that EP4 is involved in other relevant processes during pregnancy and delivery. Glycosaminoglycan redistribution is an important process involved in cervical ripening; Schmitz et al. (2001) demonstrated that, of the four subtypes of PGE_2 receptors, only EP4 mediated PGE_2-induced glycosaminoglycan synthesis in human cervical fibroblasts in a PKA-independent manner.

The EP4 receptor may regulate endometrial function. PGE_2 promotes the survival of human endometriotic cells through the EP4 receptors by activating ERK, Akt, NF-κB, and the β-catenin signaling pathway (Banu et al., 2009). Inhibition of EP4 may suppress proliferation and induce apoptosis of human endometriotic cells. In addition, Lee et al. (2012) found that EP4 was expressed in the ovine endometrium, especially during pregnancy. Interferon-τ, a pregnancy recognition signal in ruminants, increased EP4 receptors in the endometrium.

H. Lungs

1. Expression. The lung is an organ in which the EP4 receptor is abundantly expressed in many species, including humans, mice, rats, and rabbits (An et al., 1993; Honda et al., 1993; Bastien et al., 1994; Sando et al., 1994; Breyer et al., 1996b). Anatomically, the lung is composed of the bronchial tree, the alveoli, and a dense vascular network, including a variety of cell types. EP4 is highly expressed in airway smooth muscle cells, pulmonary fibroblasts, and smooth muscle cells of the pulmonary vein. In particular, together with the EP2 receptor, EP4 transcripts and proteins are abundantly expressed in human airway smooth muscle cells (Bradbury et al., 2005; Clarke et al., 2005; Mori et al., 2011; Benyahia et al., 2012). It is also known that EP4 activation causes potent relaxation in human and rat bronchial preparations (Lydford and McKechnie, 1994; Benyahia et al., 2012).

Pulmonary fibroblasts are important in the development and maintenance of lung structure and function. Their proliferation and phenotypic changes play critical roles in normal tissue repair as well as the development of pulmonary fibrosis (Ramos et al., 2001). The EP4 receptor is expressed in both fetal (Choung et al., 1998; Li et al., 2011) and adult lung fibroblasts (Huang et al., 2007; Nikam et al., 2011) in humans. Togo et al. (2008) demonstrated that the EP2 and EP4 receptors were expressed in normal pulmonary fibroblasts and that these receptors were increased in fibroblasts from patients with chronic obstructive pulmonary disease where they contribute to the pathogenesis of emphysema.

In the human pulmonary vasculature, the EP4 receptor is mostly expressed in the smooth muscle layer of the vein and only weakly in the artery (Walch et al., 1999; Foudi et al., 2008), suggesting that EP4 induces relaxation of the vein. This expression pattern may change under disease conditions. Lai et al. (2008) reported that EP4 expression in the artery is readily detectable in pulmonary arterial hypertension in human and rat models. In other cell types in the lung, the EP4 receptor is found in human pulmonary microvascular endothelial cells (Aso et al., 2012), the human bronchial epithelial cell line BEAS-2B (N'Guessan et al., 2007), and human alveolar macrophages (Ratcliffe et al., 2007).

2. Function. EP4 may induce relaxation of the airway and inhibit smooth muscle cell proliferation. Buckley et al. (2011) reported that PGE_2-induced relaxation of the airway was mediated through EP4 in humans and rats. Mori et al. (2011) demonstrated that PGE_2 inhibited fetal bovine serum–induced proliferation of human airway smooth muscle cells via EP4 receptor activation. Taken together, these findings suggest that the EP4 receptor could potentially be a therapeutic target in treating pulmonary diseases such as asthma and chronic obstructive pulmonary disease. As they potentially occur downstream of EP4/cAMP signaling, both PKA and Epac are involved in anti-inflammatory (Oldenburger et al., 2012) and relaxation (Zieba et al., 2011) signaling in airway smooth muscle cells. It was also demonstrated that PGE_2 inhibits Platelet-derived growth factor-induced phenotype switching of tracheal smooth muscle cells, from a contractile to a proliferative phenotype, through the activation of the cAMP effectors PKA and Epac (Roscioni et al., 2011).

In fibroblasts, PGE_2 inhibits proliferation and collagen synthesis in human lungs (Huang et al., 2008). Proliferation and collagen synthesis were likewise attenuated by the activation of the cAMP effectors PKA and Epac, respectively. The accumulation of cAMP was promoted by EP4 receptor activation. In addition, chemotaxis of human lung fibroblasts was inhibited by EP4 (Li et al., 2011). Subtype-specific modulation of EP receptor activity could potentially be a new therapy for fibrotic lung disease.

An interesting vasodilatory effect of EP4-mediated signaling has been reported. Cyclic AMP accumulation in vascular smooth muscle cells is thought to be the main mechanism of prostanoid-induced vasorelaxation. Lai et al. (2008) demonstrated that iloprost, a stable analog of PGI_2, increased cAMP via the EP4 receptor in pulmonary arterial smooth muscle cells isolated from rats with pulmonary hypertension. Similarly, Foudi et al. (2008) reported that PGE_2-induced vasorelaxation of the human pulmonary vein was also mediated by the EP4 receptor. It has been demonstrated that IP receptors are downregulated in human pulmonary artery hypertension, whereas the EP4 receptor is stably expressed. The EP4 receptor could thus be a novel effective therapeutic target for the treatment of pulmonary artery hypertension.

I. Skin

1. Expression. Tober et al. (2007) reported that the EP4 receptor was abundant in epidermal keratinocytes, dermal leukocytes, and vascular endothelium in murine skin. UV-B exposure induced EP4 relocalization to the plasma membranes of keratinocytes, whereas its diffuse cytoplasmic staining pattern was unchanged in the rest of the epidermis. EP4 expression was also detected in sebocytes (Chen et al., 2009), hair follicles (Colombe et al., 2008), melanoma cells (Singh and Katiyar, 2011), and squamous cell carcinoma (Lee et al., 2005) in humans. Kabashima et al. (2003) reported the expression of EP4 receptor transcripts in Langerhans cells prepared from epidermis. Li et al. (2000) reported that EP4 receptor mRNA was upregulated in fetal rabbit skin wounds, yet downregulated in adult rabbit skin wounds.

2. Function. EP4 is involved in skin inflammation. It has been suggested that PGE_2 is upregulated within antigen-exposed skin (Ruzicka and Printz, 1982; Eberhard et al., 2002). Kabashima et al. (2003) demonstrated that PGE_2 promoted skin immune responses by enhancing the migration and maturation of Langerhans cells through EP4 signaling. Although the transcripts of all four PGE_2 receptor subtypes were detected in Langerhans cells, only EP4 deletion inhibited Langerhans cell accumulation in regional lymph nodes after application of fluorescein isothiocyanate to the skin. In addition, the immune responses in a dinitrofluorobenzene-induced contact hypersensitivity model were significantly attenuated in EP4 receptor KO mice and in EP4 antagonist-treated wild-type mice (Kabashima et al., 2007). Chun et al. (2007) suggested that PGE_2 exerted an antiapoptotic effect in UV-B–exposed mouse skin through EP4/PKA/Akt signaling. Thus, EP4 is suggested to promote immune response in skin, although the downstream signaling process of EP4 remains largely unknown.

J. Nervous System

1. Expression. Zhang and Rivest (1999) reported the distribution of EP4 receptor transcripts in the rat brain. The localization of the EP4 receptor was distinct from that of the EP2 receptor. EP4 receptors were mainly expressed in regions involved in the regulation of neuroendocrine and autonomic activities. Southall and Vasko (2001) demonstrated EP4 receptor expression in embryonic rat sensory neurons and adult rat dorsal root ganglia cells.

2. Function. Traditional NSAIDs exert their antinociceptive effects through the inhibition of prostaglandin. Accordingly, prostaglandin-mediated signaling has been thought to be involved in the development of inflammatory pain. Thermal and mechanical hyperalgesia, mechanical allodynia, and joint pain were suppressed by EP4 antagonists (Lin et al., 2006; Kassuya et al., 2007; Nakao et al., 2007; Clark et al., 2008; Murase et al., 2008). Southalland Vasko (2001) demonstrated that EP4 receptors mediated the PGE_2-induced sensitization of sensory neurons. PGE_2-induced accumulation of cAMP and release of immunoreactive substance P and calcitonin gene-related peptide, all of which play important roles in the development of pain and hyperalgesia, were blocked by downregulation of EP4 receptors. Because the cAMP-PKA pathway is involved in the development of hyperalgesia after injury in the dorsal root ganglion (Song et al., 2006), EP4 may activate this pathway to regulate hyperalgesia.

Fever production may involve EP4 signaling. Oka et al. (2000) demonstrated that the EP4 receptor was expressed in regions that are involved in PGE_2-induced fever responses, including the organum vasculosum of the lamina terminalis and the adjacent preoptic area. Several reports have indicated that the EP4 receptor may contribute to PGE_2-induced changes in body temperature (Oka et al., 2000, 2003).

EP4 may also play a role in neuronal degeneration and regeneration. Hoshino et al. (2007) reported that PGE_2 enhanced the production of amyloid-β through the EP4 receptors in human neuroblastoma cells. Moreover, they observed that cognitive function of mice in an Alzheimer's disease model was improved by genetic and pharmacological inhibition of EP4 (Hoshino et al., 2012). In contrast, Liang et al. (2011) reported that an EP4 receptor agonist exerted a protective effect against cerebral ischemia injury in mice. Deletion of neuronal EP4 increased the severity of cerebral injury, as did endothelial deletion of EP4. The effect of EP4 on cerebral perfusion via endothelial nitric-oxide synthase function may be involved in such beneficial roles. A neuroprotective effect of EP4 signaling has been reported in various other models, e.g., a mouse multiple sclerosis model (Esaki et al., 2010), a rat spinal cord injury model (Umemura et al., 2010), and a mouse *N*-methyl-D-aspartate–mediated acute brain damage model (Ahmad et al., 2005). Taken together, these data suggest that EP4 contributes to hyperalgesia and fever production and plays protective roles in neuronal degeneration and regeneration, although its precise downstream signaling pathways have not been reported.

K. Other Systems

Prostanoids play a critical role in the regulation of platelet function. Several reports have indicated that EP4 mediates antithrombotic signaling (Iyu et al., 2010; Kuriyama et al., 2010; Philipose et al., 2010). Philipose et al. (2010) have reported that an EP4 agonist inhibited platelet aggregation, adhesion of platelets to fibrinogen, and thrombus formation in vitro. EP4 receptor activation could thus be a novel target for antithrombotic therapy. EP4 is also expressed in the cochleae (Hori et al., 2009). Hori et al. (2009) reported that local EP4 agonist treatment improved noise-induced hearing loss in guinea pigs. Finally, Woodward et al. (2009) reported that an EP4 receptor agonist potently decreased intraocular pressure in laser-induced ocular hypertensive monkeys. EP4 could potentially be a new therapeutic target for antiglaucoma therapy.

IV. Conclusions

This article presents an overview of the functions of EP4 and its intracellular signaling pathways in physiologic and pathologic conditions. EP4 was originally identified as a G_s-coupled receptor and has been recognized to produce cAMP. Recent emerging evidence has revealed that, in addition to cAMP and its downstream signaling, EP4 also modulates a variety of signaling pathways, such as PI3K, β-arrestin, and transactivation of EGFR. The roles of these EP4-mediated pathways in physiologic and pathologic processes continue to be discovered.

Among the EP receptors, EP4 is reported to be most abundantly expressed in the heart, the ductus arteriosus, monocytes/macrophages, bone, and the colon. It maintains the physiologic functions of these organs through protein synthesis, a vasodilatory effect, regulation of immune response, anabolic effect, and mucosal barrier function, respectively. EP4 is also highly expressed in pathologic conditions, such as colorectal cancer, inflammatory bowel disease, rheumatoid arthritis, atherosclerotic plaque, and aortic aneurysm. Studies using mouse lines devoid of each of the four EP receptors further support the concept that EP4, but not the other EP receptors, plays a primary role in bone metabolism, osteoarthritis, and immune response in the skin. Therefore, the EP4 receptor appeared to be an attractive target by which to affect manifestations of various pathologic states by application of either agonists or antagonists of the receptor. In particular, EP4 agonists have drawn much attention for their promotion of osteogenesis and their suppression of colitis, and the potential usefulness of an EP4 agonist as a treatment of bone diseases or inflammatory bowel disease has been examined in clinical trials. EP4 antagonists may be suitable for use in the treatment of rheumatoid arthritis and osteoarthritis, where continuous dosing demands a drug with a superior safety profile. Traditional NSAIDs and COX inhibitors affect a number of other related prostaglandins and can cause serious side effects. The potential of an EP4 antagonist to improve prognosis in colon cancer, myocardial infarction, aortic aneurysm, neovascularization, and autoimmune encephalomyelitis is also of great interest.

Interestingly, modulating EP4 signaling could work on more than one mechanism, because EP4 is distributed in various organs and circulating immune cells. For instance, inhibition of EP4 signaling has been expected to be useful as a treatment of migraine due to its cerebral vasoconstrictive and immunosuppressive effects. The possibilities for such dual mechanisms of action of EP4 signaling in pathologic conditions of various organs should be explored. In particular, it will be important to further clarify the intracellular signaling pathways and the precise molecular mechanisms involved in EP4-mediated pathophysiologic actions. These additional studies should lead to significant opportunities for new pharmacological therapies.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Yokoyama, Iwatsubo, Umemura, Fujita, Ishikawa.

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Research Paper
Clinical Pathology

High-dose zoledronic acid narrows the periodontal space in rats

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Y. Okamoto, M. Hirota, Y. Monden, S. Murata, C. Koyama, K. Mitsudo, T. Iwai, Y. Ishikawa, I. Tohnai: High-dose zoledronic acid narrows the periodontal space in rats. Int. J. Oral Maxillofac. Surg. 2013; 42: 627–631.

Abstract. The aim of this experiment was to evaluate the histological effects of zoledronic acid on the periodontal space in rats. 40 male Wistar rats were divided into three zoledronic acid groups and a control group. Zoledronic acid was injected subcutaneously at doses of 10, 50, or 500 μg/kg once a week for 3 weeks. The rats were killed 1 or 9 weeks after the last injection. Histological examination of the periodontal space around the incisor tooth revealed that zoledronic acid did not inhibit tooth development. In the rats killed 1 week after treatment discontinuation, the periodontal space gradually narrowed in response to increasing zoledronic acid doses, and the changes were statistically significant according to ANOVA but not according to ANOVA with *post hoc* tests. The changes persisted in the high-dose zoledronic acid group despite zoledronic acid discontinuation, with significant differences identified by ANOVA and ANOVA with *post hoc* tests. Therefore, although zoledronic acid had an insignificant effect on tooth development, it had a significant effect on the periodontal space when high doses were administered. The results of this experiment may provide useful information for future investigations on the role of zoledronic acid in the osteonecrosis of the jaw.

Key words: bisphosphonate; zoledronic acid; osteonecrosis of the jaw; periodontal space; rat.

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Intravenous bisphosphonates are widely used as the first choice of treatment for bony metastasis of cancer and hypercalcaemia of malignancies.[1,2] Bisphosphonate treatment is effective in decreasing bony pain and serum calcaemia symptoms. Conversely, osteonecrosis of the jaw (ONJ) related to bisphosphonate treatment has been reported since 2003.[2–4] ONJ is defined as exposed necrotic bone in the maxillofacial region that persists for more than 8 weeks in a patient with current or previous bisphosphonate treatment and no history of radiation therapy against cancer in the jaws.[5,6] The highest incidence of ONJ has been associated with zoledronic acid (ZA).[7,8] Marx et al. suggested that bisphosphonates are directly responsible for ONJ because of their anti-angiogenic effects.[1,4,6,9,10] The main event precipitating ONJ is dental extraction.[5,7,11] Histopathological examination revealed that bisphosphonates remarkably delayed wound healing after tooth extraction by inhibiting new bone formation.[2,5,12,13] Basi et al.[5] observed aberrant wound healing of the tooth extraction socket with decreased mineralization in a rat administered ZA and suggested that the pathogenesis of ONJ is related to high matrix metalloproteinase-9 expression and osteoclast dysfunction.

Although there are numerous clinical reports on ONJ, little information is available regarding the pathogenesis of ONJ and bony changes in the jaw after bisphosphonate treatment. Hoefert et al. reported that microcracks were present within the bones in approximately 54% of ONJ patients.[14] Takahashi et al. reported that the alveolar bone around the root of a tooth showed higher density on radiographs in ONJ patients than in age-matched controls.[15] Therefore, the present authors hypothesized that there are histological changes in the periodontal space, including the teeth and alveolar bone.

The aim of this study was to observe changes in the periodontal space of ZA-administered rats.

Materials and methods

40 male Wistar rats (Nihon SLC, Shizuoka, Japan: body weight 300–350 g; 10–12 weeks old) were used in the experiment. All rats were housed in cages with free access to food and water, and a 12 h light/dark cycle was maintained. All experiments were approved and performed in accordance with the guidelines for Animal Experiments Ethic Committee of Yokohama City University.

The 40 rats were randomly divided into four groups. Groups A, B, and C received ZA at doses of 10, 50, and 500 μg/kg, respectively. The rats in the control group received injections of saline instead of ZA. The time schedule of drug administration was designed according to the literature[2,13,16] with slight modifications to ensure long-term release after ZA discontinuation. Regarding the administration dosage, the dose of ZA for adult cancer patients weighing 50–80 kg was referenced.[9,17] As these patients receive 50–80 μg/kg ZA in one administration, 50 μg/kg was selected as the middle dose for use in this experiment. 10 μg/kg (a dose fivefold smaller than the middle dose) was selected as the low dose and 500 μg/kg as the high dose.

All rats received subcutaneous injections weekly for 3 weeks. All four groups were each randomly divided into short-term and long-term groups according to the length of the observation period. The rats in the short-term groups were killed 1 week after the last injection, and the rats in the long-term groups were killed 9 weeks after the last injection (Fig. 1). To distinguish these groups, the short-term groups were designated As, Bs, Cs, and Ctls for the A, B, C, and control groups, respectively, and the corresponding long-term groups were designated Al, Bl, Cl, and Ctll, respectively.

To evaluate the effect of ZA alone, no dental procedure or pharmacological therapy was performed.

Histological analysis

After the rats were killed, their mandibles were resected. Excess soft tissues were trimmed, and the remaining mandibular bones were fixed in 4% formalin.

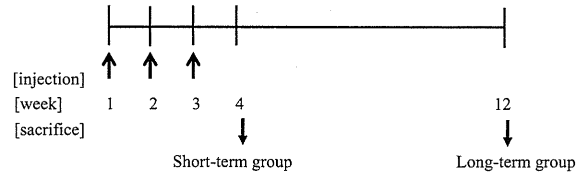

Fig. 1. Experimental design. Rats received injections of ZA or saline every Monday (↑: injection). Rats in the short-term groups were killed on the Monday of week 4, and those in the long-term groups were killed on the Monday of week 12.

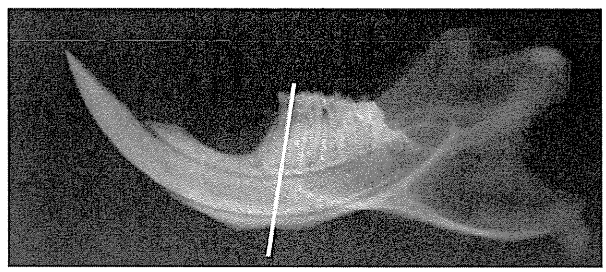

Fig. 2. To measure the periodontal space of the mandibular incisor at the same position for all specimens, cross-sections of the central portion of the first molar were used.

Following fixation, the samples were embedded in methyl methacrylate resin for histological evaluation. The embedded samples were sectioned with a microtome (30 μm thick) and stained with toluidine blue.

To measure the periodontal space of the mandibular incisor at the same position for all specimens, cross-sections of the central portion of the first molar were used (Fig. 2). On these sections, the areas of the incisor socket and the incisor were measured using MacromaX GOKO measurement software (GOKO camera Kawasaki, Japan) to calculate their cross-sectional areas (Fig. 3). To observe changes in the periodontal space, the ratio of the area of the incisor socket to that of the incisor (RSI) on the cross-section was calculated (Fig. 4).

Statistical analysis

For mean value comparisons of the incisor area and RSI between groups, ANOVA followed by Bonferroni's *post hoc* analysis for multiple comparisons was used. $P < 0.05$ indicated statistical significance.

Results

The experiment was performed without any complications, and no infection was

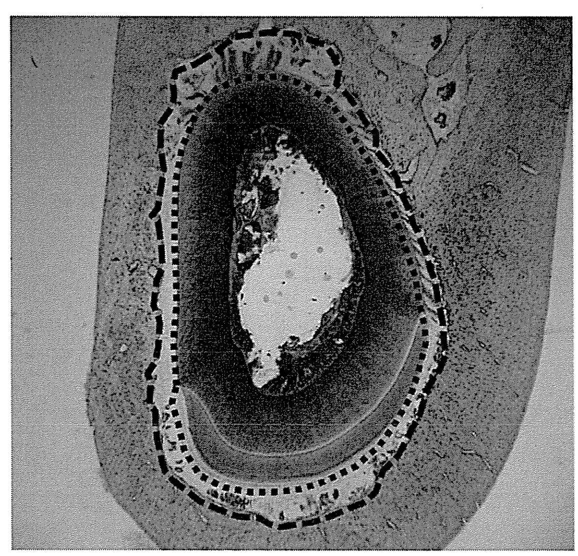

Fig. 3. The external length of the periodontal space (dotted line) and the circumference of the incisor (broken line) were measured for the statistical analysis.

$$\text{RSI} = \frac{\text{Cross-sectional area of the incisor socket [mm}^2\text{] (calculated from the external length of the periodontal space)}}{\text{Cross-sectional area of the incisor [mm}^2\text{] (calculated from the circumference of the incisor)}}$$

Fig. 4. To investigate the change in the periodontal space, the ratio of the cross-sectional area of the incisor socket to that of the incisor (RSI) was calculated.

observed in any of the rats. Histological examination revealed that neither spontaneous soft tissue necrosis nor spontaneous ONJ was observed in any of the rats.

The cross-sectional area of the incisor tended to narrow in a ZA dose-dependent manner. The narrowest and widest mean values and the standard deviations in the short-term groups were 2.340 ± 0.067 (group Cs) and 2.384 ± 0.117 mm^2 (group Ctls), respectively, whereas the corresponding values in the long-term groups were 2.389 ± 0.041 (group Cl) and 2.597 ± 0.187 mm^2 (group Ctll), respectively (Table 1). ANOVA revealed no significant differences between groups.

The mean RSI value in groups As, Bs, Cs, and Ctls was 1.480 ± 0.029, 1.444 ± 0.058, 1.429 ± 0.048, and 1.520 ± 0.053, respectively. Conversely, the values in groups Al, Bl, Cl, and Ctll were 1.456 ± 0.015, 1.481 ± 0.026, 1.413 ± 0.016, and 1.463 ± 0.006, respectively (Table 1).

In the short-term groups, the periodontal space gradually narrowed in a ZA dose-dependent manner, with statistical significance indicated by ANOVA ($F = 3.52$, $P < 0.05$) but not by ANOVA with *post hoc* tests.

In the long-term groups, ANOVA revealed a significant difference between groups with regard to the RSI, the width of the periodontal space ($F = 14.01$, $P < 0.01$). In addition, ANOVA with *post hoc* tests revealed that the RSI of the Cl group was significantly lower than that in the other groups ($P < 0.05$; Fig. 5).

Table 1. The ratio of the cross-sectional area of the socket (incisor socket) to that of the incisor (RSI). Data are presented as mean ± standard deviation. There were no significant differences among the RSI values according to ANOVA. A significant difference in RSI values was identified among the short-term groups as per ANOVA ($F = 3.52$, $P < 0.05$) but not ANOVA with *post hoc* tests. A significant difference in RSI values was identified among the long-term groups as per ANOVA ($F = 14.01$, $P < 0.01$) as well as ANOVA with *post hoc* tests ($P < 0.05$).

	Socket (mm^2)	Incisor (mm^2)	RSI (socket/incisor)
The short-term groups			
Ctls (control)	3.627 ± 0.275	2.384 ± 0.117	1.520 ± 0.053
As (ZA 10 μm/kg)	3.515 ± 0.243	2.375 ± 0.163	1.480 ± 0.029
Bs (ZA 50 μm/kg)	3.416 ± 0.346	2.363 ± 0.185	1.444 ± 0.058
Cs (ZA 500 μm/kg)	3.345 ± 0.188	2.340 ± 0.067	1.429 ± 0.048
The long-term groups			
Ctll (control)	3.799 ± 0.269	2.597 ± 0.187	1.463 ± 0.006
Al (ZA 10 μm/kg)	3.737 ± 0.136	2.567 ± 0.119	1.456 ± 0.015
Bl (ZA 50 μm/kg)	3.733 ± 0.091	2.521 ± 0.056	1.481 ± 0.026
Cl (ZA 500 μm/kg)	3.375 ± 0.067	2.389 ± 0.041	1.413 ± 0.016

Each group, $n = 5$.

Discussion

Local inflammation and connective soft tissue reactions to infection are observed in most cases of ONJ.[14,18] Stephen et al. reported that the administration of ZA with dexamethasone prior to dental extractions in rats resulted in the development of histopathological changes that were similar to ONJ in humans.[13] These findings indicate that ONJ occurs in the presence of any factor associated with bacterial infection of an intraoral wound. In the present study, the authors examined the effects of ZA, but a dental procedure that could create an intraoral wound was not performed. Therefore, spontaneous ONJ could not be observed.

In this study, narrowing of the periodontal space was observed in the high-dose ZA group; however, tooth development was not affected by systemic ZA administration. Studies reported that a topical coating of alendronate on the root surface decreased the incidence of root resorption and ankylosis in cases of tooth replantation.[19–21] In the present study, there was no root resorption and no evidence of ankylosis between the alveolar bone and

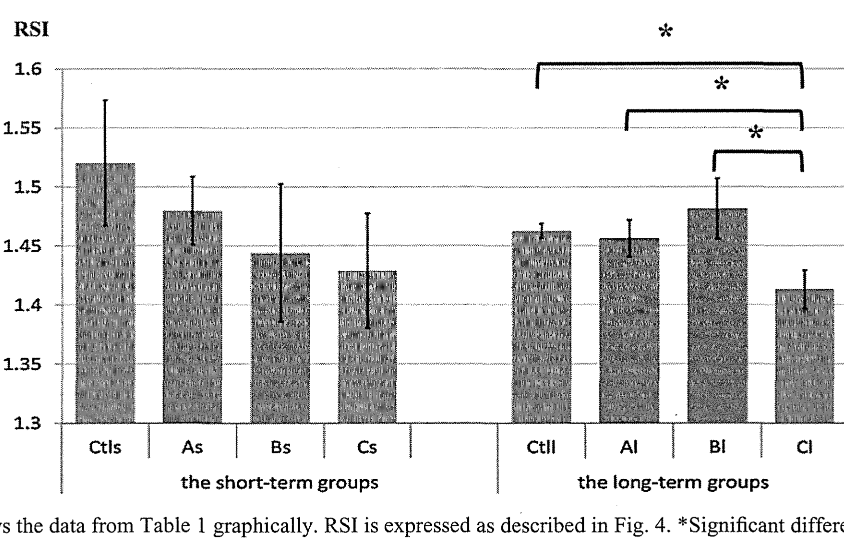

Fig. 5. The figure shows the data from Table 1 graphically. RSI is expressed as described in Fig. 4. *Significant difference as per ANOVA with *post hoc* tests ($P < 0.05$).

root, suggesting that systemic administration of ZA has an insignificant effect on tooth morphology.

In the present study, RSI decreased in response to increasing ZA doses in the short-term groups. ANOVA revealed a statistically significant difference associated with the ZA dose in the short-term group; however, ANOVA with *post hoc* tests did not confirm this significance. These results suggested that although ZA affected the width of the periodontal space, there were no significant differences between groups in this regard. The small number of samples used in the present study may explain the lack of significant differences among the short-term groups. Conversely, in the long-term groups, a significant difference was confirmed between the high-dose group Cl (500 μg/kg) and the other groups by ANOVA with *post hoc* tests, even though ZA treatment was discontinued 9 weeks previously. According to the results of both groups, the influence of ZA on the width of the periodontal space began during the early period of administration and persisted after treatment discontinuation, consequently explaining the significant difference between the high-dose group and the other groups at 9 weeks after treatment discontinuation. Then, RSI in the Bl group was higher than that in the Bs group; this finding did not hold true for the other pairs of groups. As the mean RSI values of the Bs and Bl groups had wide standard deviations, it was considered that the larger RSI of the Bl group was not a meaningful result. Considering that bisphosphonates are not metabolized and are stored within the bone for long periods of time,[2] ZA could continuously affect the width of the periodontal space in the highest dosage group. The periodontal space is tightly regulated throughout life, but certain factors such as mechanical stress and drugs may influence the width of the periodontal space. Lekic et al.[22] demonstrated that bisphosphonates decreased the width of the periodontal space by modulating the differentiation of periodontal ligament cells. Although the results in the present study were only based on histological findings, they suggested that periodontal space narrowing was induced by ZA administration, and that the narrowing was ZA dose-dependent.

The most important effect of bisphosphonates is the inhibition of bone resorption due to diminished osteoclast activity. The decrease in osteoclast activity alters the osteoclast–osteoblast interaction, according to previous reports.[2,5,8] Systematic bisphosphonate therapy would therefore be beneficial for controlling alveolar bone mass in periodontal disease.[17] Another study reported that orthodontic tooth movement is inhibited by bisphosphonates.[23]

Considering that orthodontic movement of teeth through alveolar bone requires osteoclast activity, tooth movement in the bisphosphonate-treated group was significantly less than that in the control group.[23] The findings of diminished bone resorption and remodelling at the tooth socket resulted from the altered osteoclast–osteoblast interaction. The alternation and decrease in osteoclast activity was observed to influence the differentiation of periodontal ligament cells in other studies,[20,22] suggesting that the periodontal space was narrowed by the modulated differentiation of periodontal ligament cells.[22]

Recently, ONJ associated with drugs other than bisphosphonates has been reported. One of these agents is denosumab, which is approved for use in postmenopausal women with osteoporosis and men taking androgen deprivation therapy for prostate cancer.[24] Denosumab is a humanized monoclonal antibody and antiresorptive agent that works by decreasing the activity of the nuclear factor kappa B receptor. Stopeck et al. reported that denosumab was potentially a useful medication for osteoporosis and prostate cancer; however, it induced ONJ in a similar proportion of patients as ZA.[25] Therefore, histopathological hard tissue changes caused by denosumab should be compared with those caused by ZA in further investigations to better understand the pathogenesis of ONJ.

In conclusion, ZA has an insignificant effect on tooth development and a significant effect on the width of the periodontal space, as suggested by the narrowing of the periodontal space after a short period of ZA treatment and the persistence of this effect after high-dose administration, despite ZA discontinuation. The results of this experiment may provide useful information for future investigations on the role of ZA in ONJ.

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Competing interests

None declared.

Ethical approval

Animal experiments were approved by the ethics committee at Yokohama City University (No. 10-027).

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Type 5 Adenylyl Cyclase Increases Oxidative Stress by Transcriptional Regulation of MnSOD via the SIRT1/FoxO3a Pathway

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Abstract

Background—For reasons that remain unclear, whether type 5 AC (AC5), one of two major AC isoforms in heart, is protective or deleterious in response to cardiac stress is controversial. To reconcile this controversy we examined the cardiomyopathy induced by chronic isoproterenol (ISO) in AC5 transgenic (Tg) mice and the signaling mechanisms involved.

Methods and Results—Chronic ISO increased oxidative stress and induced more severe cardiomyopathy in AC5 Tg, as left ventricular (LV) ejection fraction fell 1.9 fold more than wild type (WT), along with greater LV dilation and increased fibrosis, apoptosis and hypertrophy. Oxidative stress induced by chronic ISO, detected by 8-OhDG was 15% greater, p=0.007, in AC5 Tg hearts, while protein expression of MnSOD was reduced by 38%, indicating that the susceptibility of AC5 Tg to cardiomyopathy may be due to decreased MnSOD expression. Consistent with this, susceptibility of the AC5 Tg to cardiomyopathy was suppressed by overexpression of MnSOD, whereas protection afforded by the AC5 KO was lost in AC5 KO×MnSOD$^{+/-}$ mice. Elevation of MnSOD was eliminated by both sirtuin and MEK inhibitors, suggesting both the SIRT1/FoxO3a and MEK/ERK pathway are involved in MnSOD regulation by AC5.

Conclusion—Overexpression of AC5 exacerbates the cardiomyopathy induced by chronic catecholamine stress by altering regulation of SIRT1/FoxO3a, MEK/ERK and MnSOD, resulting in oxidative stress intolerance, thereby shedding light on new approaches for treatment of heart failure.

Keywords

Adenylyl cyclase; Adrenergic; Cardiomyopathy; Oxidative Stress

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Disclosures

Dr. David A. Sinclair is a consultant to Sirtris, a GSK company working to develop sirtuin-targeted medicines.

Introduction

Adenylyl cyclase (AC) is a key regulator of health and longevity in organisms ranging from yeast to mammals.[1–5] In the heart AC is a critical link in sympathetic control and beta adrenergic receptor (beta-AR) signaling and therefore plays a fundamental role in mediating not only baseline cardiac function, but also the cardiac response to stress, e.g., in the pathogenesis of heart failure. Type 5 AC (AC5) is one of two major isoforms in heart, the other being type 6 AC (AC6). For reasons that remain unclear, whether AC5 is protective or deleterious in response to cardiac stress is controversial, particularly with respect to the signaling mechanisms involved, and whether these mechanisms are shared by AC6. It is generally accepted that cardiac-specific AC5 overexpressed (AC5 Tg) mice exhibit enhanced cardiac performance,[6] which follows from the role of AC, which generates cyclic AMP upon beta-AR stimulation resulting in increased cardiac contractility and heart rate. However, the extent to which altered AC5 regulation is protective with chronic stress remains controversial. Prior studies examined whether overexpression or disruption of AC5 in the heart could affect the progression of cardiomyopathy induced by overexpression of Galphaq and beta1-AR. This was accomplished by mating overexpressed Galphaq and beta1-AR with AC5 Tg or AC5 knockout (KO) mice. These studies found that AC5 Tg rescued Galphaq cardiomyopathy,[6] but not beta1-AR cardiomyopathy,[7] and AC5 KO mice failed to rescue cardiomyopathy in Galphaq mice.[8] In addition, AC5 KO mice rescued cardiomyopathies from chronic pressure overload,[9] chronic catecholamine stress,[10] and aging.[1]

Since beta-AR signaling, of which AC is central, plays a key role in the pathogenesis of heart failure and since beta-AR blockade therapy is widely used in patients with heart failure, but that therapy is still far from perfect, it becomes critical to reconcile the controversy and understand the role of AC in the heart in the development of cardiomyopathy and heart failure, which would eventually be of clinical importance. Accordingly, this was the overall goal of the current investigation. We first examined the extent to which manganese superoxide dismutase (MnSOD) regulation and oxidative stress were altered in AC5 Tg at baseline and in response to chronic beta-AR stimulation, since it is known that beta-AR stimulation increases oxidative stress,[11, 12] and that MnSOD is upregulated in AC5 KO mice.[1] The results of the experiments with bigenic mice (AC5 Tg × MnSOD Tg and AC5 KO × MnSOD$^{+/-}$) led us to elucidate the signaling mechanisms linking AC5, MnSOD and oxidative stress, and the involvement of the SIRT1/FoxO3a pathway. The SIRT1/FoxO3a pathway was selected to investigate, because MnSOD is upregulated in the AC5 KO mouse, which lives longer than wild type (WT)[1] and FoxO3a is the transcriptional factor most closely related to the anti-oxidative protective effects associated with longevity, as shown in several models: *C.elegans*,[13, 14] rats[15] and human quiescent cells.[16] The final goal was to investigate whether this pathway is regulated specifically by AC5, or whether it is common to all AC signaling in the heart, which would mean that these mechanisms were shared by the other major cardiac AC isoform, AC6.

Methods

Mouse Models

Generation of AC5 Tg mice was described previously.[17] AC5 KO × MnSOD$^{+/-}$ mice were generated by crossing AC5 KO mice with MnSOD heterozygous mice. AC5 Tg × MnSOD Tg were generated by crossing AC5 Tg mice with MnSOD Tg mice (From Jackson Laboratory, Stock ID: 009438). To produce catecholamine cardiomyopathy, ISO was delivered to 3–5 month old Tg mice, bigenic mice and corresponding control littermates for 7 days at a dose of 60 mg/kg/day with a miniosmotic pump (ALZET model 2001, DURECT Corp, Cupertino, California) as described.[10] The severity of the cardiomyopathy was