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The function of microglia through purinergic receptors: Neuropathic pain and cytokine release

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Abstract

Microglia play an important role as immune cells in the central nervous system (CNS). Microglia are activated in threatened physiological homeostasis, including CNS trauma, apoptosis, ischemia, inflammation, and infection. Activated microglia show a stereotypic, progressive series of changes in morphology, gene expression, function, and number and produce and release various chemical mediators, including proinflammatory cytokines that can produce immunological actions and can also act on neurons to alter their function. Recently, a great deal of attention is focusing on the relation between activated microglia through adenosine 5'-triphosphate (ATP) receptors and neuropathic pain. Neuropathic pain is often a consequence of nerve injury through surgery, bone compression, diabetes, or infection. This type of pain can be so severe that even light touching can be intensely painful and it is generally resistant to currently available treatments. There is abundant evidence that extracellular ATP and microglia have an important role in neuropathic pain. The expression of P2X4 receptor, a subtype of ATP receptors, is enhanced in spinal microglia after peripheral nerve injury model, and blocking pharmacologically and suppressing molecularly P2X4 receptors produce a reduction of the neuropathic pain. Several cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) in the dorsal horn are increased after nerve lesion and have been implicated in contributing to nerve-injury pain, presumably by altering synaptic transmission in the CNS, including the spinal cord. Nerve injury also leads to persistent activation of p38 mitogen-activated protein kinase (MAPK) in microglia. An inhibitor of this enzyme reverses mechanical allodynia following spinal nerve ligation (SNL). ATP is able to activate MAPK, leading to the release of bioactive substances, including cytokines, from microglia. Thus, diffusible factors released from activated microglia by the stimulation of purinergic receptors may have an important role in the development of neuropathic pain. Understanding the key roles of ATP receptors, including P2X4 receptors, in the microglia may lead to new strategies for the management of neuropathic pain.

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Keywords: ATP; P2X4; Microglia; Neuropathic pain; Allodynia; Spinal cord; p38

Abbreviations: ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; ATP γ S, adenosine 5'-O-(3-thiotriphosphate); BDNF, brain-derived neurotrophic factor; BzATP, 2'- and 3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate; [Ca²⁺]_i, intracellular Ca²⁺ concentration; CD11b, clusterdeterminant 11b; CNS, central nervous system; CR3, complement receptor 3; ERK, extracellular signal-regulated protein kinase; Iba1, ionized calcium binding adaptor molecule 1; ICE, IL-1 β -converting enzyme; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; InsP3, inositol 1, 4, 5-trisphosphate; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, mitogenactivated protein kinase kinase; MHC, histocompatibility complex; oATP, oxidized ATP; PK11195, [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolincisoquinoline carboxamide]; PKC, protein kinase C; PLC, phospholipase C; PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid; PTK, protein tyrosine kinase; PTX, pertussis toxin; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SOC, store-operated Ca²⁺ entry; SP600125, anthra[1,9-cd]pyrazol-6(2H)-one; TNF- α , tumor necrosis factor- α ; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate; U0126, 1,4-diamino-2,3-dicyano-1,4-bis [2-amino-phenylthio] butadiene; UTP, uridine 5'-triphosphate.

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1. Introduction

Microglia are often considered to be resident macrophages and to play an important role as immune cells in the central nervous system (CNS; Kreutzberg, 1996; Stoll & Jander, 1999; Nakajima & Kohsaka, 2001). In adults, microglia are distributed throughout CNS and represent 5–10% of glia. They have a small soma bearing thin and branched processes under normal conditions. Such microglia are said to be ‘resting’, but acting as sensors for a range of stimuli that threaten physiological homeostasis, that is, CNS trauma, apoptosis, ischemia, inflammation, and infection. Once activated by these stimuli, that is, bioactive substances, cytokines and neurotransmitters, including adenosine 5'-triphosphate (ATP), microglia show a stereotypic, progressive series of changes in morphology, gene expression, function, and number (Perry, 1994; Kreutzberg, 1996; Stoll & Jander, 1999; Streit et al., 1999; Nakajima & Kohsaka, 2001). Activated microglia change their morphology from a resting, ramified shape into an active, amoeboid shape (Perry, 1994; Kreutzberg, 1996; Stoll & Jander, 1999; Streit et al., 1999; Nakajima & Kohsaka, 2001). They up-regulate expression of a variety of cell-surface molecules, including the complement receptor 3 (CR3; also known as clusterdeterminant [CD] 11b (integrin α M)/CD18 (integrin β 2), or as Mac-1; Kreutzberg, 1996; Stoll & Jander, 1999; Ehlers, 2000; Nakajima & Kohsaka, 2001), which is recognized by the antibody OX-42 (Robinson et al., 1986). Activated microglia also express immunomolecules such as major histocompatibility complex (MHC) class I and II (Kreutzberg, 1996; Stoll & Jander, 1999; Streit et al., 1999), which have a role in antigen presentation to T lymphocytes. Activated microglia produce and release various chemical mediators, including proinflammatory cytokines that can produce immunological actions and can also act on neurons to

alter their function (Kreutzberg, 1996; Stoll & Jander, 1999; Nakajima & Kohsaka, 2001; Hanisch, 2002). Recently, a great deal of attention has been focused on the relation between neuropathic pain and microglia activated through ATP receptors (Tsuda et al., 2005).

ATP is released from damaged cells as a result of ischemia or inflammation and serves as a cell-to-cell mediator through cell surface P2 receptors, which are widely distributed throughout the nervous system, including microglia (Inoue, 2002). P2 receptors are divided into 2 subtypes: P2X and P2Y (Abbracchio & Burnstock, 1994; Fig. 1). P2X receptors (P2X1–P2X7) are coupled to nonselective cation channels, allowing the influx of Na⁺ and Ca²⁺ (North, 2002), whereas

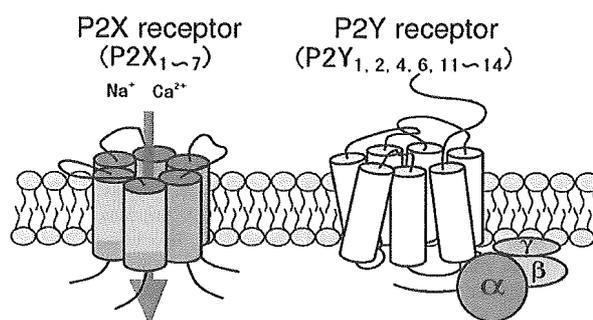


Fig. 1. P2 receptors. P2 receptors are divided into 2 subtypes: P2X and P2Y. P2X receptor subtypes (P2X1–P2X7) are 40–50% identical in amino acid sequence. Each subtype has 2 transmembrane domains. Nonselective cation channels form as multimers (presumably 3 multimers) of several subunits. Homomeric P2X1, P2X2, P2X3, P2X4, P2X5, and P2X7 channels and heteromeric P2X2/3 and P2X1/5 channels have been most fully characterized following heterologous expression. P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) are G-protein coupled, and their activation leads to inositol lipid hydrolysis, intracellular Ca²⁺ mobilization, or modulation of adenylate cyclase activation, through G_{q/11} (P2Y1, P2Y2, P2Y4, P2Y6), G_s (P2Y6) and G_{i/o} (P2Y11, P2Y12, P2Y13, and P2Y14), respectively.

P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) are G-protein coupled, and their activation leads to inositol lipid hydrolysis, intracellular Ca^{2+} mobilization, or modulation of adenylate cyclase activation (Inoue, 2002). ATP strongly activates microglial to show chemotaxis via the Gi - and Go -coupled P2Y12 receptor (Honda et al., 2001) and stimulates the release of plasminogen, interleukin (IL)-6, tumor necrosis factor- α (TNF- α), and IL-1 β (Ferrari et al., 1997a, 1997b; Inoue et al., 1998; Hide et al., 2000; Shigemoto-Mogami et al., 2001; Inoue, 2002; Suzuki et al., 2004) by means of different types of P2 receptor and intracellular signals.

Neuropathic pain is a type of pathological pain that often develops when nerves are damaged through surgery, bone compression, diabetes or infection, also which does not resolve even when the overt tissue damage has healed (Aldskogius & Kozlova, 1998; Carson, 2002; Eikelenboom et al., 2002). Neuropathic pain can be so severe that even light contact with clothing can be intensely painful (tactile allodynia: an abnormal hypersensitivity to innocuous stimuli) and is often resistant to most current treatments, including a narcotic analgesia, although a number of drugs produce some relief. Accumulating evidence concerning how peripheral nerve injury creates neuropathic pain has suggested that molecular and cellular alterations in primary sensory neurons and in the spinal dorsal horn after nerve injury have an important role in the pathogenesis of neuropathic pain (Aldskogius & Kozlova, 1998; Carson, 2002; Eikelenboom et al., 2002). While there is an increasing body of evidence suggesting that P2X3Rs, a subtype of ionotropic ATP receptors, in primary sensory neurons have a role in neuropathic pain (Colburn et al., 1999; Banati, 2002; Garden, 2002), other P2XR and P2YR subtypes of ATP

receptors are also beginning to be investigated in terms of their changes in expression using cDNA microarray (Visentin et al., 1999; Inoue, 2002; Suzuki et al., 2004). Recently, it was reported that astrocyte and microglia are activated strongly in neuropathic model animals, suggesting the role of glial cells in the pain sensation (Watkins et al., 2001). However, there was no direct evidence supporting this suggestion. More recently, we have revealed that the P2X4R subtype in the activated spinal microglia is required for the expression of neuropathic pain after nerve injury (Tsuda et al., 2003). This review shows the progress in the current understanding of how the ATP receptor participates in the activation of microglia leading into neuropathic pain.

2. Purinergic receptors expressing in microglia

ATP evokes currents in rat microglia (Nörenberg et al., 1994; Illes et al., 1996) and increases in intracellular calcium ($[\text{Ca}^{2+}]_i$) in mouse and human microglia (Walz et al., 1993; Toescu et al., 1998; Moller et al., 2000). ATP induces the release of IL-1 β (Ferrari et al., 1996, 1997b) and IL-6 (Shigemoto-Mogami et al., 2001) from mice microglia. ATP causes chemotaxis (Honda et al., 2001) and the release of plasminogen (Inoue et al., 1998) and TNF- α (Hide et al., 2000; Morigiwa et al., 2000) from rat microglia. ATP activates nuclear factor of activated T-cells (NFAT; Ferrari et al., 1999), which modulates the early inflammatory gene expression and transcriptional activator NF- κB , which controls cytokine expression and apoptosis (Ferrari et al., 1997a). ATP also stimulates the phosphorylation of mitogen-activated protein kinase (MAPK; Hide et al., 2000; Honda et al., 2001; Shigemoto-Mogami et al.,

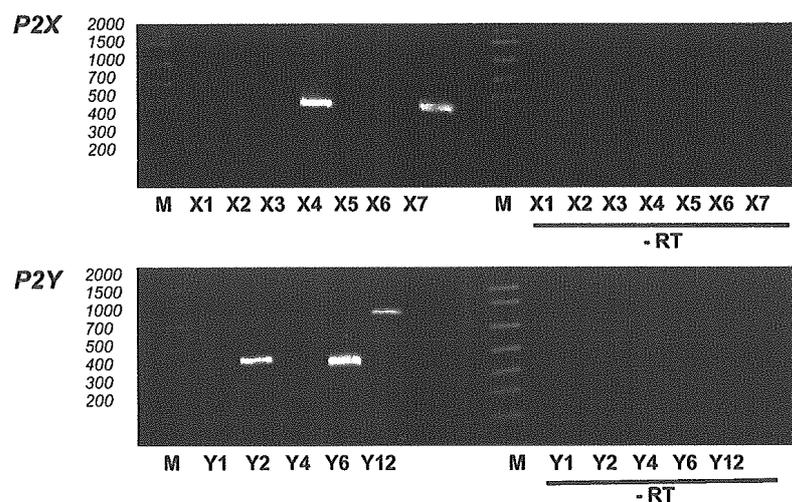


Fig. 2. mRNA expression of P2 purinergic receptors in primary culture microglia from rat brain. **Upper:** Electrophoresis photograph after a quantitative RT-PCR using specific primers for P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7. Clear bands were detected in the P2X4 and P2X7 lanes. **Lower:** Electrophoresis photograph after a quantitative RT-PCR using specific primers for P2Y1, P2Y2, P2Y4, P2Y6, and P2Y12 receptors. Clear bands were detected in the P2Y2, P2Y6, and P2Y12 lanes.

2001). These data suggest that microglia possess functional receptors for purines and pyrimidines, that is, P2X receptors, ligand-gated ion-channels (Cook et al., 1998; Li et al., 1999; Ueno et al., 1999; Tsuda et al., 2000; Chizh & Illes, 2001; Dunn et al., 2001), and P2Y receptors, G protein-coupled receptors (Svichar et al., 1997; Koizumi et al., 2001; Tominaga et al., 2001; Molliver et al., 2002; Sanada et al., 2002; Moriyama et al., 2003). There, however, are very few reports available indicating the mRNA expression of P2 receptor subtypes in microglia. We examined using a quantitative RT-PCR method and found that microglia in a primary culture from rat brain express mainly mRNAs of P2X4 and P2X7, and P2Y2, P2Y6, and P2Y12 (Shigemoto-Mogami et al., personal communication), as shown in Fig. 2.

3. Activated microglia in neuropathic pain

3.1. Microglia activation in patients bearing a kind of neuropathic pain

Since the peripheral benzodiazepine binding site practically absent in the normal brain parenchyma is strongly and preferentially expressed by activated microglia around the soma of the injured neuron, [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline isoquinoline carboxamide] (PK11195), a ligand for the peripheral benzodiazepine binding site, binds with relative cellular selectivity to activated microglia, not to residential microglia. Thus, (*R*)-PK11195 labeled with carbon-11 and positron emission tomography (PET) have been used for the study of inflammatory and neurodegenerative brain disease in vivo, even in human. This technology is highly useful to reveal the retrograde and anterograde projection areas (Banati et al., 1997, 2000). For example, increased microglial (*R*)-PK11195 binding is seen in the motor facial nucleus after peripheral facial nerve transection (Banati et al., 1997), in the gracile nucleus after sciatic nerve lesion (Banati et al., 1997), in the ipsilateral thalamus after cerebral cortical ischemic stroke (Pappata et al., 2000), and in the lateral geniculate bodies in multiple sclerosis patients with optic neuritis (Banati et al., 2000). The potentially useful clinical application of the specific ligand PK11195 is based on 3 observations (Banati et al., 2001): (1) normal brain shows only minimal binding of PK11195; (2) in CNS pathology, in vivo PK11195 binding is predominantly found on activated microglia; and (3) when labeled with carbon-11, PK11195 can be used as a ligand for PET (Benavides et al., 1988; Junck et al., 1989; Myers et al., 1991a, 1991b, 1999; Cremer et al., 1992; Ramsay et al., 1992; Sette et al., 1993; Banati et al., 1999).

The cortical plasticity developed after the amputation of a limb may be associated with the development of abnormal sensations, such as phantom pain, a kind of neuropathic pain, and referred cutaneous sensations (Banati et al., 2001). It was reported that cortical reorganization may be the consequence

of a reorganization of the thalamus following changes of afferent inputs from the amputated limb (Jones, 2000). The cause of the sustained representational plasticity in the thalamus has recently been suggested to be transneuronal atrophy in the thalamus that, in turn, would mediate cortical plasticity (Woods et al., 2000). Acute or chronic neuronal injury after the amputation of a limb evokes a rapid, transient and localized activation of microglia (Kreutzberg, 1996). Banati et al. (2001) reported that limb amputation induces a trans-synaptic increase in [¹¹C](*R*)-PK11195 binding in the human thalamus but not somatosensory cortex, suggesting the activation of microglia in the thalamus of a patient with phantom pain. The increased thalamic signal is detectable many years after nerve injury, and this means persistent reorganization of the thalamus. The microglial activation, beyond the first-order projection area of the injured neurons, may reflect continually altered afferent activity. The activation of microglia can therefore be used as a sensor of neuronal injury.

3.2. Microglia activation in neuropathic pain model rats

Clinical evidence that neuropathic pain results from damage to peripheral nerves in humans led to the development of a variety of models for studying neuropathic pain in laboratory animals. Evidence from studies using such models has revealed that peripheral nerve injury leads to a dramatic change in microglia within the spinal dorsal horn (Eriksson et al., 1993; Colburn et al., 1997, 1999; Coyle, 1998; Stuesse et al., 2000). Spinal microglia become hypertrophic in their short and thick processes within 24 hr after peripheral nerve injury (Eriksson et al., 1993; Tsuda et al., 2003). This is followed by a burst proliferation of microglia with a peak at around 2–3 days after the nerve injury (Gehrmann & Banati, 1995). Activated microglia exhibit up-regulated OX42 labeling (Eriksson et al., 1993; Liu et al., 1995; Coyle, 1998; Colburn et al., 1999; Stuesse et al., 2000; Tsuda et al., 2003), which starts to increase as early as 1 day after nerve injury and peaks at around 14 days (Coyle, 1998). The temporal pattern of OX42 up-regulation in the dorsal horn correlated with that of the development of tactile allodynia (Coyle, 1998), suggesting the role of microglia in neuropathic pain. Although there have been many studies showing that the activation of microglia in the dorsal horn is correlated with the development of pain hypersensitivity in a wide variety of nerve injury models (Eriksson et al., 1993; Liu et al., 1995; Coyle, 1998; Colburn et al., 1999; Stuesse et al., 2000; Watkins et al., 2001), it remained an open question whether spinal microglia play a causal role in neuropathic pain until the report by Tsuda et al. (2003).

3.3. High expression of P2X4 in spinal microglia in neuropathic pain model

A clue to identifying P2X4Rs in the spinal microglia required for neuropathic pain first came from pharmaco-

logical investigations of pain behaviour after nerve injury using the P2XR antagonists 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP) and pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS; Tsuda et al., 2003). It was found that the marked tactile allodynia that develops following the injury of a spinal nerve is reversed by acutely administering TNP-ATP intrathecally but is unaffected by administering PPADS (Fig. 3A). TNP-ATP has no effect on acute pain behaviour in the uninjured state nor on motor behaviour. TNP-ATP at high concentration shows the antagonistic effect on P2X1, P2X2, P2X3, P2X4, P2X5, and P2X7. PPADS inhibits the action of all these subtypes, excluding P2X4. From these pharmacological profiles of TNP-ATP and PPADS, it was inferred that tactile allodynia depends upon P2X4Rs in the spinal cord. The expression of P2X4R protein, normally low in the naive

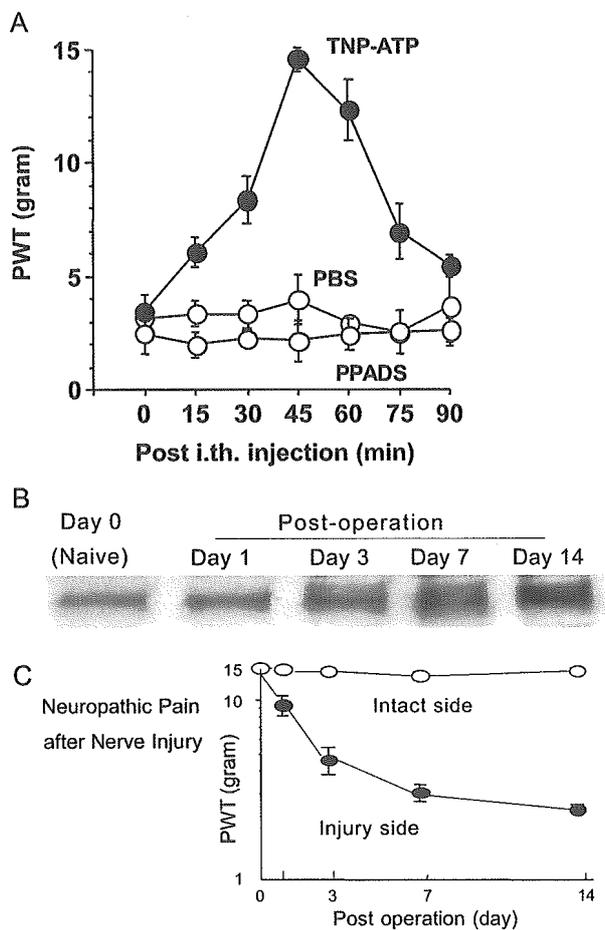


Fig. 3. Effect of TNP-ATP on tactile allodynia and expression of P2X4R in the dorsal horn after nerve injury. (A) Tactile allodynia that develops following the injury of a spinal nerve was reversed by acutely administering TNP-ATP intrathecally but was unaffected by administering PPADS. TNP-ATP had no effect on acute pain behaviour in the uninjured state or on motor behaviour. (B, C) The expression of P2X4R protein and the development of tactile allodynia after nerve injury. The expression of P2X4R protein progressively increased in the days following nerve injury with a time course parallel to that of the development of tactile allodynia.

spinal cord, progressively increases in the days following nerve injury with a time-course parallel to that of the development of tactile allodynia (Fig. 3B and C). Immunohistochemical analysis demonstrated that many small cells in the dorsal horn on the side of the nerve injury are intensely positive for P2X4R protein. These cells are identified as microglia rather than neurons or astrocytes by double immunolabelling using cell-specific markers (Fig. 5A). The cells expressing P2X4R in the nerve-injured side of the dorsal horn are more numerous than under control conditions and showed high levels of OX42 labeling and morphological hypertrophy, all of which are characteristic markers of activated microglia. Moreover, intrathecal administration with antisense oligodeoxynucleotide (AS) targeting P2X4R reduces the up-regulation of P2X4R protein in spinal microglia (Fig. 4A) and prevents the development of the nerve injury-induced tactile allodynia (Fig. 4B). The treatment with a mismatch ODN (MM) as a control does not reduce the expression of P2X4R nor prevent the tactile allodynia (Fig. 4A and B). The evidence implies that P2X4R's activation is necessary for pain hypersensitivity following nerve injury, and that microglia are required for this hypersensitivity since the expression of these receptors in the dorsal horn is restricted to this type of cell.

3.4. P2X4 stimulation causes and maintains allodynia

To demonstrate the sufficiency of P2X4R activation in microglia for the development of allodynia, Tsuda et al. (2003) performed the intrathecal administration of primary cultured microglia stimulated *in vitro* by ATP. In normal rats, intrathecal administration of cultured microglia that were preincubated with ATP to activate P2X4Rs on microglia produces tactile allodynia progressively over the 3–5 hr following the administration. In contrast, intrathecal administration of unstimulated microglia does not cause allodynia, nor does administering vehicle or ATP alone. Microglia also express another subtype of P2XR, P2X7R, but this receptor subtype appears not to be involved because the activation of P2X7Rs typically requires a higher concentration (more than 1 mM) of ATP (Surprenant et al., 1996; Khakh et al., 2001). Moreover, in the tactile allodynia caused by the administration of ATP-stimulated microglia, this allodynia is reversed by administering TNP-ATP (Tsuda et al., 2003). Thus, the stimulation of P2X4Rs is required in the tactile allodynia caused by ATP-stimulated microglia, and this tactile allodynia therefore resembles that caused by nerve injury. These findings indicate that P2X4R stimulation of microglia is not only necessary for tactile allodynia, but is also sufficient to cause the allodynia. Furthermore, this finding makes a strong case that microglia activation is not simply correlated with neuropathic pain behaviour. Rather, microglia within the dorsal horn play an active and ongoing role in the tactile allodynia produced by injury to peripheral nerves. For revealing the exact mechanism of

the P2X4-microglia-involving neuropathic pain, more deep research efforts should be endeavored.

4. Microglia activation through purinergic receptors

The variety of biological effects produced by ATP in microglia may provide hints towards clarifying the mechanisms of neuropathic pain.

4.1. Chemotaxis following membrane ruffling

The initial microglial responses that occur after brain injury and in various neurological diseases are characterized by microglial accumulation in the affected sites of the brain, which results from the migration and proliferation of these cells. The early-phase signal responsible for this accumulation is likely to be transduced by rapidly diffusible factors. Honda et al. (2001) examined the possibility that ATP released from injured neurons and nerve terminals affects the cell motility in rat primary cultured microglia. They found that extracellular ATP and adenosine 5'-diphosphate (ADP) induces membrane ruffling and markedly enhances chemokinesis in a Boyden chamber assay. Further analyses using the Dunn chemotaxis chamber assay, which allows direct observation of the cell movement, revealed that both ATP and ADP induce chemotaxis of microglia. The elimination of extracellular calcium or treatment with PPADS or suramin does not inhibit ATP- or ADP-induced membrane ruffling, whereas AR-C6931MX, a P2Y12 and P2Y13 receptor blocker (Hollopeter et al., 2001; Fumagalli et al., 2004), or pertussis toxin (PTX) treatments clearly inhibit the ruffling. As an intracellular signaling molecule underlying these phenomena, the small G-protein Rac is activated by ATP and ADP stimulation, and its activation is also inhibited by pretreatment with PTX. These findings suggested that the membrane ruffling and chemotaxis of microglia induced by ATP or ADP are mediated by G(i/o)-coupled P2Y receptors (P2Y12 and/or P2Y13).

4.2. Function and release of plasminogen

It was shown that ATP stimulates the release of plasminogen from primary cultured rat microglia in a concentration-dependent manner from 10 to 100 μ M, with a peak response at 5–10 min after the stimulation (Inoue et al., 1998). A 1-hr pretreatment with BAPTA-AM completely inhibits the plasminogen release evoked by ATP. The Ca^{2+} ionophore A23187 induces plasminogen release in a concentration-dependent manner (0.3 to 10 μ M). ATP induces a transient increase in the $[Ca^{2+}]_i$ in a concentration-dependent manner, which is very similar to the ATP-evoked plasminogen release. A second application of ATP (100 μ M) stimulates an increase in $[Ca^{2+}]_i$ similar to that of the first application (21 out of 21 cells). The ATP-evoked increase in $[Ca^{2+}]_i$ is totally dependent on extracellular Ca^{2+} . 2-

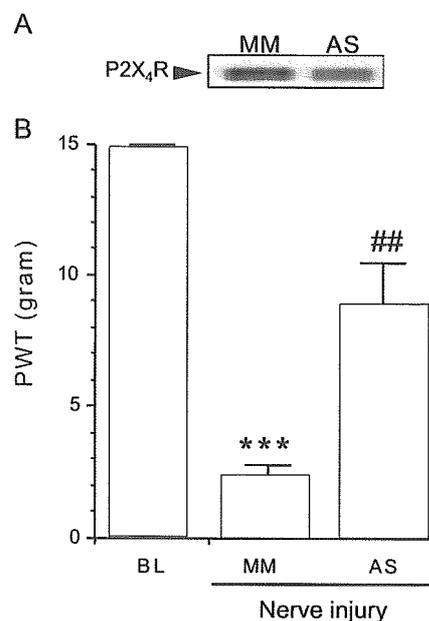


Fig. 4. Effects of antisense oligodeoxynucleotide (AS) targeting P2X4R on the expression of P2X4 protein and the development of tactile allodynia after nerve injury. (A) Western blotting analysis of the expression of P2X4 receptor protein in the spinal dorsal horn 7 days after nerve injury. The animals were treated with intrathecal administration of antisense oligodeoxynucleotide (AS) targeting P2X4R for 7 days, beginning on the day of the nerve lesion. Intrathecal administration of antisense oligodeoxynucleotide (AS) targeting P2X4R reduced the up-regulation of P2X4R protein in spinal microglia. The treatment with a mismatch ODN (MM) as a control did not reduce the expression of P2X4. (B) AS treatment prevented the development of the nerve injury-induced tactile allodynia. The paw withdrawal threshold in animals treated with MM was not different from that of untreated controls, suggesting that MM did not prevent the tactile allodynia.

Methylthio ATP is effective (7 out of 7 cells), but α,β -methylene ATP was ineffective (7 out of 7 cells) at inducing an increase in $[Ca^{2+}]_i$. Suramin (100 μ M) is shown not to inhibit the ATP-evoked increase in $[Ca^{2+}]_i$ (20 out of 20 cells). 2'- and 3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP) evokes a long-lasting increase in $[Ca^{2+}]_i$, even at 1 μ M, a concentration at which ATP does not evoke the increase. One hour pretreatment with oxidized ATP (oATP; 100 μ M), a selective antagonist of P2X7 receptors, blocks the increase in $[Ca^{2+}]_i$ induced by ATP (10 and 100 μ M). These data suggest that ATP may transit information from neurones to microglia, resulting in an increase in $[Ca^{2+}]_i$ via the ionotropic P2X7 receptor which stimulates the release of plasminogen from the microglia. However, the possible involvement of P2X4 in this release cannot be excluded because BzATP can affect on P2X4. It has been found that uridine 5'-triphosphate (UTP) also stimulates plasminogen release from a subpopulation of microglia (about 20% of total cells), presumably through store-operated Ca^{2+} entry (SOC) activated by ATP stimulation of G protein-coupled receptors, since the release evoked by UTP is also dependent on extracellular Ca^{2+} (Inoue et al., unpublished data).

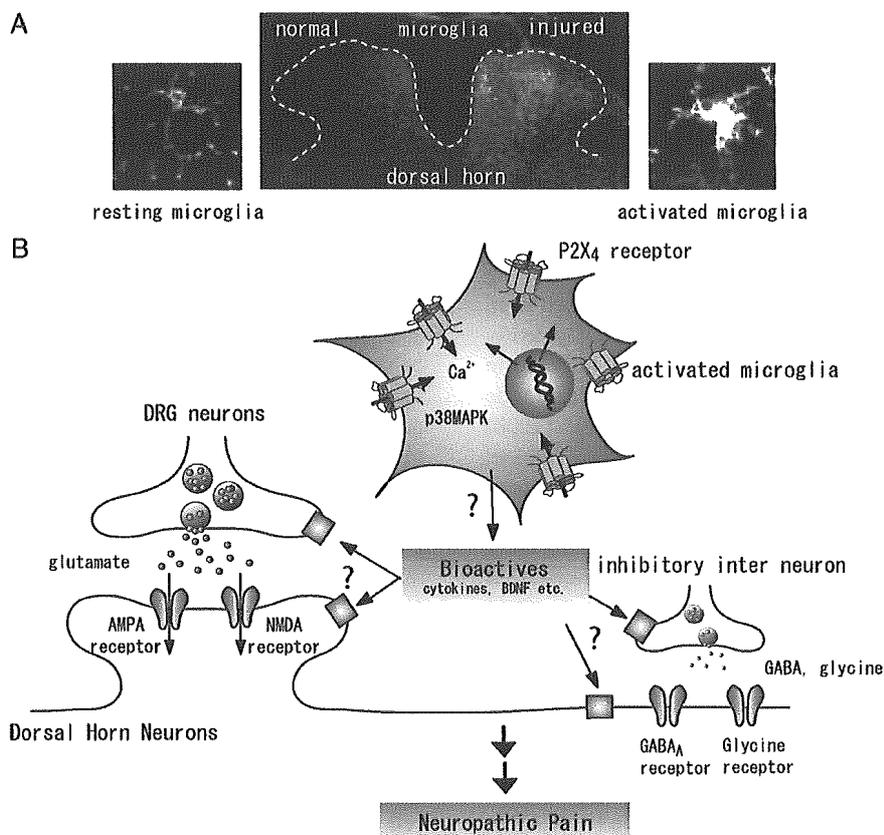


Fig. 5. Schematic illustration of possible mechanisms by which P2X4R in activated microglia modulate pain signaling in the dorsal horn. (A) OX42, a marker of activated microglia, labeling of dorsal horn 7 days after the nerve injury. Injury side was labeled highly with OX42, suggesting the existence of large number of microglia which were activated. Activated microglia were double-immunolabelled by anti-P2X4R antibody at extremely high level than that in resting microglia. (B) Peripheral nerve injury leads to molecular and cellular alterations in the spinal dorsal horn. Activated microglia after nerve injury increase the expression of P2X4R. The P2X4R are activated by ATP, which is presumably released from primary sensory neurons or astrocytes and, in turn, cause a rise in the intracellular calcium and activate p38MAPK, resulting in the release of bioactive diffusible factors such as cytokines and neurotrophic factors, including BDNF. The diffusible messengers released from the microglia may then interact with excitatory and inhibitory synapses of neighboring dorsal horn neurons and modify the excitability in dorsal horn neurons. The facilitation of glutamatergic synaptic transmission or the suppression of GABA/glycinergic inhibition will be postulated. These effects, in turn, lead to increasing transmission in pain signaling neurons.

Kohsaka's group reported the physiological significance of plasminogen in the CNS. They first reported the effects of plasminogen on the development of primary cultured mesencephalic neurons from 16-day-old embryonic rats (Nagata et al., 1993; Nakajima et al., 1993). Plasminogen markedly increases dopamine uptake and the dopamine content of cultured neurons. Furthermore, the number of mesencephalic dopaminergic neurons is significantly increased in the presence of plasminogen. However, plasminogen has only a slight effect on gamma-aminobutyric acid (GABA) uptake and glutamic acid decarboxylase activity in mesencephalic neurons. The effect on dopaminergic neurons is also observed in the culture in the presence of cytosine arabinoside. In addition, plasminogen has no effect on the number of glial fibrillary acidic protein (GFAP)-positive cells. These results suggest that plasminogen acts directly on neurons and enhances the development of dopaminergic neurons in a rather selective manner. In other words, plasminogen mediates an interaction

between microglia and dopaminergic neurons. Plasminogen also affects on the glutamatergic synaptic transmission through the modulation of NMDA receptors (Inoue et al., 1994). It was reported that plasminogen (100 nM) does not increase $[Ca]_i$ in the presence of tetrodotoxin (TTX, 3 μ M) but increased $[Ca]_i$ in the absence of TTX. In all the cells which responded to plasminogen (100 nM), NMDA (5 μ M) also increased $[Ca]_i$ in the presence of TTX. Furthermore, plasminogen (100 nM) enhances the NMDA-evoked $[Ca]_i$ increase, and the potentiation by plasminogen is blocked by an NMDA receptor blocker, 2-amino-phosphonovalerate (APV). These data suggest that plasminogen enhances glutamate-evoked $[Ca]_i$ increase through the modulation of NMDA receptor in hippocampal neurons. This is very interesting because the synaptic transmission between primary afferent sensory neurons and dorsal horn neurons is mainly glutamatergic. Plasminogen released from microglia may be able to stimulate the synaptic transmission of pain sensation in neuropathic condition.

4.3. Function and the release of tumor necrosis factor- α

TNF- α is a proinflammatory cytokine increased in the brain in response to various insults or injury (Merrill & Benveniste, 1996). Although TNF- α has been implicated in the acceleration of injury and the pathology of neurodegenerative diseases, recently emerging evidence suggests that TNF- α may also serve a protective role. The precise mechanisms involved in these 2 seemingly conflicting activities are still undetermined. This issue is discussed later.

Microglia are a major source of TNF- α . ATP potently stimulates the release of TNF- α following an increase in the TNF- α mRNA expression by activating MAPKs (Hide et al., 2000; Suzuki et al., 2004). Hide et al. (2000) demonstrated that ATP potently stimulates TNF- α release, resulting from TNF- α mRNA expression in rat cultured brain microglia. The TNF- α release is maximally elicited by 1 mM ATP and also induced by a P2X7 receptor selective agonist, BzATP, suggesting the involvement of P2X7 receptor. ATP-induced TNF- α release is Ca²⁺ dependent, and a sustained Ca²⁺ influx correlates with the TNF- α release. ATP-induced TNF- α release seems to need MAPK activation because the release is inhibited by PD 098059, an inhibitor of extracellular signal-regulated protein kinase (ERK) kinase 1 (MEK1) and by 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB 203580), an inhibitor of p38 MAPK. Interestingly, ATP rapidly activates both ERK and p38 even in the absence of extracellular Ca²⁺, suggesting that ATP-evoked TNF- α release from microglia is dependent on the sustained Ca²⁺ influx and Ca²⁺-independent ERK/p38 cascade. However, the action of P2X7 does not seem to be a channel but a novel signal-transduction system (Suzuki et al., 2004). The inhibitors 1,4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio] butadiene (U0126), anthra[1,9-cd]pyrazol-6(2H)-one (SP600125), and SB 203580, which target mitogenactivated protein kinase kinase (MEK; MAPK kinase), c-Jun N-terminal kinase (JNK), and p38, respectively, all potently suppress the production of TNF- α in ATP-stimulated microglia, whereas the production of TNF- α mRNA is strongly inhibited by U0126 and SP600125 but not by SB203580. This suggests that a transcription of TNF- α mRNA is dependent on both of ERK and JNK, but not on p38. SB203580 does not affect the increased levels of TNF- α mRNA but does prevent TNF- α mRNA from accumulating in the cytoplasm, suggesting that p38 plays an important role in the nucleocytoplasmic transport of TNF- α mRNA. The ATP-induced activation of JNK and p38, but not extracellular signal-regulated kinase (ERK), is inhibited by brilliant blue G, a P2X7 receptor blocker, and by genistein and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*D*] pyrimidine, which are general and src-family-specific tyrosine kinase inhibitors, respectively. These findings indicate that an src family acts downstream of the P2X7 receptor to activate JNK and p38 independently from the channel action.

It was not clear whether TNF- α is toxic or protective to neurons (Arnett et al., 2001; Combs et al., 2001; Fontaine et al., 2002). TNF- α appears to enhance injury, as shown by the fact that the injection of neutralizing TNF- α antibody into lesion sites significantly reduces experimental ischemic and traumatic injury (Barone et al., 1997; Meistrell et al., 1997). In the meanwhile, recent evidence indicates that TNF- α can provide protection to neurons because it is able to encourage the expression of antiapoptotic and antioxidative proteins. Moreover, it was also reported that TNF- α plays in both the long-term behavioral recovery and the histological repair of the tissues in TNF- α -deficient mice, although TNF- α has a deleterious effect during the acute response that occurs in a traumatized brain (Scherbel et al., 1999). More recent reports indicate that the dual actions of TNF- α are mediated via different TNF- α receptors, with the p55 TNF- α receptor 1 (TNFR1) eliciting neurotoxic effects and the p75 TNF- α receptor 2 (TNFR2) eliciting neuroprotection (Yang et al., 2002). How does TNF- α released from microglia by ATP stimulation act on neuronal cell death? To obtain the answer to this question, Suzuki et al. examined the effect of TNF- α on the death of cortical neurons by glutamate using a primary neuron–microglia coculture system. They demonstrated that BzATP-stimulated microglia provide neurons with effective protection against glutamate-induced cell death. This protective effect appears to be mediated by a soluble factor or factors released from P2X7-activated microglia. One such neuroprotective factor is almost certain to be TNF- α , because microglia-mediated neuroprotection is suppressed in the presence of either TACE inhibitor or anti-TNF- α antibody. ATP might be released from cells that are damaged by trauma, inflammation, or ischemia, and low ATP concentrations can lead to chemotaxis of microglia (Honda et al., 2001). These data suggest that ATP acts as an emergency molecule that recruits microglia to a damaged brain area and activates P2X7 receptors to secrete TNF- α as a neuroprotective factors from microglia.

Recently, accumulating evidence indicates the relationship between TNF- α and neuropathic pain (Wagner & Myers, 1996; Sommer & Schäfers, 1998; Sommer et al., 1998; Sorkin & Doom, 2000; Schäfers et al., 2002), and TNF- α released after injury have been proposed as initiators of abnormal pain sensation (Wagner & Myers, 1996; Sommer & Schäfers, 1998; Sommer et al., 1998; Sorkin & Doom, 2000). TNF- α is up-regulated after nerve injury in both dorsal root ganglion (DRG; Schäfers et al., 2002, 2003a, 2003b) and spinal cord (Hashizume et al., 2000), and the inhibition of TNF- α reduces the hyperalgesia in neuropathic pain models (Sommer et al., 2001a, 2001b). After peripheral nerve injury, DRG neurons robustly increase their expression of TNF- α (Schäfers et al., 2002). Exogenous TNF- α applied to intact or compression-injured DRG induces sustained mechanical allodynia (Homma et al., 2002). However, the mechanisms by which TNF- α elicits pain behavior are still unclear.

Previous studies suggest that TNF- α modulates neuronal activity in neurons (Sawada et al., 1990; Soliven & Albert, 1992; Sorkin et al., 1997; Furukawa & Mattson, 1998; Junger & Sorkin, 2000; Diem et al., 2001; Leem & Bove, 2002). Schäfers et al. (2003a, 2003b) investigated the responses of intact and nerve-injured DRG neurons to locally applied TNF- α using parallel *in vivo* and *in vitro* paradigms. *In vivo*, TNF- α (0.1–10 pg/mL) or vehicle was injected into L5 DRG in naive rats and in rats that had received L5 and L6 spinal nerve ligation (SNL) immediately before injection. In naive rats, TNF- α elicits long-lasting allodynia. In SNL rats, subthreshold doses of TNF- α synergize with nerve injury to elicit faster onset of allodynia and spontaneous pain behavior. Preemptive treatment with etanercept, a TNF- α antagonist, reduces SNL-induced allodynia by almost 50%. Perfusion of TNF- α (100–1000 pg/mL) to naive DRG neuron evokes short-lasting discharges. In injured DRG, TNF- α elicits higher and longer lasting neuronal discharges in earlier onset at much lower concentrations. In naive DRG, which is adjacent to injured DRG, TNF- α also elicits high-frequency discharges at subthreshold concentrations. These data suggest that injured and adjacent uninjured DRG neurons are sensitized to TNF- α after SNL, and sensitization to endogenous TNF- α may be essential for the development and maintenance of neuropathic pain.

The mechanisms by which TNF- α elicits pain behavior are still unclear. In cultured DRG neurons, exogenous TNF- α activates p38 MAPK (Pollock et al., 2002). Recently, p38 activation is shown to play a major role in the maintenance of pain (Ji et al., 2002; Jin et al., 2003; Milligan et al., 2003; Tsuda et al., 2004). It was speculated that the activation of the p38 cascade may represent a route correlating the development of pain after nerve injury. To obtain the answer to the question, it was investigated whether TNF- α activates the p38 cascade *in vivo* to trigger pain behavior after SNL (Schäfers et al., 2003a, 2003b). As a result, etanercept treatment starting 2 days before SNL attenuates mechanical allodynia. Interestingly, the treatment starting 1 or 7 days after SNL is ineffective. Similarly, intrathecal infusion of a p38 inhibitor (SB203580, 4 mg/day) is effective only when it was started before but not 7 days after SNL. In DRG, activated p38 is transiently elevated 5 hr after SNL and returns to baseline by 1 day after SNL. Phosphorylated p38 is localized in small TNF- α -positive DRG neurons. In the spinal cord, p38 is activated between 5 hr and 3 days after SNL and returns to baseline level within 5 days. Pretreatment with etanercept blocks p38 activation only in DRG, but not in the spinal cord. These data indicate that phosphorylated p38 levels in spinal cord and DRG are transiently elevated after SNL treatment. In DRG, p38 activation is blocked by systemic TNF- α inhibition. Another report suggests the mechanism of TNF- α -induced pain in the line of the interaction with brain-derived neurotrophic factor (BDNF), which is thought to be a modulator of pain. Onda et al.

(2004) investigated the effect of infliximab, a chimeric monoclonal antibody to TNF- α , on the induction of BDNF using an experimental herniated nucleus pulposus (NP) model. The application of NP induces a marked increase of BDNF immunoreactivity in number in the DRG neurons and within the superficial layer in the dorsal horn compared with the sham group. Infliximab treatment (intraperitoneal injection) reduces the BDNF induction in both the DRG and spinal cord.

It was noted that ATP activates microglia, leading to the phosphorylation of MAPK, including p38, and the release of TNF- α . There, however, is little report answering whether TNF- α from glial cells affects the neuropathic pain. For this question, Ohtori et al. (2004) examined changes in TNF- α and TNF- α receptor 1 (p55 receptor) in microglia, astrocytes, and neural cells in the dorsal root ganglion and spinal cord after sciatic nerve injury in mice. In the dorsal root ganglion, glial fibrillary acidic protein (GFAP)-immunoreactive (IR) satellite cells become evident after injury and are also immunoreactive for both TNF- α and p55 receptor. Dorsal root ganglion neurons express p55 receptor after injury, and these neurons are surrounded by TNF- α and GFAP-IR satellite cells. Furthermore, the number of GFAP-IR astrocytes, which surrounded p55 receptor-IR neurons, dramatically increase in the spinal cord after nerve injury, and some astrocytes are also TNF- α -IR and p55 receptor-IR. These data demonstrate that the up-regulation of glial TNF- α is associated with the expression of the p55 receptor on adjacent neurons.

4.4. Function and the release of interleukin-6

The cytokine IL-6 is an important mediator of inflammatory and immune responses in the periphery. In addition to these peripheral actions of IL-6, recent studies indicate that IL-6 is also produced in the CNS and may play an important role in a variety of CNS functions such as cell-to-cell signaling, coordination of neuroimmune responses, protection of neurons from insult, as well as neuronal differentiation, growth, and survival (Gruol & Nelson, 1997; Marz et al., 1999). IL-6 may also contribute to the etiology of neuropathological disorders. Elevated levels of IL-6 in the CNS are found in several neurological disorders, including AIDS, dementia complex, Alzheimer's disease, multiple sclerosis, systemic lupus erythematosus, CNS trauma, and viral and bacterial meningitis. Moreover, several studies have shown that chronic overexpression of IL-6 in transgenic mice can lead to significant anatomical and physiological changes in the CNS similar to that commonly observed in various neurological diseases. Thus, it appears that IL-6 may play a role in both physiological and pathophysiological processes in the CNS (Gruol & Nelson, 1997). It was reported previously that TNF- α increases neuronal survival to a toxic influx of calcium mediated through neuronal *N*-methyl-D-aspartic acid (NMDA) glutamate-gated ion channels (Carlson et al.,

1999), suggesting the neuroprotective effect of TNF- α on the neuronal death following ischemia or stroke. A similar effect was obtained by the treatment with IL-6 to excessive NMDA challenge. IL-6-mediated neuroprotection is antagonized by pretreatment with IL-1 receptor antagonist, and it was not affected by nicotine. Neutralizing anti-NGF only partially blocks IL-6-mediated protection.

A de novo synthesis and release of IL-6 is also induced by ATP through pathways mediated by both Ca²⁺-dependent protein kinase C (PKC) and p38 MAPK (Shigemoto-Mogami et al., 2001). It was reported that ATP evokes the release of IL-6 at 24 hr in a concentration-dependent manner (10–1000 μ M) in MG-5, a mouse microglial cell line (Ohsawa et al., 1997; Shigemoto-Mogami et al., 2001) and that the release is observed from 6 hr after the stimulation with ATP. There is a possibility that ATP might evoke the release of IL-6 secondarily by releasing TNF- α and IL-1 β because TNF- α and IL-1 β are reported to stimulate IL-6 production in other glial cells (Norris et al., 1994). In MG-5, a significant amount of TNF- α appears at 1 hr and peaks at 3 hr after the stimulation by 1000 μ M ATP, but TNF- α (up to 10,000 pg/mL) lonely never evokes the release of IL-6 in MG-5. This is well in accord with a previous report that TNF- α fails to stimulate IL-6 production in microglia (Sawada et al., 1992). Moreover, the release of IL-1 β , a potent inducer of IL-6 gene (Lee et al., 1993), is not evoked by 1000 μ M ATP alone in MG-5. ATP induces an \sim 7-fold increase in the expression of IL-6 mRNA, which is inhibited by 1 mM suramin, indicating that ATP stimulates the de novo synthesis and the subsequent production of IL-6. ATP can activate 2 distinct MAP kinases, that is, ERK1/2 and p38, in MG-5. The ATP-stimulated IL-6 release is inhibited by SB203580 but not by an inhibitor of ERK1/2. These results suggest that p38 but not ERK1/2 MAP kinase is responsible for the IL-6 production. The ATP-evoked p38 activation is dependent on the extracellular Ca²⁺. ATP produces a phospholipase C (PLC)-dependent transient Ca²⁺ release by inositol 1, 4, 5-trisphosphate (InsP3), which is followed by sustained Ca²⁺ entry via both SOC and P2X7 receptors. Several groups have reported that P2X7 receptors have a central role in the production of cytokines in microglia (Ferrari et al., 1996, 1997b; Hide et al., 2000). In fact, BzATP evokes sustained Ca²⁺ entry via P2X7 receptors, leading to the phosphorylation of p38 in MG-5. However, BzATP induces only a very small amount of IL-6 production in the MG-5 (Shigemoto-Mogami et al., 2001). Brilliant Blue G (up to 10 μ M), a specific P2X7 antagonist (Jiang et al., 2003), does not inhibit the release of IL-6 induced by ATP from MG-5. These data suggest that a P2Y receptor(s) rather than P2X7 receptor seems to have a major role in the IL-6 production in MG-5 cells. This idea is supported by the recent observation that ATP may evoke IL-6 production in bacterial endotoxin lipopolysaccharide (LPS)-primed P2X7-deficient mice (P2X7R^{-/-}; Solle et al., 2001). A Ca²⁺-dependent PKC may be an additional signal since the ATP-evoked IL-6 production is attenuated

by Gö6976, an inhibitor of Ca²⁺-dependent protein kinase C (PKC). The P2Y receptor responsible for these responses is insensitive to PTX and is linked to phospholipase C. Some transcriptional factors, such as NF- κ B p65 (RelA; Ferrari et al., 1997a), Jun, and Fos (Neary et al., 1999), are known to be activated by ATP. Berghe et al. (1998) have described the involvement of MAP kinase pathways in NF- κ B trans-activation, which leads to the induction of IL-6 gene expression. Although such transcriptional factors may work as the downstream signals of either p38 or Ca²⁺-dependent PKC, the detailed mechanism underlying such a cooperative regulation of IL-6 production remains to be clarified. Although the definitive classification of P2Y receptors for the ATP-evoked IL-6 release remains to be determined, it was suggested that ATP, acting on the PTX-insensitive P2Y receptors linked to PLC, stimulates the synthesis of IL-6 through pathways involving p38 and Ca²⁺-dependent PKC.

4.5. Function and the release of interleukin-1 β

IL-1 β is one of the principal proinflammatory cytokine in CNS as a result of systemic or local insult (Hopkins & Rothwell, 1995; Rothwell et al., 1997). Microglia as well as meningeal macrophages produce IL-1 β in the ischemic area after stroke and brain damage (Minami et al., 1992; Liu et al., 1993; Buttini et al., 1994) and is thought to be the major source of IL-1 β secreted in response to neuronal damage (Giulian et al., 1986; Rothwell, 1999). It is suggested that IL-1 β plays a direct role in the pathophysiology of stroke because IL-1 receptor antagonist or IL-1 β antibodies significantly reduced cerebral ischemia and neuronal loss in rats (Relton & Rothwell, 1992; Yamasaki et al., 1992; Garcia et al., 1995). It was also reported that IL-1 β is implicated in several neurodegenerative diseases, that is, multiple sclerosis (Martin & Near, 1995; Schijver et al., 1999), amyotrophic lateral sclerosis (Pasinelli et al., 1999; Li et al., 2000), and epilepsy (Vezzani et al., 1999, 2000).

Although the mechanisms underlying post-translational processing of IL-1 β are not fully understood, the depletion of cytoplasmic K⁺ has been shown to be crucial for the induction of IL-1 β -converting enzyme (ICE) activity and IL-1 β release (Perregaux & Gabel, 1994). ATP lonely does not evoke a release of IL-1 β but can stimulate the release from the microglia after priming by LPS. This LPS-primed release of IL-1 β from mouse microglial cells is a very inefficient process. It is slow and leads to the accumulation of only a modest amount of extracellular cytokine. This evidence suggested that a second stimulus is needed to elicit the IL-1 β secretion from microglia. The Di Virgilio and Ferrari group has examined the mechanism of IL-1 β secretion from microglia (Ferrari et al., 1996, 1997b). They first reported that extracellular ATP triggers IL-1 β release from LPS-treated microglia or macrophages (Ferrari et al., 1996, 1997b; Sanz & Di Virgilio, 2000). They confirmed that ATP is a powerful stimulus for IL-1 β release from LPS-treated macrophages or microglia and showed that IL-1 β

release is not necessarily associated with cell death, as it occurs at lower ATP concentrations and much earlier than the leakage of cytoplasmic markers. Sanz and Di Virgilio (2000) examined the kinetics and mechanism of ATP-dependent IL-1 β release from microglial cells. The addition of extracellular ATP to LPS-primed microglia causes a burst of release of a large amount of processed IL-1 β . ATP has no effect on the accumulation of intracellular pro-IL-1 β in the absence of LPS. In LPS-treated cells, ATP slightly increases the synthesis of pro-IL-1 β . The optimal ATP concentration for IL-1 β secretion is between 3 and 5 mM, but significant release can be observed at concentrations as low as 1 mM. At all ATP concentrations, the IL-1 β release can be inhibited by increasing the extracellular K⁺ concentration. The ATP-dependent IL-1 β release is also inhibited by the caspase inhibitors. Accordingly, in ATP-stimulated microglia, the p20 proteolytic fragment derived from the activation of the IL-1 β -converting enzyme can be detected by immunoblotting. Authors concluded that ATP triggers accelerated maturation and the release of intracellularly accumulated IL-1 β by activating the IL-1 β -converting enzyme/caspase 1 in mouse microglia. Extracellular ATP is the only endogenous compound known to cause a significant reduction in intracellular K⁺ and consequent release of IL-1 β (Perregaux & Gabel, 1994; Sanz & Di Virgilio, 2000).

Substantial evidence suggests a key role of P2X7 in the ATP-induced IL-1 β release from LPS-primed microglia, that is, (1) P2X7 antagonist α ATP inhibits the release from microglia (Ferrari et al., 1997a, 1997b), (2) microglia lacking P2X7 does not release IL-1 β after ATP stimulation (Ferrari et al., 1996), (3) macrophages pretreated with a monoclonal anti-P2X7 antibody fails to induce IL-1 β release by ATP (Buell et al., 1998), and (4) ATP fails to induce IL-1 β release from macrophages expressing mutant P2X7 receptors, both in vivo and in vitro (Solle et al., 2001). The activation of P2X7 by ATP or BzATP induces the formation of cytolitic pores, permitting the movement of K⁺, Na⁺, and Ca²⁺ through cell membrane and provokes the release of IL-1 β from microglia. The process of pore-forming of P2X7 by nucleotides in microglia is very interesting, according to the report by Chakfe et al. (2002). Previous studies have shown that ADP (Perregaux & Gabel, 1994; Ferrari et al., 1997b), but not AMP, UTP, or GTP (Perregaux & Gabel, 1994), also triggers the secretion of significant amounts of IL-1 β from LPS-primed microglia. If ADP is not an antagonist of P2X7, then the mechanism underlying the ADP-stimulated IL-1 β release remains unclear. They investigated the actions of other endogenous nucleotides on recombinant and microglial P2X7 receptors using electrophysiology, fluorescence imaging, and interleukin-1 β release measurement. They found that an initial application of ADP or AMP to *Xenopus* oocytes expressing P2X7 receptors is ineffective to activate P2X7. When ADP and AMP, but not UTP or adenosine, are applied after a brief exposure to ATP or BzATP, ADP and AMP activate

P2X7 receptors in a dose-dependent manner. They also found that responses to ADP and AMP are also elicited after exposure to low concentrations of ATP and are recorded several minutes after the removal of ATP from the extracellular medium. A whole-cell recording from mouse microglial cells shows that significant responses to ADP and AMP are elicited only after ATP application. ADP and AMP stimulate the release of IL-1 β after the short ATP treatment from LPS-primed mouse and human microglial cells. These data indicate that selective sensitization of P2X7 receptors to ADP and AMP requires priming with ATP. This novel property of P2X7 leads to activation by ATP metabolites and proinflammatory cytokine release from microglia without cytotoxicity.

Recently, accumulating evidence indicates the relationship between inflammatory cytokines including IL-1 β and neuropathic pain (Robertson et al., 1997; Deleo & Yeziarski, 2001; Sweitzer et al., 2001; Watkins et al., 2001; Winkelstein et al., 2001; Raghavendra et al., 2003). The expression of IL-1 β and TNF- α are up-regulated in the spinal cord in several rat mononeuropathy models (Sweitzer et al., 2001; Winkelstein et al., 2001; Raghavendra et al., 2003). Sweitzer et al. (2001) investigated whether blocking the action of central IL-1 β and TNF- α attenuates mechanical allodynia in a gender-specific manner in a rodent L5 spinal nerve transection model of neuropathic pain with/without glial activation. Soluble TNF- α receptor treatment attenuates mechanical allodynia, although the glia remain activated. IL-1 receptor antagonist treatment alone does not affect allodynia. When treated with soluble TNF-(alpha) receptor antagonist, however, allodynia decreases in a dose-dependent manner and the glial cells are still activated. At days 3 and 7 post-transection, the level IL-6, but not IL-1 β , in the L5 spinal cord of animals receiving daily IL-1 receptor antagonist in combination with soluble TNF- α receptor is significantly less than that of control animals. These findings further support a role for central IL-1 β and TNF- α in the development and maintenance of neuropathic pain through the induction of a proinflammatory cytokine cascade.

The exogenous application of IL-1 β enhances NMDA receptor-mediated Ca²⁺ responses via activating tyrosine protein kinase Src (Viviani et al., 2003), which is known to enhance NMDA receptor activity in dorsal horn neurons (Yu et al., 1997; Woolf & Salter, 2000). IL-1 β also decreases GABA_A receptor-mediated currents (Wang et al., 2000). Thus, many cytokines from activated microglial are involved in neuropathic pain. These effects may account for some part of the mechanisms of IL-1 β on evoking neuropathic pain.

4.6. Function and the release of ionized calcium binding adaptor molecule 1/microglial response factor-1/allograft inflammatory factor-1

Ionized calcium binding adaptor molecule 1 (Iba1) is an EF hand calcium binding protein whose expression is

restricted to macrophages/microglia, suggesting the important function of Iba1 in microglia. Iba1 is called as microglial response factor-1 (MRF-1) or allograft inflammatory factor-1 (AIF-1) in independent research groups, respectively. Three genes of these proteins may actually be a single gene (Utans et al., 1995; Tanaka et al., 1998; Ohsawa et al., 2000). Iba1 is shown to colocalize with F-actin in membrane ruffles induced by macrophage colony-stimulating factor and in phagocytic cups formed during zymosan phagocytosis (Ohsawa et al., 2000). The expression of mutant Iba1 carrying either N- or C-terminal deletions or carrying a substitution in the calcium binding domain suppresses the membrane ruffling and the phagocytosis. These data suggest that Iba1 is a key molecule of microglia activation resulting in membrane ruffling and phagocytosis (Ohsawa et al., 2000). It was reported that the gene of MRF-1 is up-regulated in activated microglia in response to apoptosis of cerebellar granule neurons in culture (Tanaka et al., 1998). It has been shown that *mrf-1* is up-regulated in response to neuronal death and degeneration in vivo (Tanaka et al., 1998, 2000; Kato et al., 2000) and that MRF-1 then may play a significant role in both developmental programmed cell death and recovery from brain injuries (Tanaka & Koike, 2002). Recently, it was reported that microglia constitutively express MRF-1 and up-regulate its level in response to inflammation (Tanaka et al., 1998, 2000; Kato et al., 2000). Altogether, these data suggest that MRF-1/AIF-1/Iba1 may play a significant role in all inflammatory lesions. However, the exact role of MRF-1 remains unknown. For this question, there is an interesting paper that MRF-1 is released from cultured rat microglia, and its release is greatly enhanced under inflammatory conditions. Interestingly, ATP stimulation increases the release by 10-fold compared with the basal level of MRF-1 release (Kaya et al., 2002). ATP-enhanced MRF-1 release is induced within 10 min and peaks within 1 hr; after 4 hr, the MRF-1 release returns to normal. MRF-1 release is stimulated by 2-methyl-thio-ATP and a P2X7 selective agonist, 2'- and 3'-O-(4-benzoylbenzoyl)-ATP. The ATP-stimulated MRF-1 release is inhibited by a P2X7-selective antagonist, oxidized ATP (oATP), and in extracellular Ca²⁺-free condition. These results indicate that the effects of ATP are dependent on Ca²⁺ influx through P2X7 receptors. MRF-1 release is enhanced by Ca²⁺-ionophore A23187 (6-fold), thapsigargin. However, it was not enhanced by glutamate or lipopolysaccharide. These data indicate that selective inflammatory stimulations including ATP enhance MRF-1 release from microglia through a Ca²⁺-dependent mechanism. It was reported that some fractions of AIF-1 are released into sera, and the levels in the sera increase at the preclinical stage of experimental autoimmune neuritis (Pashenkov et al., 2000). These data also suggest that microglial MRF-1 release is enhanced in response to the apoptosis of cerebellar granule neurons, and the release is significantly dependent on ATP. We

have very few information on the relationship between Iba1 and neuropathic pain.

5. Conclusion and consideration

As mentioned above, ATP is able to activate MAPK, resulting in the release of cytokines and many active substances from activated microglia. Interestingly, it was found that nerve injury leads to persistent activation of p38 MAPK, and, importantly, that the activation of p38 MAPK in the spinal cord is entirely restricted to microglia (Jin et al., 2003; Tsuda et al., 2004). Furthermore, an inhibitor of this enzyme, SB203580, administered intrathecally, reverses mechanical allodynia following spinal nerve ligation (Jin et al., 2003; Tsuda et al., 2004). Also, continuous infusion of SB203580 starting before the nerve injury prevents the development of tactile allodynia (Tsuda et al., 2004). These results indicate that p38MAPK can be an important intracellular event in the development of nerve injury-induced pain. Moreover, it is reported that several cytokines, such as IL-1 β , IL-6, and TNF- α , in the dorsal horn are increased after nerve lesion (Sweitzer et al., 2001; Winkelstein et al., 2001; Raghavendra et al., 2003) and have been implicated in contributing to nerve-injury pain (Robertson et al., 1997; Deleo & Yezierski, 2001; Sweitzer et al., 2001; Watkins et al., 2001; Winkelstein et al., 2001; Raghavendra et al., 2003), presumably by altering synaptic transmission in the CNS, including the spinal cord (Kerr et al., 1999; Thompson et al., 1999; Vikman et al., 2003). For example, the exogenous application of IL-1 β enhances NMDA receptor-mediated Ca²⁺ responses via activating tyrosine protein kinase Src (Viviani et al., 2003), which is known to enhance NMDA receptor activity in dorsal horn neurons (Yu et al., 1997; Woolf & Salter, 2000). IL-1 β also decreases GABA_A receptor-mediated currents (Wang et al., 2000). Thus, many cytokines from activated microglial are involved in neuropathic pain. This is supported by the report that the inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy (Raghavendra et al., 2003). Ledebner et al. (2005) also reported that intrathecal administration of minocycline, a selective inhibitor of microglia activation, inhibits low threshold mechanical allodynia. In a rat model of neuropathic pain induced by sciatic nerve inflammation (sciatic inflammatory neuropathy, SIN), minocycline delays the induction of allodynia in both acute and persistent paradigms. Minocycline attenuates an established SIN-induced allodynia at 1 day, but not 1 week later. In a model of spinal immune activation by intrathecal HIV-1 gp120, it was found that the anti-allodynic effects of minocycline are associated with decreased microglial activation, attenuated mRNA expression of IL-1 β , TNF- α , IL-1 β -converting enzyme, TNF- α -converting enzyme, IL-1 receptor antagonist, and IL-10 in lumbar dorsal spinal cord, and reduced IL-1 β and

TNF- α levels in the CSF. In contrast, no significant effects of minocycline are observed on gp120-induced IL-6 and cyclooxygenase-2 expression in spinal cord or CSF IL-6 levels. Taken together, these data highlight the importance of microglial activation in the development of exaggerated pain states.

More recently, Moriguchi et al. (2003) have shown in acute cortical slices that the application of a microglial-conditioned medium potentiates NMDA receptor-mediated postsynaptic responses, but not when the medium is boiled or incubated with proteinase K. They fractionated the medium into 6 sharp peaks by anion-exchange chromatography and found that the fraction contains a relatively strong protein band with a molecular mass of ~ 70 kDa that showed the most potent enhancing activity on the NMDA receptor-mediated responses. Although they have not identified the molecule(s) yet, they suggested that both heat- and protease-labile molecules released from microglia regulate NMDA receptor-mediated excitatory synaptic transmission in the CNS. Thus, diffusible factors released from activated microglia by activating P2X4Rs may also have modulatory effects on neurons in the pain processing network within the dorsal horn as shown in Fig. 5B. Novel molecules should be added to the hypothesis in the near future with a great progress in this research area.

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Osteoclastic function is accelerated in male patients with type 2 diabetes mellitus: the preventive role of osteoclastogenesis inhibitory factor/osteoprotegerin (OCIF/OPG) on the decrease of bone mineral density

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Abstract

To clarify the pathogenesis of altered bone metabolism in diabetic state and its underlying mechanisms, the bone mineral content and fasting levels of serum intact parathyroid hormone (i-PTH), intact osteocalcin (i-OC), tartrate-resistant acid phosphatase (TRAP) and osteoclastogenesis inhibitory factor/osteoprotegerin (OCIF/OPG) were measured in male type 2 diabetic patients and their age-matched controls. In addition, urine levels of osteoclastic markers, C-telopeptide of type I collagen (CTx), deoxypyridinoline (DPD), and N-telopeptide of type I collagen (NTx) were simultaneously determined. Serum levels of i-PTH and i-OC in diabetic patients were significantly lower than those in the controls. Conversely, serum concentrations of TRAP were significantly elevated in diabetic patients. However, no clear correlation was observed between serum i-OC and TRAP. It was also observed that urinary excretion of CTx, DPD, and NTx was significantly increased in the diabetics as compared with the controls. Unexpectedly, serum levels of OCIF/OPG tended to be higher in the diabetic group, and these values exhibited a significantly positive correlation with those of serum TRAP. There was found a significantly negative correlation between serum TRAP and bone mineral density (BMD) and also between serum OCIF/OPG and bone mineral

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density. It seems probable that OCIF/OPG has a suppressive role on the increased bone resorption to prevent further loss of the skeletal bone mass in type 2 diabetic patients.

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1. Introduction

It has been recognized that the alterations in mineral and bone metabolism are associated with diabetes mellitus and that the resulting bone loss is one of the chronic complications of diabetic patients [1–6]. Regarding the altered bone metabolism found in patients with type 2 diabetes, we and others have demonstrated the evidence that the osteoblastic function was decreased whereas the osteoclastic function was conversely elevated [5,7,8]. In order to further confirm the enhancement of osteoclastic function in type 2 diabetes, we measured the urine markers for bone resorption, cross-linked C-telopeptide of type I collagen (CTx), deoxypyridinoline (DPD), and cross-linked N-telopeptide of type I collagen (NTx) [9–11], as well as the circulating osteoclastic marker.

In terms of the molecular mechanisms for functional coupling between osteoblasts and osteoclasts, the bone coupling factors have been recently cloned from osteoblastic cells. Osteoclast differentiation factor (ODF) has been identified as a membrane-bound ligand mediating an essential signal to osteoclast progenitors for their differentiation into osteoclasts [12,13]. On the other hand, osteoclastogenesis inhibitory factor/osteoprotegerin (OCIF/OPG)

inhibits the osteoclastogenesis by interrupting the binding of ODF to ODF receptor of osteoclast progenitors [14,15].

In this study, we confirmed that the osteoclastic function is significantly accelerated in type 2 diabetic patients. For the purpose of clarifying its underlying pathogenesis, the circulating levels of OCIF/OPG were measured to investigate their interrelationship with the increased osteoclastic markers, and then the putative role of OCIF/OPG on the altered bone metabolism in diabetes was analyzed.

2. Subjects

A total of 169 male type 2 diabetic patients (55. ± 0.9 years of age and 6.1 ± 0.5 years of disease duration; means ± S.E.) was examined in this study. Ninety-five age-matched male healthy subjects (54.7 ± 1.1 years of age) were also studied as the controls with fasting blood glucose level less than 7.0 mmol/l (Table 1). The female type 2 diabetic patients were excluded from the present study to prevent the influences of sex hormones from the evaluations of bone mineral content. Those who had obvious renal dysfunction (serum creatinine levels of 1.32 ± 10⁻⁴ mmol/l or more, urine corrected albumin levels

Table 1
Clinical profile of male patients with type 2 diabetes and their age-matched controls

	Number	Body mass index (kg/m ²)	Duration (years)	Glucose (nmol/l)	HbA1c (%)
Diabetic patients					
Diet	61	23.5 ± 0.5	5.4 ± 0.9	9.37 ± 0.51 ^a	8.3 ± 0.3 ^a
Oral hypoglycemic agent	64	21.8 ± 0.4 ^b	6.5 ± 0.8	10.41 ± 0.48 ^a	10.1 ± 0.3 ^a
Insulin	44	21.0 ± 0.5 ^a	7.0 ± 0.9	12.43 ± 0.66 ^a	10.6 ± 0.4 ^a
Totals	169	22.3 ± 0.2	6.1 ± 0.5	10.46 ± 0.29 ^a	10.0 ± 0.5 ^a
Controls	95	22.8 ± 0.2	–	5.41 ± 0.05	5.0 ± 0.1

Values were presented as mean ± S.E.

^a $p < 0.01$.

^b $p < 0.05$ vs. controls.

of 5.04 ± 10^{-6} mg/mmol creatinine or more) were excluded to avoid possible influence of renal osteodystrophy and retardation of hormone excretion [16]. In addition, the patients with retinopathy, neuropathy and coronary artery disease were also excluded. None had taken oral calcium supplementation, drugs such as statins to affect bone metabolism [17] or any vitamin preparation containing Vitamin D, or K. Each subjects had given informed written consent to participate in this study, and the study was carried out in accordance with the Declaration of Helsinki as revised in 1996.

3. Methods

3.1. Biochemistry

A Hitachi 7350 autoanalyzer (Hitachi Co. Ltd., Tokyo, Japan) was used to measure circulating levels of glucose, calcium, magnesium, inorganic phosphorus, creatinine and albumin, and urinary excretion of calcium and creatinine. Corrected values of serum calcium were calculated according to the formula $10y/x$ mmol/g albumin, where x denotes albumin (g/dl) and y denotes calcium (mmol/l) [18]. HbA1c was determined with a high-performance liquid chromatography [19]. Urine C-peptide (CPR) was analyzed by a radioimmunoassay (RIA) technique as reported previously [20]. Serum insulin was determined by enzyme immunoassay (AxSYM Insulin Kit, Dinabott Co. Ltd., Tokyo, Japan) using monoclonal antibody which cross-reacted neither with human proinsulin nor CPR. The sensitivity of this assay is 1.0 pmol/l, and the coefficients of variance (CV) of intra- and inter-assay were 2.2–2.7% and 3.5–7.2%, respectively. Serum intact osteocalcin (i-OC) was determined by one-step enzyme immunometric assay (Osteocalcin test Teijin, Teijin Co. Ltd. Tokyo, Japan) [21]. The sensitivity of this assay is 8.5 pmol/l, and the CV of intra- and inter-assay were 2.5–7.5% and 4.0–7.6%, respectively. Serum TRAP was determined by using paranitrophenol phosphate as previously described [22]. Intact molecules of parathyroid hormone (i-PTH) in serum were determined by a highly sensitive two-site immunoradiometric assay [6]. The detection limit for i-PTH was 10 pmol/l, and CV of intra- and inter-assay were 2.6–2.9% and 3.0–4.0%, respec-

tively. Circulating 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃) were measured by RIA (1,25(OH)₂D RIA Kit; Immunodiagnostic Systems Co. Ltd., Boldon, UK) [23]. The sensitivity of this assay is 3.3 pmol/ml, and the CV of intra- and inter-assay were 8.7–10.4% and 11.3–11.9%, respectively.

Urinary concentrations of CTx were quantified by RIA (α -CrossLaps[®]; Osteometer Co. Ltd., Copenhagen, Denmark) using a specific monoclonal antibody [9]. The detection limit for CTx is 44 nmol/l, and CV of the intra- and inter-assay were 2.9–9.7% and 4.6–9.6%, respectively. Urinary DPD were quantified by ELISA (Osteolinks “D”[®]; Sumitomo Pharmaceutical Co. Ltd., Osaka, Japan) using a specific monoclonal antibody [10]. The detection limit for DPD is 1.1 nmol and CV of the intra- and inter-assay were 3.5–4.3% and 4.4–5.7%, respectively. The DPD assay used determines only the free component of DPD in urine since samples are not hydrolysed. Urinary NTx were measured using ELISA assay (Osteomark[®], Ostex International Inc., Seattle, WA) and were expressed as nanomoles of bone collagen equivalent (BCE)/l/mM creatinine [11]. The detection limit for NTx is 5.0 nMBCE/mMcre, and CV of the intra- and inter-assay were 3.5–4.4% and 2.9–4.8%, respectively.

Serum levels of OCIF/OPG were determined by two steps sandwich ELISA (OCIF/OPG Kit; Cosmo Bio. Co. Ltd., Tokyo, Japan) according to the method of Tsuda et al. [14]. The detection limit for OCIF/OPG is 0.52 pmol/l, and CV of the intra- and inter-assay were 4.3–4.9% and 8.7–10.5%, respectively.

After overnight fasting, blood samples were collected from the antecubital vein and centrifuged at 3000 rpm for 5 min. Serum was immediately separated and stored at -40°C until assayed. The first morning urine samples were collected and centrifuged at 1500 rpm for 5 min immediately after collection in order to eliminate the debris, and were also stored at -40°C .

3.2. Measurement of bone mineral density

Teijin Bonalyzer (Teijin Co. Ltd.) was utilized to measure the bone mineral density (BMD) by means of computed X-ray densitometry (CXD) at the center of the right second metacarpal bone [6]. The principle of this method is based on the microdensitometric method using X-ray films of hands with aluminum