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Prostaglandin E₂ Increases Both Osteoblastic and Osteoclastic Activity in the Scales and Participates in Calcium Metabolism in Goldfish

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Using our original *in vitro* assay system with goldfish scales, we examined the direct effect of prostaglandin E₂ (PGE₂) on osteoclasts and osteoblasts in teleosts. In this assay system, we measured the activity of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) as respective indicators of each activity in osteoblasts and osteoclasts. ALP activity in scales significantly increased following treatment at high concentration of PGE₂ (10⁻⁷ and 10⁻⁶ M) over 6 hrs of incubation. At 18 hrs of incubation, ALP activity also significantly increased in the PGE₂ (10⁻⁹ to 10⁻⁶ M)-treated scale. In the case of osteoclasts, TRAP activity tended to increase at 6 hrs of incubation, and then significantly increased at 18 hrs of incubation by PGE₂ (10⁻⁷ to 10⁻⁶ M) treatment. At 18 hrs of incubation, the mRNA expression of osteoclastic markers (TRAP and cathepsin K) and receptor activator of the NF-κB ligand (RANKL), an activating factor of osteoclasts expressed in osteoblasts, increased in PGE₂ treated-scales. Thus, PGE₂ acts on osteoblasts, and then increases the osteoclastic activity in the scales of goldfish as it does in the bone of mammals. In an *in vivo* experiment, plasma calcium levels and scale TRAP and ALP activities in the PGE₂-injected goldfish increased significantly. We conclude that, in teleosts, PGE₂ activates both osteoblasts and osteoclasts and participates in calcium metabolism.

Key words: prostaglandin E₂, osteoblasts, osteoclasts, goldfish scales, plasma Ca, RANKL

INTRODUCTION

Prostaglandin E₂ (PGE₂) is an important local regulator in bone and promoter of osteoclastogenesis (Kaji et al., 1996;

Gardner, 2007; Kaneko et al., 2007). The bone-resorbing activity of mature osteoclasts in osteoblast-containing mouse bone cell cultures is increased by PGE₂ although it does not affect osteoclast-like cell formation in osteoblast-free mouse spleen cell cultures (Kaji et al., 1996). The function of PGE₂ must therefore be examined using a co-culture system of osteoclasts and osteoblasts.

In fish, the osteoblastic function of PEG₂ has been examined using salmon (Ytteborg et al., 2010). However,

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there has been no report on the osteoclastic function of PGE₂ due to a lack of a suitable *in vitro* assay system. Scales are calcified tissue that contains osteoclasts and osteoblasts (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Suzuki et al., 2007). Scales have been reported to be a better potential internal calcium reservoir than body skeletons, jaws, and otoliths, as examined in a ⁴⁵Ca-labeling study for the calcified tissues of goldfish and killifish (Mugiya and Watabe, 1977). In the scale, as in mammalian bone, type I collagen (Zylberberg et al., 1992), bone γ -carboxyglutamic acid protein (Nishimoto et al., 1992), osteonectin (Lehane et al., 1999; Redruello et al., 2005), and hydroxyapatite (Onozato and Watabe, 1979) are present. Moreover, we have previously demonstrated that the osteogenesis of regenerating scale is very similar to that of mammalian membrane bone, and serves as a good model of osteogenesis (Yoshikubo et al., 2005). Thus, the teleost scale has a number of features in common with mammalian bone.

Using goldfish scales, we recently developed a novel *in vitro* assay system (Suzuki et al., 2000; Suzuki and Hattori, 2002), as the scale is a very active tissue of calcium regulation in fish, as described above. Using this system in the present study, we examined the effect of PGE₂ on scale osteoblasts and osteoclasts. In addition, the mRNA expression of osteoclastic markers (tartrate-resistant acid phosphatase (TRAP) and cathepsin K) and the receptor activator of the NF- κ B ligand (RANKL), an activating factor of osteoclasts expressed in osteoblasts, was investigated *in vitro*. To confirm the results of the *in vitro* experiment, furthermore, an *in vivo* experiment was performed.

To our knowledge, our study is the first to indicate the effect of PGE₂ on osteoclasts in teleosts.

MATERIALS AND METHODS

Animals

In a previous study (Suzuki et al., 2000), we indicated that the sensitivity to a calcemic hormone, such as estrogen and calcitonin, was higher in mature female than in mature male goldfish (*Carassius auratus*). Mature female goldfish were purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) and used in the scale *in vitro* assay. To examine the effect of PGE₂ on the calcium metabolism, immature goldfish (4–6 g), in which the endogenous effects of sex steroids are negligible, were used for the *in vivo* study. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Effect of PGE₂ on ALP and TRAP activities in the cultured scales of goldfish

Scales collected from goldfish after anesthesia with ethyl 3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich, Inc., MO, USA) were cut into two approximately equal pieces. One piece was treated with PGE₂ (Cayman Chemical, MI, USA, 10⁻¹¹ to 10⁻⁶ M), and the other was used as a control. Using these scales, we examined the effects of PGE₂ on the osteoblasts and osteoclasts with alkaline phosphatase (ALP) and TRAP as markers because, in mammals, the effects of hormones and some bioactive substances on osteoclasts and osteoblasts have been investigated using ALP and TRAP as respective markers (Vaes, 1988; Dimai et al., 1998; Suda et al., 1999). Sixteen scales (ALP activity: eight scales; TRAP activity: eight scales) were used for the evaluation of each concentration of PGE₂. The pieces of scales were incubated for 6 and

18 hrs in Eagle's modified minimum essential medium (MEM; ICN Biomedicals Inc., OH, USA). HEPES (20 mM) was added to MEM. The pH was adjusted to 7, and the incubation temperature was 15°C.

The measurement methods of ALP and TRAP activity reported by Suzuki and Hattori (2002) were modified as follows. The incubated scale was transferred to its own well in a 96-well microplate after washing with saline. An aliquot of 100 μ l of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM MgCl₂; 0.1 mM ZnCl₂) was added to each well. This microplate was frozen at -85°C immediately and then kept at -20°C until analysis. After thawing, an aliquot of 100 μ l of 20 mM para-nitrophenyl-phosphate in an alkaline buffer was added to each well. This plate was then incubated at 20°C for 30 min while being shaken. After incubation, the reaction was stopped by adding 50 μ l of a 3 N NaOH-20 mM EDTA solution. Aliquots of 150 μ l of a colored solution were transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced para-nitrophenol (pNP) using a standard curve for pNP. After measurement of the absorbance, the ALP activity was normalized by the dry weight (mg) of each goldfish scale. The results are shown as the means \pm SE of eight scales.

TRAP activity was measured using an acid-tartrate buffer (20 mM tartrate in a 0.1 M sodium acetate buffer (pH 5.3)). Other conditions were the same as those for the measurement of ALP activity.

Changes in TRAP, cathepsin K and RANKL mRNA expressions in PGE₂-treated goldfish scales

Scales were collected from goldfish under anesthesia with ethyl 3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich) and cut into halves. One half of a piece was then put into a microtube in MEM (1 ml) supplemented with PGE₂ (10⁻⁶ M). The other half was also put into another microtube in a PGE₂-free medium as a control. To examine changes in TRAP, cathepsin K and RANKL mRNA expressions in response to PGE₂, the scales were incubated for 6 and 18 hrs in MEM (containing an antibiotic and 20 mM HEPES) at 15°C. The mRNA expression in the control and experimental scales in the same individual was compared. After incubation, the scales were frozen at -85°C for mRNA analysis.

Total RNAs were prepared from goldfish scales using a total RNA isolation kit for fibrous tissue (Qiagen GmbH, Hilden, Germany). Complementary DNA synthesis was performed using a kit (Qiagen GmbH). Gene-specific primers for TRAP (sense: 5'-AACTTCCGCATTCCTCGAACAG-3'; antisense: 5'-GGCCAGC-CACCAGGAGATAA-3') (Azuma et al., 2007), cathepsin K (sense: 5'-GCTATGGAGCCACACAAAAGG-3'; antisense: 5'-CTGCGCT-TCCAGCTCTCAT-3') (Azuma et al., 2007), and RANKL (sense: 5'-GCGCTTACCTGCGGAATCATATC-3'; antisense: 5'-AAGTG-CAACAGAATCGCCACAC-3') (Suzuki et al., 2011a) were used. The amplification of β -actin cDNA using a primer set (5': CGAGCGT-GGCTACAGCTTCA; 3': GCCCGTCAGGGAGCTCATAG) (Azuma et al., 2007) was performed. PCR amplification was analyzed by real-time PCR apparatus (Mx3000p, Agilent Technologies, CA, USA) (Suzuki et al. 2011a). The annealing temperature of TRAP, cathepsin K, RANKL, and β -actin was 60°C. The TRAP, cathepsin K and RANKL mRNA levels were normalized to the β -actin mRNA level.

Changes in the plasma calcium and scale TRAP and ALP activities in PGE₂-injected goldfish (*in vivo* experiment)

In the experimental group, goldfish (body weight: 4–6 g) were anesthetized in the same manner as above, and PGE₂ (500 ng/g body weight) was then injected intraperitoneally. The goldfish in the control group were injected with saline (0.9% NaCl) in the same manner as experimental goldfish. These goldfish were kept in the aquarium for one and two days, respectively (each *n* = 8). During the experimental periods, these goldfish were not given any food to exclude intestinal calcium uptake from diets. Each day after injec-

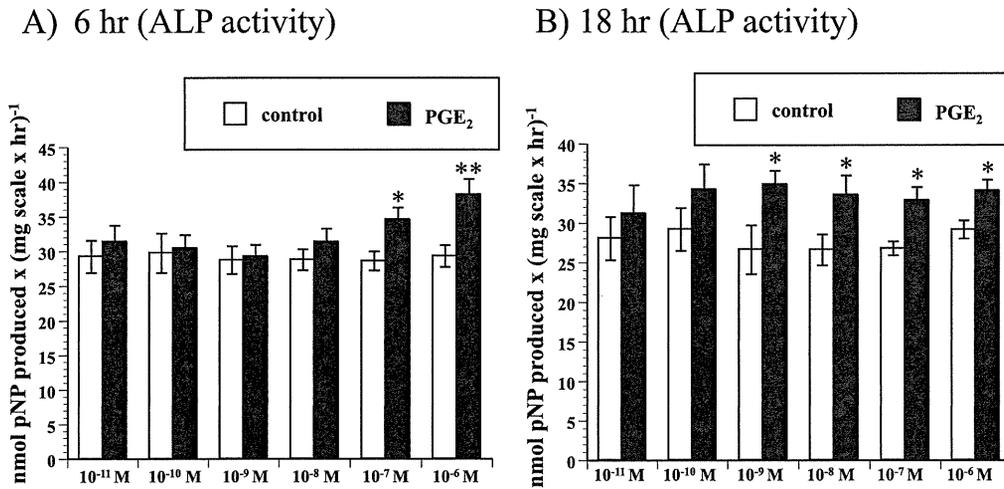


Fig. 1. Effect of PGE₂ (10⁻¹¹ to 10⁻⁶ M) on ALP activity in cultured scales incubated for 6 (A) and 18 (B) hrs. * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values in the control scales ($n = 8$).

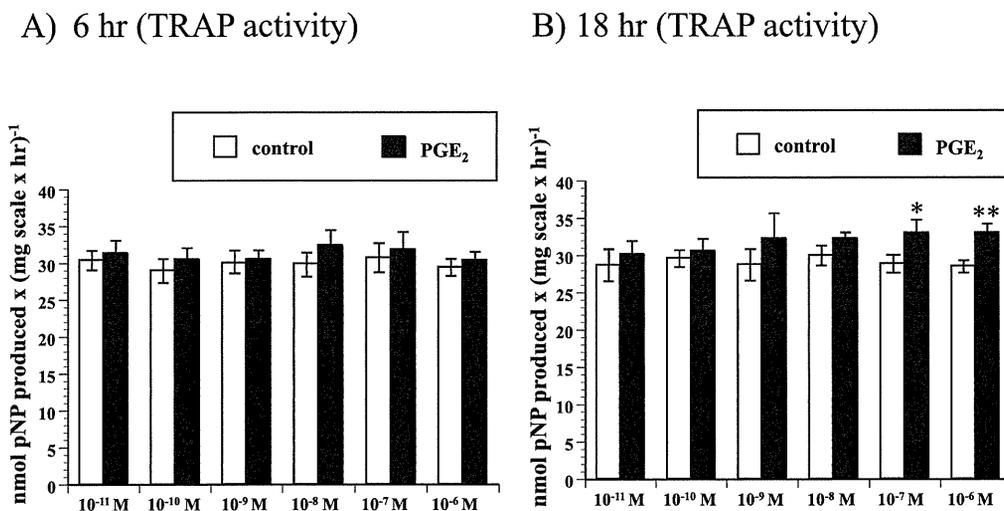


Fig. 2. Effect of PGE₂ (10⁻¹¹ to 10⁻⁶ M) on TRAP activity in cultured scales incubated for 6 (A) and 18 (B) hrs. * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values in the control scales ($n = 8$).

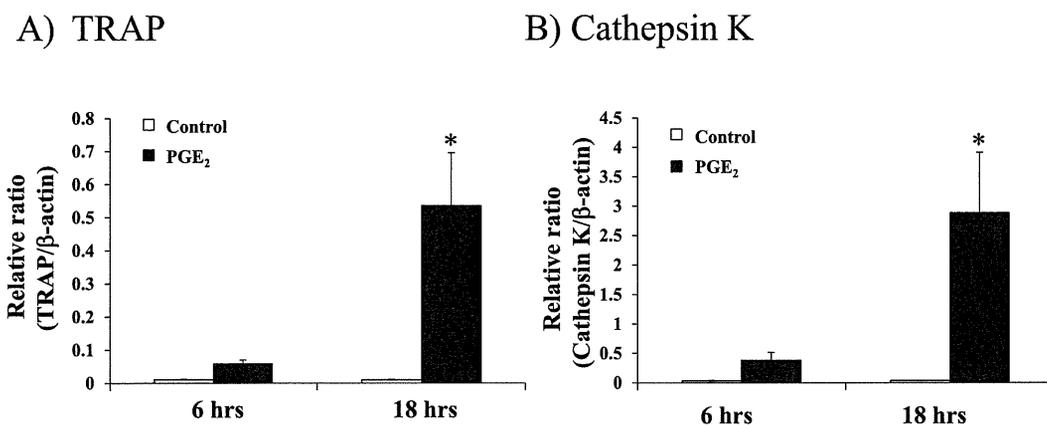


Fig. 3. Expression analysis of osteoclastic markers: TRAP (A) and cathepsin K (B) mRNAs in the PGE₂ (10⁻⁶ M)-treated scale. The TRAP and cathepsin K mRNA levels were normalized to the β-actin mRNA level. * indicates a statistically significant difference at $P < 0.05$ from the values in the control scales ($n = 8$).

tion, blood samples were collected from the gill using a heparinized capillary from individual, anesthetized goldfish. After centrifugation at 15,000 rpm for 3 min, the plasma was immediately frozen and kept at -80°C until use. The plasma total calcium level (mg/100 ml) was determined using an assay kit (Calcium C, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Then, we measured the ALP and TRAP activities in the scale. As the scales were very small, the respective marker enzyme activity was normalized by the surface area (mm^2) of each goldfish scale (Suzuki et al., 2009).

Statistical analysis

All results are expressed as the means \pm SE ($n = 8$). The statistical significance between control and experimental group was assessed by Student's *t*-test or paired *t*-test. In all cases, the selected significance level was $P < 0.05$.

RESULTS

Effect of PGE₂ on ALP activity in the cultured scales of goldfish

PGE₂ significantly increased the ALP activities of the scales by 6 hrs of incubation ($P < 0.05$ for 10^{-7} M; $P < 0.01$ for 10^{-6} M) (Fig. 1A). At 18 hrs of incubation, the ALP activities in the PGE₂-treated scales also significantly increased

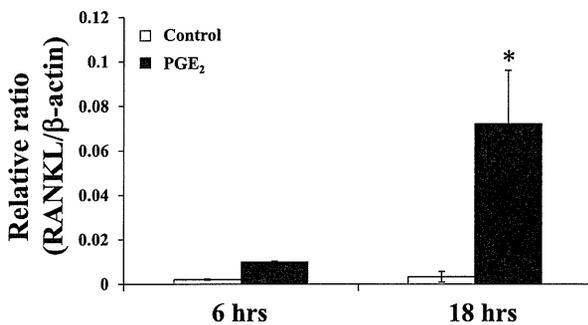


Fig. 4. Expression analysis of RANKL mRNA in the PGE₂ (10^{-6} M)-treated scale. The RANKL mRNA levels were normalized to the β -actin mRNA level. * indicates a statistically significant difference at $P < 0.05$ from the values in the control scales ($n = 8$).

by PGE₂ treatment ($P < 0.05$ for 10^{-9} to 10^{-6} M) (Fig. 1B).

Effect of PGE₂ on TRAP activity in the cultured scales of goldfish

The TRAP activity of the PGE₂-treated scales by 6 hrs of incubation tended to be higher than that in the control scales, although there was no significant difference between the PGE₂-treated and control scales (Fig. 2A).

However, the TRAP activity in the PGE₂-treated scales was significantly higher by 18 hrs ($P < 0.05$ for 10^{-7} M; $P < 0.01$ for 10^{-6} M) of incubation relative to the values of the control scales (Fig. 2B).

Changes in TRAP, cathepsin K and RANKL mRNA expressions in PGE₂-treated goldfish scales

The results of TRAP and cathepsin K are shown in Fig. 3. The mRNA expression of osteoclastic markers (TRAP and cathepsin K) in the PGE₂-treated scales tended to

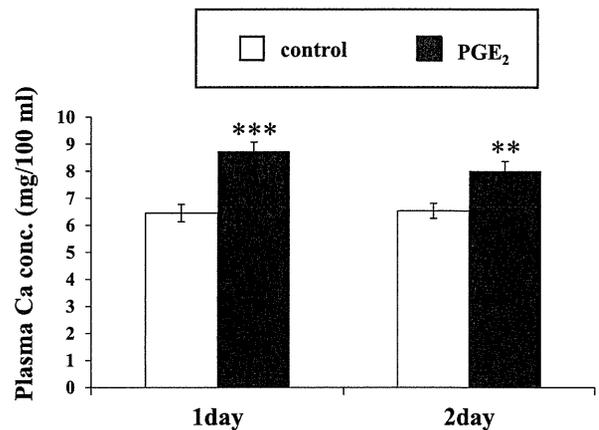


Fig. 5. Effects of PGE₂ on plasma calcium (mg/100 ml) in an in vivo experiment using immature goldfish. ** and *** indicate statistically significant differences at $P < 0.01$ and $P < 0.001$, respectively, from the values in the control ($n = 8$).

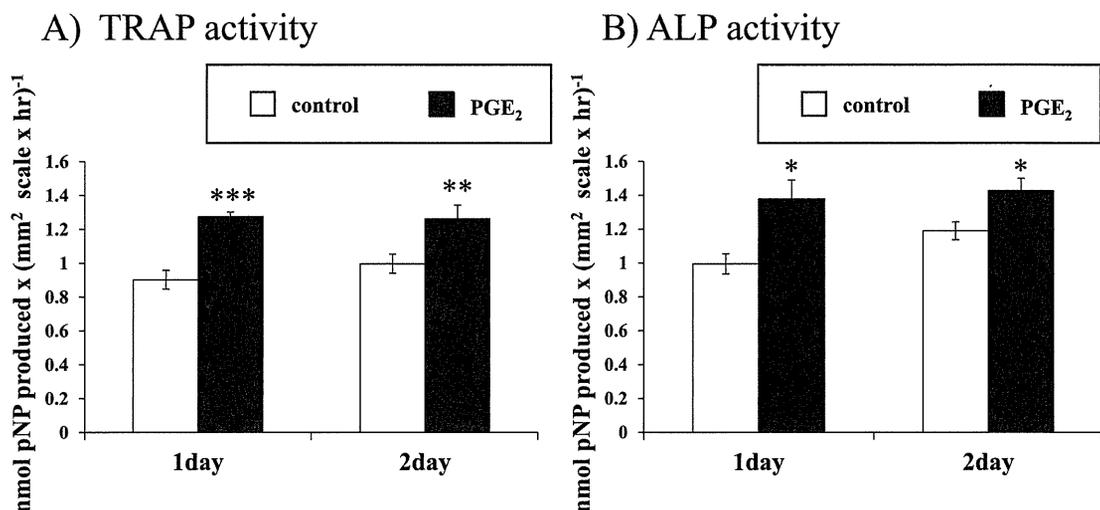


Fig. 6. Effects of PGE₂ on scale TRAP (A) and ALP (B) activity in an in vivo experiment using immature goldfish. *, **, and *** indicate statistically significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, from the values in the control ($n = 8$).

increase at 6 hrs of incubation compared with those in control scales. At 18 hrs incubation, mRNA expression of both osteoclastic markers increased significantly following PGE₂ treatment.

Similar results were obtained in RANKL. The mRNA expression of RANKL, an activating factor of osteoclasts expressed in osteoblasts, increased significantly by PGE₂ treatment at 18 hrs of incubation although RANKL mRNA in PGE₂-treated scales tended to be higher than that in the control scales at 6 hrs of incubation (Fig. 4).

Changes in the plasma calcium and scale TRAP and ALP activities in PGE₂-injected goldfish (in vivo experiment)

The plasma calcium levels increased significantly at both one and two days after PGE₂ injection (Fig. 5). In addition, the scale TRAP and ALP activities in PGE₂-injected goldfish were significantly promoted on both days promoted (Fig. 6).

DISCUSSION

The present study is the first to demonstrate that PGE₂ promoted both osteoblastic and osteoclastic activity in the scales of goldfish in an in vitro experiment. Furthermore, we found that mRNA expression of osteoclastic markers, such as TRAP and cathepsin K, was significantly promoted in PGE₂-treated scales. An in vivo experiment reconfirmed the findings from the in vitro experiments. In teleosts, the functions of calcemic hormones in both osteoblasts and osteoclasts have not been elucidated because of the lack of a suitable in vitro assay system. Using our system, we first demonstrated that calcitonin, a hypocalcemic hormone, suppressed osteoclastic activity in teleosts as well as in mammals (Suzuki et al., 2000) and that melatonin, a major hormone secreted from the pineal gland, suppresses the functions in both osteoclasts and osteoblasts (Suzuki and Hattori, 2002). Osteoblasts in the scale responded to estrogen as they do in mammals (Yoshikubo et al., 2005). The effects of endocrine disrupters, such as bisphenol-A (Suzuki and Hattori, 2003) and tributyltin (Suzuki et al., 2006), and heavy metals, i.e., cadmium (Suzuki et al., 2004a), organic mercury (Suzuki et al., 2004a), and inorganic mercury (Suzuki et al., 2011b), on osteoblasts and osteoclasts have also been sensitively evaluated. Considering these results with the present data, our scale assay system may be useful for the analysis of various substances, including hormones, in bone metabolism.

RANKL produced by cells in the osteoblast lineage binds to the receptor activator of NF- κ B (RANK) in mononuclear hemopoietic precursors and promotes the formation and activity of multinucleated osteoclasts (Teitelbaum, 2000). Our present study indicated that RANKL mRNA expression was promoted by PGE₂ treatment. In mammals, it has been reported that PGE₂ promotes osteoclastogenesis via the RANK-RANKL system (Gardner, 2007; Kaneko et al., 2007; Blackwell et al., 2009). We therefore strongly believe that, in teleosts as well as mammals, osteoclastogenesis is induced by the RANK-RANKL pathway.

In our in vivo experiment, the plasma calcium levels increased significantly at both one and two days after PGE₂ injection. In the reproductive period, the plasma calcium level in female teleosts increases remarkably (Watts et al.,

1975; Yamauchi et al., 1978; Norberg et al., 1989; Suzuki et al., 2004b; Guerreiro et al. 2007). This calcium is bound to vitellogenin, which is a major component of egg protein and the calcium-binding protein (Tinsley, 1985; Kwon et al., 1993). We previously demonstrated, in an in vivo experiment with goldfish, that fugu PTH1-induced hypercalcemia results from an increase of both osteoblastic and osteoclastic activity in the scale as well as the decrease of scale calcium contents after fugu PTH1 injection (Suzuki et al., 2011a). In estrogen-injected goldfish, the scale TRAP and ALP activities (Suzuki and Hattori, 2003) and plasma calcium level (Suzuki et al., 2003) increased. In estrogen-injected goldfish, we demonstrated that estrogen promoted calcitonin secretion and that estrogen directly acts on the ultimobranchial gland (UBG), a CT-secreting organ since the estrogen receptor was detected in UBG (Suzuki et al., 2004b). Our preliminary data indicate that, during reproductive periods, the plasma calcium level in female goldfish increases corresponding to the plasma level of PGE₂. In addition, the plasma TRAP level also increased at this period (N Suzuki Personal Communication). There may therefore be an interaction among calcemic hormones, such as PGE₂, PTH, estrogen, and calcitonin, in the scales of goldfish. As a result of the hormonal interaction, scale resorption may occur. We are currently developing an original array system for goldfish on the basis of EST analysis for goldfish scales. In the future, we will examine the interaction among calcemic hormones and describe in detail mechanism of teleost bone metabolism.

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魚類のウロコを用いた評価系の開発と骨代謝研究への応用

Development of an Assay System Using Fish Scales and Its Application to the Study of Bone Metabolism

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Magnetic fields are known to enhance bone formation. However, basic data concerning the mechanism are limited because there are no satisfactory model systems of human bone, which consists of osteoblasts, osteoclasts, and a bone matrix. The bone matrix, consisting of substances including hydroxyapatite and type I collagen, has an important function in the response to physical stress. Few techniques to co-culture osteoclasts and osteoblasts that include the bone matrix have been developed. Fish scales are calcified tissue that contains osteoclasts, osteoblasts, and a bone matrix, similar to human bone. We recently developed an *in vitro* assay system using fish scales and then examined the effects of physical stimuli, such as hypergravity, microgravity, and magnetic fields, on bone metabolism. We describe here our recent results regarding bone metabolism.

Key words: extremely low-frequency magnetic fields, fish scales, hyper-gravity, micro-gravity, bone metabolism

1. はじめに

ヒトの骨には骨を作る細胞である骨芽細胞と骨を壊す細胞である破骨細胞があり、それらが骨形成を調節している。骨芽細胞は細胞株があるため培養は容易であるが、破骨細胞は多核の活性型に誘導する必要があるため、培養は難しい。さらに磁場や重力等の物理的な刺激には、骨基質が重要な役割を果たしていることも報告されている^{1),2)}。したがって、人体の骨組織を再現させるためには、これらすべて共存させて培養する必要がある。しかしながら、現時点ではこのような共存培養は困難な状況である。また骨粗鬆症などの骨疾患に対する治療薬を開発するためには、卵巣を除去し、骨が折れやすくなったラットを用いて、時間と多額の費用を投資して開発している。そこで磁場のよう

な物理的な刺激により、骨疾患の治療を行うことができれば、薬を使用する必要がなく、さらに副作用もなく、高齢者には適した治療になる。しかしながら、磁場の刺激に対する骨形成機構の研究は、モデルシステムの欠如によって遅れ、そのメカニズムに関する基礎的なデータも少なく、骨形成に最適な磁場の条件は決定されていない。

魚類のウロコは、直接化骨をする点で哺乳類の頭蓋骨などの膜性骨に似ており、I型コラーゲンからなる線維層と、I型コラーゲンとハイドロキシアパタイトからなる石灰化層の二つを主体とする(Fig. 1)。そして石灰化層の上に、骨芽細胞と破骨細胞が共存し(Fig. 2)、添加的石灰化による骨形成や破骨細胞による骨吸収を行っている。

進化のうえで、初めて骨の原型を備えた動物は、古生代の海に生息した甲冑魚(甲皮類)である。甲冑魚の体制は極めて原始的で、顎もなく、歯もなく、体内の骨もまだ軟骨で形成されていたが、外表を覆う装甲(甲皮)は、構造的には象牙質と骨の性格を併せ持つ硬組織で、その主成分はハイドロキシアパタイトであった。甲皮類はやがて棘魚類を経て硬骨魚(=現生の魚類)へと進化するが、この過程で甲皮も形を変え、頭部では膜性骨として残り、体表部では運動性向上と軽量化のためにウロコとなったと考えられている。また、顎では歯が、体内部では硬骨(軟骨性化骨)もできるようになった。このように、ウロコは甲皮の直系の子孫器官である点で、進化的に興味深い硬組織である。

このような骨のモデルとなりうるウロコを用いて評価システムを開発した^{3),4)}。本稿では、①ウロコを用いた評価システムの特徴を記載し、②過重力、③擬似微小重力、④微小重力(宇宙)、さらに⑤磁場に対する応答解析について述べる。

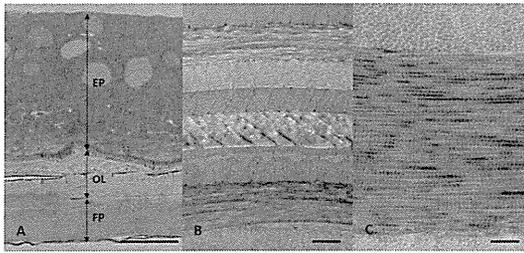


Fig. 1 Morphological characteristics of goldfish scales. Light-microscopic image of goldfish scales by toluidine blue stain (A). The main parts of the scale are the osseous layer (OL) and the fibrillary plate (FP), and they are partially covered by the epithelium (EP). The electron microscopical image shows a sheetlike structure composed of multiple layers of lamellae within FP (B); each of the layers is filled with parallel collagen fibers, and their orientation differs from that of the adjacent lamellae (C). The bars shown in Figs. 1A, 1B, and 1C are 50 μm , 2 μm , and 500 nm, respectively.

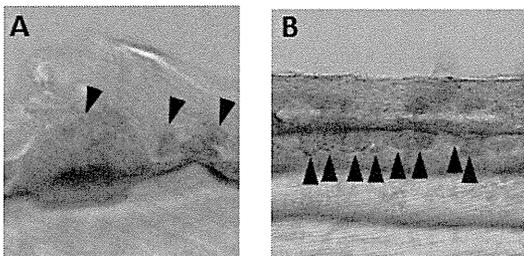


Fig. 2 TRAP for osteoclast (A) and ALP for osteoblast (B) staining of goldfish scales.

2. ウロコを用いた評価システムの特徴

ウロコには破骨細胞と骨芽細胞が共存し、その骨質層からカルシウムを出し入れして、血液中のカルシウム濃度を調節している⁵⁾。したがってウロコを用いることで、従来の材料や方法では難しい骨芽細胞と破骨細胞の相互作用を、生体内に近い状態で再現できる。そこでウロコを用いた培養系を確立し、これを用いた新規測定系を開発した^{3), 4)}。以下にこの系の利点を挙げる。

- 1) ウロコは容易に抜去でき、培地の中に抜去したウロコを入れて培養するのみで、非常に簡便である。
- 2) 破骨細胞や骨芽細胞はウロコ表面に存在するため、そのまま培養に用いることができる。コラゲナーゼなどの処理による細胞の調整が不要なため、細胞の状態は極めて良い。
- 3) 短時間の培養には血清が不要であり、低コストである。炭酸ガスも要らない。
- 4) キンギョでは、1個体からほぼ同じ細胞活性をもつウ

ロコを約100枚取れ、同時にさまざまな条件で検討できる。また、キンギョ（主として用いる）はラットやマウスに比べ、格段に安価な実験材料である。

- 5) 短時間（6時間から24時間）の培養で結果が得られ、迅速に解析できる。
- 6) ウロコ1枚でも、破骨細胞や骨芽細胞の活性測定が可能である。培養実験後のウロコをマイクロプレート（96穴）に1枚ずつ入れ、破骨細胞の場合は酒石酸抵抗性酸フォスファターゼ (TRAP)、骨芽細胞の場合はアルカリフォスファターゼ (ALP) を指標として、それぞれの酵素活性をプレートリーダーで容易に測定することができる。

3. 遠心機の過重力に対する応答解析

水棲生物用の麻酔薬 (MS-222) で麻酔したキンギョ (*Carassius auratus*) からウロコを取り、そのウロコを半分に切り、片方を実験群とし、他方をコントロール群 (1G) とした。これらのウロコを96穴のマイクロプレートに入れた。次に、そのwellに HEPES (20 mM), (pH 7.0) および抗生物質 (1%) を含む培地 (MEM, ICN Biomedicals Inc.) を 200 μL 加えた。このマイクロプレートを 2, 4 および 7G で遠心機 (LIX-130, トミーデジタルバイオロジー (株)) で 5 および 10 分間遠心した後、6 および 24 時間培養し、ウロコの骨芽細胞および破骨細胞の活性を測定した。なお、それぞれの実験は 3 回行い、ウロコは各群とも 12 枚使用した。

骨芽細胞の活性は、2G という低強度の重力負荷でも応答し、5 および 10 分間処理により、その活性が上昇した。4G では 2G とあまり変わらないが、7G では顕著に骨芽細胞の活性が上昇した。さらに、破骨細胞の活性も 2G で 5 分間処理しても応答し、その活性が低下した。破骨細胞活性の低下率は、強度が上がるにつれて下がり、7G では破骨細胞の活性が上昇する傾向にあった。

以上のように、非常に弱い強度で短時間の過重力処理でも、ウロコの骨芽細胞と破骨細胞は応答することが判明した。

4. 3次元クリノスタットによる擬似微小重力に対する応答解析

次に擬似微小重力に対する応答を解析した。培養細胞に対する影響を3次元クリノスタットで調べる場合は、培地の流動が細胞活性に影響を及ぼす可能性がある。そこで筆者らは、エッペンドルフチューブ内に綿球を詰めてウロコを固定し、ウロコの動きを抑え、かつ培地の流動をできるだけ低減する工夫を施した。結果として、ウロコは非常に感度よく3次元クリノスタットに応答した。

キンギョ (8 個体) を使用して、過重力と同様にウロコを抜き、エッペンドルフチューブに入れ、綿球を詰めて、

擬似微小重力下で6および24時間培養した。その結果、骨芽細胞の活性は、6時間の処理により8個体中6個体において低下し、24時間の処理により8個体中7個体において有意に下がった。一方、破骨細胞の活性は、6時間の処理により8個体中5個体において上昇し、24時間の処理により8個体中6個体において有意に上がった。

次に1軸(2次元)でクリノスタットを6時間回転させ、骨芽細胞および破骨細胞の応答を解析した。その結果、骨芽細胞の活性は3次元では低下するのに、2次元では変化しなかった。また破骨細胞の活性は3次元ではその活性が上昇したが、2次元では変化しなかった。

したがって、3次元クリノスタットに対するウロコの骨芽細胞および破骨細胞に現れた現象は、微小重力に特異的な反応である可能性が高い。

5. ウロコを用いた宇宙実験

擬似微小重力に対する応答が見られ、微小重力に対する応答を解析する優れた骨モデルであると評価され、「きぼう」の国際宇宙ステーションを用いた宇宙実験を2010年5月に実施することができた。

日本でパッキングした試料を冷蔵状態で米国NASAケネディ宇宙センターに輸送した。試料は冷蔵を保ったまま、スペースシャトルアトランティスSTS-132によって国際宇宙ステーション(ISS)に向けて2010年5月14日に打ち上げられ、5月16日にISSに到着、同日のうちに「きぼう」内の細胞培養装置にセットされ、22°Cで86時間培養された(Fig. 3)。5月20日、野口宇宙飛行士により細胞培養装置から取り出された培養試料は、冷凍保存および化学固定後、冷蔵保存により、地上に帰還した。

これらのウロコを解析した結果、破骨細胞の活性が上昇し、また、細胞が多核化するという形態学的な変化が引き起こされた。このことは、微小重力がウロコの骨吸収を引き起こしたことを示している。これは、長期間の低温保管

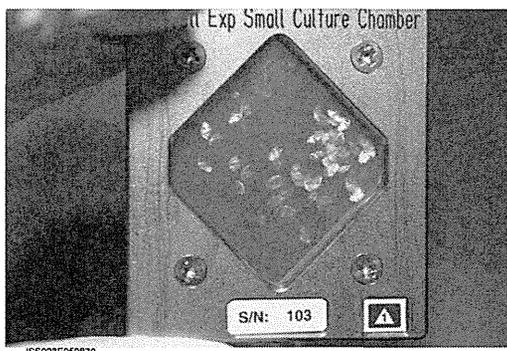


Fig. 3 JAXA astronaut Soichi Noguchi took a shot of cultured goldfish scales in KIBO of the International Space Station. The photograph indicates scales being cultured in space.

も可能であり、かつ物理的的刺激に対して応答性の良いウロコだからこそ達成することができた快挙である。

6. 極低周波磁場による破骨および骨芽細胞に及ぼす影響

以上のように、過重力および微小重力に対して非常に感度よく応答する骨モデルを用いて、60 Hzの極低周波磁場に対する影響を解析した。

麻酔したキンギョからウロコを取り、2 mL用のエッペンドリフトチューブ(BM機器)に入れた。次に、そのチューブにHEPES(20 mM)、(pH 7.0)および抗生物質(1%)を含む培地(MEM, ICN Biomedicals Inc.)を500 μ L加えた。そのチューブを、3、5、10および30 mTの極低周波磁場(60 Hz)に15°C、24時間暴露し、破骨および骨芽細胞の活性に及ぼす影響を調べた。磁場発生装置の詳細は、Miyakawa *et al.*⁶⁾に示してある。本研究では、破骨細胞の活性の指標としてTRAPを用い、骨芽細胞の活性の指標としてALPを使用し、磁場の骨組織に対する作用を調べた。また、培地中に放出された酵素活性も同様にして測定した。さらに、30 mTで処理したウロコからRNAを抽出して、遺伝子発現解析も行った。

3 mTの磁場により、ウロコの破骨細胞の活性が有意に低下した。また、細胞の活性と同様に培地中のTRAP活性もコントロールに比べて有意に低下していた。一方、ウロコの骨芽細胞の活性は変化しなかったが、培養液中のALP活性が、コントロールと比較して上昇していた。したがって、3 mTの磁場刺激でも骨形成が進行中であると推測される。また、5 mTでも3 mTと同様な変化が見られた。

10および30 mTでは、骨芽細胞の活性が上昇し、それに伴い破骨細胞の活性も上昇していた。これら2種類の細胞は、密接に連絡しており、骨芽細胞で発現しているリガンドであるRANKLと破骨細胞にあるレセプターであるRANKが結合することにより、破骨細胞が活性化し、多核の活性型の破骨細胞に分化する⁷⁾。したがって、10および30 mTで24時間暴露することにより、骨芽細胞が活性化し、RANK-RANKLを通して破骨細胞も活性化された可能性が高い。

さらに、30 mTで処理したウロコの遺伝子発現解析を調べると、膜レセプターであるカルシトニンレセプターmRNAの発現は上昇したが、核レセプターであるエストロゲンレセプターmRNAの発現は変化しなかった。カルシトニンレセプターは破骨細胞に特異的に発現している遺伝子なので、細胞活性と一致している。以上のことから、膜に何らかの影響を与えて、磁場は骨形成を促している可能性が高い。

7. おわりに

ウロコは、非常にシンプルで骨モデルとして有効である

ことがわかった。ウロコは、60 Hzの極低周波磁場に対しても非常に感度よく応答して、磁場強度より異なった応答をしていることがわかった。今後、磁場に対する応答を解析するため、キンギョのオリジナルアレイも開発中である。さらにこのモデルを用いて、物理的刺激のほか、骨代謝に関与するホルモン（カルシトニン³⁾、副甲状腺ホルモン⁸⁾、メラトニン⁴⁾、プロスタグランジンE₂⁹⁾）に対する応答も解析している。今後、ホルモンと磁場との相互作用についても解析していく予定である。

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Distribution of PAHs in the northwestern part of the Japan Sea

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ABSTRACT

Surface water samples were collected at 13 stations in the Japan Sea and filtered through a glass fiber membrane (pore size 0.5 μm). The vertical distribution of PAHs (nine points from depths between 2 m and 3300 m) were measured at one station. Thirteen PAHs having 3–6 rings in the dissolved and particulate phases were determined by HPLC with fluorescence detection. The mean concentration of total PAHs on the seawater surface was 8.5 ng/L. The mean concentrations in the dissolved and particulate phases for surface seawater were 5.6 ng/L and 2.7 ng/L, respectively. In the dissolved phase, 3-ring PAHs were the largest contributor, and 5-ring or more PAHs were in low ratio. Also, concentrations of PAHs in the particulate phase were dominated by 3-ring and 4-ring PAHs, but ratio of 3-ring PAHs was lower than in dissolved phase. Maximum of concentrations of PAHs in the dissolved (6.5 ng/L) and particulate (10.6 ng/L) phases were found for a depth 300 m. The possible source of PAHs is the atmosphere.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) containing from 2 to 8 fused aromatic rings are persistent organic pollutants. These hydrocarbons may be released into the environment as a result of human activity, and as a result of natural processes. PAHs may be formed by a variety of mechanisms: pyrogenic, petrogenic, biogenic and diagenic (Neff, 2002). As a result of human activity, most of these hydrocarbons are formed during the incomplete combustion of organic matter (coal, oil, timber, tobacco, etc.) (WHO, 2000).

The physical properties of these compounds vary widely depending on the number of benzene rings and chemical structures (McGowin, 2006). The data on the persistence of these substances are different. For the process of partial destruction of PAHs average half-life ranges from 2 to 700 days (Haritash and Kaushik, 2009; Johnsen et al., 2005; Shuttleworth and Cerniglia, 1995). Also important property of PAHs is the bioaccumulation (D'Adamo et al., 1997). Some PAHs (benzo[a]pyrene, benzo[b]-fluoranthene, etc.) are known to be carcinogenic and mutagenic (WHO, 2001), and therefore, the distribution and fate of these substances in the environment should be determined. Additionally PAHs are excellent markers to determine their origin,

ways of transport and transformation processes of these substances in different environmental conditions.

The main sources of PAHs in seas and oceans are mainly river runoff and atmospheric input (Dachs et al., 1997; Dickhut et al., 2000; Lipiatou and Salot, 1991).

The Japan Sea is a relatively closed ocean system because the each strait connecting the Japan Sea with ocean and other seas is narrow and shallow, and the maximum and average depths of Japan Sea are 3796 and 1350 m, respectively (Kosuke et al., 2005). So pollutants may accumulate long-term in the bottom of the Japan Sea and the pollution can be assumed to be of serious proportions. The surface circulation of the sea is well described in many works (e.g., Talley et al., 2006). The main circulation features are the strait inflows (Tsushima Strait and Tatar Strait) and outflows (Tsugaru Strait and Soya Strait), major currents (including the western and eastern boundary currents), the Subpolar Front and vigorous eddies.

The Amur River's waters are imported into the Japan Sea by Primorye (Liman) Current through Tatar Strait. Depending on the season the Amur River runoff are going to the Sea of Okhotsk (summer period) or to the Japan Sea (winter period). Approximately one-third of the annual Amur River discharge flow into the Japan Sea that is half of total river runoff (Dobrovol'skii and Zalogin, 1982; Ogi et al., 2001; Yakunin, 1978).

The Japan Sea is connected with waters of the East China Sea by Tsushima Warm Current and East Korean Warm Current through Tsushima Strait (Talley et al., 2006). The total concentrations of PAHs in surface waters of the East China Sea were observed from 70.22 to 120.29 ng/L (Ren et al., 2010).

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Monsoon climate is typical for the Japan Sea, south-eastern winds in summer and north-western winds in winter are dominant over the sea (Kaneyasu et al., 2000). The winter season is characterized by the transfer of air masses from China to the waters of the sea (Hayakawa et al., 2007; Tang et al., 2005). The last 20 years China's economy has been developing rapidly, which resulted in the significant growing consumption of energy derived mainly from fuel combustion (Feng et al., 2006; Huang et al., 2009). Hence during the heating season, air masses over China are most heavily contaminated with PAHs (Yang et al., 2007). Spatial distribution of atmospheric PAHs from the Bohai Sea to the high Arctic was studied by Ding et al. (2007). They have demonstrated a decreasing latitude trend for gas-phase PAHs and have noted higher levels of total concentration of atmospheric PAHs occurred in Far East Asia (34–48N, 122–148E) including the Japan Sea with an arithmetic mean of 47.9 ng/m³.

There are a few investigations and reports of PAHs in the seawater of the Japan Sea. Nemirovskaya (2007) has reported total concentrations of PAHs in the Amurskiy Bay (Peter the Great Bay, northwestern part of the Japan Sea) widely varied, from 5 to 85 ng/L. There is a gap in our knowledge about PAHs contents in the open part of the Japan Sea. This paper presented data about dissolved and particulate forms of PAHs contents in seawater samples collected in late summer in 2010.

2. Materials and methods

2.1. Sampling site

Surface water samples in all stations were collected in the Japan Sea from August 11 to September 5, 2010 by cruise of R/V "Akademik M.A. Lavrent'ev". Also on the station D1 the vertical distribution of PAHs were studied. The sampling sites are shown in Fig. 1. All stations are located in northern from Subpolar Front. Five liters of sea water was collected by a Niskin bottle and conductivity and temperature depth profiler (CTD), respectively, were monitored.

2.2. Sample treatment

Water samples were filtered through a 0.5 µm pore glass fiber filter (Advantec GC50) to separate the dissolved phase from the particulate phase. The filters containing SPM samples were dried in air conditions for 1 h in the dark.

2.3. PAH extraction

Prior to HPLC analysis, PAHs in the dissolved phase (DP) and in the particulate phase (PP) were extracted by different methods.

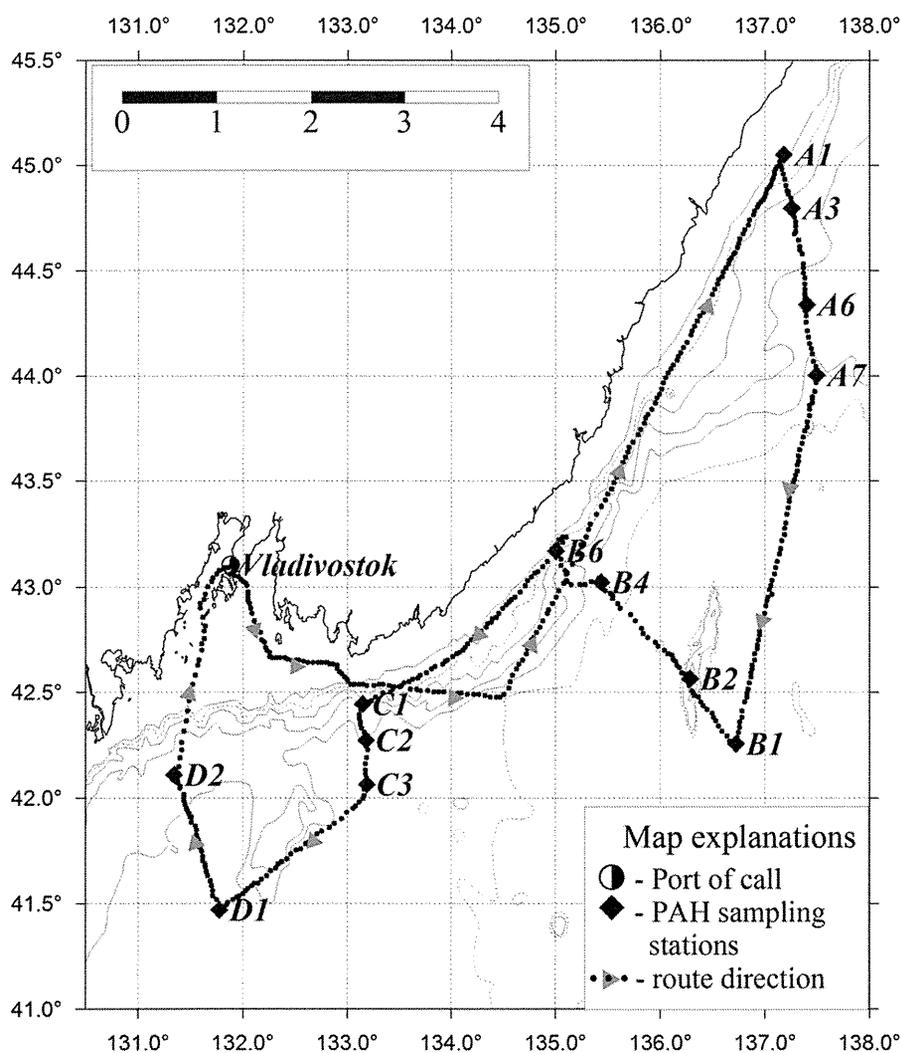


Fig. 1. The map of the cruise Lav-51.

Extraction of PAHs in DP: PAHs in water were extracted by solid phase extraction using a C18 cartridge (Waters Sep-Pak C-18 Cartridge). The cartridges were preconditioned with 5 mL methanol and by 5 mL distilled water. After preconditioning, water samples were applied to the cartridges. The flow rate of sample through the cartridge was 10 mL/min. After all samples were percolated, the cartridges were dried under vacuum condition. PAHs in the cartridge were eluted with 15 mL of dichloromethane (Kiss et al., 1996). Surrogate recovery standards (Nap-*d*₈, Ace-*d*₁₀, Phe-*d*₁₀, Pyr-*d*₁₀ and BaP-*d*₁₂) were added to the cartridge just before the elution. PAHs in the extracts were concentrated by rotary evaporation to dryness and then the residue was dissolved in 1 mL hexane for the clean-up procedure.

Extraction of PAHs in PP: the glass fiber filters were soaked in a 40 mL mixture of benzene and ethanol (3:1, v/v). Surrogate recovery standards (Nap-*d*₈, Ace-*d*₁₀, Phe-*d*₁₀, Pyr-*d*₁₀ and BaP-*d*₁₂) were added to the sample just before the extraction. The mixtures were extracted in an ultrasonic bath (NSD Japan, US-106, 38 kHz, 300 W) for 30 min. Extracts were then filtered. The extraction was repeated once without adding surrogate recovery standard. PAHs in the extracts were concentrated by rotary evaporation to dryness and then the residue was dissolved in 1 mL hexane for the clean-up procedure.

2.4. Clean-up

After extraction, the samples were cleaned up with a silica gel cartridge (Water Sep-Pak Silica Cartridge). The cartridges were preconditioned with 5 mL of hexane. Samples were applied to the cartridges and the PAHs were eluted with a mixture of hexane and acetone (9:1, v/v). After clean-up, 200 μ L DMSO was added. PAHs in the extracts were concentrated by rotary evaporation and then the residue was dissolved in 800 μ L acetonitrile. An aliquot (20 μ L) of the solution was injected into the HPLC.

2.5. HPLC

The HPLC system consisted of two Hitachi L-2130 pumps, a Hitachi degasser, a Hitachi L-2485 fluorescence detector and a Hitachi organizer. The analytical column and guard column were Inertsil ODS-P (4.6 i.d. \times 250 mm, 5 μ m, GL Sciences) and Inertsil ODS-P (4.0 i.d. \times 10 mm, 5 μ m, GL Sciences), respectively. Both of the columns were kept at 20 °C. The mobile phase was a mixture of acetonitrile and distilled water set in a linear gradient program. The flow rate of mobile phase was kept at 1.0 mL/min. The sample was detected by the fluorescence detector for which

the excitation and emission wavelengths were automatically set by a time program.

Fifteen PAHs from USEPA's 16 priority PAHs list were quantified: the two-ring PAH was Naphthalene (Nap); three-ring PAHs were Acenaphthene (Ace), Fluorene (Fle), Anthracene (Ant) and Phenanthrene (Phe); four-ring PAHs were Fluoranthene (Flu), Pyrene (Pyr), benz[a]anthracene (BaA) and Chrysene (Chr); five-ring PAHs were Benzo[b]fluoranthene (BbF), Benzo[k]fluoranthene (BkF), Benzo[a]pyrene (BaP) and Dibenz[a,h]anthracene (DBA); six-ring PAHs were Benzo[ghi]perylene (BgPe) and Indeno[1,2,3-cd]pyrene (IDP). Nap and Phe could not be quantified because of too low recovery rate and poor resolution in chromatography results, respectively.

The wavelengths of fluorescence were set according to the optimization excitation and emission of each PAH. The excitation and emission wavelengths were set at 280 and 340 nm for Nap, Ace, Fle, and Phe; 250 and 400 nm for Ant; 286 and 433 nm for Flu; 331 and 392 nm for Pyr; 264 and 407 nm for BaA, Chr, BbF, BkF, BaP, DBA, and BgPe; and 294 and 482 nm for IDP, respectively.

3. Results and discussion

3.1. Distribution of PAHs in the surface waters of the Japan Sea

Table 1 shows the concentrations of the 13 PAHs identified in the surface waters of the Japan Sea. The total concentrations of the 13 PAHs ranged from 7.4 to 10.2 ng/L with a mean concentration of 7.9 ng/L. These data are significantly lower as compared with those of the studies for the other seas in the Far East Asia, e.g., for the Western Taiwan Strait connecting the East China Sea and the South China Sea the total concentrations of PAHs varied from 23.3 to 70.9 ng/L (Wu et al., 2011) and for the East China Sea—70.22–120.29 ng/L (Ren et al., 2010). Excepting stations A1 and B1, the values of the total concentration of PAHs were similar for all other stations.

The highest concentrations (about 10 ng/L) were observed at stations A1 (the northernmost station of the cruise) and B1 (the station of the cruise closest to Japan). High concentration of PAHs at station A1 may be related to the proximity of the Amur River waters, which are probably indicated lower salinity of surface seawater at this station (32.398 psu). As for high concentration of PAHs at station B1 it may be associated with the Tsushima Current from East China Sea. The surface waters of the warm Tsushima Current spread over the southern Japan Sea to the Subpolar Front (approximately at 40°N) and converge around the western side of the Tsugaru Strait. Then current flows out through

Table 1
Concentrations of PAHs in the surface samples of the Japan Sea. Cruise Lav-51.

PAHs	DP (ng/L)				PP (ng/L)				Total (DP + PP) (ng/L)			
	Max.	Min.	Mean	Std. dev.	Max.	Min.	Mean	Std. dev.	Max.	Min.	Mean	Std. dev.
Ace	1.0	0.8	0.9	0.09	1.0	0.6	0.7	0.13	1.92	1.48	1.52	0.15
Fle	2.6	1.8	2.1	0.24	0.64	0.35	0.42	0.08	3.13	2.25	2.39	0.28
Ant	0.50	0.26	0.34	0.06	0.093	0.051	0.064	0.011	0.59	0.31	0.38	0.07
Flu	0.90	0.24	0.63	0.16	0.69	0.12	0.31	0.15	1.32	0.59	0.90	0.18
Pyr	2.3	0.8	1.3	0.35	1.10	0.17	0.60	0.26	3.17	1.36	1.80	0.5
BaA	0.16	0.03	0.07	0.035	0.083	0.029	0.048	0.017	0.19	0.07	0.11	0.037
Chr	0.078	0.022	0.036	0.014	0.292	0.036	0.09	0.069	0.32	0.06	0.12	0.07
BbF	0.065	0.025	0.044	0.012	0.11	0.07	0.09	0.012	0.18	0.11	0.13	0.02
BkF	0.016	0.010	0.012	0.002	0.055	0.035	0.043	0.005	0.068	0.048	0.054	0.006
BaP	0.027	0.007	0.016	0.006	0.052	0.024	0.037	0.008	0.072	0.035	0.051	0.011
DBA	0.063	0.023	0.039	0.011	0.13	0.07	0.09	0.018	0.18	0.09	0.12	0.023
BgPe	0.063	0.030	0.042	0.009	0.17	0.07	0.11	0.025	0.21	0.11	0.15	0.026
IDP	0.051	0.008	0.027	0.012	0.29	0.03	0.13	0.079	0.333	0.032	0.151	0.08
Σ PAHs	6.521	4.758	5.605	0.882	3.725	2.126	2.731	0.807	10.246	7.387	7.880	1.681

the Tsugaru and Soya Straits, and the rest trends northward (Kosuke et al., 2005). Therefore the surface waters of the East-China Sea can be considered as source of PAHs for the Japan Sea.

Table 1 and Fig. 2 show the concentrations of the 13 PAHs in DP and PP ranged from 4.8 to 6.5 ng/L (mean—5.6 ng/L) and 2.1 to 3.7 ng/L (mean—2.7 ng/L), respectively. In the both phases, DP and PP, 3-ring and 4-ring PAHs were dominant. But concentrations of PAHs with 3-ring and 4-ring (excepted Chr) were higher in DP than in PP. While concentrations of Chr and 5,6-rings PAHs were 2–5 times higher in PP than in DP. These results indicate that DP and PP contained 67% and 33% of the total PAHs, respectively. Similarly, in the atmosphere low molecular weight PAHs are mainly in the gas phase, while high molecular weight species are in particulate phase (Dickhut et al., 2000). For the Mediterranean Sea, which similar to the Japan Sea, due to connecting with adjacent basins by straits, the concentrations of PAHs in DP ranged from 0.4 (in the open sea) to 2.2 ng/L (in the estuaries). The concentrations of PAHs in PP ranged from 0.3 to 1.1 ng/L accounting for 30–40% of total PAHs (Dachs et al., 1997). This correlation agrees with our data. The higher concentrations of PAHs in this investigation can be explained that the sampling for the Mediterranean Sea's study was conducted in 1993 and 11 PAHs were analyzed.

3.2. PAHs ratios as markers of PAHs contamination

Different genetic sources of PAHs have the various ratios between the individual compounds themselves. Therefore these ratios have been selected as markers to determine the sources of pollution of ecosystems by PAHs. Yunker et al. (2002) use PAH isomer pairs ratios Ant/(Phe+Ant), Flu/(Flu+Py), BaA/(BaA+Chr)

and IDP/(IDP+BgPe) to elucidate possible sources of PAHs in marine environment. They proposed that for IDP/(IDP+BgPe), a ratio < 0.2 probably implies petroleum (petrogenic source), 0.2–0.5 implies petroleum combustion (liquid fossil fuels combustion), and > 0.5 implies grass, wood or coal combustion; for Flu/(Flu+Pyr) the petroleum boundary ratio appears close to 0.4, and the ratio between 0.4 and 0.5 is characteristic of petroleum combustion, whereas a ratio > 0.5 is characteristic of implies grass, wood or coal combustion. Similarly, the BaA/(BaA+Chr) ratio of less than 0.2 implies the petroleum input, between 0.2 and 0.35 suggests liquid fossil fuel combustion and more than 0.5 infers grass, wood and coal combustion (Wu et al., 2011). As shown in Fig. 3A and B, the ratios IDP/(IDP+BgPe) were ranged from 0.2 to 0.8, and the values in PP were a little higher than those in DP. On the contrary the values of BaA/(BaA+Chr) ratios in DP were a little higher than those in PP and were ranged from 0.4 to 0.9 for DP and from 0.2 to 0.6 for PP (Fig. 3C and D). Flu/(Flu+Pyr) ratios were mostly found in the range from 0.2 to 0.4; the values in PP and DP are very similar (Fig. 3A–D). This suggests that PAHs mainly descended from the mixed sources both with the inputs from petrogenic and pyrolytic origins.

3.3. Vertical distribution of PAHs at the station D1

The main feature of hydrophysical characteristics determining the vertical structure of the Japan Sea is that all of the subtropical water from about 300 m to the bottom and all of the subpolar water in the Japan Sea is very ventilated north of the Subpolar Front. All of this subpolar-ventilated water can be referred to as Japan Sea Proper Water, once thought to be a nearly homogenous water mass (Talley et al., 2006). Station D1 is located about 50 km

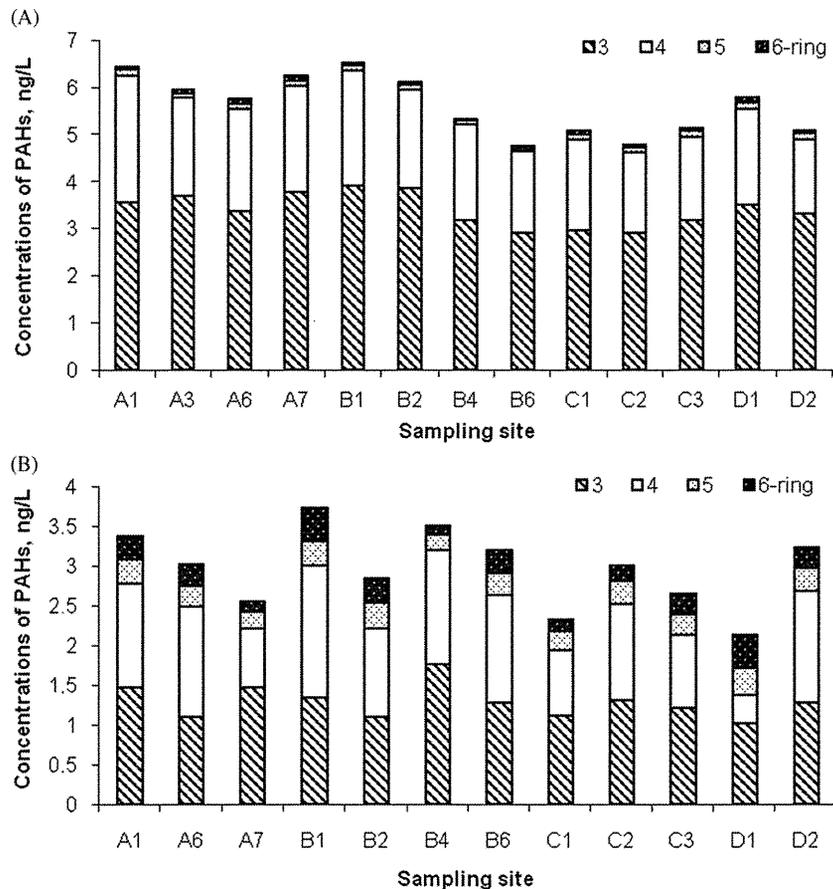


Fig. 2. Concentrations of PAHs in the surface seawater in DP (A) and PP (B).

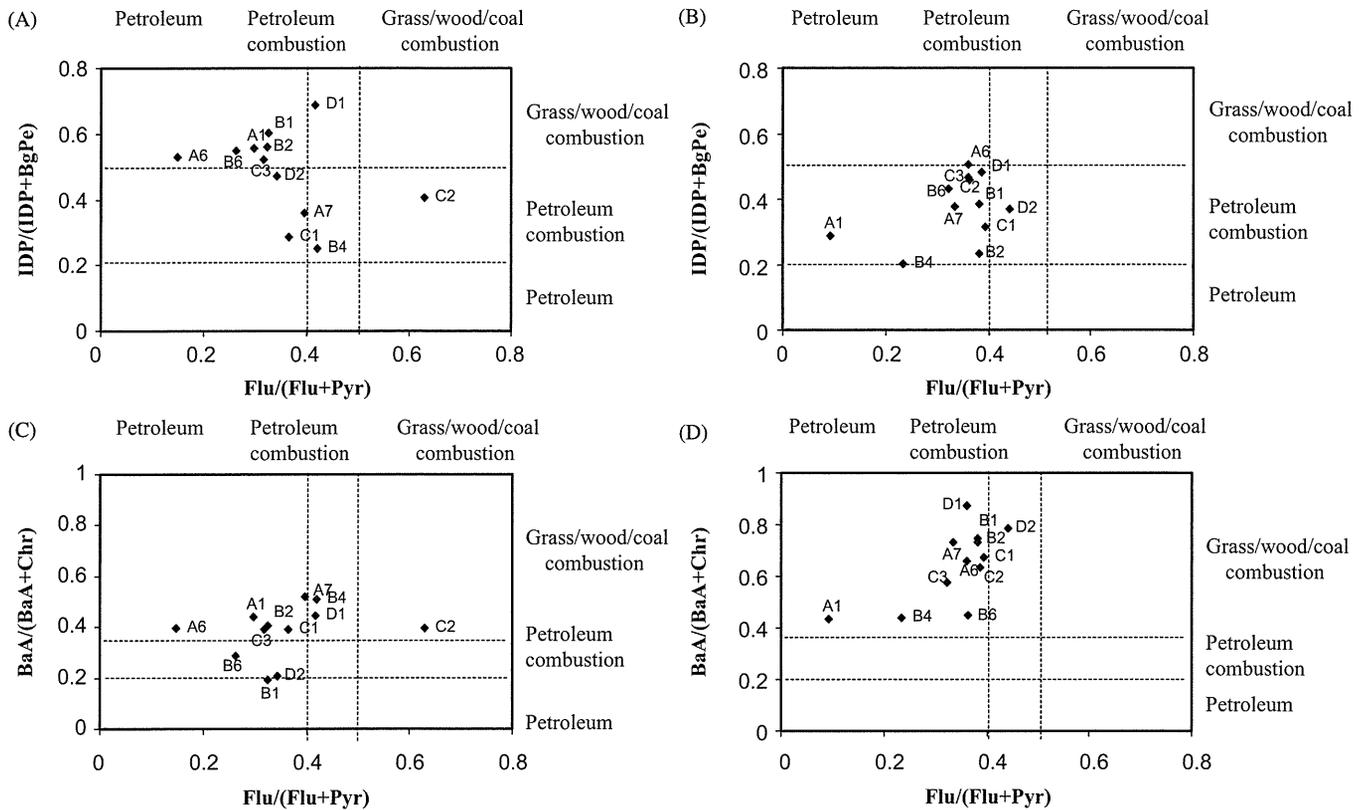


Fig. 3. Ratios of IDP/(IDP+BgPe) vs. Flu/(Flu+Pyr) in PP (a) and DP (b); BaA/(BaA+Chr) vs. Flu/(Flu+Pyr) in PP (c) and DP (d) in the stations of the cruise Lav-51.

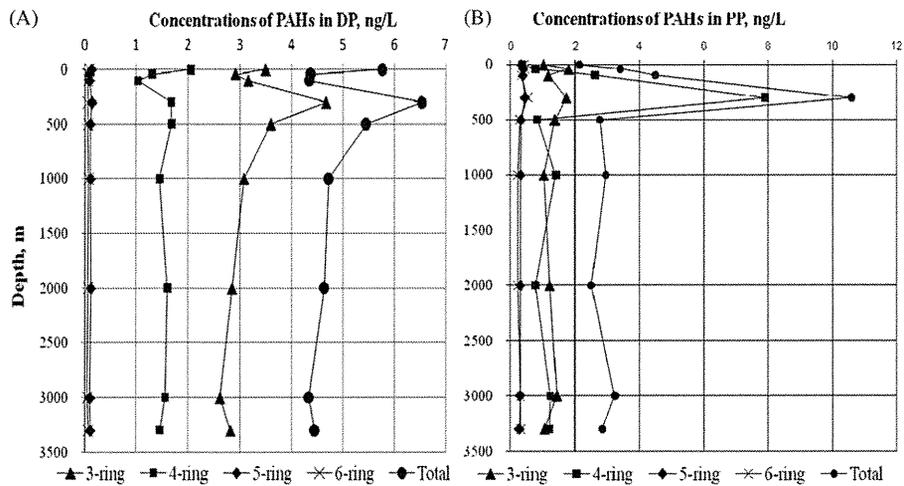


Fig. 4. Vertical distribution of PAHs in DP (a) and PP (b) at the station D1.

far from northern edge of the Subpolar Front. This area is characterized as relatively high productive unit. Vertical distribution of PAHs in PP and DP is almost homogenous below 500 m (Fig. 4). The maximum concentrations of PAHs in DP and PP were detected on the sea surface and at a depth of 300 m. The high concentrations of PAHs for the sea surface indicate the main source of PAHs is the solid particles of the atmosphere (aerosols). Sufficiently high concentrations of PAHs for greater depths lead us to the same conclusion. We assume the basic mechanism of penetration of PAHs in the deep horizons of the sea is the settling of solid particles with adsorbed PAHs. Otherwise, the penetration of these compounds in the intermediate and deep waters of the sea only by the mechanism of turbulent diffusion would take

several decades, during which low molecular PAHs with a half-life of several tens of days are destroyed. During the settling of the solid particles, PAHs partially transfers from the particulate phase to the dissolved phase. The rate of deposition of particles varies widely from a few meters to several hundred meters per day (Burd and Jackson, 2009). A depth of 300 m showed the highest concentrations of PAHs. Particulate phase on this depth contains a high concentration of Pyr (4 ng/L). As observed zooplankton fecal pellets are important vector of vertical flux of PAHs (Lipiatou et al., 1993). In an earlier authors have found the plankton samples contain high concentrations of Pyr and Flu (Lipiatou and Saliot, 1992). Therefore we suggest the relationship between the maximum of the Pyr-concentration at the depth 300 m and zooplankton fecal pellets.

4. Conclusions

This investigation first provides the data of PAHs spatial distribution in the northwestern part of the Japan Sea and contributes to the present status of PAHs contamination in studied region.

Thirteen PAHs were quantitatively analyzed in this study. The values of total PAHs concentration in the Japan Sea were similar for surface seawater at all stations (excepting stations A1 and B1) and were ranged from 7.4 to 10.2 ng/L with a mean concentration of 7.9 ng/L. The level of PAHs contamination was founded lower in comparison with others seas of the Far East Asia.

Data PAHs isomer pair ratios Flu/(Flu+Py), BaA/(BaA+Chr) and IDP/(IDP+BgPe) suggest that PAHs mainly descended from the mixed sources both with the inputs from petrogenic and pyrolytic origins.

Vertical distribution of total PAHs was detected maxima on the sea surface and depth of 300 m. We assume the former originate from atmosphere and the later relate with zooplankton fecal pellets.

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[Review]

Biological Effects of Polycyclic Aromatic Hydrocarbon Derivatives

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Abstract : Polycyclic aromatic hydrocarbons (PAHs) are included in various environmental pollutants such as airborne particles and have been reported to induce a variety of toxic effects. On the other hand, PAH derivatives are generated from PAHs both through chemical reaction in the atmosphere and metabolism in the body. PAH derivatives have become known for their specific toxicities such as estrogenic/antiestrogenic activities and oxidative stress, and correlations between the toxicities and structures of PAH derivatives have been shown in recent studies. These studies are indispensable for demonstrating the health effects of PAH derivatives, since they would contribute to the comprehensive toxicity prediction of many kinds of PAH derivatives.

Key words : PAH derivative, structure activity relationship, estrogenic/antiestrogenic activity, reactive oxygen species.

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Introduction

An important group of pollutants associated with airborne particulate matter (PM) are polycyclic aromatic hydrocarbons (PAHs), which are constructed of two or more aromatic rings and are produced by incomplete combustion of fossil fuels. PAHs have carcinogenicity and mutagenicity [1], and have been classified according to International Agency for Research for Cancer (IARC) as carcinogenic or probably carcinogenic compounds [2, 3]. PAHs are believed to be the main causal compounds in the health effects of ambient air pollutants. PAHs generate various derivatives both in the atmosphere and in the body. PAH derivatives are becoming known to have particular effects, such as oxidative stress and endocrine disruption.

Many studies give information on the possible roles of PAH derivatives in several diseases which have been increasing for several decades worldwide. However, a comprehensive assessment of the toxicities of these compounds is not easy, since numerous PAH derivatives exist in the atmosphere and they have different toxicities.

In recent years, studies of the structure and activity relationship have developed in the study of environmental science. This study can predict the possibility of the toxicities of compounds according to the relationship between chemical three-dimensional (3D) structures, even though the toxicities of their compounds have not been measured. Therefore, an analysis of the structure and activity relationship is a key to know the health risk of numerous compounds in the

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atmosphere, including PAH derivatives.

This review provides information mainly about our recent observation assessing the relationship between structural and biological activities of PAH derivatives.

Generation of PAH derivatives

It is well known that PAH derivatives such as hydroxylated PAHs (OHPAHs) and PAH quinones (PAHQs) are generated in the atmosphere through chemical reactions with nitrogen radicals ($\bullet\text{NO}^3$), hydroxide radicals ($\bullet\text{OH}$) and ultraviolet light [4–6]. These PAH derivatives are also generated in the body. After entering the body, PAHs bind to one of the nuclear receptors, the aryl hydrocarbon receptor (AhR), and then induce the cytochrome P450 drug-metabolizing enzymes such as *Cyp1a1*, *Cyp1a2* and *Cyp1b1*, which metabolize PAHs into various PAH derivatives.

Toxicities of PAH derivatives

Concerning the toxicities of PAH derivatives, the mutagenicity induced by nitrated PAHs (NPAHs) has been well known for many years [7]. In recent years, it has been shown that other PAH derivatives also show various toxicities. For instance, PAHQs produce reactive oxygen species (ROS) through redox cycle, leading to ROS-related toxicities, such as physical DNA damage, oxidative stress and cell death [8–10]. The most important information is that PAH derivatives-induced oxidative stress might be involved in various diseases, such as allergic reaction, circulatory organ system disease, infection and aging [11–17]. Cho *et al.* have recently reported that phenanthrenequinone (PQ) induced the recruitment of inflammatory cells, such as eosinophils and neutrophils, into the lung with the lung expression of pro-inflammatory molecules such as interleukin (IL)-5 and eotaxin *in vivo* [19]. PQ also aggravates antigen-related airway inflammation in mice, and PQ has adjuvant activity for antigen-specific immunoglobulin G (IgG), leading to aggravation of antigen-related airway inflammation in mice [20]. Because PQ is a major quinone in diesel exhaust particles (DEP) [18], which have been reported to cause lung inflammatory-related impacts, these reports suggest a key role of PQ in lung diseases by air pollutants.

Interestingly, there are several reports suggesting that PAH derivatives have endocrine disruptor-like activities. DEP extracts including numerous PAH derivatives exhibit estrogenic and/or antiestrogenic activities in human MCF-7 breast cancer cells and recombinant yeast cells [21–23]. These samples also exhibited a significant antiandrogenic effect in PC3/AR human prostate carcinoma cells [24]. Actually, one of the OHPAHs, hydroxyphenanthrene (OHPhe) and hydroxyfluoranthene (OHFrt), constructed with three or four rings, were determined in the DEP extracts as antiandrogenic compounds. Furthermore, strong estrogenic activities of several OHPAH isomers, hydroxybenz[*a*]anthracene (OHBaA) and hydroxychrysene (OHCh), were also detected by screening evaluation using yeast two-hybrid assay [25].

Structure activity relationship of estrogenic/ antiestrogenic activity of PAH derivatives

It has gradually become known that the endocrine disruptor-like activities of PAH derivatives are related to their structure. It has been reported theoretically that the common structure of estrogenic compounds is a phenol with a hydrophobic moiety at the *para*-position without a bulky group at the *ortho*-position [26]. This theory could be applied to the activities of PAH derivatives. In our recent study, we investigated whether OHPAHs, PAHQs and PAH ketones (PAHKs) having two to six rings show estrogenic or antiestrogenic activities [25, 27] by using the yeast two-hybrid assay system [28], in order to elucidate the characteristics of PAH derivatives in more detail.

Among the OHPAHs we tested, strong estrogenic activity was observed mainly in OHPAHs having 4 rings. We also observed strong antiestrogenic activity in several OHPAHs having 4 and 5 rings [25]. Because PAHs can't bind to the active site of human estrogen receptor (hER), it is strongly suggested that the hydroxyl modification and its location are key factors for the large difference in estrogenic activities between PAHs and OHPAHs. At this time, relative binding affinity (RBA) is also correlated with estrogenic or antiestrogenic activity [29]. On the other hand, we have found that several PAHQs also showed strong antiestrogenic activities, suggesting that exhibition of