status of DBA/2 and B6 may affect the direction of the effect of the ablation of P2X7.

The observed lack of mechanical stimulation upon bone formation in the Pfizer P2X7−/−, would probably not have been found in a pure B6 background since the DBA/2 shows increased remodeling compared to the B6 mice. The best reason for the different observations will be the escape of gene knockout due to the splice variant P2X7-k [28] in the GSK P2X7−/− model [23]. Even though no expression of the splice variant P2X7-k has been found in osteoclasts [29], and GSK P2X7−/− osteoblasts shows no dye uptake, the splice variant P2X7-k could still be found in other cells in the bone environment.

The underlying goal was to find a candidate strain of mice that could be used as new genetic background for the P2X7−/− mice, in the search for the therapeutic potential of P2X7 agonists and antagonists in a murine model for postmenopausal osteoporosis. Four strains were determined to carry the P451 allele by sequencing, however the NOD mice develop type I diabetes (during which P2X7 expression change [41]) and the NZB mice get autoimmune anaemia. Only two strains with the P451 allele of the mutation were left, namely, 129X1/SvJ and BALB/cJ. In this study we found that DBA/2 and B6 had low BMD (totally and regionally in the femur) and had a low bone strength, which could make it difficult to detect changes in the parameters. Besides carrying the P451 allele, high baseline BMD, relatively strong bones, and high trabecular bone volume should characterize the selected strain. All that fits well with the bone phenotype of 129X1/SvJ, but mice from this strain show no cancellous bone loss upon oestrogen depletion, as has been reported in postmenopausal women and oestrogen depleted rats [42, 43]. In contrast, BALB/cJ has relatively strong bones and high BMD in the femur, high Tb.Th number, and respond as predicted upon ovariectomy [44].

In conclusion, we find that the P451 strains have stronger femurs and higher BMD, but the cellular basis could not be established in this study. The major finding in this study is that the genetic background could be of significant importance when determining the effect of the P2X7 ablation and that an optimal strain for studying the bone-specific effect of the P2X7 ablation could be the BALB/cJ mice.

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Review Article

Modulating P2X7 Receptor Signaling during Rheumatoid Arthritis: New Therapeutic Approaches for Bisphosphonates

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P2X7 receptor-mediated purinergic signaling is a well-known mechanism involved in bone remodeling. The P2X7 receptor has been implicated in the pathophysiology of various bone and cartilage diseases, including rheumatoid arthritis (RA), a widespread and complex chronic inflammatory disorder. The P2X7 receptor induces the release into the synovial fluid of the proinflammatory factors (e.g., interleukin-1β, prostaglandins, and proteases) responsible for the clinical symptoms of RA. Thus, the P2X7 receptor is emerging as a novel anti-inflammatory therapeutic target, and various selective P2X7 receptor antagonists are under clinical trials. Extracellular ATP signaling acting through the P2X7 receptor is a complex and dynamic scenario, which varies over the course of inflammation. This signaling is partially modulated by the activity of ectonucleotidases, which degrade extracellular ATP to generate other active molecules such as adenosine or pyrophosphates. Recent evidence suggests differential extracellular metabolism of ATP during the resolution of inflammation to generate pyrophosphates. Extracellular pyrophosphate dampens proinflammatory signaling by promoting alternative macrophage activation. Our paper shows that bisphosphonates are metabolically stable pyrophosphate analogues that are able to mimic the anti-inflammatory function of pyrophosphates. Bisphosphonates are arising per se as promising anti-inflammatory drugs to treat RA, and this therapy could be improved when administrated in combination with P2X7 receptor antagonists.

1. Introduction

Rheumatoid arthritis (RA) is a widespread chronic systemic inflammatory disorder that affects approximately 1% of the worldwide population. The female-to-male ratio of the disease is 3:1, and although it can occur at any age, it is more common between ages 40 and 70 years [1]. In this context, pharmaceutical companies are interested in developing new anti-inflammatory treatments for the disease, including the use of P2X7 receptor antagonists or bisphosphonates [2, 3]. This paper will focus on the role of P2X7 receptors in the pathophysiology of RA and the possible therapeutic connection of bisphosphonates with P2X7 receptor signaling.

2. P2X7 Receptor in Bone and Cartilage

Bone is a specialized connective tissue composed of mineralized extracellular matrix and distinct cell populations including osteoblasts, osteocytes, and osteoclasts. Under physiological conditions, bone is subjected to a continuous balance between resorption and formation. However, disturbances of this balance can lead to various diseases such as osteoporosis, RA, or periodontitis [4]. The balance is regulated in bone by a complex network of factors, including hormones and mechanical stimulation. The latter, in turn, induces nucleotide release to the extracellular space and purinergic P2-receptor signaling [5]. P2 receptors are expressed in a variety of cell types in the bone and cartilage, including osteoblasts, osteoclasts, chondrocytes, and synoviocytes and are subdivided into two classes: the P2Y family of G-protein-coupled receptors and the P2X family of ligand-gated cation channels [6].

Recent evidence reviewed by Grol et al. [7] provides specific insight into the role of the P2X7 receptor subtype in osteoblasts and osteoclasts. Additionally, P2X7 receptor
knockout mice exhibit decreased periosteal bone formation, increased trabecular bone resorption, and impaired response to mechanical stimulation, leading to a reduction in total bone content [8, 9]. P2X7 receptor activation in osteoblasts enhances differentiation and bone formation [10], whereas its activation in osteoclasts results in apoptosis [11]. These differences in the function of P2X7 receptor reflect a sophisticated mechanism whereby the skeleton responds to mechanical stimulation by simultaneously increasing bone formation and suppressing its resorption. Furthermore, genetic loss-of-function polymorphisms of the human P2X7 receptor are related with increased skeletal fragility, which is consistent with decreased susceptibility of osteoclasts to apoptosis, as well as impaired osteoblast differentiation and bone formation [12, 13]. P2X7 receptor modulation could also play an important role in regulating bone-cell response, and ATP appears to mediate internalization of P2X7 receptors in osteoblast-like cells [14].

Mechanical stimulation of different cell types, including osteoblasts and chondrocytes, induces ATP release through hemichannel opening [15–17]. Osteoblast-like cells constitutively release nucleotides into the extracellular environment [18]. ATP released into the extracellular compartment of the bone could activate P2X7 receptors on osteoblasts and osteoclasts and the function of P2X7 receptor in bone is consistent with the altered skeleton phenotype of P2X7 receptor knockout mice described by Ke et al. [8]. In this regard, pores formed in response to P2X7 receptor activation induces additional ATP release, initiating a positive purinergic feedback loop [19]. Although the physiological importance of this phenomenon remains unknown, in vitro mechanical stimulation of osteoblasts leads to cell permeabilization via a mechanism dependent on P2-receptor signaling [9].

3. P2X7 Receptor in the Pathophysiology of RA

Purinergic signaling has been implicated in the pathophysiology of various bone and cartilage diseases, including bone loss, RA, osteoarthritis, and bone cancer pain [20–24]. RA is a widespread and complex chronic inflammatory disorder with no current successful treatment [1]. Because it is a complex multifactorial disease, its pathophysiology is not fully understood; however, there is evidence to suggest that T lymphocytes and macrophages play a critical role in the initiation and perpetuation of synovial inflammation [25]. Interleukin (IL)-1β and tumor necrosis factor (TNF)-α are macrophage-derived cytokines that play a primary role in the pathogenesis of RA. One effect of these cytokines is to regulate the production of matrix metalloproteinases (MMPs), which are directly involved in extracellular matrix degradation during RA [26]. In fact, the serum and synovial concentrations of TNF-α and IL-1β are high in patients with active RA [27], and drugs targeting TNF-α were the first biologics to be approved and widely used to treat RA. At present, five TNF inhibitors are approved for use by the U.S. Food and Drug Administration; all of these agents have been shown to be effective in reducing the clinical signs of inflammation in RA patients [27].

Experimental injection of IL-1β into the knee joints of rabbits resulted, within hours, in leukocyte accumulation in the synovial fluid as well as substantial proteoglycan loss from joint cartilage [28, 29]. Unlike TNF-α, which is predominantly detected in the early stages of disease, IL-1β is detected long after the onset of RA [30]. IL-1β is a potent stimulator of leukocytic infiltration, synovial hyperplasia, cell activation, cartilage breakdown, and inhibition of cartilage matrix synthesis [31].

A two-step process tightly regulates IL-1β production. The first step regulates the synthesis of IL-1β precursor (pro-IL-1β) by engagement of innate pattern recognition receptors such as toll-like receptors (TLR), activating nuclear factor-κB (NF-κB), and mitogen-activated protein kinase (MAPK) transcription pathways [32]. The second stimulation signal leads to the formation of a multiprotein complex called the inflammasome, which culminates in caspase-1 activation and pro-IL-1β maturation. The best-characterized inflammasome is formed by the NLRP3 receptor (nucleotide-binding domain and leucine-rich repeat receptor containing pyrin domain 3), the adaptor protein ASC (apoptotic speck-like protein with a caspase-activating recruiting domain), and caspase-1. Numerous stimuli trigger the NLRP3 inflammasome, including pathogen- or danger-associated molecular patterns (PAMPs and DAMPs, resp.) [33]. Extracellular ATP activating P2X7 receptor is one of the most widely investigated DAMPs that activate the NLRP3 inflammasome and is probably one of the most important pathways for IL-1β release in RA. Extracellular ATP is found at high concentrations in the synovial fluid of patients with RA [34], and P2X7 receptor deficiency leads to a lower incidence and lower severity of joint inflammation in animal models of arthritis induced by anticollegen antibodies [35]. RA-associated synoviocytes express functional P2X7 receptors, and their activation upregulates the production of proinflammatory cytokine IL-6 from these cells [36].

Interest in developing new drugs that target the synthesis, processing, and/or release of IL-1β has risen in recent years [23]. Blocking antibodies for soluble IL-1β have been shown to reduce joint destruction in several animal models of RA [37]. In a phase II clinical trial with RA patients, 24 weeks of treatment with the recombinant IL-1 receptor antagonist (IL-1Ra or anakinra) provided significantly greater clinical improvement of RA symptoms than placebo [38]. Anakinra treatment is safe and highly effective for patients with systemic-onset juvenile idiopathic arthritis, adult-onset Still’s disease, hereditary autoinflammatory syndromes, Schnitzler’s syndrome, and gouty attacks [39]. However, this treatment has been associated with liver toxicity, and long-term follow-up analyses are essential to guide appropriate management strategies [40]. Selective drug-like P2X7 receptor antagonists have already been tested in clinical trials for RA. Nonetheless, after initial positive outcomes in phase II trials, AstraZeneca and Pfizer discontinued the development of their compounds following negative phase IIb/III results in subjects receiving methotrexate [2, 41]. The poor pharmacokinetics and pharmacodynamics of AstraZeneca’s and Pfizer’s P2X7 receptor antagonists could partially explain the lack of effectiveness. However,
as described above, the fact that RA pathophysiology could be strongly mediated by TNF-α might also explain such poor outcomes. Recently, a new generation of P2X7 receptor antagonists with better drug-like properties is entering early-phase clinical studies [40].

New evidence suggests that P2X7 receptor activation also triggers inflammasome- and cytokine-independent pathways that play an active role in the pathogenesis of RA. In fact, an effective anti-P2X7 receptor therapy would be more beneficial than anticytokine directed therapy. P2X7 receptor activation has been associated with the release of the tissue-destroying proteases MMPs and cathepsins [22, 42, 43]. Cathepsin is a family of lysosomal proteases implicated in cartilage joint destruction and found in the synovial fluid of patients with active RA [44, 45]. Treatment with P2X7 antagonist, but not with other active RA drugs such as methotrexate, abolished cathepsin release from ATP-activated macrophages [22]. P2X7 receptor activation has also been associated with the release of prostaglandin E2 (PGE2) and other autacoids [46]. In particular, PGE2 induces the release of MMPs in the joint during RA and is a key mediator of pain sensation [26].

4. Bisphosphonates: Anti-Inflammatory Compounds in RA

Because of their anti-inflammatory properties, bisphosphonates may represent a promising new drug for the treatment of RA. Bisphosphonates are metabolically stable analogues of pyrophosphate, where the central oxygen atom is replaced by a carbon atom (Figure 1). The R1 and R2 side chains bonded to the central carbon confer different properties to the bisphosphonate molecule, whereas R1 is usually short and is involved in binding to bone mineral and R2 is responsible for its biological effects [47, 48]. Depending on the side-chain structure of R2, bisphosphonates are classified into nitrogen-containing bisphosphonates (N-bisphosphonates, such as pamidronate, risedronate, ibandronate or zolendronate) and nonnitrogen-containing bisphosphonates (non-N-bisphosphonates, such as etidronate or clodronate) (Figure 1).

Bisphosphonates are able to bind divalent ions, such as Ca$^{2+}$, and therefore target exposed bone mineral surfaces in vivo. After bone binding, bisphosphonates are incorporated in bone-resorbing osteoclasts, resulting in osteoclast function inhibition and apoptosis [49]. Bisphosphonates that selectively affect osteoclasts have become a major class of antiresorptive drug for the treatment of osteoporosis and Paget’s disease [50, 51]. Bisphosphonates can also be encapsulated in liposomes and then selectively used to target phagocytic cells in vivo [52]. Liposome-encapsulated clodronate is widely used to eliminate macrophages in vivo and has been shown to reduce RA-associated inflammation in animal models and in patients [3].

Several molecular mechanisms have been described for the action of bisphosphonates. Non-N-bisphosphonates can be metabolized to nonhydrolyzable and β,y-methylene-containing (AppCp-type) analogues of ATP [53]. Intracellular accumulation of these metabolites arrest osteoclast and macrophage metabolism and function. The in vivo accumulation of AppCCl2p in osteoclasts induces apoptosis and inhibits bone resorption [54, 55]. N-bisphosphonates are also known to affect normal cellular function by inhibiting farnesyl diphosphate synthase and thereby preventing the prenylation of small GTPases [47, 56].

Additionally, several studies demonstrate that bisphosphonates are antioxidant compounds. Reactive oxygen species (ROS) are known to contribute to the inflammatory process in RA by degrading cartilage and bone [57]. In an inflamed joint, ROS are produced by macrophages, neutrophils, and chondrocytes [58]. Serretti et al. [59] showed that bisphosphonates inhibit ROS production in human neutrophils by acting before or on NADPH oxidase. Bisphosphonates also have antioxidant properties as iron chelators and block chondrocyte lipid peroxidation, suggesting a protective role for bisphosphonates in RA [60].

5. Bisphosphonates, Extracellular Metabolism of Nucleotides, and P2X7 Receptors

Our group recently identified clodronate and its physiological analogue pyrophosphate as a new molecule structure able to block IL-1β release [61]. This inhibition was also found by using high ATP concentrations during macrophage polarization towards anti-inflammatory or alternatively activated M2 states, where P2X7 receptor signaling was uncoupled from inflammasome activation. In proinflammatory M1 macrophages, this effect was achieved when the P2X7 receptor was absent or pharmacologically blocked. We previously proposed a model for inflammasome regulation by pyrophosphates and extracellular ATP during macrophage polarization gradient towards M2 where P2X7 receptor uncouples from both ROS production and the NLRP3-inflammasome/caspase-1 pathway, while P2X7 receptor remains functional in terms of its ion channel activity [60]. Under
these conditions, pyrophosphates act to inhibit ROS and cluster actin dynamics induced by other inflammasome activators (e.g., maitotoxin), resulting in blockage of caspase-1 and IL-1β release. This effect could be beneficial in enhancing the resolving phase of inflammation.

Our evidence suggested that the diphosphate group resident in nucleotides was blocking inflammasome activation, because pyrophosphates and triphosphates but not monophosphates (inorganic phosphate) inhibited caspase-1 activation and IL-1β release, with a potency order of clodronate > diphosphate > ATP ≫ ADP. It is still unknown if pyrophosphates act by phosphate chains remaining attached to the nucleotide molecule or if they result from ATP metabolism by ectonucleotidases. Ectonucleotidases might play a role in this process, because the expression of ectonucleotidases and other genes involved in the extracellular generation of pyrophosphate is highly expressed by M2 compared to M1 macrophages [62]. M2 macrophages accumulate the transcript for ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), which primarily degrades ATP to produce AMP and pyrophosphate [63]. M2 macrophages also accumulate the transcript for ANK, a plasma-membrane protein involved in progressive ankylosis that mediates cytosolic pyrophosphate release into the extracellular space [64].

Pyrophosphates were also able to alter the LPS-induced proinflammatory gene expression profile in a similar manner to the action of IL-4 (cytokine responsible for polarizing macrophages to M2). Interestingly, pyrophosphates were also able to upregulate ENPP1 expression after LPS stimulation, suggesting that over the course of the inflammatory process, extracellular pyrophosphate production could promote the initiation of resolution by shifting macrophage polarization to M2. In M1 macrophages, pyrophosphate and clodronate significantly inhibited gene expression dependent on NF-κB activation, such as the LPS-induced production of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and the recovery of IκBα protein [65]. Redox balance has been extensively implicated in NF-κB activation [66], and it has been found that pyrophosphates, so as clodronate, chelating the actions of ROS, could specifically affect the nuclear actions of translocated NF-κB to dampen proinflammatory gene expression and enhance ENPP1 gene expression [62]. Additionally, pyrophosphates are able to enhance the resolution of peritonitis in vivo by reducing proinflammatory cytokine production [62].

Taken together, these results suggest that extracellular ATP and its metabolism to pyrophosphate are key triggers in the switch from a proinflammatory macrophage towards its alternative functions in the resolution of inflammation. Pharmacologically, this effect could be mimicked and enhanced by the use of bisphosphonates as anti-inflammatory compounds.
6. Conclusions
Several approaches can be taken to reduce synovial inflammation in RA, and many of them use the P2X7 receptor as a central signaling molecule. These approaches include direct P2X7 receptor antagonism and extracellular IL-1β blocking (Figure 2). In fact, there is increasing evidence that bisphosphonates may be useful as novel anti-inflammatory drugs in RA. These compounds present various modes of action; for example, they deplete macrophages when administered encapsulated in liposomes. In contrast, free bisphosphonates present pyrophosphate-like activities that chelate ROS and block inflammosome activation during the resolving phase of inflammation, when P2X7 receptor stimulation is uncoupled from proinflammatory signaling or when the P2X7 receptor is pharmacologically inhibited. In the latter case, bisphosphonates produce a switch in P2X7 receptor signaling and extracellular nucleotide metabolism to pyrophosphates during the resolution of inflammation [61, 62]. Therefore, a combination of P2X7 receptor antagonists with bisphosphonates could be more successful in treating chronic inflammation in RA.

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