$CuSO_4 \cdot 5H_2O$ in 1% sodium tartrate and tri-sodium citrate. The Folin phenol solution was made: The Folin-Ciocalteu's reagent solution (Nacalai Tesque, Inc.) was diluted with distilled water to make it 1 N in acid.

One hundred microliters of the cell lysates and 2 ml of the alkaline copper solution were mixed and kept at room temperature for 20 min. In addition, 200 μl of the Folin phenol solution was added and the mixture was kept at room temperature for 30 min. The absorbance of the reaction solution was read at 750 nm. Bovine serum albumin was used as a reference standard.

DNA content

DNA content of cell lysates was measured by the fluorescence assay using Hoechst 33258 dye (Wako Pure Chemicals Industries Ltd., Osaka, Japan). Twenty microliters of the cell lysates, 2 ml of PBS(−) and 100 μl of 1 μg/ml Hoechst 33258 solution were mixed and kept at room temperature for 30 min. The fluorescence of the reaction solution was read at an excitation wavelength of 356 nm and an emission wavelength of 458 nm. Calf thymus DNA (Sigma Chemical Co., St. Louis, Missouri) was used as a reference standard.

HYP content

HYP content of cell lysates was measured by the methods of Huszar *et al.* [13]. The chloramines T solution was made: Sodium N-chloro-*p*-toluenesulfonamide, 1.41 g, was dissolved in 10 ml of 1-propanol, and then 10 ml of water and 80 ml of pH 6.6 buffer (citric acid monohydrate 5 g, acetic acid 1.2 ml, sodium acetate trihydrate 12 g, sodium hydroxide 3.4 g to 100 ml of water) were added. Aldehyde-perchloric acid solution was freshly made: *p*-Dimethylaminobenzaldehyde, 15 g, was dissolved in 62 ml of 1-propanol and then 26 ml of perchloric acid was added slowly.

Two hundred microliters of the cell lysates were pipetted into the polypropylene tubes of the 2 ml capacity and dried in an oven at 100°C for 4 h. Fifty microliters of 4 N NaOH was added into the tube and the tube was autoclaved at 121°C for 10 min. After cooling to room temperature, 50 μl of 1.4 N citric acid and 1 ml chloramines T solution were added. After the mixture was kept at room temperature for 20 min, it was incubated with 1 ml of aldehyde-perchloric acid solution at 65°C for 15 min. The absorbance of the reaction solution was read at 550 nm. The standard solutions that diluted HYP at the various concentrations were also operated by the same manner in order to make calibration curve.

ALP activity

ALP activity was measured according to the method of Ikarashi *et al.* [6]. The same quantity of 2 mM $MgCl_2$ in 0.1 M carbonate buffer (pH 10.2) and 20 mM *p*-nitrophenylphosphate solution were mixed, and then, the substrate solution was

pre-incubated at 37°C. Twenty microliters of the cell lysates was incubated with 1 ml of the substrate solution at 37°C for 30 min. The enzymatic reaction was stopped by adding 2 ml of 0.25 N NaOH, and the absorbance of p-nitrophenol liberated was read at 410 nm. The calibration curve of ALP activity was made by the standard solutions that diluted calf intestine ALP (Boehringer Mannheim GmbH, Germany) at the various concentrations. The ALP activity of cell lysate was calculated from the calibration curve.

Statistical analysis

All measured values were collected in 4 sets and expressed in means ± standard deviation (SD). Differences among the groups were evaluated with one-way analysis of variance (ANOVA). When significant differences among the groups were found, Turky–Kramer test was applied for multiple comparisons. A difference was considered to be significant if $p < 0.05$.

RESULTS

Molecular weight of γ-ray irradiated PLLA

The molecular weights of γ-ray irradiated PLLA at the dose of 10, 25 or 50 kGy were determined by GPC (Table 1). The molecular weights (M_w and M_n) of irradiated PLLA decreased with increasing irradiation dose. The M_w of 271 000 of unirradiated PLLA was decreased to 95 000 by irradiation up to 50 kGy. The polydispersity index (M_w/M_n) of irradiated PLLA increased from 1.56 of unirradiated PLLA to 1.78 with increasing irradiation dose (Table 1). The γ-ray irradiation decreased the molecular weight of PLLA.

Proliferation and calcification cultured on γ-ray irradiated PLLA

The proliferation and calcification of MC3T3-E1 cells cultured on γ-ray irradiated PLLA for 2 weeks were determined. Figure 1 shows the effect of irradiated PLLA on the proliferation of MC3T3-E1 cells. The irradiation dose of 10, 25 and

Table 1.
Effect of γ-ray irradiation on the molecular weight and polydispersity index of PLLA

Irradiation dose (kGy)	M_w	M_n	Polydispersity index
0 (unirradiated)	271 000	174 000	1.56
10	195 000	119 000	1.64
25	142 000	82 700	1.72
50	95 000	53 500	1.78

The PLLA was γ-irradiated at the dose of 10, 25 or 50 kGy, and the molecular weight of irradiated PLLA was measured by GPC.

50 kGy did not affect the proliferation of the cells cultured on irradiated PLLA. The calcium depositions of cell cultures were stained by alizarin red S solution (Fig. 2). The alizarin red S stained areas were measured to determine the extent of the calcification of MC3T3-E1 cells cultured on irradiated PLLA. Figure 3 shows the effect of irradiated PLLA on the calcification of MC3T3-E1 cells. The calcifications of MC3T3-E1 cells cultured on irradiated PLLA were significantly increased ($p = 0.02$ by ANOVA) for that on unirradiated PLLA. The calcification was increased by irradiation up to 25 kGy, but not further increased even at the irradiation of 50 kGy. The calcifications were increased 1.4-fold by the irradiations at 25 and 50 kGy.

Cell activity cultured on γ-ray irradiated PLLA

Protein and DNA contents were determined as the indicators of the cell proliferation of MC3T3-E1 cells cultured on γ-ray irradiated PLLA for 2 weeks (Table 2). The irradiation dose of 10, 25 and 50 kGy did not affect the protein and DNA contents cultured on irradiated PLLA. HYP amount expresses collagen synthesis of osteoblastic cells in the process for the differentiation, and ALP is the representative enzyme of osteoblastic differentiation. Thus, HYP content and ALP activity were determined as the indicator of the osteoblastic differentiation of MC3T3-E1 cells

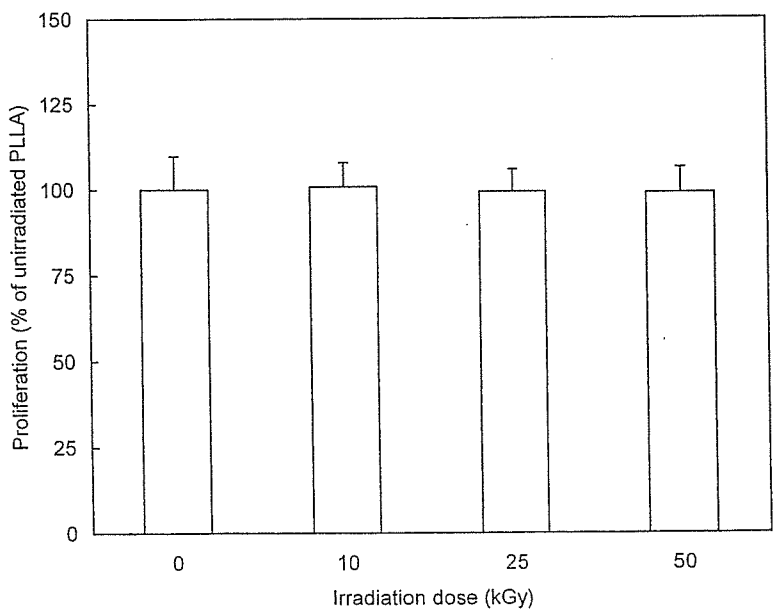

Figure 1. The proliferation of MC3T3-E1 cells cultured on γ-ray irradiated PLLA. The PLLA was γ-irradiated at the dose of 10, 25 or 50 kGy, and then MC3T3-E1 cells were cultured on irradiated PLLA for 2 weeks using the micromass culture. The proliferation of MC3T3-E1 cells cultured on irradiated PLLA was determined. Values are means ± SD for 4 dishes.

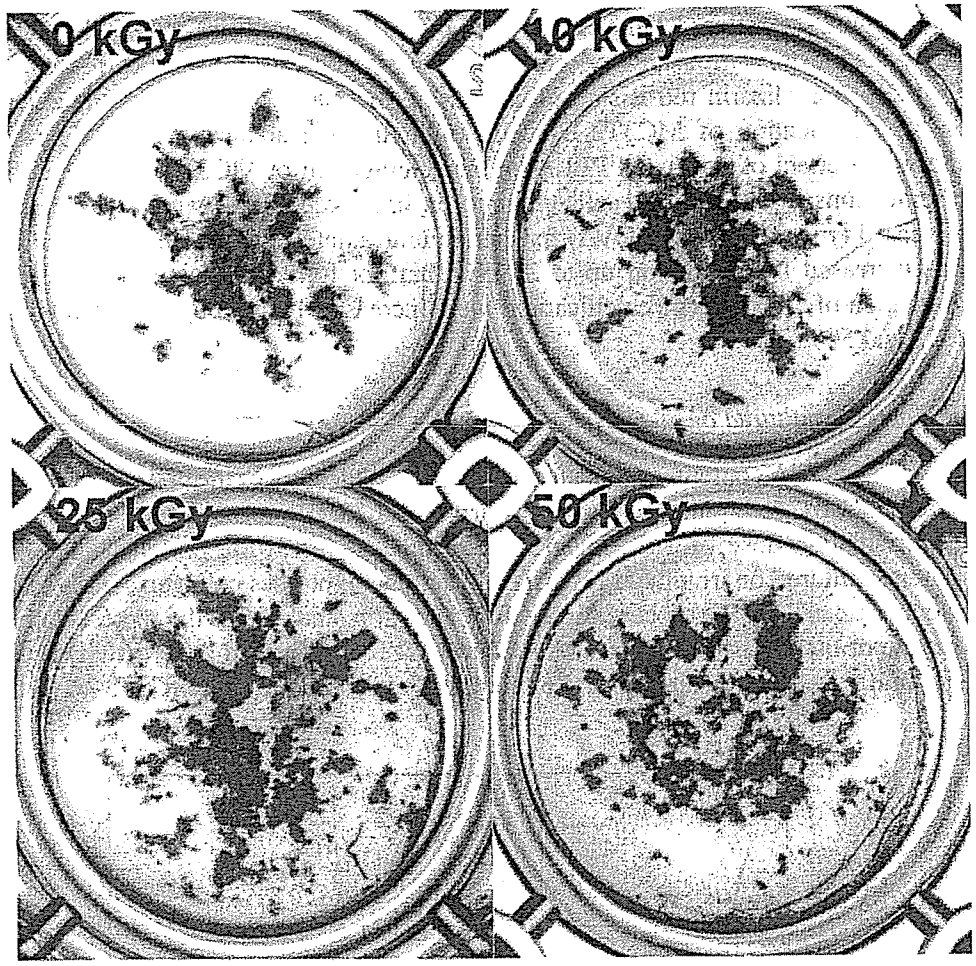

Figure 2. Appearance of alizarin red S stained cultures of MC3T3-E1 cells on γ-ray irradiated PLLA. The PLLA was γ-irradiated at the dose of 10, 25 or 50 kGy, and then MC3T3-E1 cells were cultured on irradiated PLLA for 2 weeks using the micromass culture. The culture on γ-irradiated PLLA was stained by alizarin red S solution. (This figure is published in colour on http://www.vsppub.com/jconts/JBS)

cultured on irradiated PLLA (Table 2). HYP content increased, and ALP activity significantly increased ($p = 0.04$ by ANOVA), with γ-irradiation to PLLA dose-dependently. The irradiated PLLA had no effect on the proliferation but stimulated the differentiation of MC3T3-E1 cells.

Effects of low molecular weight PLLA on the proliferation and calcification

The proliferation and calcification of MC3T3-E1 cells cultured in the medium containing low molecular weight PLLA for 2 weeks were determined (Figs 4 and 5).

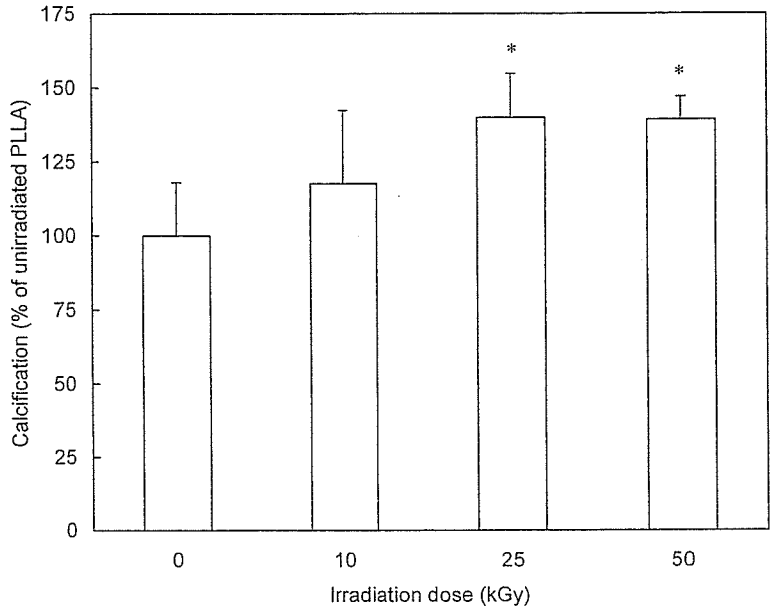

Figure 3. The calcification of MC3T3-E1 cells cultured on γ-ray irradiated PLLA. The PLLA was γ-irradiated at the dose of 10, 25 or 50 kGy, and then MC3T3-E1 cells were cultured on irradiated PLLA for 2 weeks using the micromass culture. The calcification of MC3T3-E1 cells cultured on irradiated PLLA was determined. Values are means ± SD for 4 dishes. Significant difference compared with unirradiated PLLA at $^*p < 0.05$.

Table 2.
Protein, DNA, HYP contents and ALP activity of MC3T3-E1 cells cultured on γ-ray irradiated PLLA

Irradiation dose (kGy)	Protein (μg/dish)	DNA (μg/dish)	HYP (μg/dish)	ALP (mU/dish)
0 (unirradiated)	144.9 ± 18.7	8.26 ± 0.50	4.96 ± 0.38	171.2 ± 29.1
10	143.9 ± 11.2	8.52 ± 0.91	5.57 ± 0.64	215.8 ± 22.8
25	143.5 ± 13.8	8.83 ± 1.64	5.86 ± 0.62	231.9 ± 47.7
50	143.2 ± 3.3	8.82 ± 1.03	5.90 ± 0.35	244.6 ± 25.7*

The PLLA was γ-irradiated at the dose of 10, 25 or 50 kGy, and then MC3T3-E1 cells were cultured on irradiated PLLA for 2 weeks using the micromass culture. The cell lysates were prepared, and protein, DNA, HYP contents and ALP activity of the cell lysate cultured on irradiated PLLA were determined. Values are means ± SD for 4 dishes. Significant difference compared with unirradiated PLLA at $^*p < 0.05$.

PLLA 5000 and PLLA 10 000 did not affect the cell proliferation (Fig. 4), but increased the cell calcification significantly (Fig. 5). The calcification of MC3T3-E1 cells was higher in the medium containing PLLA 5000 than PLLA 10 000.

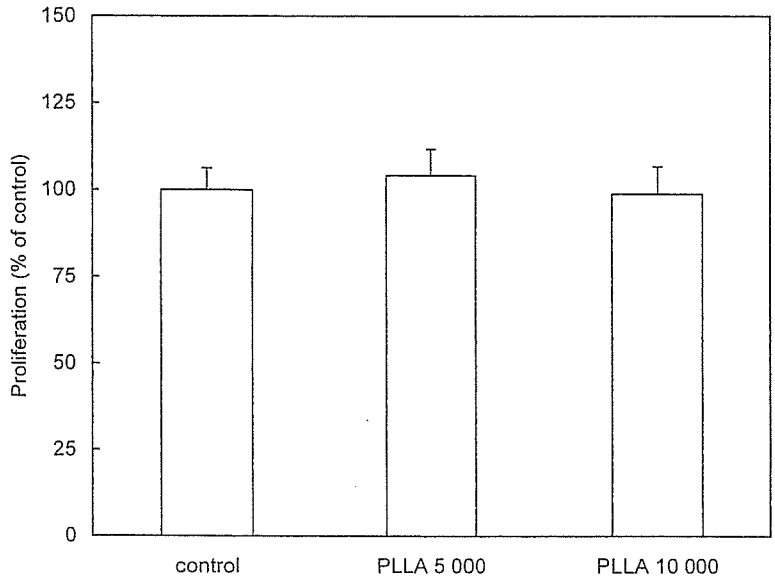

Figure 4. The proliferation of MC3T3-E1 cells cultured in the medium containing low molecular weight PLLA. MC3T3-E1 cells were cultured in the medium containing PLLA 5000 and PLLA 10 000 for 2 weeks using the micromass culture. The proliferation of MC3T3-E1 cells cultured in the medium containing low molecular weight PLLA was determined. Values are means ± SD for 4 dishes.

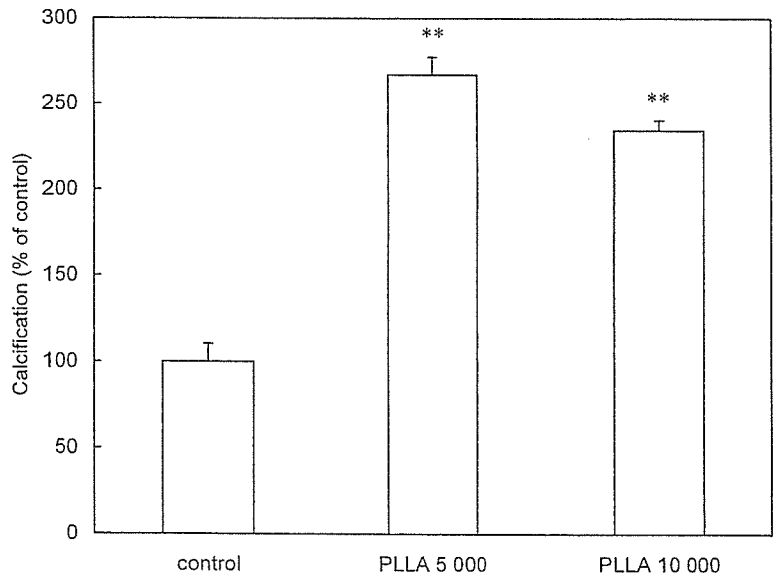

Figure 5. The calcification of MC3T3-E1 cells cultured in the medium containing low molecular weight PLLA. MC3T3-E1 cells were cultured in the medium containing PLLA 5000 and PLLA 10 000 for 2 weeks using the micromass culture. The calcification of MC3T3-E1 cells cultured in the medium containing low molecular weight PLLA was determined. Values are means ± SD for 4 dishes. Significant difference compared with control at $^{**}p < 0.01$.

DISCUSSION

The purpose of the present study was to clarify the effects of γ-ray irradiation to high molecular weight PLLA on the proliferation and differentiation of mouse osteoblasts *in vitro*. If the molecular weight of PLLA is decreased by γ-irradiation, the physiochemical properties of PLLA are changed resultingly. Yoshioka *et al.* [7, 8] reported that γ-irradiation caused random cleavage of molecular chain with hydrolysis of ester bonds, and the molecular weight of PDLLA decreased with increasing irradiation dose. In addition, they detected decomposition products having a molecular weight higher than lactic acid in alkali hydrolysis products of irradiated PDLLA, and they suggested crosslinkage of molecular chain also occurred. However, they did not detected tartronic acid which is a decomposition product formed by oxidation of the methyl group of lactic acid. Otto *et al.* also observed that the molecular weight of PLLA was decreased from 160 000 to 35 200 by γ-irradiation with the dose of 25 kGy [2]. In our study, the molecular weights (M_w and M_n) of irradiated PLLA decreased with increasing irradiation dose up to 50 kGy (Table 1). There was no change in FT-IR/ATR spectra of PLLA by γ-irradiation (data not shown). Zhang *et al.* [9] and Slivka *et al.* [10] reported that the γ-irradiation of poly(glycolide-lactide) copolymer and calcium phosphate/PLLA composite accelerated the rate of hydrolysis degradation *in vitro*, because of molecular chain scission by γ-irradiation. Thus, γ-ray irradiation decreases the molecular weight and changes the bioabsorbability of PLLA.

The proliferation and differentiation of MC3T3-E1 cells cultured on γ-ray irradiated PLLA were evaluated in this study. β-GP displays synergistic action with ascorbic acid to further stimulate collagen accumulation and ALP activity in osteoblasts, and mature osteoblasts require β-GP for mineralization [14]. The α-MEM medium already has contained the ascorbic acid of 50 μg/ml concentration. Then, we added 10 mM β-GP to the culture medium of MC3T3-E1 cells. Thompson and Puleo observed that the ALP activity, osteocalcin content and calcium amount of bone marrow stromal cells greatly rose in the later stage of culture. They indicated that the osteoprogenitor cells first differentiated into immatute osteoblasts characterized by the expression of ALP and then into mature osteoblasts characterized by the expression of osteocalcin and calcification [15]. Quarles *et al.* also reported that before attaining confluence MC3T3-E1 cells actively proliferated, but failed to express ALP activity and did not accumulate mineralized extracellular collagenous matrix at this stage. After the cultures underwent growth arrest with the attainment of confluence, ALP activity and mineralized extracellular collagenous matrix were expressed [14]. The cell density of the micromass culture is high, and the situation of the micromass culture is similar to the state of confluence at the initial stage of culture and formed 3-dimensional spheroids similar to the *in vivo* tissues. In fact, when equal number cells were cultured, ALP activity rose earlier in the micromass culture than low density monolayer culture (data not shown). Therefore,

the micromass culture was carried out for the present experiment, and the detection of the calcification of MC3T3-E1 cells succeeded in the short period of 2 weeks (Fig. 2).

Figure 1 suggested γ-ray irradiation to PLLA did not affect the proliferation of MC3T3-E1 cells cultured on irradiated PLLA. No changes in protein and DNA contents also proved that there was no effect of γ-irradiation on the proliferation of MC3T3-E1 cells cultured on irradiated PLLA (Table 2). On the other hand, the calcification of MC3T3-E1 cells cultured on irradiated PLLA increased with γ-irradiation to PLLA dose-dependently (Fig. 3). The increase of HYP content and ALP activity also proved that the application of γ-irradiation to PLLA stimulated the differentiation of MC3T3-E1 cells cultured on irradiated PLLA (Table 2). The molecular weights of PLLA were decreased by γ-irradiation; thus lower change in the molecular weight of PLLA would be responsible for enhancing the differentiation of MC3T3-E1 cells cultured on irradiated PLLA. Otto *et al.* reported when mouse osteoblastic cells were cultured with γ-irradiated PLLA wire for 48 h, DNA content did not change, but ALP activity increased by 28% [16]. They examined only PLLA in which the molecular weight was 21 500 after the γ-irradiation, and their results corresponded to our present results. Ikarashi *et al.* reported that heat treatment of PLLA did not affect protein and DNA contents of MC3T3-E1 cells cultured on heat treated PLLA, but HYP content and ALP activity were increased. The weight average molecular weight of PLLA decreased from 1 000 000 to 150 000 at 200°C for 2 h and to 20 000 at 250°C for 2 h, respectively. Further, they examined MC3T3-E1 cell responses cultured on the PLLA with low molecular weight of 20 000 for 2 weeks. HYP content and ALP activity remarkably increased in comparison with protein and DNA contents. They also described that lower change in the molecular weight of PLLA was a cause of stimulation of MC3T3-E1 cells cultured on the heat treated PLLA [6].

Low molecular weight PLLA, which was eluted from the PLLA disk to the culture medium, may influence the proliferation and calcification of MC3T3-E1 cells. Therefore, MC3T3-E1 cells were cultured in the medium containing low molecular weight PLLA. PLLA 5000 and PLLA 10 000 did not affect the proliferation (Fig. 4), but stimulated the calcification of MC3T3-E1 cells (Fig. 5). In our recent studies, low molecular weight PLLA were found to increase the ALP activity of MC3T3-E1 cells, dose-dependently, with no effect on the protein and DNA contents [17]. Ikarashi *et al.* reported that the PDLLA with molecular weight of 5000 and 10 000 did not affect protein, DNA and HYP contents, but increased ALP activity of MC3T3-E1 cells. They also indicated that low molecular weight PDLLA stimulated the differentiation of MC3T3-E1 cells [18].

Our results may have suggested that γ-ray sterilization is suitable for PLLA devices on the differentiation of osteoblasts. Otto *et al.* reported that newly bone was formed around the PLLA wire that was γ-irradiated with the dose of 25 kGy after 2 months as well as 6 months with intramedullary implantation [2]. Their

results indicated that γ-irradiated PLLA had satisfactory osteoconductive property *in vivo*. However, there were some reports that the mechanical properties were lowered with γ-irradiation of PLLA [9, 10]. We also reported that the tensile strength of PLLA and the particle size of the wear debris derived from PLLA were decreased by γ-irradiation of PLLA [19]. The suitability of γ-ray sterilization of PLLA devices should be judged by the synthetic evaluation of osteoconductivity and mechanical property of irradiated PLLA.

CONCLUSION

The effects of γ-ray irradiated PLLA on the proliferation and differentiation of mouse osteoblast-like MC3T3-E1 cells were examined. The molecular weight of irradiated PLLA decreased with increasing irradiation dose, and the γ-irradiation of PLLA stimulated the differentiation of MC3T3-E1 cells cultured on irradiated PLLA with no effect on the proliferation. These results suggested that lower change in molecular weight of PLLA was responsible for enhancing the differentiation of MC3T3-E1 cells cultured on γ-irradiated PLLA. In addition, the low molecular weight PLLA also did not affect the proliferation, but stimulated the calcification of MC3T3-E1 cells. The γ-ray sterilization would be suitable for PLLA devices, when satisfactory mechanical properties were maintained.

Acknowledgements

We thank Dr. Yoshiaki Ikarashi (Division of Medical Devices, National Institute of Health Sciences) for his helpful advice on this study.

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Materials Transactions, Vol. 43, No. 12 (2002) pp. 3122 to 3127
Special Issue on Biomaterials and Bioengineering

Biocompatibility of Various Kinds of Polymer Microspheres Estimated from Their Effect on Gap Junctional Intercellular Communication of Fibroblasts

Ryusuke Nakaoka * and Toshie Tsuchiya

Division of Medical Devices, National Institute of Health Sciences, Tokyo 158-8501, Japan

Gap junctional intercellular communication is a function that plays an important role in maintaining cell and tissue homeostasis and in regulating cell growth, development and differentiation. Change in this function when contacting fibroblasts with various polymer microspheres was estimated using the fluorescence recovery after photobleaching (FRAP) assay system. When the cells were in contact on test dishes, the inhibition level increased as the diameters of polystyrene microspheres decreased, except for a microsphere with 0.5 μm diameter. The function was inclined to be recovered with the increase of the incubation time, while it was not recovered when the cells were cultured with pre-coated polystyrene microspheres. As well as inhibitory activities of the function, cytotoxicity potentials of tested microspheres depended on their diameter and their composition. These findings suggest that the size and the physico-chemical character of polymer microspheres, and how cells recognize them plays important roles in causing influences of the microspheres on both gap junctional intercellular communication and their cytotoxicity. Therefore, estimating the inhibitory effect of biomaterials on the gap junctional intercellular communication will provide valuable information about the biocompatibility of materials even in the form of particles.

(Received August 26, 2002; Accepted September 9, 2002)

Keywords: *biocompatibility, gap junctional intercellular communication, polymer microspheres, fluorescence recovery after photobleaching analysis, cytotoxicity, mitochondrial activity*

1. Introduction

It is important for biomaterials to make them safer and more effective in order to use them for not only for tissue engineering studies but also studies of artificial organs or orthopedic devices. It is indispensable for biomaterial development to estimate their toxicity and dysfunction on cellular level as well as human body.

It is well known that biomaterials implanted into body, due to a severe environment of inside of body, cannot maintain their original shape, and even their desired function in the body, sometimes resulting in undesirable side effects. The most famous case is aseptic loosening of artificial joint observed in many patients who underwent operation of total joint replacement 5 to 25 years ago. Many researchers have reported that the aseptic loosening with associated periprosthetic bone resorption is partly due to activation of macrophages and osteoclasts by wear debris from artificial joint.[1–14] Macrophages stimulated with wear debris *in vitro* release significant amounts of inflammatory mediators such as interleukin-1, interleukin-6, prostaglandin E2, collagenase, and tumor necrosis factor.[6–14] The biological effect of wear debris may depend on the type of materials used as well as the shape, size, and the amounts of the debris.[4–11] Therefore, it is very important to estimate biocompatibility of biomaterials with not only their original shape but also possible transformed shapes after their usage.

During the last decade, we have been researching the inhibitory potential of many kinds of biomaterials on gap junctional intercellular communication (GJIC) to use the potential as an index for their biocompatibility.[15–18] GJIC is a function that plays an important role in maintaining cell and tissue homeostasis by exchanging low molecular weight molecules, that results in regulating cell growth, development and differentiation of cells.[19,20] Therefore, it is reasonable that disruption of this function is the cause of many kinds of diseases. In the previous report,[18] we have estimated an inhibitory activity of polymer microspheres, which were used as model wear debris from biomedical polymer *in vivo*, on the GJIC using a metabolic cooperation assay system that is a method for estimating the GJIC function of Chinese hamster-derived fibroblasts. We concluded that estimating the inhibitory activity of the microspheres on the GJIC might be useful for considering their side effects in body. In other words, it may be possible to predict whether wear debris causes aseptic loosening of artificial joint from estimating their effect on GJIC function. However, it must be noted that a distribution of coated microspheres in each test wells may be different in spite of the same amounts of coated microsphere, because the method in previous study was carried out without any special equipments useful for homogeneous coating. Different distribution of coated microspheres makes reproducibility of experiments poor because the probability of contacting cell and microspheres will be affected by the distribution. To solve this problem, we have employed fluorescence recovery after photobleaching (FRAP) analysis technique for estimating the GJIC function.[21] Using the FRAP analysis, the inhibitory potential of many kinds of polymer microspheres, as model wear debris, on the GJIC was assessed, and effects of characteristics of the microspheres and conditions of interaction between cells and microspheres on the GJIC were discussed.

2. Materials and Methods

2.1 Microspheres

Monodispersed polystyrene (PS) microspheres with different diameter were purchased and kindly supplied from Japan Synthetic Rubber Co., Ltd. (Tokyo, JAPAN). Low-density polyethylene (PE) and ethylene-acrylic acid copolymer (EA) microspheres were kindly supplied from Sumitomo Seika

*Corresponding author: E-mail: nakaoka@nihs.go.jp

chemicals Co., Ltd. (Tokyo, JAPAN). Poly (L-lactic acid) (PLLA, Mw = 3.3×10^5) was kindly supplied from Shimazu Co. (Kyoto, JAPAN) and its microspheres were prepared by a conventional solvent evaporation method.[22] The diameter of the microspheres was determined by Multisizer II (Coulter Electronics Inc., Hialeah, FL, USA).

2.2 Cell culture

Balb/3T3 clone A31-1-1 cells were kindly provided by Dr. T. Kuroki, University of Tokyo. The standard cell culture, cytotoxic assay and GJIC assay were performed using Eagle's MEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10 vol% of fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo Japan) in incubators under standard conditions (37°C, 5%-CO_2-95%-air, saturated humidity).

2.3 Cytotoxicity of microspheres

To estimate the effect of microspheres on cell function in detail, microspheres contacted cells with those upper or bottom side, as in the previous report.[18] Briefly, polymer microspheres were sterilized by dispersing in 70% ethanol solution, followed by centrifugation in sterile condition to remove ethanol. The microspheres were dispersed in sterile methanol or test medium at specified concentration. To contact microspheres from bottom of cells, suspension of microspheres in methanol was added into 35 mm glass bottom microwell dish (MatTek Co., Ashland, MA, USA), followed by drying the plates for overnight at room temperature. The obtained microsphere-coated dishes were subjected to the assays. For estimating effects of microspheres contacting upper side of cell on cytotoxicity and the GJIC function, the suspension in test medium was added to the dishes after adhesion of test cells.

Cytotoxicity of the microspheres was estimated by staining the cells in contact with the microspheres using crystal violet dye. A31-1-1 cells (1×10^4/100 μL Eagle's MEM-10% FCS) were cultured on microsphere-coated 96 well cell culture plates, or with microspheres after their adherence onto the non-coated plates. After 3-day incubation, aliquots of 100 μL methanol containing 4% crystal violet dye were added to each well to stain survived cells. After extraction of the dye from the stained cells into methanol, the absorbance of the extract at 600 nm was measured with Titertek Multiscan Plus MK II (Flow Laboratories Inc., McLean, VA, USA) and the absorbance of subjected extracts were compared with that of extracts without microspheres.

2.4 Measurement of GJIC activities

The obtained dishes were subjected to fluorescence recovery after photobleaching (FRAP) analysis in order to estimate inhibitory activity of microspheres on the GJIC. FRAP analysis was carried out according to a procedure reported by Wade *et al.*[21] with some modifications. Briefly, A31-1-1 cells (2.5×10^5/2.5 mL Eagle's MEM-10% FCS) were plated on untreated and microsphere-coated 35 mm glass bottom dishes. After cell attachment, microsphere suspension in the medium was added to the untreated dishes for estimating the effect of microsphere addition over test cells, and the dishes were incubated for 1 to 3 days. After washing with phosphate buffer saline solution containing $MgCl_2$ and $CaCl_2$ (PBS(+)), the cells were incubated for 10 min at room temperature in PBS(+) containing 5,6-carboxyfluorescein diacetate (7 μg/mL, excitation 488 nm and emission 515 nm). After washing excess extracellular dye by PBS(+), the cells in the test dishes in PBS(+) were subjected to the FRAP analysis. For a control experiment, cells were inoculated on an untreated glass bottom dish and treated with the same procedure with the tested cells. Cells contacting with test microspheres and at least two other cells were subjected to FRAP analysis under Ultima-Z confocal microscopy (Meridian Instrument, Okemos, MI) with a 10 times objective lens at room temperature. The cells were photobleached with 488 nm beam and recovery of their fluorescence intensity was subsequently monitored at 1-minute intervals for a total period of 4 minutes. The results were calculated from obtained data from more than 7 independent cells and expressed as the average of fluorescence recovery rate ± their standard deviation. The results were treated statistically with Student's t-test.

2.5 Western blotting of connexin 43

Immunoblots of connexin 43, which A31-1-1 cells express as gap junction protein, were performed according to the method proposed by Hayashi *et al.*[23] The cells on the test film were lysed by treatment with 20% SDS aqueous solution containing 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM leupeptin, 1 μM antipain, 10 mM iodoacetamide, 0.1 mM sodium orthovanadate and 5 mM sodium fluoride. After sonicating the lysates with 10 seconds pulse for three times using a probe sonicator (Sonics and Materials Inc., Danbury, CT, USA), their protein concentrations were determined by using BCA protein assay reagent (Pierce, Rockford, IL, USA). The lysate was mixed with equal volume of Laemmli sample buffer and proteins were separated on 7.5% SDS polyacrylamide gels and transferred to PVDF membranes. Connexin 43 was detected using rabbit polyclonal anti-connexin 43 antibody (Zymed, San Francisco, CA, USA), followed by incubation with secondary horseradish peroxidase-conjugated antibody and detection with the ECL chemiluminescent detection reagent (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK).

3. Results

3.1 Cytotoxicity of microspheres

Figures 1 and 2 showed cytotoxicity of three kinds of PS microspheres after 3-day incubation when they were added to A31-1-1 wells after cell adhesion or coated onto test wells before cell seeding. When cells were attached to the microspheres after adhering to the bottom of the wells, it was obvious that microspheres' cytotoxicity depended on their amounts and sizes. An increase in the amounts of microspheres added resulted in an enhancement of their cytotoxicity. In addition, their cytotoxicity also became stronger if their diameter was decreased. On the other hand, when the microspheres were coated onto test wells, their cytotoxicity did not depend on their amounts when their diameter was larger than 1 μm. When the microspheres with 10 μm diameter were precoated, the cell viability increased slightly. Other kinds of microspheres, PE, EA and PLLA, showed less cytotoxicity compared with the PS microspheres of comparable diameters

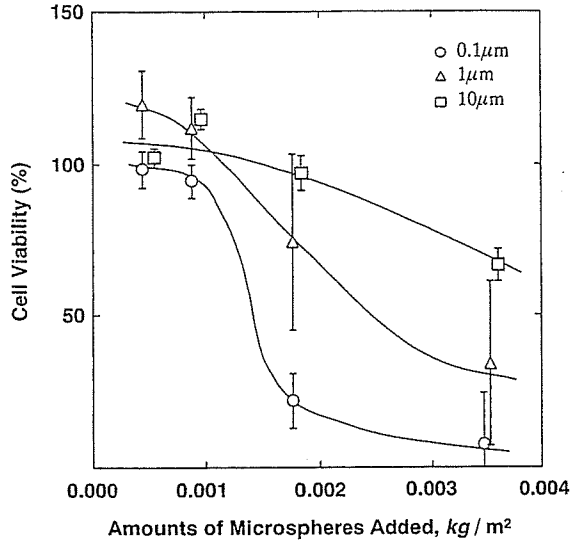

Fig. 1 Cytotoxicity of added PS microspheres on A31-1-1 cells detected after 3-day incubation using 96 well culture plates. The diameter of tested microspheres was 0.1 (circle), 1 (triangle) and 10 μm (square), respectively.

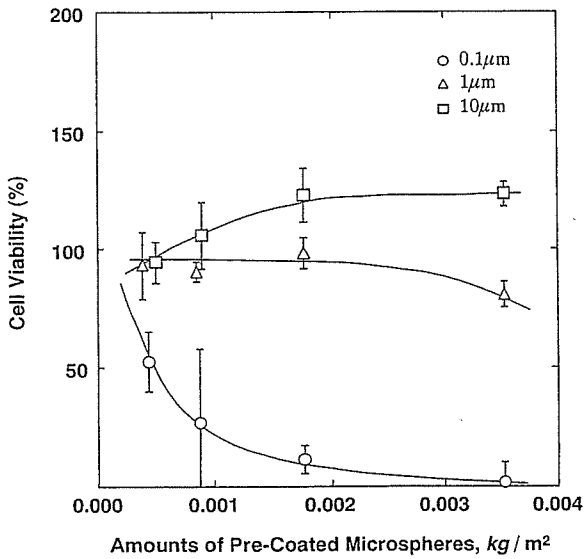

Fig. 2 Cytotoxicity of pre-coated PS microspheres on A31-1-1 cells detected after 3-daysincubation using 96 well culture plates. The diameter of tested microspheres was 0.1 (circle), 1 (triangle) and 10 μm (square), respectively.

(data not shown). Calculating from these results, we adopted 0.0001 kg/m^2 (0.1 mg/35 mm glass bottom dish) as an amount of microspheres for FRAP assay, in which amount more than 70% of the cells in contact with the microspheres might survive during the assay.

3.2 Effect of microspheres on GJIC function

By using the FRAP technique, a slight inhibitory effect on the GJIC was observed after 1-day incubation when PS microspheres of 1 μm diameter were added directly into the medium after cell adhesion, although the activity disappeared after 3-day incubation, as shown in Fig. 3. When test cells were cultured on wells pre-coated by PS microspheres with various diameters, the GJIC function was inhibited, as shown in Fig. 4. This figure indicates that the inhibitory activity

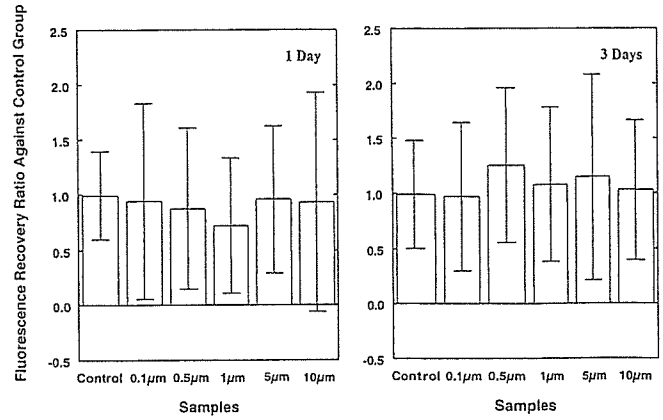

Fig. 3 Effect of the diameter of added PS microspheres on GJIC function of A31-1-1 cells detected by FRAP analysis.

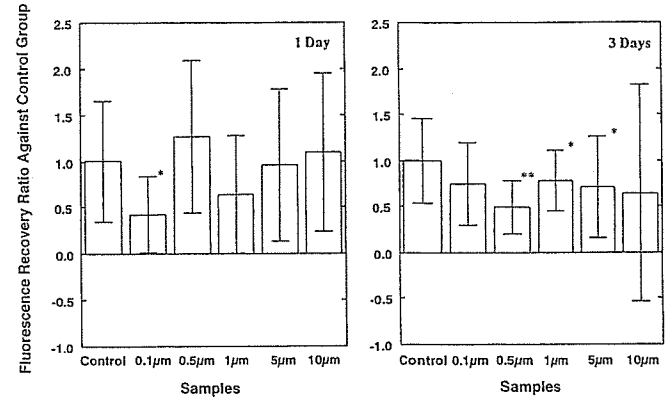

Fig. 4 Effect of the diameter of pre-coated PS microspheres on GJIC function of A31-1-1 cells detected by FRAP analysis. (*$p < 0.05$, **$p < 0.01$).

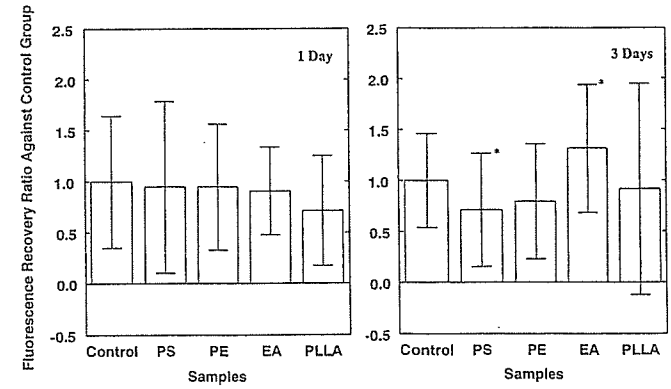

Fig. 5 Effect of various compositions of pre-coated microspheres on GJIC function of A31-1-1 cells. (*$p < 0.05$) The diameter of PS, PE, EA and PLLA microspheres was 5, 6.4, 9 and 6.8 μm, respectively.

of the microspheres decrease as their diameter increase after 1-day incubation, except for 0.5 μm PS microsphere. After incubation for 3 days, the microspheres inhibited the GJIC function, irrespective to their diameter.

Figure 5 shows the results of the GJIC inhibition induced by various kinds of pre-coated microspheres with diameter ranging from 5 to 9 μm. After 1-day incubation, only PLLA microspheres showed inhibitory activity on the GJIC function. After 3-day incubation, while PS and PE microspheres showed the inhibitory activity, the GJIC function inhibited by

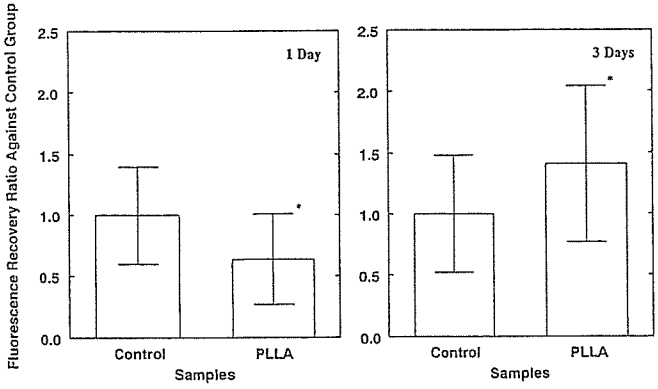

Fig. 6 Effect of added PLLA microspheres on GJIC function of A31-1-1 cells. (*$p < 0.01$) The diameter of PLLA microspheres was 6.8 μm.

PLLA was recovered to a normal level. On the other hand, EA microspheres did not show the inhibitory activity after 1-day incubation and were likely to enhance the GJIC function after 3-day incubation.

Figure 6 shows the effect of PLLA microspheres added to adherence cells on the GJIC function. After 1-day incubation, the GJIC function was suppressed to about 70% of normal cells. However, after 3-day incubation, the function was enhanced rather than inhibited.

3.3 Expression of connexin 43 in A31-1-1 cells contacting with various microspheres

Figure 7 shows an expression of connexin 43 protein in A31-1-1 cells interacted with various pre-coated PS microspheres for 1 to 3 days. The expression levels of connexin 43 in the cells contacting with 0.1 μm PS microspheres, of which inhibitory activity on the GJIC was found the strongest, were decreased after 1-day incubation. Although differences in the inhibitory activity of 5 kinds of the PS microspheres on the GJIC were observed after 1-day incubation in Fig. 4, phosphorylated states of expressed connexin, which are related with its function in the GJIC, were almost similar after 1-day incubation. On the other hand, after 3-day incubation, inhibitory level on the GJIC function was the strongest with 0.5 μm PS microspheres as shown in Fig. 4. However, differences of connexin 43 expression in the cells contacting with polystyrene microspheres with different diameters could not be observed.

4. Discussion

Because a coating method in this study was carried out without any special equipments, there may be differences in microsphere distribution on test wells that affect a reproducibility of experimental results of the GJIC. We applied the FRAP analysis in this study for estimating the GJIC function of cells, because the FRAP analysis can choose the cell contacting with microspheres and can be used to estimate time profiles of the GJIC changes, as shown in this study. Before adapting this analysis, we checked cytotoxicity of microspheres in order to determine the adequate amounts of coating for the analysis. In this study, we found out that a cytotoxicity of PS microspheres with 10 μm diameter decreased as the pre-coated amounts increased. Microscopic observation suggested that the microsphere with 10 μm diameter could not be phagocytosed by the cell, resulting in an increase of surface area where the cell could adhere. From these results, we employed 0.1 mg of microspheres per 35 mm glass bottom dish as the amounts for estimating their inhibitory activity on the GJIC function without any cytotoxic effects.

The PS microspheres showed inhibitory activities on the GJIC function when they were pre-coated onto a test dish although their cytotoxicity was not observed at this amount. The result obtained after 1-day incubation shown in Fig. 4 is consistent with our previous study.[18] However, the expression status of connexin 43 in the cells contacted with PS microspheres for 1 day was almost similar in all cases, inconsistent with their inhibitory activities on the GJIC function. In addition, a change in the phosphorylated status of the connexin was hardly indicated after 3-day incubation with PS microspheres. It suggests that inhibitory action of PS microspheres is not due to the change in the connexin status. From our previous study,[24] however, the expression of connexin 43 in fibroblasts cultured on a PE film was apparently different from that on a normal culture dish, suggesting that an interaction between the cell and PE surface resulted in irregular intracellular signal transduction, followed by abnormal expression of connexin. It was reported that the phosphorylated state of connexin 43 was important for its accurate assembly and distribution inside the cell. Why the different expression status could not be observed from Fig. 7, as observed in the previous study, may be due to a small contact area of the microspheres with the fibroblasts, compared to the polymer film. This small contact area might induce a small change in the connexin status that is hardly observed by western blotting. However, it is probable that another mechanism including other protein molecules has a responsibility for the inhibition of the GJIC in contact with the microspheres. To discuss the effect of their diameter on the GJIC function, we estimated the change in an activity of A31-1-1 cells contacting with the PS microspheres with various sizes using Alamar Blue™ reagent[25] (BioSource International, Inc., Camarillo, CA, USA), which incorporates an oxidation-reduction indicator based on detection of a mitochondrial activity in a cell. Compared with the results from cytotoxic study of the microspheres shown in Figs. 1 and 2, the mitochondrial activity of each cell was enhanced when the cell contacts with microspheres for 3 days that is suggested from the results shown in Figs. 8 and 9. Especially, when the microspheres were pre-coated, their effect on this mitochondrial activity was enhanced. When the microspheres were pre-coated, a decrease in microsphere diameter enhanced the metabolic activity, while cells in contact with 0.1 μm added PS microspheres showed little change in the activity per cell. In addition, it is known that the size of the microspheres plays an important role in phagocytosis[26] and fibroblasts can phagocyte the small substances like particles. Therefore, this size effect may also play an important role in different GJIC inhibition induced by pre-coated microspheres of different sizes. The synergistic effect of their size effect on phagocytosis and the mitochondrial activity may result in lower GJIC inhibition of 0.5 μm PS microspheres.

It is interesting to note that increase in interacting time of cells with microspheres may influence microsphere effects on

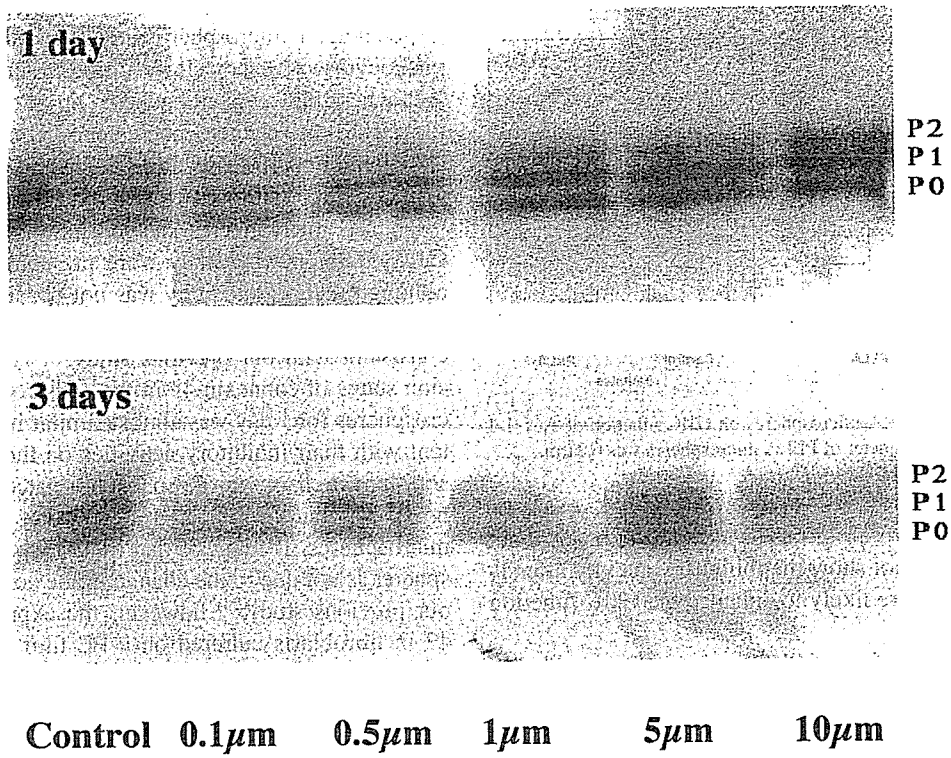

Fig. 7 Effect of pre-coated PS microspheres' diameter on the expression of connexin 43 in A31-1-1 cells. Non-phosphorylated connexin is expressed as P0 and phosphorylated connexin is expressed as P1 or P2.

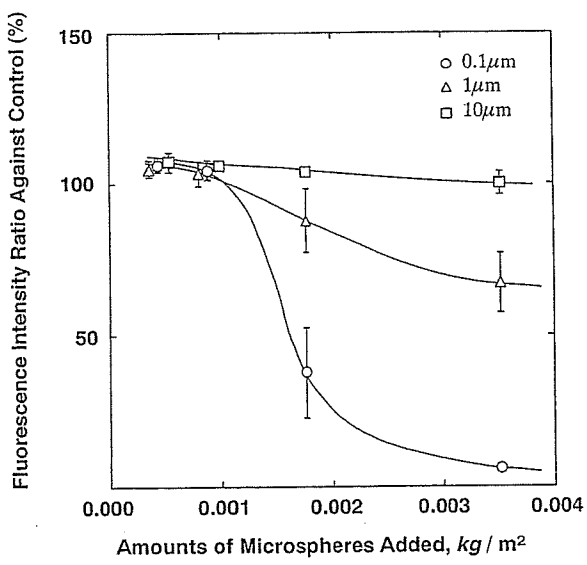

Fig. 8 Effect of added PS microspheres on a mitochondrial activity of A31-1-1 cells detected after 3-day incubation. The diameter of tested microspheres was 0.1 (circle), 1 (triangle) and 10 μm (square), respectively.

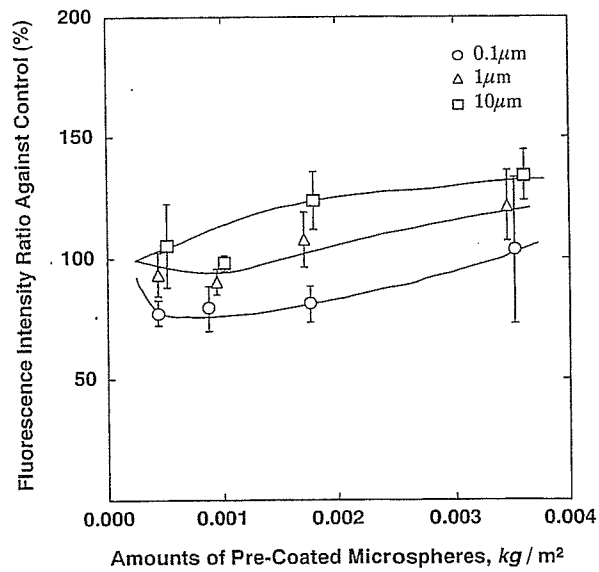

Fig. 9 Effect of pre-coated PS microspheres on a mitochondrial activity of A31-1-1 cells detected after 3-day incubation. The diameter of tested microspheres was 0.1 (circle), 1 (triangle) and 10 μm (square), respectively.

cell function. Recovery of the inhibitory activity induced by PLLA and EA microspheres after 3-day incubation indicates that interacting time of cells with microspheres is an important factor on the GJIC function. On the other hand, PE and PS increase the inhibitory activity as the interacting time increases. These findings suggest that a characteristic of microspheres, as well as the interacting time, affects the GJIC inhibition induced by contact with the microspheres. In addition, this study indicated that added microspheres showed different inhibitory activity from pre-coated microspheres on the GJIC function although other experimental factors were the same. This suggests that the condition of cellular contact to the materials is also one of the important factors to arise the effect of the microspheres on cell function. In our previous study, the inhibitory activity of added PLLA microspheres could not be detected by metabolic cooperation (MC) assay system.[18] Moreover, the inhibitory activity of microspheres could not be found out when the microspheres were added after cell attachment on test dishes by the MC assay system. However, the inhibitory activity was detected using the FRAP analysis in this study. These suggest that the FRAP analysis is a suit-

able method for detecting the GJIC changes of test cells in contact with microspheres.

Osteoblastic cells are not only important cells in bone resorption as they play an important role in bone formation, but also an essential role for differentiation of precursor cells into osteoclasts,[27] which play an important role in bone resorption induced by wear debris from artificial hip joint. To consider the bone resorption induced by wear debris, it is natural to study not only macrophages and osteoclasts but also osteoblastic cells. It has been reported that osteoblastic cells have the GJIC function and the function is believed to be critical for the coordinated cell behavior necessary in bone tissue development.[28–30] It was reported that enhancing the GJIC function of osteoblasts by connexin gene transfection induced up-regulation of osteocalcin and bone sialoprotein gene transcription.[30] In addition, wear debris particles have been reported to suppress collagen and glycosaminoglycan synthesis from osteoblasts when the particles are included in the osteoblasts.[14,31] Therefore, it should be important to discuss effects of wear debris on the GJIC function using microspheres as model wear debris and fibroblasts as model cells which make gap junction of connexin 43 with neighbor cells. Recently, we have been studying effects of microspheres made from many biomaterials on the GJIC and differentiation of normal human osteoblastic cells. The study has suggested that effects of the microspheres on the GJIC within 1-day contact have more critical influences on the differentiation of the osteoblastic cells than those on the GJIC during the later contact period. This indicates that estimating the effects of biomaterials on the GJIC of the osteoblastic cells is very useful to determine the biocompatibility, including the effect on cell differentiation, of biomaterials, even in particle form. In the near future, we will report and discuss about these results.

5. Conclusion

The size and the composition of polymer microspheres, and their contact condition with cells affects the GJIC function of the cells, which plays an important role in maintaining cell and tissue homeostasis and regulating cell growth, development and differentiation. This finding suggests the possibility that biocompatibility of biomaterials, even in a particle form, can be estimated by analyzing their effect on the GJIC function of cells.

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Neuroscience Letters 324 (2002) 141–144

Neuroscience Letters

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A highly conserved tryptophane residue indispensable for cloned rat neuronal P2X receptor activation

Ken Nakazawa[a,*], Hiloe Ojima[a], Yasuo Ohno[b]

[a]Cellular and Molecular Pharmacology Section, Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

[b]Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

Received 31 January 2002; received in revised form 27 February 2002; accepted 28 February 2002

Abstract

The role of a tryptophane residue (Trp^{256}) in the extracellular loop of a neuronal P2X receptor clone ($P2X_2$ receptor/channel) was investigated using site-directed mutagenesis and *Xenopus* oocyte expression. When Trp^{256} was replaced with leucine, serine or phenylalanine (W256L, W256S or W256F), a current response to adenosine triphosphate (ATP) mediated through the $P2X_2$ receptor/channel was abolished. When replaced with tyrosine (W256Y), the response was not abolished, but a reduced current response to ATP was observed. The insertion of a tryptophane residue in W256L at positions close to position 256 failed to recover the responsiveness to ATP. These results suggest that an amino acid residue with a side chain of an aromatic ring with a hydroxy group (tryptophane or tyrosine) is necessary exactly at position 256 for $P2X_2$ receptor/channel activation. © 2002 Published by Elsevier Science Ireland Ltd.

Keywords: P2X receptor; Adenosine triphosphate; Ion-channel; *Xenopus* oocyte; Site-directed mutagenesis; Amino acid side chain; Protein expression

P2X receptors are ion channel-forming proteins that are activated by extracellular adenosine 5′-triphosphate (ATP), and its roles in excitatory neurotransmission have been demonstrated in both brain and peripheral neurons [3,9]. To date, at least seven subclasses ($P2X_{1-7}$) have been cloned, and they have been shown to form homo- or heteromeric receptors which act as functional ionic channels [14]. The analysis of the hydropathy profiles of amino acid sequences of P2X receptors has shown that each subclass consists of two transmembrane domains (TM1 and TM2) and one long extracellular loop between them (E1). As the ATP molecule is highly hydrophilic, it is assumed that ATP binds to the E1 region, and this assumption has also been supported by amino acid sequence homology between the E1 loop of P2X receptors and the ATP-binding domain of aminoacyl-tRNA synthetases [7]. Recent reports have demonstrated that positively charged amino acid residues in the E1 loop near the channel pore are involved in the activation by ATP, presumably by recognizing the negative charge of the phosphate group of an ATP molecule [6,8]. P2X receptors are highly selective to ATP among nucleotides, suggesting that the adenine moiety is recognized upon the receptor activation. However, the region recognizing this moiety has not been determined. We previously reported that two highly conserved glycine residues (Gly^{247} and Gly^{248}) in a glycine-rich region of the E1 loop are necessary for the activation of the $P2X_2$ receptor [11], a neuronal clone of P2X receptors [2]. In aminoacyl-tRNA synthetases, it has been speculated that an aromatic amino acid residue (phenylalanine) serves for the recognition of adenine moiety of an ATP molecule through aromatic–aromatic interaction from crystal structure analysis [1,4,10]. For the present study, we examined the roles of two aromatic amino acid residues (Phe^{240} and Trp^{258}), the residues closest to the glycine-rich region in either side that are completely conserved throughout seven subclasses of P2X receptor.

The expression of cloned and mutant $P2X_2$ receptor and the recordings of ionic current through the channels were performed according to our previous reports [12,13]. Briefly, $P2X_2$ receptor mutants were constructed from the cloned $P2X_2$ receptor [2] by site-directed mutagenesis. The wild type and the mutant channels were expressed in *Xenopus* oocytes for a 4-day-incubation at 18 °C, and the oocytes were served for membrane current measurements. Oocytes

* Corresponding author. Tel.: +81-3-3700-9704; fax: +81-3-3707-6950.

E-mail address: nakazawa@nihs.go.jp (K. Nakazawa).

0304-3940/02/$ - see front matter © 2002 Published by Elsevier Science Ireland Ltd.

PII: S0304-3940(02)00234-3

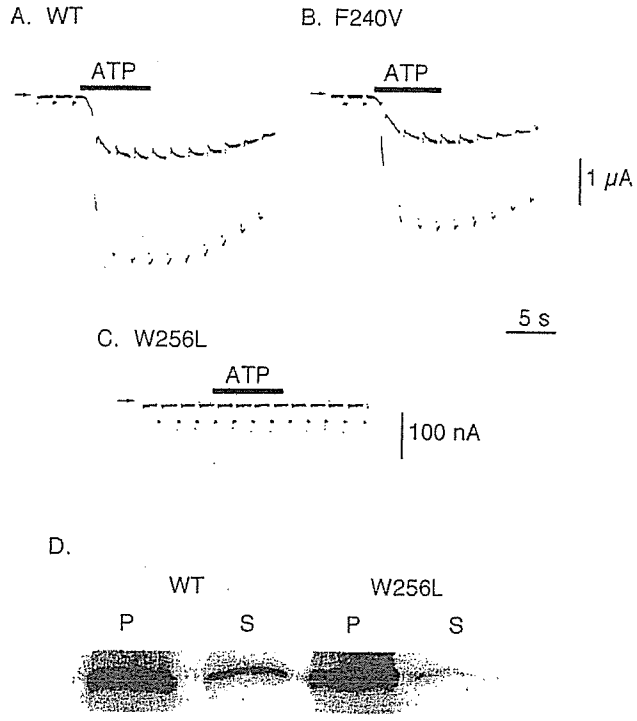

Fig. 1. (A–C) Membrane current recorded from *Xenopus* oocytes injected with cRNAs encoding: (A), wild type (WT); (B), F240V; and (C), W256L $P2X_2$ receptor/channels. The oocytes were held at −50 mV and stepped to −80 mV for 400 ms every 2 s, and ATP (100 μM) was applied. ATP evoked an inward current in the oocytes injected with the wild type or F240V channel cRNA, but not in those with the W256L cRNA. Arrows indicate zero current level. (D) Immunoblotting analysis using anti-$P2X_2$ receptor antibody. A section corresponding to $P2X_2$ receptor protein (about 65 kD) was shown to compare the expression in precipitation (P) and supernatant (S) fractions prepared from the oocytes injected with the WT or W256L channel cRNA.

were bathed in ND96 solution containing: 96 mM NaCl; 2 mM KCl; 1.8 mM $CaCl_2$; 1 mM $MgCl_2$; 5 mM HEPES (pH 7.5 with NaOH). ATP (ATP disodium salt; Sigma, St. Louis, MO) was applied by superfusion for about 6 s with a regular interval of 1 min. The expression of channel protein was also confirmed by immunoblotting analysis. The homogenate of oocytes (20 oocytes for the wild type channel and each mutant) was suspended in a protease and phosphatase inhibitor cocktail (Sigma, general use) diluted in a buffer containing 20 mM Tris–HCl, 2 mM EDTA (disodium salt), 0.5 mM EGTA and 320 mM sucrose, and centrifuged at 1000 × *g* for 10 min. The supernatant was then centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant was stocked and the precipitation was rinsed with a sucrose-free cocktail buffer by centrifuging again at 20,000 × *g* for 30 min at 4 °C. The precipitation was suspended in a sucrose-free cocktail solution, and this suspension and the stocked supernatant were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting. By using $P2X_2$ receptor antibodies (Oncogene, Boston, MA) and anti-rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey; Amersham, Little Chalfont, UK), correct channel expression was detected as a 65 kD band.

Fig. 1A–C shows membrane currents measured from oocytes injected with cRNAs encoding: (A), wild type; (B), F240V; and (C), W258L channels. The oocytes were held at −50 mV and stepped to −80 mV for 400 ms every 2 s. ATP (100 μM) activated an inward current of about 1 μA at −50 mV in the oocytes injected with the wild type channel cRNA (Fig. 1A). A similar inward current was observed in the oocytes injected with F240V channel cRNA (Fig. 1B), but not in those injected with W258L channel cRNA (Fig. 1C). Fig. 2 compares the concentration–response relationship for the ATP-activated current. The current mediated through the F240V channel was comparable with that

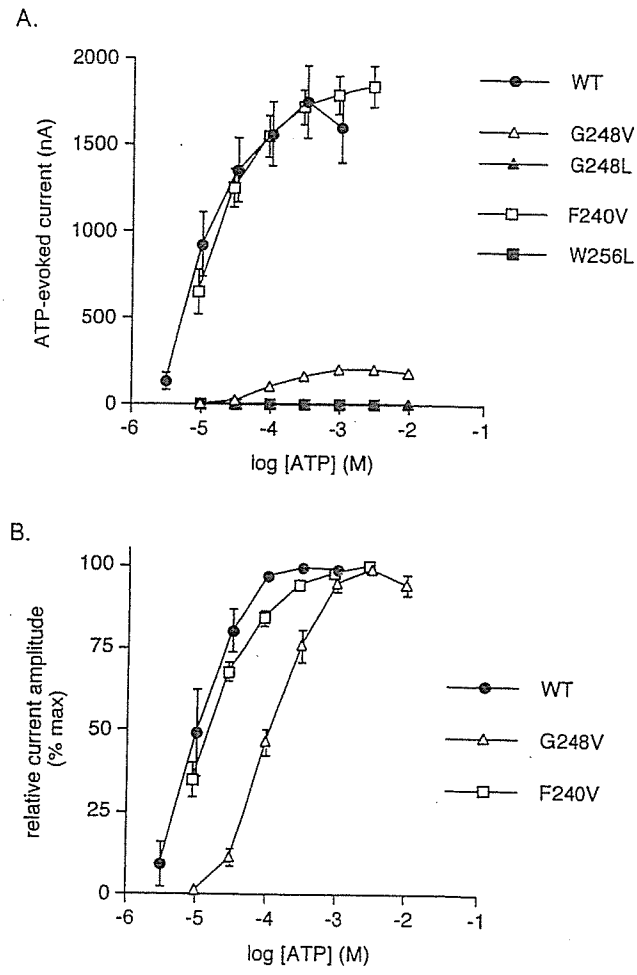

Fig. 2. (A) Concentration–response relationships for ATP-evoked current in oocytes injected with cRNAs encoding the wild type and mutant $P2X_2$ receptor/channels. ATP-evoked current was recorded as in Fig. 1A–C, and the current responses at −80 mV were compared. The oocytes injected with G248L or W256L channel cRNA did not respond to ATP. Each symbol and bar indicate mean and SE obtained from 4–6 oocytes tested. (B) Normalized concentration–response relationships. The data shown in (A) were normalized to the maximal ATP-evoked current in individual oocytes.

through the wild type channel in both amplitude (Fig. 2A) and the sensitivity to ATP (Fig. 2B). In contrast, ATP failed to activate a detectable inward current up to 10 mM in oocytes injected with W258L channel cRNA (Fig. 2A). Mutant channels with substitutions of Gly^{248} were also examined for comparison. When Gly^{248} was replaced with valine (G248V), the maximal current amplitude was reduced to about one-tenth (Fig. 2A), and the sensitivity to ATP was reduced by about ten-fold (Fig. 2B). The ATP-activated current was abolished when Gly^{248} was replaced with leucine (G248L; Fig. 2A). These results support our previous finding of essential roles of this as well as neighboring Gly^{247} residue in channel activation by ATP [11].

Tests were made to determine which amino acid residue can compensate for the channel activation in the place of Trp^{256}. The side chain of tryptophane involves an aromatic ring (benzimidazole) and a hydroxy group substituted at the ring. When replaced with serine (W256S) or phenylalanine (W256F), an amino acid whose side chain involves either a hydroxy group or an aromatic ring (benzene), the channel activity was not observed (Fig. 3A), suggesting that either a hydroxy group or an aromatic ring alone in the side chain is not sufficient for the channel activation. In contrast, when replaced with tyrosine (W256Y), an amino acid whose side chain involves both a hydroxy group and an aromatic ring (benzene), the channel activity appeared; the maximal amplitude of the ATP-activated current through W256Y channel was about half as large as that through the wild type channel (Fig. 3A), and the sensitivity of this mutant channel to ATP was about five-fold lower than that of the wild type channel (Fig. 3B).

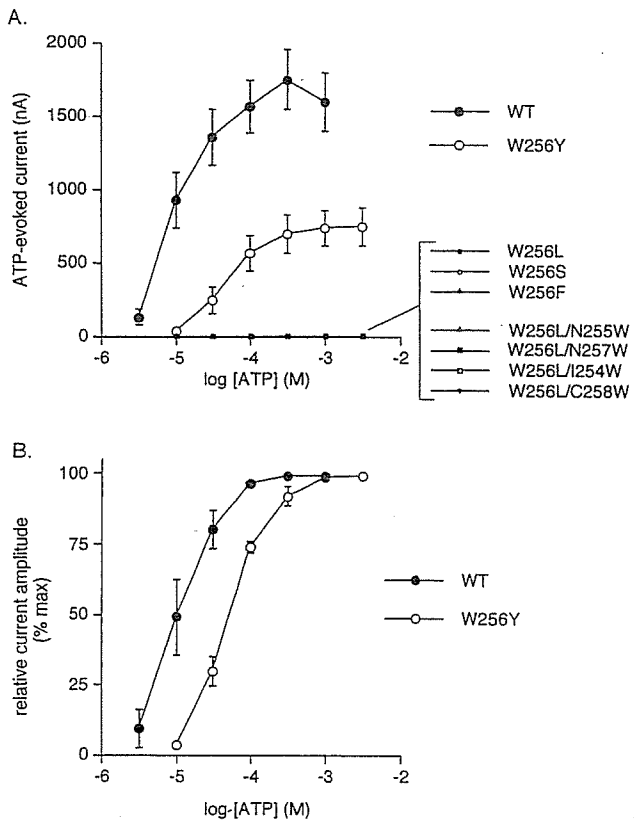

Fig. 3. (A) Comparison of ATP-evoked current responses for the wild type and W256 mutant $P2X_2$ receptor/channels. Concentration–response relationships were shown as in Fig. 2A. Each symbol and bar indicate mean and SE obtained from 4–6 oocytes tested. (B) Normalized concentration–response relationships. The data shown in (A) were normalized to the maximal ATP-evoked current in individual oocytes.

We next examined whether or not a tryptophane residue inserted near the position 256 can substitute for the channel activation role of Trp^{256}, by replacing amino acid residues in W256L mutant channel. When the neighboring residues (Asn^{255} and Asn^{257}) were replaced with tryptophane (W256L/N255W and W256L/N257W), the channel activity was not observed (Fig. 3A). It has been speculated that the region involving Trp^{256} forms a β-sheet [7]. If this is the case, the residues closest to Trp^{256} are not neighboring residues that are stretched to the opposite direction, but the residues next to the neighboring ones (Ile^{254} and Cys^{258}) are closest. Thus, we also examined the channel activity of the mutants with the replacement of these residues with tryptophane (W256L/I254W and W256L/C258W). The channel activity was, however, not found for these mutant channels (Fig. 3B).

The expression of the W256L channel was confirmed by immunoblotting analysis (Fig. 1D). A 65 kD band corresponding to $P2X_2$ receptor subunits was found in the precipitation prepared from the oocytes injected with W256L channel cRNA, which was comparable with that from the oocytes expressing the wild type channel. This result suggests that processes until protein translation are not hampered by this mutation, and that W256L channel protein is expressed but not functional. Similar 65 kD bands were also found for W256L/N255W, W256L/N257W and G247A, which were mutants with no channel activity.

The present study has shown that the regular activation by ATP of $P2X_2$ receptor/channel requires a tryptophane residue exactly at the position 256. Since tyrosine, but not serine or phenylalanine, partially substituted for this tryptophane (Fig. 3A), both an aromatic ring and a hydroxy group may be necessary in the side chain at this position for the channel activation. These findings may provide structural information about P2X receptors, which is helpful for better understanding of neuronal modulations of neuronal and non-neuronal cells through these receptors. It is not clear at present whether or not this indispensable role of Trp^{256} in the channel activation is related to the recognition of ATP molecules as shown for phenylalanine residues in aminoacyl-tRNA synthetases [1,4,10]. Even if Trp^{256} contributes to the molecular recognition of ATP, the intermolecular relation, however, may not be simple aromatic–aromatic interaction because phenylalanine failed to substitute for Trp^{256} (Fig. 3A). As tryptophane is the most bulky side chain [5] with a hydroxy group, Trp^{256} may form a hydrogen bond

with some distant amino acid residue, and thereby may stabilize the structure of the functional $P2X_2$ receptor, which leads to physiological roles of this receptor/channel, including membrane depolarization and Ca^{2+} permeation [9]. The indispensable role of Trp^{256} for the function of the $P2X_2$ receptor may be characteristic for this or other P2X receptors, which enables for extracellular ATP to activate the receptors without affecting other membrane proteins. Alternatively, a similar (and unidentified) role of tryptophane residues may also be found for other ATP-binding proteins as a common feature.

The authors are grateful to Dr N. Murayama and Y. Shigemoto-Mogami for helpful suggestions on experiments, and Professor K. Takeuchi and Dr R. Ishii-Nozawa of Meiji Pharmaceutical University for providing H.O. an opportunity to join this work and continuous encouragement. This work was partly supported by a Health Science Research Grant for Research on Environmental Health and a "Nanomedicine" project grant from the Ministry of Health, Labour and Welfare, Japan awarded to Y.O. and K.N. and a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture, Japan awarded to K.N.

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