morphology of fibroblast on these surfaces will be discussed.

MATERIALS AND METHODS

Preparation of the copolymers and copolymer-coated surfaces

D,L-lactide was kindly supplied by Musashino Chemical Laboratory (Tokyo, Japan) and was recrystallized from ethyl acetate. BMA (Wako Pure Chemical Co. Ltd., Osaka, Japan) and 2-isocyanate ethyl methacrylate (IEMA, Showa Denko Co., Tokyo, Japan) were distilled at reduced pressure. *n*-Dodecanol, stannous octoate (Sn-(oct)$_2$), and dibutyltin dilaurate (DBTL) were purchased from Wako Pure Chemical Co. Ltd. and used without further purification. 2-Methacryloyloxyethyl phosphorylcholine (MPC) was synthesized and purified by a method previously reported (5). Other reagents were commercially available and used without further purification.

Poly(D,L-lactic acid) (PDLA) macromonomer was prepared as follows: polymerization of D,L-lactide using *n*-dodecanol as an initiator and methacrylation of the terminal hydroxyl group of PDLA via a urethane bond. The preparative route is shown in Fig. 1. A typical example for the synthesis is given below. A total of 10 g (69 mmol) of D,L-lactide and 0.65 g (3.5 mmol) of *n*-dodecanol were added into a round-bottomed flask with a magnetic stirring bar, and the flask was evacuated overnight. After removing water, 130 µl (0.1 g/mL, 0.045 mol% based on D,L-lactide) of a toluene solution of Sn-(oct)$_2$ was added, and the flask was evacuated again for several hours. After removing the toluene, the reaction mixture was heated at 150°C for 1 h. After cooling, the mixture was dissolved in chloroform and then poured into an excess of hexane to obtain PDLA as a white powder.

As shown in Fig. 1, 8.0 g (2.7 mmol) of the PDLA was dissolved in 40 mL of toluene, and IEMA (1.9 mL, 13.7 mmol) and DBTL (0.5 mL) were added to the solution. The mixture was kept at 65°C for 6 h. After the reaction had finished, toluene was evaporated under reduced pressure and the residual fluid was poured into an excess of hexane to obtain the PDLA macromonomer. The structure of the PDLA macromonomer was confirmed by Fourier transform infrared spectroscopy (FT-IR) (VALOR-III, Jasco, Tokyo, Japan) and proton nuclear magnetic resonance (^{1}H-NMR) (JEOL α-500, Tokyo, Japan). Yield: 90%. IR (KBr, cm^{-1}): 1758 (ester), 1638 (double bond), 1560 (urethane). ^{1}H-NMR ($CDCl_3$, ppm): δ 0.85 (t, 3H, CH_3 for *n*-dodecanol), 1.23 (m, 2H ×11, CH_2 for *n*-dodecanol), 1.52–1.55 (m, 3H ×30, CH_3 for PDLA), 1.92 (s, 3H, CH_3 for methacryloyl), 3.48–4.20 (t, 2H ×2, CH_2CH_2 for IEMA), 4.72 (s, 1H, urethane), 5.11–5.20 (m, 1H ×30, CH for PDLA), 5.50–6.12 (s, 1H ×2, methylene for double bond).

A typical example for the copolymerization is given as follows (Fig. 2). The desired amounts of MPC, BMA, and PDLA macromonomer were placed in a glass tube, and the mixture was diluted with ethanol–tetrahydrofuran (THF) (1/1 by volume) to 0.5 mol/L. 2,2′-Azobisisobutyronitrile (AIBN) was dissolved (2.0 mmol/L) into the solution, and then the glass tube was cooled in liquid nitrogen with Ar bubbling. The glass tube was sealed, and kept at 60°C for 24 hours. After the polymerization, the product was poured into an excess of ether–hexane–chloroform (4/4/1 by volume) mixture to obtain the copolymer. The precipitate was filtered and dried in vacuo. The chemical structure of the copolymers was confirmed by ^{1}H-NMR and FT-IR.

The copolymers were coated on cell culture polyethylene terephthalate (PET) films (diameter: 14 mm) (Wako Pure Chemical Co. Ltd.). PET films were immersed into a chloroform solution containing 1 wt% of the copolymer. The solvent was evaporated under chloroform atmosphere at room temperature, and the films were dried in vacuo. The surface of the coating was analyzed with an X-ray

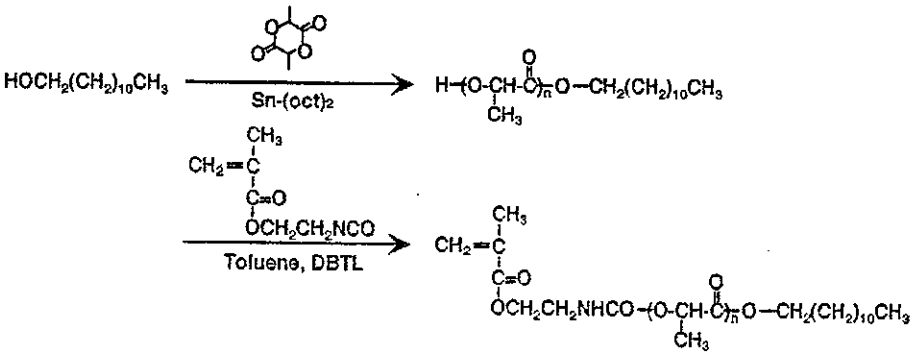

FIG. 1. Shown is the route for the preparation of poly(D,L-lactic acid) macromonomer.

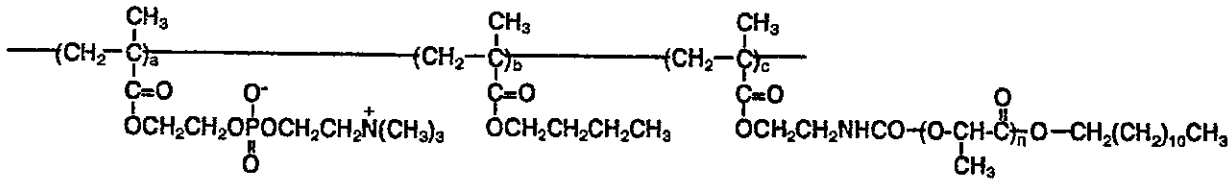

FIG. 2. The chemical structure of the copolymer is illustrated.

photoelectron spectroscope (XPS, AXIS-Hsi, Shimadzu/KRATOS, Kyoto, Japan) with MgKα. The analyzer was placed perpendicular to the surface of the sample.

Cell culture and fibronectin adsorption

L-929 cells (mouse fibroblasts) were used to evaluate the interaction between polymer-coated surfaces and the cells. The fibroblasts were routinely cultured in Eagle's Minimum Essential Medium (E-MEM, Nissui, Tokyo, Japan), supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, U.S.A.) at 37°C in a 5% CO_2 atmosphere. After treatment with 0.25% trypsin (Gibco), the cell density was adjusted to 5×10^5 cells/mL and the cells were seeded on the surface (5×10^3 cells/cm^2). After 24 h and 48 h, the numbers of adhering cells were determined using the lactate dehydrogenase (LDH) assay. The cell morphology was evaluated by using a phase-contrast microscope (BX60, OLYMPUS, Tokyo, Japan).

The amount of fibronectin adsorbed on copolymer surfaces from E-MEM (including FBS) was determined by the antigen-antibody reaction using enzyme-immunoglobulin conjugate (11). Coated films were placed in a 24-well plate, and then equilibrated with Dulbecco's phosphate buffered saline (D-PBS). After equilibration, the films were immersed into E-MEM containing 10% FBS for 3 h at 37°C. The medium was removed, and anti-bovine fibronectin rabbit polyclonal anti-serum (Yagai, YU-B0004) was added as a primary antibody. After treatment with the blocking reagent (1 wt% of ovalbumin D-PBS solution), horseradish peroxydase (HRP)-conjugated immunoglobulins ([anti-rabbit IgG] goat IgG) (A-6154, Sigma) were added to the films as a secondary antibody. After rinsing, *o*-phenylenediamine hydrochloride (ML-1120T, Sumitomo) was added as a substrate for HRP, and then the absorbance of the solution was measured at 450 nm.

RESULTS AND DISCUSSION

Synthesis of the copolymers

PDLA macromonomer was first synthesized and obtained with high yield (ca. 80%) as a white powder. From the ^{1}H-NMR spectrum of the macromonomer, the degree of polymerization of lactic acid was determined to be ca. 40 (as repeating units of lactic acid residues). Methacrylation of the terminal hydroxyl groups of the macromonomer was carried out with IEMA. The conversion was almost quantitative based on the ^{1}H-NMR spectrum. The chemical structure and the results of the synthesis of the copolymers are shown in Fig. 2 and Table 1, respectively. The polymerization was carried out for 24 hours because the reactivity of the PDLA macromonomer is lower than that of the other comonomers. Tetrahydrofuran and ethanol were used as a mixed solvent. Here the copolymers are designated by the following abbreviation, for

TABLE 1. *Synthesis of the copolymers*

	Mole fraction in feed			Mole fraction in copolymer*				
Abb.	MPC	BMA	PDLA macro-monomer	MPC	BMA	PDLA macro-monomer	Time (h)	Yield (%)
PMBLA5	0.05	0.90	0.05	0.10	0.85	0.05	24	40
PMBLA10	0.05	0.85	0.10	0.13	0.77	0.10	24	46
PBLA5	—	0.90	0.10	—	0.95	0.05	24	37
PMB	0.05	0.95	—	0.05	0.95	—	24	56

[Monomer] = 0.5 mol/L, [AIBN] = 2.5 mmol/L.
Solvent: THF : EtOH = 1 : 1.
Polymerization temperature = 60°C.
*Determined by ^{1}H-NMR.

instance PMBLA where M, B, and LA refer to MPC, BMA, and PDLA macromonomer, respectively. Further, the last number such as PMBLA5 means the mole fraction (0.05) of the PDLA macromonomer in the copolymer. The PDLA mole fractions in PMBLA were calculated to be 0.05 (PMBLA5) and 0.1 (PMBLA10), respectively. This result suggests that the PDLA mole fraction can be controlled by the feeding ratio. For MPC, both mole fractions in PMBLA were determined to be ca. 0.1. PBLA5 and PMB only consisting of two monomer units were synthesized as reference samples.

Surface characterizations with XPS

For the polymer coating, 1 wt% of a chloroform solution (PMBLA and PBLA) and 1 wt% of an ethanol solution (PMB) were prepared. PET films were immersed into the polymer solution and dried in the solvent atmosphere. Figure 3 shows XPS results of coated films before and after contact with water. The releasing angle of the photoelectron for each element was fixed at 90 degrees, and then 10–20 nm of the depth was evaluated. From this, the obtained spectra will give information of the copolymer coating. In the case of N1s core level spectra of PMBLA5 before contact with water, nitrogen peaks of both urethane bonds and choline groups were observed at 399.6 and 402.0 eV, respectively. On the other hand, only nitrogen peaks of urethane bonds for PBLA5 and only nitrogen peaks of choline groups for PMB were found for the corresponding surfaces. For PET as a coating substrate, no nitrogen peak was found as expected. Further, the phosphorus peak corresponding to the phosphate ester in PMBLA5 and PMB was found at 133.5 eV. For PBLA5 and PET surfaces, no phosphorus peak was detected. These results indicate that the chemical compositions of the surfaces correspond with their copolymers. In order to estimate the surface conditions after contact with water, the polymer-coated films and PET films were immersed into pure water at 37°C for 48 hours. After swelling, the films were lyophilized. From the XPS data (N1s and P2p) of these films, similar peaks were observed as for the polymer-coated films. Based on these results, it can be concluded that PLA chains and MPC units of the copolymer are also located on the surface of coatings on PET films after contact with water.

Characterization of cell adhesion on polymer-coated films

The stability of the polymer-coated surface was estimated by C1s and O1s core levels of XPS spectra

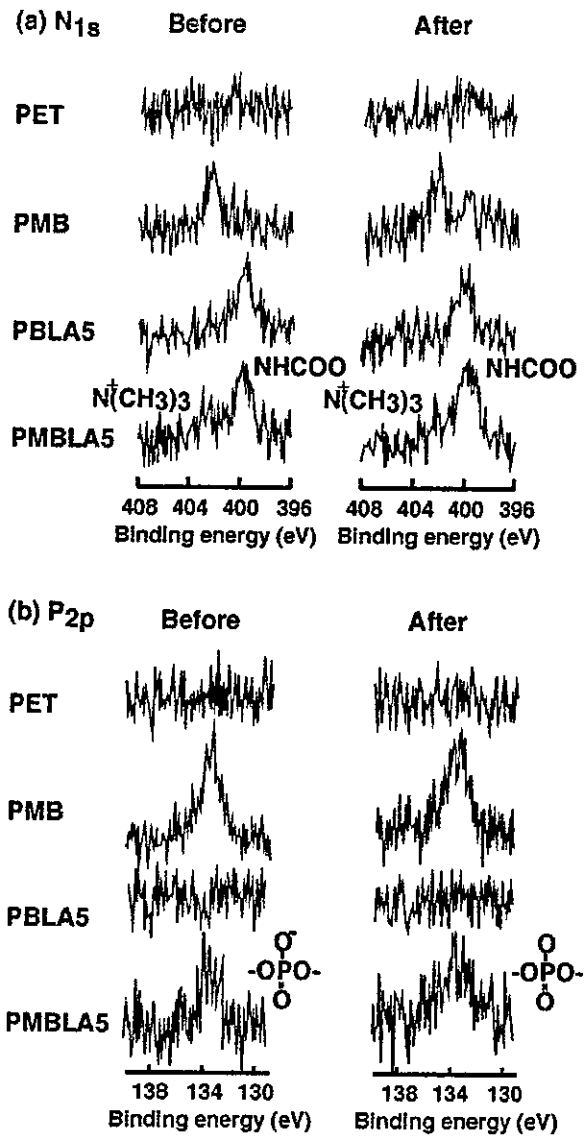

FIG. 3. Shown is X-ray photoelectron spectroscopy of copolymer-coated films before and after contact with water; (a) N_{1s} Core level of spectra, (b) P_{2p} Core level of spectra.

(data not shown). From the results, COC and C=O spectra attributed to the ester group (PLA chain) were not changed after contact with water for 48 hours. Further, the C1s and O1s spectra were significantly different from PMB. These results indicate that PLA was stable during the cell culture period. L-929 fibroblast cells were used for preliminary cell adhesion experiments. The number of fibroblast cells after 24 and 48 hours on the films is presented in Fig. 4. After 24 hours' culture, the number of fibroblast cells on PMBLA was found to be 0.2×10^3 cells/cm^2 (PMBLA5) and 3.4×10^3 cells/cm^2 (PMBLA10). This

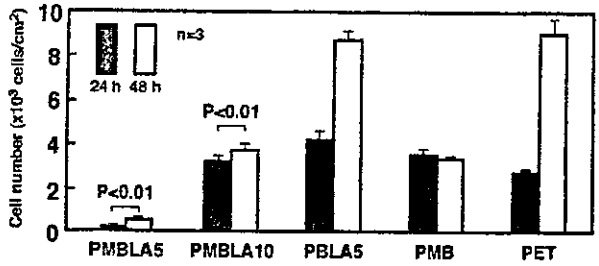

FIG. 4. Fibroblast cell adhesion on copolymer-coated films after 24 and 48 hours is depicted. Mean values of three measurements and standard deviation are indicated.

result indicates that the number of fibroblast cells increases with the concentration of PLA chains in the copolymer. The highest cell number on the films after 24 hours was observed for PBLA5 (4.2×10^3 cells/cm^2). The cell number for PMBLA5 was significantly lower than that of PBLA5. It is considered that cell adhesion is suppressed by the introduction of MPC units. After 48 hours' culture, the number of cells increased to 0.5×10^3 cells/cm^2 (PMBLA5) and 3.7×10^3 cells/cm^2 (PMBLA10). On the other hand, the cell number on PMB was found to be 3.5×10^3 cells/cm^2 (24 hours) and 3.3×10^3 cells/cm^2 (48 hours). Relatively higher cell adhesion on the PMB was observed in comparison with PMBLA5. In this case the MPC units in the copolymer play an important role for cell adhesion. The MPC unit mole fraction in PMBLA5 (0.10) is higher than that of PMB (0.05). Based on these results it seems that the increase of cell adhesion by PDLA in PMBLA5 is effectively reduced by the large amount of MPC unit in the copolymer. There was no significant difference in cell number between 24 and 48 hours, though the cell number was almost at the same level comparing with PMBLA10 (24 hours). In the case of PBLA5, the number of cells increased to 8.7×10^3 cells/cm^2 at 48 hours in spite of 0.05 of PDLA mole fraction. These results indicate that PDLA chains in the copolymer are effective for cell growth.

After 48 hours, the cell morphology was studied by using phase contrast microscopy (Fig. 5). From the pictures, the number of adhering cells on PMBLA5 seems to be higher than that for PMB. In the case of the copolymers with MPC units (PMBLA and PMB), a round shape is observed. On the other hand, spread cells are observed on PBLA and PET. These results suggest that the interaction between cells and polymer surfaces of PMBLA and PMB is

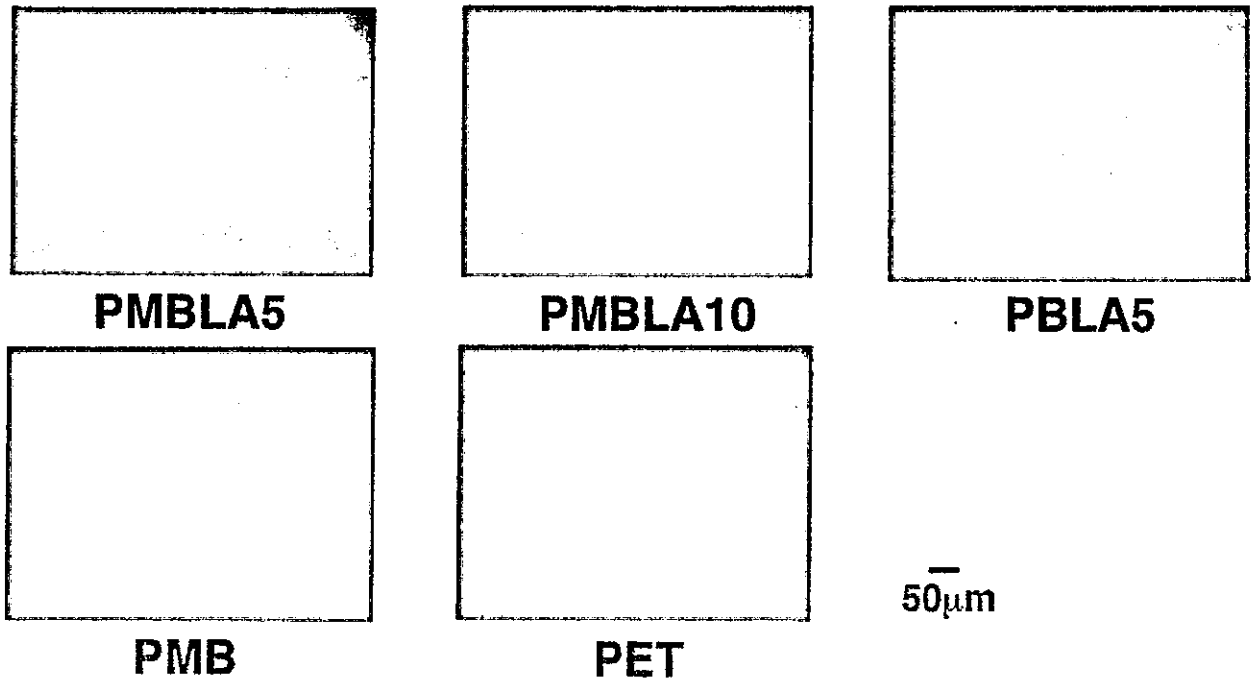

FIG. 5. Phase-contrast microscopic pictures of adhered fibroblast cells on copolymer-coated films after 48 hours' incubation are shown.

milder than that for PBLA and PET. Based on these results, it is considered that the cell morphology could be regulated by the composition of the copolymers.

Fibronectin adsorption on polymer-coated films

The results of the enzyme-linked immunoassay for fibronectin adsorption from cell culture medium are shown in Fig. 6. The amount of fibronectin adsorption was compared by using the relative intensities of the absorbance at 450 nm. The relative amount of fibronectin adsorption was divided into two groups, PMBLA5 and the others. For the group showing relatively high fibronectin adsorption, there was no significant relationship between cell adhesion and fibronectin adsorption. In our previous study, fibroblast cell adhesion and fibronectin adsorption on the PMB surfaces were examined (11). It was found that the amount of fibronectin adsorbed on PMB decreased with increasing MPC mole fraction in the copolymer. The fibroblast cell adhesion on PMB also decreased with increasing MPC mole fraction in the copolymer. These phenomena suggest that for the present copolymers the adsorbed fibronectin was over- or underestimated due to conformational changes of fibronectin adsorbed on the films. Furthermore, other proteins such as vitronectin from the medium might play an important role for cell adhesion. In the case of PMBLA5, the amount of fibronectin adsorption was quite low with respect to all the polymer-coated films. Based on these results, it can be concluded that cell adhesive proteins such as fibronectin first adsorbed on the films, and the amount of fibronectin adsorption was controllable by the chemical composition of the copolymers.

CONCLUSIONS

Fibroblast cell adhesion and morphology on a copolymer composed of poly(D,L-lactic acid) (PDLA) macromonomer, 2-methacryloyloxyethyl phosphorylcholine (MPC), and butyl methacrylate (BMA) was evaluated. PDLA chains and MPC units are located at the copolymer-coated films. From the results of cell culture, the number of adhering fibroblasts on the films increased with PDLA content, and cell growth was observed. On the other hand, cell growth was not observed on control films composed of MPC and BMA in spite of similar numbers of adhering cells. As for the cell morphology, a round shape was observed by introduction of MPC units. These results suggest that the cells recognize the surface chemical composition due to the monomers, and the number of adhering cells and morphology are controllable by the chemical composition of the copolymer. These copolymers coated on films will be promising materials for cell culture in which the adhesion and morphology can be regulated by the chemical composition of the coating.

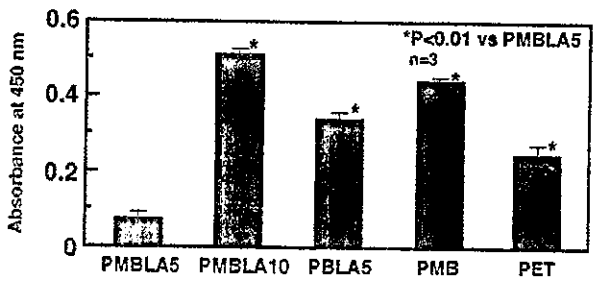

FIG. 6. The graph shows the amount of fibronectin adsorbed on copolymer-coated films from cell culture medium. Mean values of three measurements and standard deviation are indicated.

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Contribution of Membrane-Associated Prostaglandin E_2 Synthase to Bone Resorption

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This study initially confirmed that, among prostaglandins (PGs) produced in bone, only PGE_2 has the potency to stimulate osteoclastogenesis and bone resorption in the mouse coculture system of osteoblasts and bone marrow cells. For the PGE_2 biosynthesis two isoforms of the terminal and specific enzymes, membrane-associated PGE_2 synthase (mPGES) and cytosolic PGES (cPGES) have recently been identified. In cultured mouse primary osteoblasts, both mPGES and cyclooxygenase-2 were induced by the bone resorptive cytokines interleukin-1, tumor necrosis factor-α, and fibroblast growth factor-2. Induction of mPGES was also seen in the mouse long bone and bone marrow in vivo by intraperitoneal injection of lipopolysaccharide. In contrast, cPGES was expressed constitutively both in vitro and in vivo without being affected by these stimuli. An antisense oligonucleotide blocking mPGES expression inhibited not only PGE_2 production, but also osteoclastogenesis and bone resorption stimulated by the cytokines, which was reversed by addition of exogenous PGE_2. We therefore conclude that mPGES, which is induced by and mediates the effects of bone resorptive stimuli, may make a target molecule for the treatment of bone resorptive disorders. J. Cell. Physiol. 197: 348–356, 2003. © 2003 Wiley-Liss, Inc.

Prostanoids are important mediators that play a variety of roles in biological events, such as fever, pain, inflammation, tumorigenesis, gastrointestinal protection, vascular circulation, and bone metabolism (Raisz, 1995; Kawaguchi et al., 1995a; Funk, 2001; Harris et al., 2002). Prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, PGI_2, and thromboxane B_2 are accumulated in bone and are produced by cells of the osteoblast lineage (Raisz, 1995; Kawaguchi et al., 1995a). These PGs are multifunctional regulators with both stimulatory and inhibitory effects on bone formation and resorption. However, the major effect of PGs has been recognized as the stimulation of bone resorption since PGE_2 was first shown to increase cyclic AMP and stimulate resorption in cultured fetal rat long bones more than 30 years ago (Klein and Raisz, 1970). Many factors including cytokines and systemic hormones, which are involved in regulation of bone resorption, also regulate PG production in bone (Kawaguchi et al., 1994, 1995a,b; Raisz, 1995). In addition, resorptive responses to cytokines and hormones are often, at least in part, dependent on PG production since nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit these responses (Akatsu et al., 1989, 1991; Shinar and Rodan, 1990; Kawaguchi et al., 2000). In vivo studies in animals and humans also suggest that PGs could be responsible for bone resorptive disorders, such as postmenopausal bone loss (Kawaguchi et al., 1995c), hypercalcemia of malignancy (Tashjian et al., 1977; Minkin et al., 1981), inflammatory bone loss in periodontal disease (Harris et al., 1973; Harvey et al.,

Abbreviations: PG, prostaglandin; mPGES, membrane-associated prostaglandin E_2 synthase; cPGES, cytosolic prostaglandin E_2 synthase; COX, cyclooxygenase; IL, interleukin; TNF, tumor necrosis factor; FGF, fibroblast growth factor; NSAIDs, nonsteroidal anti-inflammatory drugs; LPS, lipopolysaccharide; MGST1-L1, microsomal glutathione S-transferase 1-like 1; RA, rheumatoid arthritis; αMEM, alpha modified-minimum essential medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; TRAP, Tartrate resistant acid phosphatase; SDS, sodium dodecyl sulfate; RANKL, receptor activator of nuclear factor-κB ligand; RT-PCR, reverse transcriptase-polymerase chain reaction; PVDF, polyvinyl difluoride; ELISA, enzyme-linked immunosorbent assay; ECL, enhanced chemiluminescence.

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1984), loosening of joint replacements (Goldring et al., 1983; Horowitz et al., 1994), and joint destruction in rheumatoid arthritis (RA) (Robinson et al., 1975; Manabe et al., 1999).

PGE_2 is the most abundant prostanoid among PGs in bone and has been believed to be the most potent bone resorber (Raisz, 1995; Kawaguchi et al., 1995a). PGE_2 exerts its resorptive action by inducing receptor activator of nuclear factor κB ligand (RANKL) on osteoblastic cells through an autocrine/paracrine mechanism, which elicits the support of osteoclast differentiation from hemopoietic precursors (Suda et al., 1999). PGI_2 is the next most abundant PG in bone (Raisz, 1995; Kawaguchi et al., 1995a), but it does not appear to be an important mediator of the bone resorptive response (Tashjian et al., 1988). Small amounts of $PGF_{2\alpha}$ are produced by bone cells and exogenous $PGF_{2\alpha}$ can stimulate bone resorption; however, its effect appears to be due to an increase in endogenous PGE_2 production (Raisz et al., 1990). In the present study we initially performed a comprehensive and comparative examination among prostanoids accumulated in bone on their potency to resorb bone using two mouse assay systems, and confirmed that only PGE_2 can stimulate osteoclastogenesis and bone resorption.

PGE_2 production from arachidonic acid, which is released by phospholipase A_2 from membrane phospholipids, is regulated by two rate-limiting steps. The first step is catalyzed by cyclooxygenase (COX), which converts arachidonic acid to the intermediate prostanoid PGH_2. Two isoforms of the COX enzyme have been identified: COX-1, which is a constitutive enzyme; and COX-2, which is induced by various stimuli, including cytokines, hormones, and lipopolysaccharide (LPS) (Smith and Langenbach, 2001). The second step is the terminal conversion reaction of PGH_2 to PGE_2, which is catalyzed by PGE_2 synthase (PGES). At least two forms of the PGES enzyme have recently been identified (Jakobsson et al., 1999; Tanioka et al., 2000). Cytosolic PGES (cPGES), which is identical to the heat shock protein 90-associated protein p23, is expressed ubiquitously and unaltered by proinflammatory stimuli in a wide variety of cells and tissues and promotes COX-1-mediated immediate PGE_2 production (Tanioka et al., 2000). This COX-1 and cPGES coupling is assumed to contribute to the production of PGE_2, which plays a role in the maintenance of tissue homeostasis. Contrarily, membrane-associated PGES (mPGES), which was originally designated microsomal glutathione S-transferase 1-like 1 (MGST1-L1), is an inducible enzyme, which is coordinately induced with COX-2 on the perinuclear membrane and is functionally coupled with COX-2 in marked preference to COX-1 (Jakobsson et al., 1999; Murakami et al., 2000). mPGES expression is induced by proinflammatory stimuli in various tissues and cells and was down-regulated by dexamethasone, accompanied by changes in COX-2 expression and subsequent PGE_2 production (Jakobsson et al., 1999; Murakami et al., 2000). COX-2 and mPGES are therefore thought to be essential components for PGE_2 biosynthesis, which may be linked to several pathological conditions such as inflammation, fever and cancer (Satoh et al., 2000; Filion et al., 2001; Mancini et al., 2001).

We have reported that COX-2 is abundantly expressed in osteoblasts and plays a central role in the biosynthesis of PGE_2 in response to several stimuli of bone resorption (Pilbeam et al., 1993; Kawaguchi et al., 1994, 1995b, 1996, 2000; Chikazu et al., 2001). A previous mouse co-culture experiment revealed that osteoclast formation was significantly reduced in cultures of cells derived from mice lacking COX-2 relative to wild-type cultures, an effect that could be reversed by providing exogenous PGE_2 (Okada et al., 2000a). We also demonstrated the involvement of COX-2 induction and subsequent PGE_2 production by cytokines: interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and fibroblast growth factor-2 (FGF-2), in the bone resorptive disorders postmenopausal osteoporosis and RA joint destruction (Kawaguchi et al., 1995c; Manabe et al., 1999). In this study we examined the expression of mPGES and its regulation by these bone resorptive cytokines in bone in vitro and in vivo. To further investigate the possibility of mPGES as a novel target for the development of drugs that are highly selective for bone resorptive diseases with low side effects on other tissue homeostasis, we examined the contribution of endogenous mPGES to bone resorption using an antisense oligonucleotide against mPGES.

MATERIALS AND METHODS

Materials

Neonatal and 8-week-old ddY mice were purchased from Shizuoka Laboratories Animal Center (Shizuoka, Japan). Human recombinant FGF-2 was provided by Kaken Pharmaceutical Co., Ltd. (Chiba, Japan). Mouse TNF-α was purchased from Genzyme (Cambridge, MA). Rabbit anti-mouse mPGES, COX-1, COX-2 antibodies were purchased from Cayman Chemical (Ann Arbor, MI). A rabbit anti-human cPGES antiboby, which has been confirmed to cross-react with mouse cPGES, was prepared by the immunization of rabbits as previously reported (Tanioka et al., 2000). Anti-rabbit IGG HRP Conjugate was purchased from Promega (Madison, WI). The reverse transcriptase-polymerase chain reaction (RT-PCR) kit was from Takara Biomedicals (Shiga, Japan). Bacterial collagenase and $1,25(OH)_2$ vitamin D_3 were purchased from Wako Pure Chemicals Co. (Osaka, Japan), and dispase from Nitta Gelatin Co. (Osaka, Japan). LPS and NS-398 were purchased from Calbiochem (San Diego, CA). Alpha modified-minimum essential medium (αMEM) was purchased from GIBCO BRL (Rockville, MD), and fetal bovine serum (FBS) was from the Cell Culture Laboratory (Cleveland, OH). Other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Mouse primary osteoblast culture

All animal experiments were performed according to the guidelines of the International Association for the Study of Pain (Zimmermann, 1983). In addition, the experimental work was reviewed by the committee of Tokyo University, charged with confirming ethics. Calvariae dissected from 1- to 4-day-old mice were washed in phosphate buffered saline (PBS) and digested with 1 ml of trypsin/EDTA (GIBCO BRL) containing 10 mg collagenase (Sigma, type 7) for 10 min five times, and cells from fractions 3 to 5 were pooled. Cells were

plated in 6-multiwell dishes at a density of 5,000 cells/cm^2 and grown to confluence in αMEM containing 10% FBS, penicillin (50 U/ml), streptomycin (50 mg/ml), and 0.2 mM L-ascorbic acid phosphate ester sodium salt in a humidified CO_2 incubator.

Tartrate resistant acid phosphatase (TRAP)-positive multinucleated osteoclast formation in the coculture of mouse primary osteoblasts and bone marrow cells

Mouse calvarial primary osteoblasts described above (2×10^4 cells/well) and bone marrow cells prepared from 8-week-old mice (1×10^6 cells/well) were cocultured in 24-multiwell dishes containing αMEM/10% FBS with and without prostanoids, $1,25(OH)_2$ vitamin D_3, IL-1α, and oligonucleotides as described below for 6 days with a medium change at 3 days. After 6 days of culture, the cells were fixed with 3.7% formaldehyde in PBS and ethanol-acetone (50:50, vol/vol), and stained at pH 5.0 in the presence of L(+)-tartaric acid using naphtol AS-MX phosphate in N,N-dimethyl formamide as the substrate. The number of TRAP-positive-multinucleated cells containing more than three nuclei was counted as osteoclasts.

Resorbed pit formation assay in the coculture of mouse primary osteoblasts and bone marrow cells

Mouse osteoblasts (1×10^6 cells/dish) and bone marrow cells (5×10^7 cells/dish) prepared as above were cocultured on 10-cm culture dishes coated with 0.24% collagen gel matrix (Nitta Gelatin, Tokyo, Japan) containing αMEM/10% FBS with and without prostanoids, $1,25(OH)_2$ vitamin D_3, IL-1α, and oligonucleotides as described below for 6 days with a medium change every 3 days. Non adherent cells were then washed with PBS and adherent cells were stripped by 0.2% bacterial collagenase. An aliquot of the crude cell preparation (0.1 ml) was further cultured on a dentine slice placed in each well of 96-well dishes containing αMEM/10% FBS with and without the same agents as the previous culture. After 24 h of culture, cells were removed with 1 N NH_4OH solution, and stained with 0.5% toluidine blue. Total area was estimated under a light microscope with a micrometer to assess osteoclastic bone resorption using an image analyzer (System Supply Co., Nagano, Japan).

Northern blot analysis

Mouse primary osteoblasts were cultured as described above, changed to the same medium 24 h before cytokines stimulations {IL-1α (10 ng/ml), TNF-α (10 ng/ml), and FGF-2 (10 nM)} and cultured for the indicated periods. Total RNA was extracted using ISOGEN (Wako Pure Chemicals Co.) according to the manufacturer's instructions. Equal amounts (10–20 μg) were electrophoresed in 1.2% agarose-formaldehyde gel and transferred onto nylon membrane filters (Hybond-N; Amersham International, Little Chalfont, UK). The resulting blots were then probed with the respective cDNA probes shown in previous reports (Murakami et al., 2000; Tanioka et al., 2000) that had been labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech, Inc., Tokyo, Japan) by random priming (Takara Biomedicals) according to the manufacturer's protocol. After washing, the filter was exposed to films at −80°C for 24 h to 1 week.

Western blot analysis

Mouse primary osteoblasts were cultured for indicated periods as described above, and were then collected by trypsin/EDTA and lysed in PBS containing 0.1% sodium dodecyl sulfate (SDS) to the final concentraion of 1×10^7 cells/ml. Equivalent amounts of cell lysates (10 μl containing 1×10^5 cells) were electrophoresed on the 4–20% gradient gel (Tris-Glysine Gel, Invitrogen, Carlsbad, CA), and transferred onto PVDF membrane (BIO-RAD, Hercules, CA). After blocking with 10 mM Tris-HCl containing 150 mM NaCl, 0.1% Tween 20 (TBS-T), and 5% skim milk, the membrane was incubated with antibodies against mPGES, cPGES, COX-2, and actin in TBS-T overnight at 4°C, and further with an anti-rabbit IgG horseradish peroxidase conjugate for 2 h. Reactive proteins were visualized using the enhanced chemiluminescence (ECL; Amersham Co., Arlington Heights, IL) following the manufacturer's instructions.

Intraperitoneal LPS injection

Eight-week-old ddY mice were intraperitoneally injected with 0.5 or 5.0 mg/kg of bacterial endotoxin LPS, which is known to evoke an acute inflammatory response (Fry et al., 1980). Control mice were injected with equivalent amounts of the vehicle PBS. After 3 and 6 h, animals were sacrificed and the whole femora and tibiae were excised. To extract RNA from the bone marrow and the residual bone from which bone marrow was removed, epiphyses at both ends were cut off, bone marrow was flushed with PBS, collected cells were suspended in ISOGEN extraction buffer, and total RNA was extracted. The residual bones were washed with PBS and immediately put into ISOGEN extraction buffer.

Semiquantitative RT-PCR and Southern blotting

Semiquantitative RT-PCR was performed within an exponential phase of the amplification. Total mRNA (1 μg) was reverse transcribed using Super Script reverse transcriptase with random hexamer (Takara Biomedicals), and 5% of the reaction mixture was amplified with LA-Taq DNA polymerase (Takara Biomedicals) using specific primer pairs: 5′-ATGCCTTCCCCGGGCCTGGTG-3′ and 5′-TGGTCACAGATGGTGGGCCAC-3′ for mPGES; 5′-AGGAAGCGATAATTTTAAGC-3′ and 5′-ACCCATGTGATCCATCATCTC-3′ for cPGES; 5′-GCATCGCTCTGTTCCTGTA-3′ and 5′-GTGCTCCCTCCTTTCATCA-3′ for RANKL; 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ for G3PDH. The cycling parameters were 5 sec at 94°C and 4 min at 65°C for 25 cycles for mPGES; 45 sec at 94°C, 1 min at 49°C and 1 min at 72°C for 28 cycles for cPGES; 30 sec at 94°C, 30 sec at 56°C and 1.5 min at 72°C for 20 cycles for RANKL and G3PDH. Following PCR amplification, samples were electrophoresed on 1.0% agarose gels and transferred to nylon membrane filters. For the Southern blotting, the resulting blots were then probed with the mPGES cDNA probe above that had been labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech) by random

priming (Takara Biomedicals) according to the manufacturer's instruction.

Antisense oligonucleotide

To examine the role of endogenous mPGES, its antisense oligonucleotide was synthesized. This was designed on the basis of the sequence of targeted to first ATG in mPGES: 5′-GGCCCGGGGAAGGCAT-3′. A random sequence oligonucleotide was used as a control: 5′-CCTCTTACCTCAGTTA-3′. To protect the oligonucleotide from degradation, a phosphorothioate oligonucleotide was synthesized commercially by Amersham Pharmacia Biotech. These oligonucleotides at the final concentration of 1 μg/ml were added to cultured primary osteoblasts and then cocultured with bone marrow cells for 4 h before IL-1α (10 ng/ml) or PGE_2 (10^{-6} M) treatment. RANKL mRNA level and PGE_2 in the supernatants of were measured by semiquantitative RT-PCR and enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical), respectively, at 12 h after the stimulation in the osteoblast culture. The number of TRAP-positive multinucleated osteoclasts and the resorbed pit formation on a dentine slice were evaluated as described above.

Statistical analysis

Means of groups were compared by ANOVA and significance of differences was determined by post-hoc testing using Bonferroni's method.

RESULTS

Osteoclastogenesis and bone resorption by prostanoids in bone

Among prostanoids, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane B_2 are known to be produced and accumulated in bone (Raisz, 1995; Kawaguchi et al., 1995a). To begin with, we performed a comprehensive and comparative study to investigate, which of these prostanoids can affect osteoclastogenesis and bone resorption by measuring the number of TRAP-positive multinucleated osteoclasts (Fig. 1A) and the area of resorption pit on a dentine slice (Fig. 1B), respectively, in the coculture system of mouse primary osteoblasts and bone marrow cells. PGE_2 dose-dependently stimulated osteoclastogenesis up to a level similar to that of $1,25(OH)_2D_3$, although other prostanoids hardly affected them (Fig. 1A). When osteoclasts formed in the coculture were transferred onto the dentine slice and further cultured with and without these prostanoids, only PGE_2 showed the potency to stimulate resorbed pit formation. $PGF_{2\alpha}$ showed slight stimulation of osteoclastogenesis and pit formation as reported previously (Raisz et al., 1990), although the effects were not significant. These results suggest that among prostanoids in bone only PGE_2 has the potency to stimulate osteoclastic bone resorption. Hence, to elucidate the regulation of bone resorption through PGE_2, we further investigated the regulation and function of the terminal and specific enzyme PGES in bone.

Induction of mPGES in cultured osteoblasts by bone resorptive cytokines

We first examined the regulation of expressions of mPGES and cPGES by bone resorptive cytokines, IL-1α, TNF-α, and FGF-2, in cultured mouse primary osteoblasts. Figure 2A shows the time course of effects of the bone resorptive cytokines on the mRNA levels determined by Northern blotting in primary osteoblasts. mPGES induction by IL-1α and TNF-α was observed at 1–2 h, peaked around 3 h, and decreased thereafter, while that by FGF-2 was detected at 3–6 h and increased up to 48 h. This time course of mPGES transcript induction was similar to that of COX-2. Western blot analysis confirmed induction of the mPGES protein level by these cytokines (Fig. 2B). It was stimulated by bone resorptive cytokines at 6–12 h and maintained or increased thereafter up to 48 h, while the COX-2 protein level was stimulated earlier at 3 h and decreased after 12 h. In contrast to the induction of mPGES, both mRNA and protein level of cPGES was expressed constitutively without being much affected by these cytokines (Fig. 2A,B).

Induction of mPGES in bone marrow and the residual bone by intraperitoneal injection of LPS

To investigate PGES expression in vivo, an intraperitoneal injection of LPS (0.5 and 5 mg/kg) or PBS was administered to mice. After 3 and 6 h of injection, tibiae and femora were extracted, and the bone marrow and the residual bone were separated. mPGES mRNA level determined by RT-PCR/Southern blotting was up-regulated by LPS injection dose-dependently in both bone marrow and the residual bone (Fig. 3). The induction seemed transient because it was stronger at 3 h than at 6 h in both tissues. cPGES mRNA level determined by RT-PCR was not affected by LPS injection in either tissue.

Contribution of mPGES to osteoclastic bone resorption

To examine the contribution of mPGES to bone resorption, we synthesized a phosphorothioate antisense oligonucleotide against mPGES. The antisense oligonucleotide was confirmed by Western blot analysis to inhibit the protein level of mPGES, but not that of COX-2, in the primary osteoblast culture with and without IL-1α stimulation (Fig. 4A). It also reduced medium PGE_2 and RANKL mRNA levels in the same cultures as compared to the control oligonucleotide (Fig. 4B). To learn the effect of this antisense oligonucleotide on osteoclastic bone resorption, it was added to cultured primary osteoblasts, which were then cocultured with bone marrow cells with and without IL-1α or PGE_2. In the control and IL-1α-stimulated cultures, the antisense oligonucleotide significantly reduced both osteoclastogenesis and resorbed pit formation on the dentine slice, while the control oligonucleotide changed neither of them (Fig. 4C). These inhibitions by the mPGES antisense oligonucleotide were similarly seen in the cocultures stimulated by TNF-α and FGF-2 (data not shown). NS-398, a specific COX-2 inhibitor, also decreased osteoclastogenesis and resorbed pit formation to levels similar to those of the mPGES antisense oligonucleotide. Neither the mPGES antisense oligonucleotide nor NS-398, however, could inhibit the osteoclastogenesis and resorbed pit formation stimulated by exogenous PGE_2. These results demonstrate that mPGES as well as COX-2 is an essential mediator of

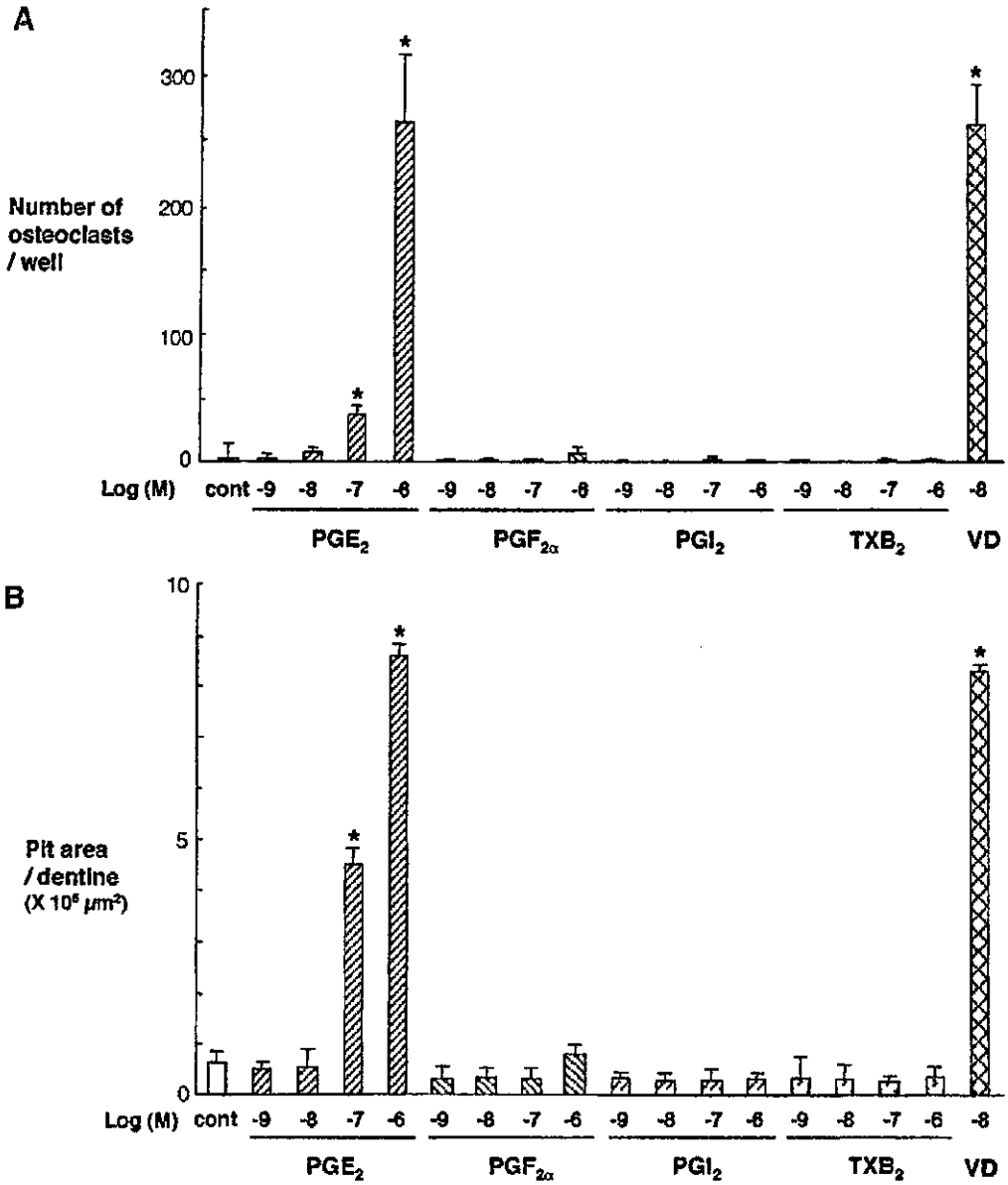

Fig. 1. Osteoclastogenesis (A) and bone resorption (B) by prostanoids in bone. A: Effect of prostanoids on osteoclastogenesis determined by the number of TRAP-positive multinucleated osteoclasts in the co-culture system of mouse primary osteoblasts and bone marrow cells. These cells were cocultured with and without various concentrations (10^{-9}–10^{-6} M) of prostanoids (PGE_2, $PGF_{2\alpha}$, PGI_2, and TXB_2) or a positive control 1,25(OH)$_2$ vitamin D_3 (10^{-8} M) for 6 days. The number of TRAP-positive cells containing more than three nuclei was counted as osteoclasts. B: Effect of prostanoids on bone resorption determined by the area of resorption pit on a dentine slice by the crude fraction of osteoclasts formed in the coculture above. An aliquot of the cell preparation formed in the coculture on collagen gel matrix was stripped and further cultured on a dentine slice for 24 h with the same agent as the previous coculture. *$P < 0.01$, significant difference from the control culture. Data are expressed as means (bars) ± SEMs (error bars) for eight cultures/group.

bone resorption by several cytokines through the production of PGE_2.

DISCUSSION

Based on the fact that PGE_2 was the only prostanoid that had the potency to stimulate osteoclastic bone resorption, in this study we examined the expression and the function of the terminal and specific enzyme of PGE_2 biosynthesis, PGES, in bone. Since mPGES was shown to be induced by and to mediate effects of bone resorptive stimuli, this enzyme may play an important role in physiological and pathological bone resorption.

Accumulated evidence has shown that mPGES is an inducible enzyme, the expression of which is markedly increased just like COX-2 in various cells and tissues following several stimuli (Jakobsson et al., 1999; Murakami et al., 2000; Soler et al., 2000; Stichtenoth et al., 2001; Yamagata et al., 2001; Han et al., 2002). Inducible genes usually contain particular nucleotide

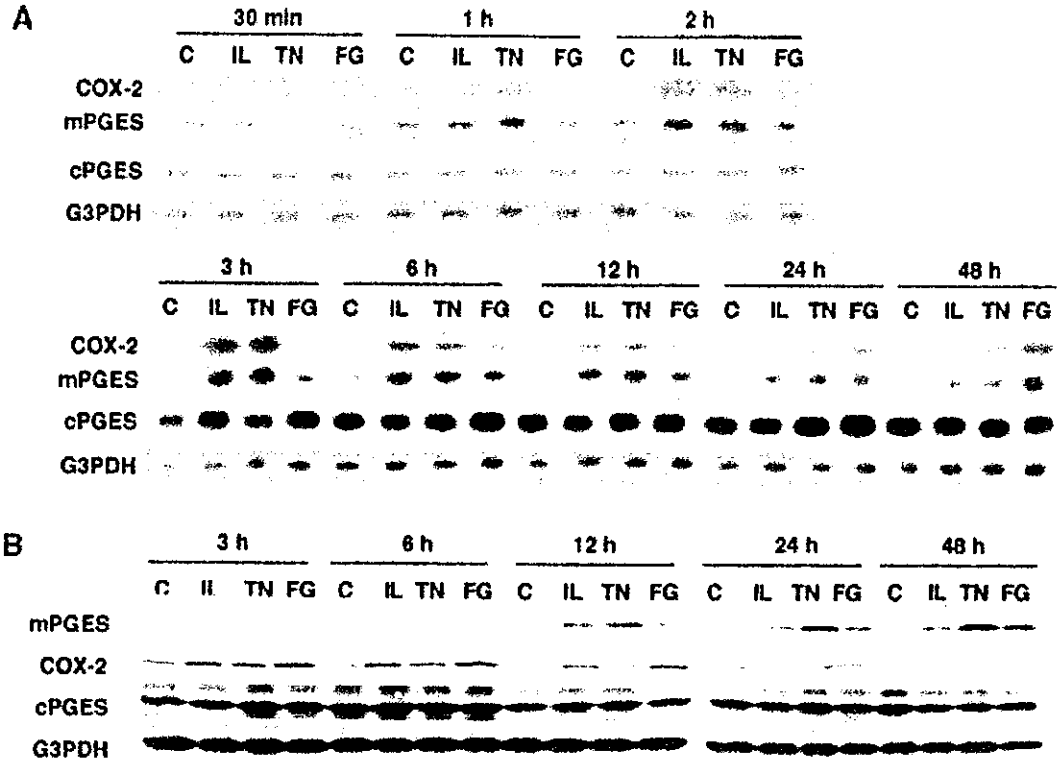

Fig. 2. Time course of effects of bone resorptive cytokines on mPGES, cPGES and COX-2 expressions in cultured mouse primary osteoblasts. A: mRNA levels of these enzymes in the control culture (C) and the IL-1α (IL, 10 ng/ml), TNF-α (TN, 10 ng/ml) or FGF-2 (FG, 10 nM)-stimulated cultures of primary osteoblasts for the indicated periods (30 min to 48 h) determined by Northern blot analysis. B: Protein levels of these enzymes in the control and stimulated cultures of primary osteoblasts for the indicated periods (3–48 h) determined by Western blot analysis.

elements within their promoter regions that are responsible for regulated transcription. In fact, a number of studies have reported the transcriptional regulation of COX-2 expression, in which several consensus sites in the COX-2 promoter have been identified as regulatory sequences for COX-2 induction in response to various stimuli in various cells. In osteoblasts, NF-κB, NF-IL6, AP-1, C/EBP-α, -β, CRE and Runx2 are known to be involved in the transcriptional induction of COX-2 (Yamamoto et al., 1995; Harrison et al., 2000; Okada et al., 2000b; Chikazu et al., 2002). Regarding mPGES, however, although the structure of the human gene, including a 632-bp 5′-flanking region, has been reported (Forsberg et al., 2000), transcription factors or transcriptional regulatory elements have been studied little. Our colleagues recently reported that the binding of Egr-1, an inducible transcription factor, to the proximal GC box is an essential event that directs the regulatory

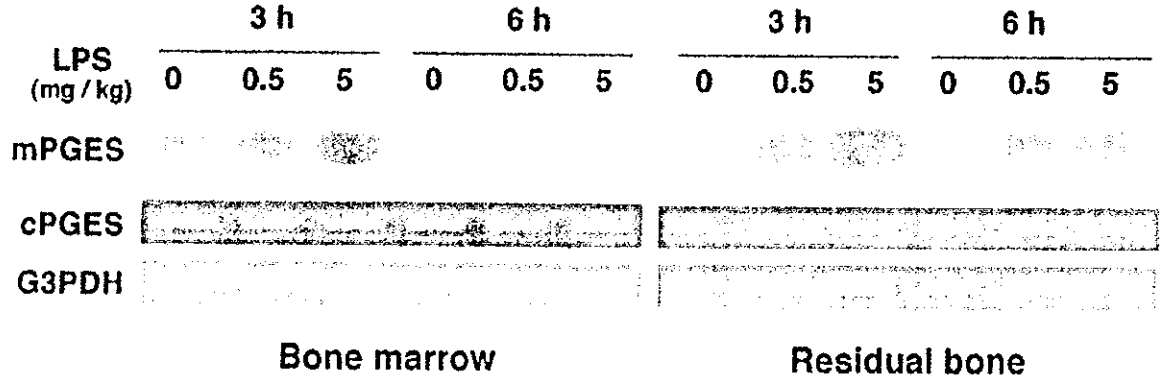

Fig. 3. Effect of intraperitoneal injection of LPS on mPGES and cPGES mRNA levels in bone marrow and the residual bone. After 3 and 6 h the intraperitoneal injection of PBS (0) or LPS (0.5 and 5 mg/kg) to mice, mRNA was extracted separately from the bone marrow of long bones and the residual bone. mPGES and cPGES mRNA levels were determined by RT-PCR/Southern blotting and RT-PCR, respectively.

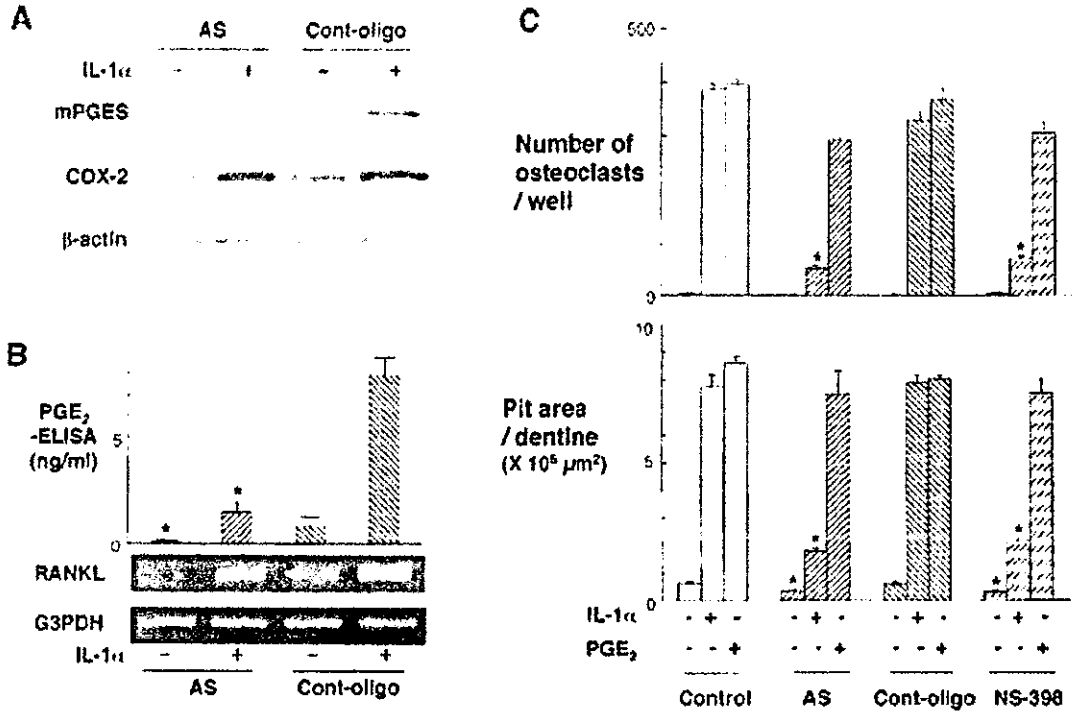

Fig. 4. Inhibitory effects of an mPGES antisense oligonucleotide on mPGES expression (A), PGE_2 production and RANKL expression (B), and osteoclastic bone resorption (C). A: Effects of the antisense and the control oligonucleotides on mPGES and COX-2 protein levels determined by Western blotting in the primary osteoblast culture with and without IL-1α (1 ng/ml) stimulation for 12 h. B: Effects of the antisense and control oligonucleotides on medium PGE_2 level and RANKL mRNA level measured by ELISA and semiquantitative RT-PCR, respectively, in the primary osteoblast culture with and without IL-1α stimulation. C: Effects of the oligonucleotides and NS-398, a specific COX-2 inhibitor, on osteoclastogenesis and resorbed pit formation on the dentine slice determined as described above (Fig. 1) in the coculture with and without IL-1α or PGE_2. These oligonucleotides and NS-398 were first added to cultured primary osteoblasts, and after 4 h, cocultured with bone marrow cells with and without each agent. $^*P < 0.01$, significant inhibition from the control-oligonucleotide culture (B) and from the control culture (C). Data are expressed as means (bars) ± SEMs (error bars) for eight cultures/group.

expression of mPGES under proinflammatory stimulations by LPS, IL-1, and TNF-α in mouse osteoblastic MC3T3-E1 cells (Naraba et al., 2002). Although the mouse mPGES promoter contains several other consensus binding sites such as C/EBP-α, -β, and AP-1, which have been implicated in COX-2 induction, the report failed to show the involvement of these sites in the promoter activation. Since there was no binding site for NF-κB, NF-IL6, CRE or Runx2 in the mPGES promoter, transcriptional mechanisms for inducible expression of mPGES and COX-2 are likely to be distinct. In addition, the time course of the stimulation of protein levels of mPGES and COX-2 by bone resorptive cytokines was different: the former kept increasing during the culture periods while the latter was transient with the peak around 3–6 h. This discrepancy also implies a difference in translational or post-translational regulations between mPGES and COX-2 by these cytokines. Even though both enzymes are stimulus-inducible and function sequentially in the same PGE_2-biosynthetic pathway, the kinetics of the induction seems not to be entirely identical in osteoblasts.

A synthesized antisense oligonucleotide inhibited osteoclastic bone resorption in the coculture of osteoblasts and marrow cells. We suspect that the target of this antisense oligonucleotide was osteoblasts because it inhibited RANKL expression in cultured primary osteoblasts. In this system, however, the possible involvement of the down-regulation of mPGES in bone marrow hemopoietic cells that are osteoclast precursors in this effect cannot be denied. In fact, mPGES is reported to be expressed and up-regulated transcriptionally by proinflammatory cytokines in the macrophage cell line RAW264.7 (Naraba et al., 2002). Furthermore, previous studies showed that PGE_2 has a potency to stimulate osteoclast formation from precursors directly in the absence of osteoblasts/stromal cells (Wani et al., 1999; Li et al., 2000). The cellular mechanism of cytokine-induced osteoclastic bone resorption through mPGES induction and PGE_2 production still remains to be investigated.

It is expected that the mPGES pathway will be a target for therapeutic intervention for patients with bone resorptive diseases like postmenopausal osteoporosis and RA joint destruction. Bone resorptive cytokines used in this study are actually known to contribute to these disorders. We and others have reported that up-regulation of IL-1 and TNF-α production in bone marrow are implicated in bone loss with estrogen withdrawal at least partly through PGE_2 production (Kawaguchi et al., 1995c; Pacifici, 1996; Pfeilschifter et al., 2002). Although our preliminary studies so far have failed to detect the regulation of mPGES expression in bone or bone marrow by ovariectomy in mice, possibility of the involvement

of a subtle change of endogenous mPGES level in the etiology of postmenopausal osteoporosis cannot be ignored. We also reported that another cytokine FGF-2 in the synovial fluid plays a role in the RA joint destruction (Manabe et al., 1999) and that FGF-2 stimulates osteoclastogenesis through PGE_2 production in osteoblasts (Kawaguchi et al., 1995c, 2000; Chikazu et al., 2001). Since proinflammatory cytokines are reported to induce mPGES in synovial cells as well (Stichtenoth et al., 2001), mPGES might also possibly involved in the pathophysiology of the RA joint destruction. Unlike NSAIDs, which inhibit COX activity and suppress not only PGE_2 but also other essential prostanoids, which maintain physiological functions, an inhibitor of this PGE_2 specific enzyme could provide a highly selective treatment for these diseases with low side effects.

It should be noticed that the present study is limited to in vitro experiments. In fact, PGs, particularly those of the E series, are known to have potent effects not only on bone resorption, but also on bone formation in vivo (Miller and Marks, 1994; Jee and Ma, 1997). The osteogenic effects are mainly seen when a substantial amount of exogenous PGEs were applied locally or systemically. Regarding the role of endogenous PGEs, recent reports on knockout mice of PGEs-related molecules including PGE_2 receptors or COX-2 failed to demonstrate their abnormalities of skeletal development or growth, indicating that endogenous PGEs are not important for physiologic osteogenesis; however, under pathologic conditions such as fracture healing and mechanical stimulation, osteogenesis is reported to be impaired by the deficiency of COX-2 and EP2, respectively (Akhter et al., 2001; Simon et al., 2002). Contrarily, studies on knockout mice have also shown the essential role of COX-2 and EP4 in the stimulation of bone resorption by parathyroid hormone and LPS in vivo (Sakuma et al., 2000; Okada et al., 2000a). Hence, further studies using mPGES-deficient mice will be needed to clarify the in vivo roles of endogenous mPGES in bone formation and resorption under physiologic and pathologic conditions.

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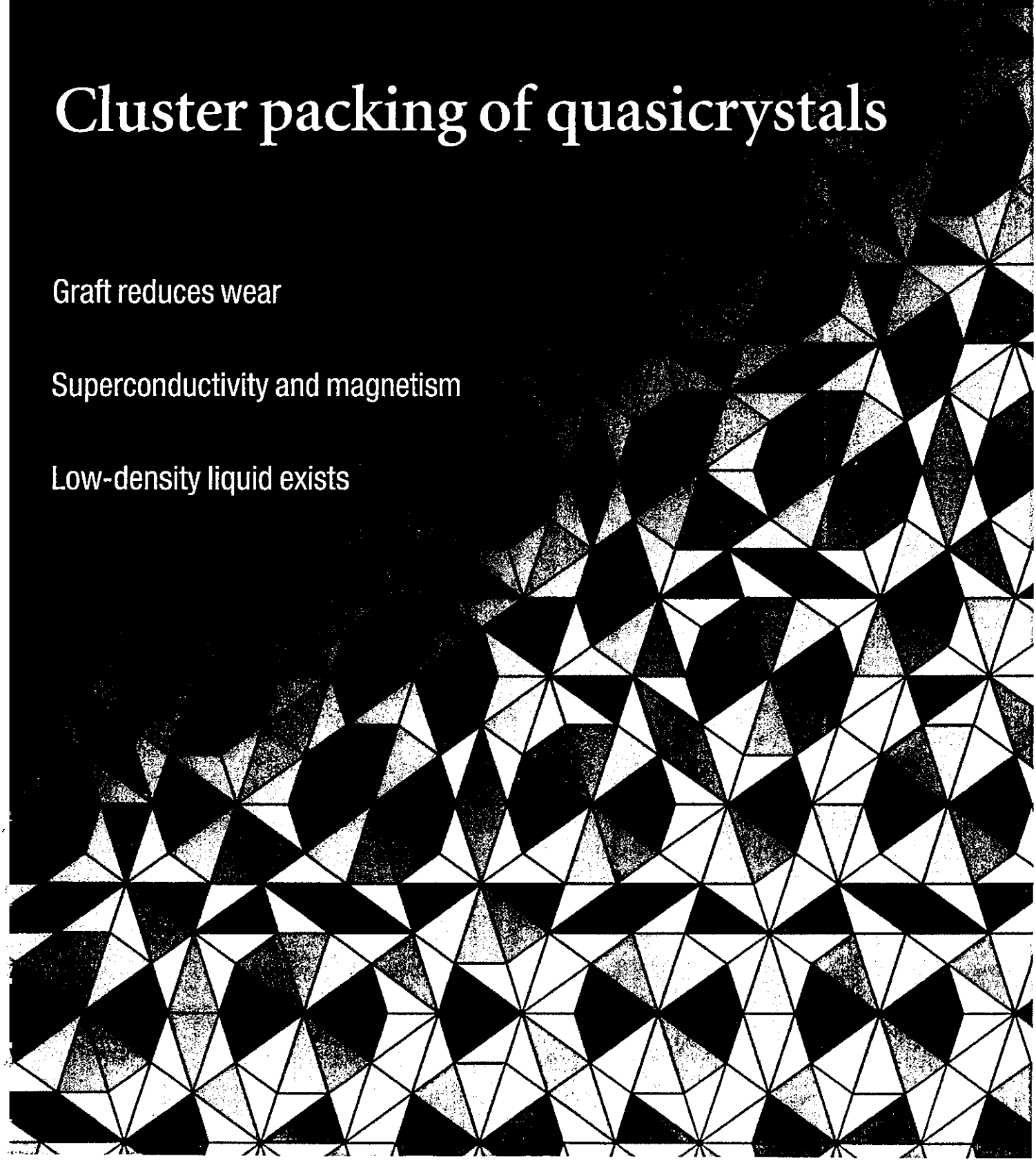
Cluster packing of quasicrystals

Graft reduces wear

Superconductivity and magnetism

Low-density liquid exists



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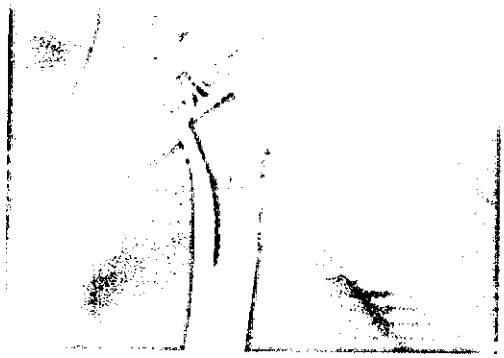
COVER STORY

CHEMISTRY IMPROVES WEAR IN ARTIFICIAL JOINTS

Friction of metallic hip prostheses against polyethylene-lined cups in artificial joints causes bone loss through an inflammatory response to wear particles. It is a serious problem with dire consequences that ultimately can undermine improvements in implant design and surgical techniques. H. Kawaguchi and colleagues have now found a way to overcome this problem through a chemical modification of the polyethylene surface. Through a photoinduced polymerization technique they grafted a biocompatible phospholipid polymer, 2-methacryloyloxyethyl phosphorylcholine, to the polyethylene surface. This has a surprisingly positive effect on the amount of wear. Moreover, biological experiments suggest that the bone-resorption response will be avoided for wear particles from the phospholipid-grafted surface.



Grafting of a biocompatible phospholipid polymer on the articulating surface of polyethylene artificial joints drastically reduces wear and may prevent periprosthetic bone loss.

Article

Surface grafting of artificial joints with a biocompatible polymer for preventing periprosthetic osteolysis

TORU MORO, YOSHIO TAKATORI, KAZUHIKO ISHIHARA, TOMOHIRO KONNO, YORINOBU TAKIGAWA, TOMIHARU MATSUSHITA, UNG-IL CHUNG, KOZO NAKAMURA & HIROSHI KAWAGUCHI

Nature Materials **3**, 829–836

Surface grafting of artificial joints with a biocompatible polymer for preventing periprosthetic osteolysis

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Periprosthetic osteolysis—bone loss in the vicinity of a prosthesis—is the most serious problem limiting the longevity of artificial joints. It is caused by bone-resorptive responses to wear particles originating from the articulating surface. This study investigated the effects of graft polymerization of our original biocompatible phospholipid polymer 2-methacryloyloxyethyl phosphorylcholine (MPC) onto the polyethylene surface. Mechanical studies using a hip-joint simulator revealed that the MPC grafting markedly decreased the friction and the amount of wear. Osteoclastic bone resorption induced by subperiosteal injection of particles onto mouse calvariae was abolished by the MPC grafting on particles. MPC-grafted particles were shown to be biologically inert by culture systems with respect to phagocytosis and resorptive cytokine secretion by macrophages, subsequent expression of receptor activator of NF-κB ligand in osteoblasts, and osteoclastogenesis from bone marrow cells. From the mechanical and biological advantages, we believe that our approach will make a major improvement in artificial joints by preventing periprosthetic osteolysis.

Total joint replacement is the most significant advance in the treatment of osteoarthritis, rheumatoid arthritis and other arthritic diseases affecting major joints of the upper and lower extremities[1]. Despite improvements in implant design and surgical techniques, periprosthetic osteolysis causing aseptic loosening of artificial joints remains the most serious problem limiting their survival and clinical success[2].

Pathogenesis of the periprosthetic osteolysis is known to be a consequence of the host inflammatory response to wear particles originating from the prosthetic devices[1,2]. Many clinical and animal studies have shown that the most abundant and bone-resorptive particle within the periprosthetic tissues is polyethylene (PE) generated from the interface between the PE and metal components[3–5]. A key role has generally been attributed to the phagocytosis of the PE particles by macrophages, followed by secretion of prostaglandin E_2 (PGE_2) and the cytokines tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 (ref. 6). These bone-resorptive factors induce the expression of a receptor activator of NF-kB ligand, the key member-associated molecule for osteoclastogenesis, in osteoblasts, consequently resulting in osteoclastic bone resorption[2,7,8]. Hence, reducing the production of wear particles and bone-resorptive responses may lead to the elimination of periprosthetic osteolysis. Based on this hypothesis, we prepared a novel hip PE component grafted with MPC onto its surface. The MPC polymer is our original biocompatible polymer whose side chain is composed of phosphorylcholine resembling phospholipids of biomembranes (Fig. 1a)[9]. The MPC grafting onto the surface of medical devices has already been shown to suppress biological reactions even when they are in contact with living organisms[10,11], and is now clinically used on the surfaces of intravascular stents, intravascular guide wires, soft contact lenses and the oxygenator (artificial lung) under the authorization of the Food and Drug Administration of the United States[12–14]. The present study investigated the mechanical and biological effects of the MPC grafting onto the surface of the PE component of artificial joints.

Grafting of the MPC onto the PE surface of hip acetabular liners was performed by a photoinduced polymerization technique, producing a covalent bond between the MPC and PE polymers (Fig. 1a)[15]. The stable grafting of MPC on the PE was confirmed using highly sensitive X-ray photoelectron spectroscopy (XPS; PHI5400MC, Perkin Elmer, USA) (Fig. 1b). The peaks in the carbon atom region (C_{1s}) at 286.5eV and 289 eV, indicating the ether

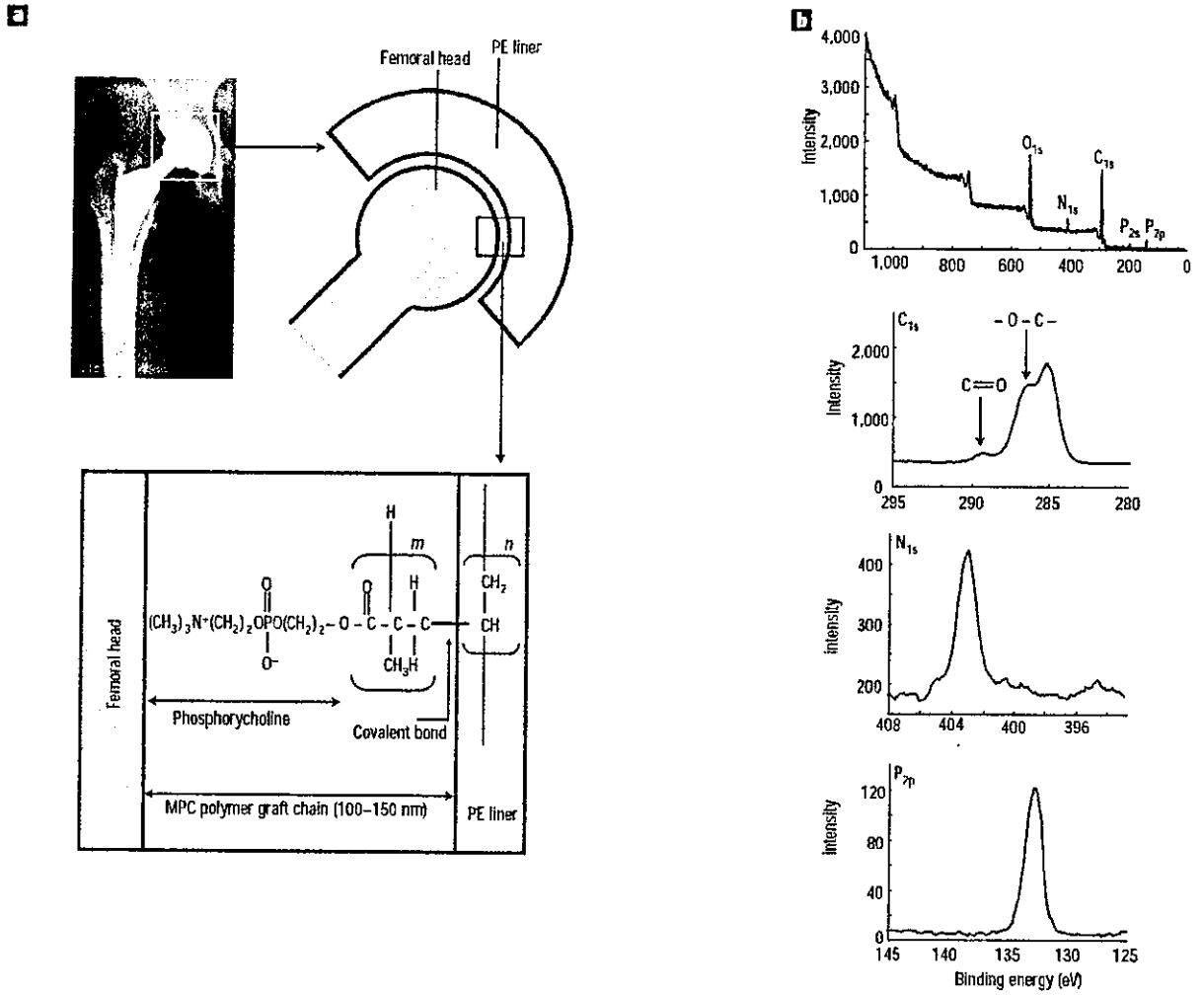

Figure 1 Surface analyses of the MPC grafted PE. **a**, Upper left shows an X-ray of a replaced hip joint in which the relationship between the femoral head and the PE liner is indicated at upper right. MPC is bound to the PE liner by the covalent bond with a photoinduced graft polymerization technique. **b**, XPS charts of the PE liner surface with the MPC grafting. The peaks in the carbon (C_{1s}), nitrogen (N_{1s}) and phosphorus (P_{2p}) atom regions are specific to the MPC, indicating successful grafting. **c**, Lubricity determined by the friction coefficient of PE plates with and without the MPC grafting (MPC-PE and PE, respectively). **d**, Hydrophilicity determined by the contact angle of a water drop with PE and MPC-PE plates. Representative pictures are shown below. Data are expressed as means (bars) ± s.e.m. (error bars) for 12 plates per group. * significant difference from PE; $P<0.01$.

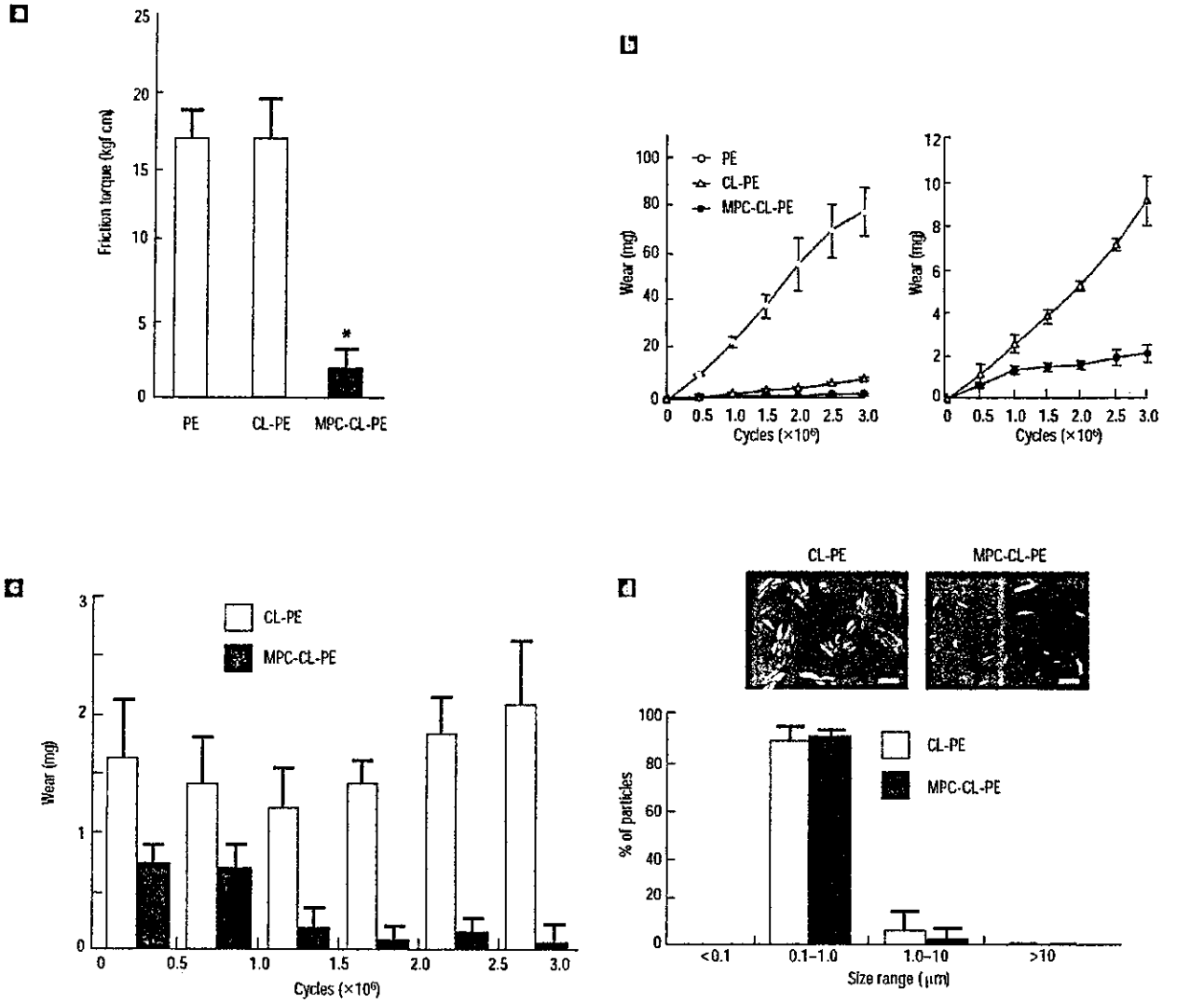

Figure 2 The friction torque and the wear amount in the hip-joint simulator with three kinds of PE liners. a, Friction torque of the three liners against the femoral heads measured before the loading test. **b**, Time course of the wear amount produced from the three liners during 3×10^6 cycles of loading. The CL-PE and MPC-CL-PE data are shown in an expanded scale on the right. **c**, The wear amount from the CL-PE and MPC-CL-PE liners for every 5×10^5 cycle intervals. **d**, Representative SEM images of the wear particles isolated from lubricants of the simulators with CL-PE and MPC-CL-PE liners. The graph below shows the distribution of particles in each size range. Data are expressed as means (symbols and bars) ± s.e.m. (error bars) for 10 liners per group. * significant difference from PE; $P<0.01$.

bond and the ester bond, respectively, and those in the nitrogen atom at 403 eV (N_{1s}) and phosphorus atom at 133 eV (P_{2p}) were specific to the phosphorylcholine group in the MPC unit.

To assess the lubricity and hydrophilicity, the MPC was grafted onto the PE plate (MPC-PE plate). The friction coefficient measured using a tensile test device and the contact angle of a water drop measured using the sessile drop method with a goniometer on the MPC-PE plate were about 1/7 and 1/5, respectively, of those on the non-grafted PE plate (Fig. 1c,d). These results indicate that the MPC grafting on PE greatly increases both lubricity and hydrophilicity.

Mechanical effects of the MPC grafting on the hip prosthesis were examined using a hip-joint wear simulator[16] under the conditions recommended by the International Organization for Standardization (ISO). We prepared crosslinked acetabular PE liners with photoinduced grafting of MPC onto their surface (MPC-CL-PE liner), and compared them with crosslinked PE liners without the MPC grafting (CL-PE liner) and non-crosslinked PE liners without the MPC grafting (PE liner). The friction torques of the three liners against the femoral head were compared before the loading test. There was no difference between PE and CL-PE liners; however, the MPC-CL-PE liner showed 80–90% lower torque than these two (Fig. 2a). Throughout the 3×10^6 cycles of gravimetric loading by the hip-joint simulator, the wear amount of the MPC-CL-PE liner was about 4 and 40 times less than those of the CL-PE and the PE liners, respectively (Fig. 2b). Clinically, the wear rate at the initial stage after a total hip replacement is thought to be well correlated with the incidence of periprosthetic osteolysis, because the wear particles may gain access to the articulation and accelerate the additional wear by a three-body mechanism[17]. In fact, the time-course analysis of the wear amount for every 5×10^5 cycle intervals