with the Ti–Zr or Zr specimens. A few giant cells were found in the Ti- and Cr-implant groups, but not in the Zr-implant and Ti–Zr alloy-implant groups. Increased numbers of neutrophils, eosinophils, and lymphocytes were found especially in the Cr-implant group compared with the Ti–Zr alloy- and Zr-implant groups. The total scores in the Ti–Zr alloy- and Zr-implant groups were significantly lower than that in the Cr-implant group.

The capsule membrane formed around the Ti specimen was thinner than those around the Zr and Cr specimens. Individuals varied considerably in membrane thickness in the Ti-implant group, so no statistically significant differences were obtained between the Ti- the Ti–Zr alloy-implant group. The membrane around the Cr specimen was the thickest ($170 \pm 36\,\mu m$) among the materials tested and differed significantly from that around the Ti–Zr alloy specimen ($126 \pm 16\,\mu m$). A statistical difference in membrane thickness was also found between the Ti–Zr alloy- and the Zr-implant groups.

3.4 Sensitization

A patch test was performed to assess the sensitization to each metal. The animals were topically challenged with 0.02% $K_2Cr_2O_7$, 2% $TiCl_4$, or 10% $ZrCl_4$ solution on the skin. None of the animals exhibited positive skin reactions to any of these metal salt solutions, so sensitization to Cr, Ti, or Zr did not develop as a result of implantation of the metal specimens.

Spleen lymphocyte proliferation is an *in vitro* indicator in the assessment of immune function and provides information useful for diagnosing contact allergy. The proliferation activity without mitogens or metal salts was similar among the test groups. The addition of $K_2Cr_2O_7$ solution did not stimulate lymphocyte proliferation in the Cr-implant group. In the same way, the stimulation index obtained by the addition of $TiCl_4$ or $ZrCl_4$ were almost all 1.0 in the Ti-, Zr-, and Ti–Zr alloy-implant groups (Table 4). Con A-induced blastogenesis was somewhat lower in the Cr-implant group than in the other groups, but no significant difference was observed (Fig. 3).

Table 4 Lymphocyte proliferative responses by stimulation with each metal salt.

Sample	Stimulation index (mean ± SD)		
	$TiCl_4$ 10^{-6} mol/L	$ZrCl_4$ 10^{-6} mol/L	$K_2Cr_2O_7$ 10^{-9} mol/L
Control	1.04 ± 0.08	1.03 ± 0.06	1.01 ± 0.07
Ti	1.00 ± 0.09	ND	ND
Ti–Zr	0.98 ± 0.03	0.99 ± 0.05	ND
Zr	ND	0.99 ± 0.02	ND
Cr	ND	ND	0.97 ± 0.05

Spleen was collected from each animal, and a single cell suspension of spleen cells was prepared. The cells (5×10^5 cells) were cultured with each concentration of metal salt and 25 μCi [^{3}H]methyl thymidine (^{3}HTdR) for 48 h, and the ^{3}HTdR incorporation (cpm) into cells was determined. A stimulation index, the ratio of ^{3}HTdR incorporation relative to control wells, was derived for each metal salt. The data are mean ± SD for 6 animals.
ND = not determined.

4. Discussion

In a corrosive environment, component elements of metallic materials are released from the surface as ions and chemical compounds, and some of them may cause local adverse tissue reactions and the development of metal allery.[1,14] Commercially pure Ti has generally good biocompatibility,[14–17] but its mechanical strength is insufficient for use in artificial hip joints.[16,20] Furthermore, the appearance of increased wear debris from Ti has been associated with tissue inflammation.[1,2,31] Elements for Ti alloys are classified into three microstructural categories: α-stabilizers [aluminum (Al), oxygen (O), nitrogen (N), carbon (C)], β-stabilizers [molybdenum (Mo), vanadium (V), iron (Fe), Cr, Ni, Co] and neutral [zirconium (Zr)]. The properties of Ti alloys vary according to the composition of the elements. Ti alloys with α and near-α microstructures exhibit superior corrosion resistance but low ambient temperature strength. The $\alpha + \beta$ and β alloys have high strength and good formability but relatively low corrosion resistance.[18] For example, Ti–6Al–4V alloy was developed as a high-strength material, but its low corrosion resistance in the living body was problematic.[18] Kobayashi *et al.* experimented with Zr, whose chemical properties are similar to those of Ti, and formed an insoluble oxide. They developed a Ti–Zr alloy that has an $\alpha + \beta$ structure and better mechanical properties than commercially pure Ti. Because of its good mechanical properties, such as tensile strength and hardness, Ti–Zr alloy was presented as a new biomedical material for use in artificial joints or bone plates.[20]

Since orthopedic devices are generally implanted into a corrosive environment for anywhere from several months to the lifetime of the patient, long-term evaluation of biocompatibility is necessary. In a previous study, we investigated sensitization of the rat to Cr by implantation of Cr–Fe alloys into a subcutaneous position for 4 months.[26] Lewin *et al.* also evaluated the local response to bone screws in guinea pigs after 4 months' implantation.[32] Oron and Alter examined the corrosion of metal specimens by implantation into rats for 14 months, and found progressive increase in corrosion concomitant with the length of implantation period.[2] In this study, an implantation period of 8 months was used because Ti–Zr alloy and other metal specimens do not easily corrode and release metallic ions. Furthermore, the early inflammatory responses caused by surgical injury could be disregarded.

The results of body or relative organ weights, hematological examination, and mitogen-induced blastogenesis suggest that the implantation of test materials did not cause systemic toxicity or decrease immune activity (Tables 1 and 2, Fig. 3). The membrane thickness around the Cr specimen was significantly higher than that around the Ti–Zr alloy specimen (Table 3). The membranes that formed around the Ti–Zr and Ti specimens were similar in thickness to that around the 316L-type stainless steel (SUS316L) specimen, which is used clinically.[8,26] Fibroblasts migrated to the injury site around the implants in the early phase and increased in density with an increasing amount of extracellular collagen. The tightly formed fibroblast membrane inhibits circulation of biological substances to inside the membrane, possibly

with negative consequences. However, the membrane thickness is related only to fibroblasts but not to other cells, so it has debatable utility as an index of tissue response in implantation studies that cover long periods.

Local tissue response to a material is the most important aspect of the material's biocompatibility, and is mainly related to inflammatory reaction.[15] It can be evaluated by analyzing the cell population or the morphological characteristics of the tissue around the implant.[8,21,22] Ryhänen *et al.* found no qualitative differences in histology between stainless steel, Ti–6Al–4V, and Ti–Ni alloy 26 weeks after implantation in rats.[8] Macrophages are the main inflammatory cell types in a short-term implantation. They play an important role in acute inflammation and probably in determining the final biocompatibility of an implanted material.[33,34] They release various mediators that influence the activities of fibroblasts, lymphocytes, and other cells.[33,34] In addition, macrophages form multinucleated foreign-body giant cells, which constitute evidence of a specific inflammatory response evoked by a foreign substance.[35] Two of the six animals in the Ti-implant group showed a moderate inflammatory response. In all animals in the Ti–Zr alloy-implant group, the frequency of cell infiltration into the membrane was smaller than in other implanted groups (Fig. 2). The numbers of macrophages and inflammatory cells in the fibrous tissue around the Cr and Ti specimens were higher than those around the Ti–Zr and Zr specimens (Table 3). In the Ti- and Cr-implant groups, giant cells were found. These observations indicate that the Cr and Ti specimens induced inflammatory reactions more strongly than did the Ti–Zr and Zr specimens. Neutrophils, eosinophils, and lymphocytes are involved in immune responses.[8,36] The infiltration frequency of these cells into the fibrous membrane was especially high in the Cr-implant group. A small increase in these cells was observed in the Ti-implant group relative to the control group. However, the number and type of infiltrating cells did not differ among the tested materials. The histological examination did not allow us to clearly say whether or not hypersensitive response occurred in an animal. The total scores obtained in the Ti–Zr alloy- and Zr-implant groups were significantly lower than that in the Cr-implant group. The total score in the Ti–Zr alloy-implant group was the same as or less than those of the Ti-implant and SUS316L-implant groups, the latter of which scored 7.3 ± 1.5 in the previous study.[26] These results suggest that the Ti–Zr alloy has good biocompatibility.

The patch test using metal salts is the most widely used method for evaluating metal allergy.[30,37] The *in vitro* lymphocyte proliferation test is also considered useful for assessing allergic conditions or immune activity.[38,39] If a positive reaction appears following the implantation of a metallic device, the contribution of the device to the development of metal sensitivity should be considered. We previously observed that animals became sensitized by the high amount of Cr ion released from the easily corrosive Cr–Fe alloy implanted in them.[5,26] The stable oxide complex layer that formed on the surfaces of Ti, Zr, and their alloys allowed little release of their ions.[16,18,25] Although highly unusual, cases of contact sensitivity to pacemakers which are made of Ti have been reported.[27,28] A positive skin reaction to titanium chloride was obtained by a sensitization test using guinea pigs.[38] Topical exposure to Ti and Zr salt solutions in rats implanted with Ti, Zr, or Ti–Zr alloy specimens resulted in no skin responses. Ti and Zr salts did not stimulate the proliferation of lymphocytes in these implanted animals (Table 4). None of these materials caused blastogenesis of spleen lymphocytes in the groups implanted with them (Fig. 3). These results indicated that the implantation of the Ti, Zr, and Ti–Zr alloy specimens did not induce sensitization to Ti or Zr ions. This may be explained by the weak sensitization potentials of Zr and Ti ions. Or it may be that the amount of ions released from each specimen was insufficient to cause a sensitization response.

As a result, the Ti–Zr alloy and Zr had better biocompatibility than Ti and Cr. Considering the fragility of Zr, we concluded Ti–Zr alloy as the best material tested in this study. For orthopedic implants, the hardness of metallic materials is important. However, for clinical application, other factors are more important: wear resistance, fretting corrosion resistance, and mechanical/tensile strength such as Young's modulus determine a material's suitability for the targeted position or tissue. In the future, we intend to design new alloys using a Ti–Zr base to serve this purpose.

5. Conclusion

By implanting Ti, Zr, Cr, or Ti–Zr alloy into rats for 8 months, we evaluated the tissue response around the implant and the development of metal sensitization. There was no toxicological change in body or organ weights or in hematological parameters. The tissue inflammatory responses to the Ti–Zr alloy were lower than those to Ti. No sensitization response to the Ti–Zr alloy appeared. The Ti–Zr alloy has better biocompatibility than Ti for use as an artificial surgical implant.

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ORIGINAL ARTICLE

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Biodegradable polymers in chondrogenesis of human articular chondrocytes

Abstract The aim of this study was to evaluate the potential role of polyglycolic acid (PGA), poly(glycolic acid-ε-caprolactone) (PGCL), poly(L-lactic acid-glycolic acid) (PLGA), poly(L-lactic acid-ε-caprolactone, 75:25 (w/w)) [P(LA-CL)25], poly-ε-caprolactone (tetrabutoxy titanium) [PCL(Ti)], and fullerene C-60 dimalonic acid (DMA) in cartilage transplants. After 4 weeks of culture of human articular cartilage, the levels of cell proliferation and differentiation and the expression of cartilage-specific matrix genes were estimated. The relationship between cell differentiation and gap junction protein connexin 43 (Cx43) was also evaluated. All materials except PCL(Ti) retained cell proliferation activities similar to the controls. Cell differentiation levels from the highest to the lowest were in the following order: PGA >> PLGA > PGCL > Control = DMSO > P(LA-CL)25 = PCL(Ti) >> fullerene C-60 DMA. Expression of the collagen type II gene was selectively upregulated for PGA, PGCL, and PLGA and slightly increased for P(LA-CL)25 polymers but was downregulated for fullerene C-60 DMA. Aggrecan gene expression was strongest with PGA and was consistently expressed with other matrices, especially with PGCL and PLGA. However, the expression patterns of the connexin 43 gene were different from the former two genes. Multiple regression analysis revealed a high correlation between cartilage proteoglycans production and expression levels of these three genes.

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Introduction

A shortage of donor tissue restricts the successful application of tissue reconstruction for various cartilage injuries. Tissue engineering is a relatively new and promising field directed at the evolution of new tissues that will offer hope to orthopedic patients with a variety of injuries. To permit repair of cartilage defects, many researchers are turning toward a tissue engineering approach involving cultured cells and biomaterials. Although these biomaterials, especially polyglycolic acid (PGA) and poly(L-lactic acid) (PLLA), play an increasingly important role in orthopedics, adverse reactions to these biomaterials have been reported in animal experiments. PLLA produces toxic substances due to acidic degradation,[1] and long-term implants of PLLA produced tumorigenicity in rats.[2] Despite these setbacks, numerous studies have documented the biocompatibility of these bioabsorbable polymers.[3–7] PLLA, PGA, and their copolymers also have been used in clinical practice.[5,8] More recent studies have indicated that copolymers of glycolic acid promoted peripheral nerve regeneration in a rat model.[9,10] These polymers are degraded by hydrolysis and enzymatic activity and have a range of mechanical and physical properties that can be engineered appropriately to suit a particular application.

Knowledge of the biological interactions between chondrocytes and biodegradable polymers is needed to design novel biomaterials and to develop new strategies for cartilage repair. Therefore, further experimental elucidation of these polymers, their combination with other biomaterials, and new materials to find good substrates is essential to attain satisfactory conditions for their clinical application. In this study, along with PGA and poly(L-lactic acid-glycolic acid) (PLGA), we investigated the copolymer poly(glycolic acid-ε-caprolactone) (PGCL), the copolymer poly(L-lactic acid-ε-caprolactone) 75:25 (w/w) P(LA-

CL)25, and poly-ε-caprolactone (tetrabutoxy titanium) [PCL(Ti)] to determine their effects on human articular chondrocyte (HAC) proliferation, differentiation, and phenotypic expression with the aim of clarifying their suitability as carriers for future clinical cartilage transplants. Fullerene C-60 dimalonic acid (DMA) has been reported to stimulate[11] and inhibit[12] proliferation and differentiation of rat embryonic limb bud cells and mouse embryo midbrain cells, respectively, and in the present study we also investigated the effect of fullerene C-60 DMA on HACs.

Gap junctions are intercellular channels supporting direct cell-to-cell communication and tissue integration.[13] Connexins, the family of proteins that form vertebrate gap junctions, play key roles during development and in the adult. Among the 19 connexins that have been identified in mammals, the gap junction protein connexin 43 (Cx43) is the most abundant member of the channel-forming proteins in chondrocytes.[14,15] The distribution of Cx43 in hyaline cartilage and in the perichondrium of mouse and rat knee joints suggested a possible involvement of connexins in cartilage development.[16] It has been indicated that the early stage of in vitro chondrocyte differentiation is the formation of cell condensations and the ability to establish cell-to-cell communication. Cx43, together with other molecular mechanisms, mediates the condensation phase of chondrogenesis.[17] In the present study, we investigated the role of gap junctional protein Cx43 in the process of chondrocyte differentiation.

Materials and methods

Materials

HACs from knee joints and chondrocyte growth medium were commercially obtained from BioWhittaker (Walkersville, MD, USA). Chondrocyte growth medium contains bovine insulin, basic fibroblast growth factor, insulin-like growth factor-1, transferrin, gentamicin sulfate, and fetal bovine serum (5% v/v). PGA (mw 3000) and PLGA (mw 5000) were purchased from Nakalai Tesque (Kyoto, Japan) and PGCL (mw 3000) was from Taki Chemical (Hyogo, Japan). P(LA-CL)25 (mw 10000) and PCL(Ti) (mw 130000) were synthesized in our laboratory and fullerene C-60 DMA was obtained from Dr. T. Mashino.[18]

Synthesis of P(LA-CL)25

L-Lactide (Tokyo Kasei Kogyo, Tokyo, Japan) 7.5 g and caprolactone (Wako Pure Chemical Industries, Osaka, Japan) 2.5 g were put into a reactor as monomers. As a catalyst, tetrabutoxy titanium (Wako) 0.03 g was added. Furthermore, *n*-octyl alcohol (Wako) 0.001 g was added. These were completely dissolved in methylene chloride (Wako) 50 mL at room temperature. Methylene chloride was removed by decompression and a uniform mixture was left. The reactor was filled with nitrogen and was sealed. The contents were mixed and heated to 140°C. Polymerization was carried out for 4 h. After the reaction, the reactant was cooled to room temperature, and was dissolved in tetrahydrofuran 100 mL. The solution was dropped into cold methanol and a colorless precipitate was obtained. This was dried under reduced pressure and precipitation was done once again. This was again dried under reduced pressure and the polymer was obtained. The yield was 58.2% (5.82 g).

Synthesis of PCL(Ti)

Synthesis was done using the same method as described for the synthesis of P(LA-CL)25 except that the monomer was only caprolactone (Wako). The yield was 87.1% (8.71 g).

Preparation of materials

PGA, PGCL, PLGA, and P(LA-CL)25 were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 50 μg/0.8 μl of DMSO (Sigma-Aldrich, Irvine, CA, USA) and then dissolved in chondrocyte growth medium to give a final concentration of 50 μg/ml. PCL(Ti) was dissolved in tetrahydrofran (THF) at a concentration of 5 mg of PCL/ml of THF. Glass wells were coated with this solution to give a final concentration of 2 mg PCL(Ti)/well. A homogenous solution of fullerene C-60 DMA was made with the chondrocyte growth medium.

Cell culture

In vitro high-density micromass cultures of HACs were initiated by spotting 4×10^5 cells in 20 μl of medium onto each well of 12-well microplates for tissue culture (Costar Type 3513, Corning, Corning, NY, USA) and PCL(Ti)-coated glass wells (diameter 22 mm). After 2 h in a 5% CO_2 incubator at 37°C, the wells were flooded with chondrocyte growth medium (2 ml/well). The medium was supplemented with DMSO (0.8 μl/ml), PGA (50 μg/ml), PGCL (50 μg/ml), PLGA (50 μg/ml), P(LA-CL)25 (50 μg/ml), or fullerene C60 DMA (50 μg/ml). HACs cultured on tissue culture polystyrene but not exposed to any biomaterials served as a control. The media were changed in every 3 days and culture was continued for 4 weeks.

Cell morphology assay

Cell morphology was determined by inverted light microscopy. Twice weekly observations were done and photographs were taken with Fuji film.

Proliferation assay

Cell proliferation was quantitatively measured by alamar blue (Biosource International, Camarillo, CA, USA) assay after 4 weeks of culture, as previously described.[18] The assay

demonstrates the metabolic activity of the cells by detection of mitochondrial activity. The indicator dye alamar blue is incorporated into the cells and reduced and excreted as a fluorescent product. At the end of the 4-week culture period, the medium from all wells was discarded and the culture wells and three blank wells were filled with 1 ml/well of 5% alamar blue solution in fresh medium. The culture plates were incubated at 37°C for 4 h. After the incubation period, two aliquots of 100 µl of solution from each well were transferred to new wells of a Costar 96-well tissue culture microplate (Costar Type 3595, Corning). The extent of cell proliferation was quantitated by a Cytofluor II fluorescence multiwell cell reader (PerSeptive Biosystems, Framingham, MA, USA) at 535 nm for excitation and 590 nm for emission. The intensity of the blue color obtained was directly proportional to the metabolic activity of the cell populations. Blank values were subtracted from experimental values to eliminate background readings.

Proteoglycan production assay

Proteoglycans are typical components of the cartilage matrix. The extent of chondrogenesis was determined by staining the cartilage-specific proteoglycans with alcian blue (Wako) as described previously.[11,19] Briefly, the cultures and three blank wells were stained overnight at 4°C (0.5 ml/well) with 1% (v/v) alcian blue, pH 1.0. The alcian blue solution was then removed and the micromass cultures and blank wells were rinsed with 3% (v/v) acetic acid and distilled water to completely remove the free dye. The cartilage proteoglycans were extracted using 4-M guanidine hydrochloride, and the absorbance was measured at a wavelength of 600 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek Instruments, Winooski, VT, USA). Blank values were subtracted from experimental values to eliminate background readings.

RNA harvest

After the 4-week culture period, RNA was extracted from all matrices except the PCL(Ti) matrix. For the PCL(Ti) matrix, we did not have enough samples to harvest RNA because cells from 50% of the cultured wells became detached overnight following cell spotting. Total cellular RNA was extracted from cultured cells of four wells (for each material) in 0.5 ml Trizol reagent (Life Technologies, Frederick, MD, USA) according to the manufacturer's instructions. The concentration of total RNA was determined using a UV spectrophotometer (Gene Quanta, Pharmacy Biotech, Piscataway NJ, USA) at 260 nm.

Reverse transcription (RT) and polymerase chain reaction (PCR)

The matrix molecules probed as part of this study were collagen type II and aggrecan. The gap junction protein gene Cx43 was also studied. Single-strand cDNA was prepared from 1 µg of total RNA by reverse transcription (RT) using a commercially available First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After optimization of PCR conditions, subsequent PCR was performed with 4 µg of cDNA in a 20-µl reaction mixture (10 × PCR buffer 2 µl, dNTP 1.6 µl, forward and reverse primer 0.4 µl, Taq DNA polymerase 0.1 µl, and distilled water to make up 20 µl). The codon sequence used for the primer sets was as follows:

Collagen type II:
forward 5′-GGCAATAGCAGGTTCACGTACA-3′
reverse 5′-CGATAACAGTCTTGCCCCACTT-3′

Aggrecan:
forward 5′-TCGAGGACAGCGAGGCC-3′
reverse 5′-TCGAGGGTGTAGCGTGTAGAGA-3′.
Connexin 43 (*Homo sapiens*):
forward 5′-ATGGGTGACTGGAGCGCCTTAGGCAAACTC-3′
reverse 5′-GACCTCGGCCTGATGACCTGGAGATCTAG-3′

For collagen type II and Cx43, an initial denaturation step at 94°C was carried out for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. For aggrecan, an initial denaturation at 95°C was carried out for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min), with a final extension at 72°C for 5 min. The polymerization of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was accomplished by 25 cycles with a corresponding PCR program. Electrophoresis of PCR products was done on 3% agarose gel for visualization of collagen type II and aggrecan and on 1% agarose gel for Cx43 after staining with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA). The relative intensity of signals from each lane was analyzed with a computerized scanner. For relative quantitation, the signal intensity of each lane was standardized to that of a housekeeping gene, GAPDH:

forward 5′-CCCATCACCATCTTCCAGGAGCGAGA-3′
reverse 5′-TGGCCAAGGTCATCCATGACAACTTTG G-3′.

Statistical analysis

Comparing the control with samples exposed to various materials assessed the statistical significance of the cell proliferation and cartilage proteoglycans production. Student's *t* test was used to assess the statistical significance. Statistical significance was taken as $P < 0.05$. Data were indicated as the mean ± SD (standard deviation). Four or five cultures were run for each biomaterial. All experiments were repeated at least twice, and similar results were obtained.

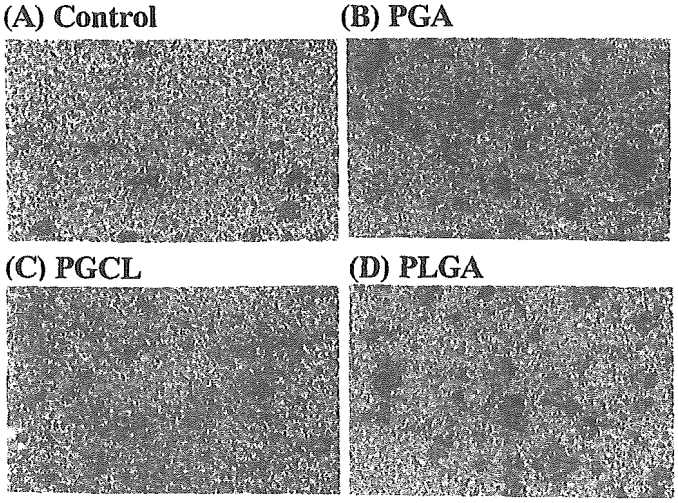

Fig. 1A–D. Light microscopic appearance of cultured human articular chondrocytes spotted as a high-density micromass culture with different biodegradable polymers for 4 weeks. **A** Control, **B** polyglycolic acid (PGA), **C** poly(glycolic acid-ε-caprolactone) (PGCL), **D** poly(L-lactic acid-glycolic acid) (PLGA). Original magnification ×200

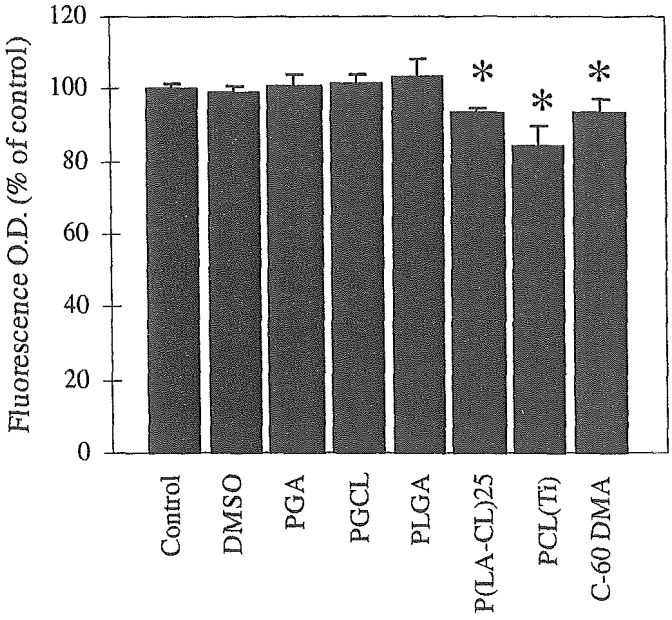

Fig. 2. Cell proliferation of human articular chondrocytes as determined by alamar blue assay after culturing with synthetic biodegradable polymers for 4 weeks. The proliferation in all samples exposed to dimethyl sulfoxide (*DMSO*) and biomaterials were calculated as a percentage of control values. *P(LA-CL)25*, poly(L-lactic acid-ε-caprolactone) 75:25 (w/w); *PCL(Ti)*, poly-ε-caprolactone (tetrabutoxy titanium); *C-60 DMA*, fullerene C-60 dimalonic acid. $*P < 0.05$ and *error bars* represent standard deviations of the mean

Results

Cell morphology

Cells were aggregated as high-density micromass cultures 2h after cell spotting. After 4 weeks of culture, the chondrocytes mainly formed a uniform sheet of chondrogenic cells with nodules. The cartilage nodules were first observed in the first week of the culture. These nodules were better visualized by staining the proteoglycans with alcian blue after 4 weeks of culture. The control cells showed less nodule formation and they were poorly defined (Fig. 1A). The cultures exposed to the PGA and PLGA had more distinct nodules and greater numbers of nodule formations than the controls (Figs. 1B and 1D). The nodules formed in the culture exposed to PGCL were less distinct and fewer in number than the nodules in the cultures exposed to PGA and PLGA, but were more distinct and numerous than the nodules of the control cultures (Fig. 1C). After alcian blue staining, light microscopic examination also revealed that PGA-, PGCL-, and PLGA-treated cultures contained denser extracellular matrix (ECM) than the controls. Cells extended from the edge of all micromass cultures, and the extending cells were spindle-shaped.

Cell proliferation assay

The proliferation rates of all the matrices are shown in Fig. 2, with error bars representing the standard deviation of the mean. All values for the samples exposed to the biomaterials were expressed as a percentage of the control average value, which was taken as 100%. The effect of DMSO on cell proliferation was not significant (99.3% ± 1.6%). The cell proliferations for PGA, PGCL, and PLGA were fairly parallel to that of control cell proliferation. The cell proliferation for P(LA-CL)25, PCL(Ti), and fullerene C-60 DMA were significantly inhibited compared to the control. The inhibitions for P(LA-CL)25 and fullerene C-60 DMA were mainly due to the small variation of the standard deviation. Despite being significantly different from the control, both proliferation values were fairly close to the control proliferation value.

Therefore, from the standpoint of cell proliferation, all materials except for PCL(Ti) remained viable candidates for tissue engineering. The values of cell proliferation for the samples exposed to PGA, PGCL, PLGA, P(LA-CL)25, PCL(Ti), and fullerene C-60 DMA were 101% ± 2.7%, 101.6% ± 2.2%, 103.5% ± 4.8%, 93.2% ± 1.4%, 84.3% ± 5.1%, and 93.6% ± 3.7%, respectively.

Proteoglycan synthesis

The proteoglycans bound with alcian blue were extracted with 4-M guanidine hydrochloride. Their levels were expressed as a percentage of the average control value, which was taken as 100% (Fig. 3). The intensity of alcian blue staining was found to be higher in PGA-, PGCL-, and PLGA-containing cultures than in the control culture. Among the biomaterials, PGA caused a significant 3.1-fold increase in cartilage proteoglycans compared to the control ($P < 0.05$). The samples exposed to PGCL (116.2% ± 10.1%) and PLGA (128.4% ± 11.1%) also produced

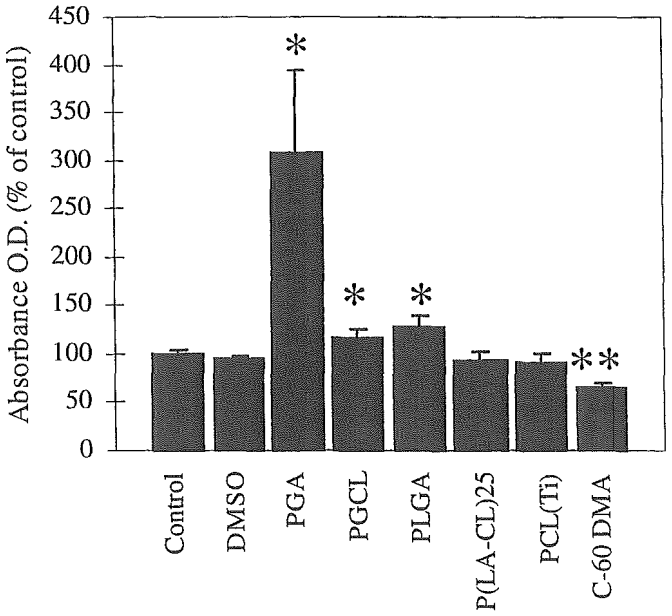

Fig. 3. Cartilage proteoglycan content of human articular chondrocytes as determined by the alcian blue staining method after culturing with synthetic biodegradable polymers for 4 weeks. The values are expressed as a percentage of control values. $*P < 0.05$ and $**P < 0.01$

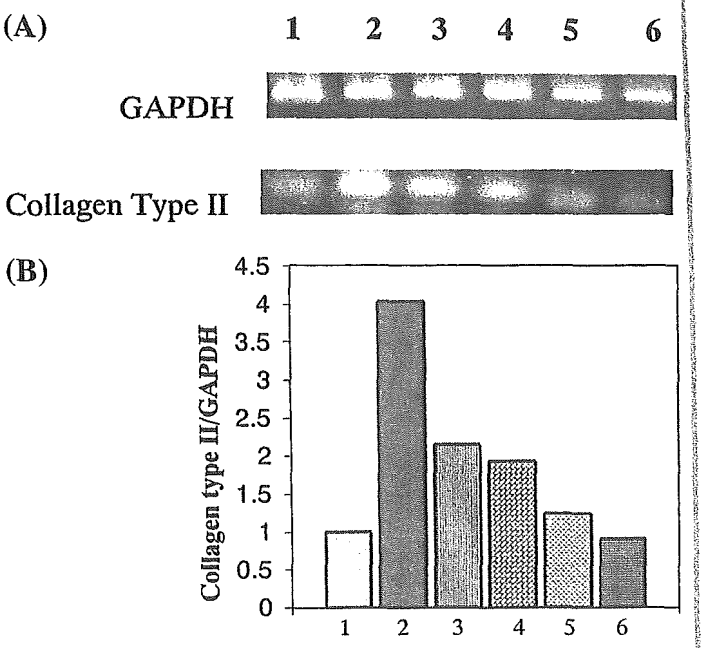

Fig. 4. Reverse transcription polymerase chain reaction (RT-PCR) analysis (**A**) and National Institutes of Health (NIH) image analysis quantitation of RT-PCR bands (**B**). In both figures, the level of collagen type-II gene expression was represented by the mRNA level of 4-week cultured human articular chondrocytes treated with different types of biodegradable polymers. The mRNA expression of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used for comparing the level of expression. **A** Lane 1, Control; lane 2, PGA; lane 3, PGCL; lane 4, PLGA; lane 5, P(LA-CL)25; lane 6, Fullerene C-60 DMA. **B** Bar 1, Control; bar 2, PGA; bar 3, PGCL; bar 4, PLGA; bar 5, P(LA-CL)25; bar 6, Fullerene C-60 DMA

significantly higher cartilage proteoglycans than the control. Copolymers P(LA-CL)25 (92.7% ± 10.5%) and PCL(Ti) (90.8% ± 9.1%) did not induce significant changes in cartilage proteoglycans compared to the control. Fullerene C60 DMA acted as a potent inhibitor (66.1% ± 4.7%) and caused a significant inhibition of cartilage proteoglycans ($P < 0.01$) compared to the control. The effect of DMSO (96% ± 1.1%) on cell differentiation was negligible.

Extracellular matrix gene expression

RT-PCR and corresponding National Institutes of Health (NIH) image analysis showed that all matrices consistently supported the expression of the collagen type II gene and that the PGA matrix had the strongest induction (Fig. 4). Slight increases in expression of the collagen type II gene were noted with PGCL, PLGA, and P(LA-CL)25 matrices. Expression of the collagen type II gene for fullerene C60 DMA was similar to the control. The PGA matrix also showed the strongest induction of the aggrecan gene (Fig. 5). Aggrecan gene expression was slightly increased in PGCL and PLGA matrices. The P(LA-CL)25 matrix caused an expression of this gene similar to that of the control, but the fullerene C60 DMA matrix caused decreased expression of this gene.

Expression of gap junction protein connexin 43 gene

To determine the expression of gap junctions during in vitro chondrocyte differentiation, RT-PCR and corresponding

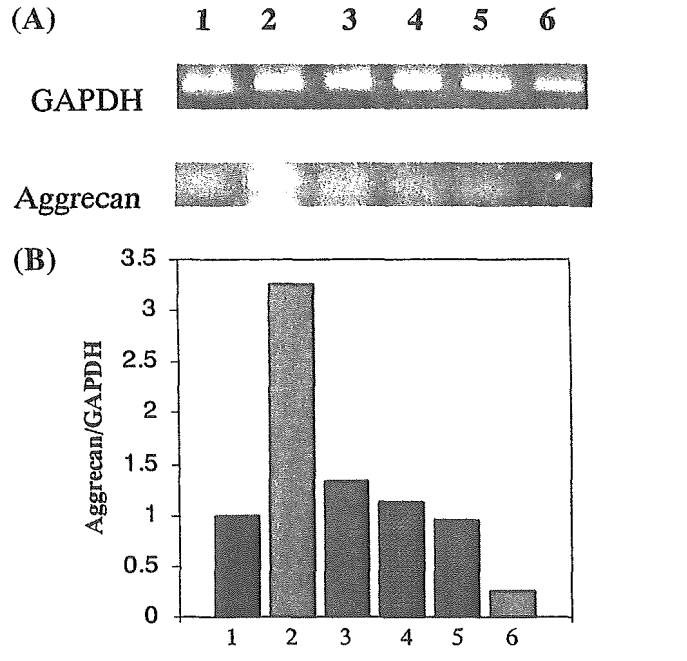

Fig. 5. RT-PCR analysis (**A**) and National Institutes of Health (NIH) image analysis quantitation of RT-PCR bands (**B**). In both figures, the level of aggrecan gene expression was represented by the mRNA level of 4-week cultured human articular chondrocytes treated with different types of biodegradable polymers. The mRNA expression of housekeeping gene GAPDH was used for comparing the levels of expression. *Lanes* and *bars* as defined in Fig. 4

Determination of Benzo[*a*]pyrene, Benz[*a*]anthracene and Dibenz[*a,h*]anthracene in Creosotes and Creosote-Treated Woods

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The amount of benzo[*a*]pyrene (BaP), benz[*a*]-anthracene (BaA), and dibenz[*a,h*]anthracene (DBA) has been restricted to a concentration of 10 μg/g each in creosotes, and 3 μg/g each in creosote-treated woods, respectively, because of the possibility of the risk of skin cancer in consumers, and creosotes can otherwise contain high concentrations of each chemical. We already reported the content of 16 polycyclic aromatic hydrocarbons (PAHs) and phenols in creosotes and creosote-treated wood as determined by gas chromatography-mass spectrometry (GC-MS) and absorptiometry [*Chemosphere*, 60, 1279–1287 (2005)]. However, the limit of determination of each PAH per sample was > 40 μg/g according to that method, the sensitivity of which was insufficient for determining the allowable levels of these 3 compounds. Moreover, a substantial amount of time was needed for GC-MS analysis. In the present study, we improved upon our previous analytical method in order to increase the sensitivity of the test and to reduce the duration of GC-MS analysis. Creosote was extracted from treated wood samples by dichloromethane-soak incubation, and was placed on a Sep-Pak silica cartridge and eluted with dichloromethane. The eluates were evaporated and dissolved in dichloromethane. The sample solution spiked with the internal standard solution was injected into the GC-MS system. The limit of determination of each chemical in the test solution was approximately 0.2 μg/ml, which corresponded to 1–2 μg/g in each sample. The duration of GC-MS analysis was approximately 17 min. A collaborative study was also carried out in order to evaluate the reproducibility of the method for determining low levels of BaP and related compounds in creosotes. The present method was applied for the analysis of certain commercially available creosotes and creosote-treated wood samples in Japan. It was confirmed that some creosotes and railway sleepers contained these compounds in high concentrations, thus exceeding the allowed control value.

Key words —— polycyclic aromatic hydrocarbon, creosote, benzo[*a*]pyrene, GC-MS, wood preservative

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INTRODUCTION

Creosote is a mid-heavy distillate of coal tar.[1] The majority of the creosote produced to date has been used as raw material for carbon black, while much of the remainder has been used as a wood preservative [The Japan Aromatic Industry Association, Inc., http://www.jaia-aroma.com/, Japan Wood Preserving Association (JWPA), http://wwwsoc.nii.ac.jp/jwpa/]. Wood treated with creosote was formerly used for railway sleepers and poles for the transport of electricity, but creosote-treated wood is now commonly used for the foundations of buildings, fences, and stakes for agricultural use, and also for the manufacture of garden furniture and outdoor recreational facilities in parks. However, direct contact with creosote can lead to skin irritation and disease,[2,3] and is likely to be carcinogenic in humans; creosote is therefore classified as belonging to Group 2A among potential human carcinogens, as established by the International Agency for Research on Cancer (IARC).[4] Creosote contains high quantities (up to 85%) of polycyclic aromatic hydrocarbons (PAHs),[1] and the U.S. Environment Protection Agency (U.S. EPA) has defined 16 PAHs as priority pollutants.[5] Benzo[*a*]pyrene (BaP) is one of the most thoroughly investigated PAHs, and is classified as belonging to Group 2A among potential human carcinogens.[6] Therefore, BaP has been chosen as a marker for creosote treatment and is taken as an indicator for the toxicity of creosote. The Scientific Committee on Toxicity, Ecotoxicity and the Environment reported that the cancer risk from exposure to creosote is greater than previously thought.[7] Such concerns have led to a new Directive (2001/90/EC) that was adopted by the European Council,[8] according to which creosote that contains BaP at a concentration of higher than 0.005% (50 μg/g) by mass, as well as water-extractable phenols at a concentration

of higher than 3% by mass, may not be used in the treatment of wood; moreover, wood treated in such a manner may no longer be placed on the market. Each country in the European Union (EU) has thus been restricting the use of creosote since 2003.

In Japan, the recycling of disused railway sleepers as exterior wood for use in gardens has recently become popular. Disused railway sleepers imported from other countries and/or new exterior wood products have been sold at retail stores that deal in carpenter's tools and gardening supplies. Consequently, the opportunity to come into contact with creosote is increasing among the general public in Japan. In our monitoring study, more than 50 μg/g of BaP was found in creosote and creosote-treated wood products available on the market in Japan.[9] To reduce the health risk posed by creosote, the Ministry of Health, Labour and Welfare decided to restrict the use of creosotes containing elevated amounts of carcinogenic PAHs. The health risks were estimated on the basis of the opportunity and time that a child could come into contact with creosote containing BaP in Japan. Based on these considerations, the amount of BaP was restricted to the following concentrations: 10 μg/g in creosotes and 3 μg/g in creosote-treated woods. Both benz[*a*]anthracene (BaA) and dibenz[*a*,*h*]anthracene (DBA) are also classified as belonging to Group 2A, due to their carcinogenic potential. The amount of DBA is lower than that of BaP in creosote, but DBA has a similar toxic equivalency factor relative to that of BaP.[10–12] The toxicity of BaA is not as great as that of BaP, but the amount of BaA in creosotes has been reported to be several times higher than that of BaP.[9] Therefore, the amount of these 2 PAHs are also restricted to the same level as that of BaP in creosotes. Creosotes that are commercially available in Japan undergo alkaline treatment after distillation (personal communication with a manufacturer), such that the content of water-extractable phenols is slight, relative to the EU control value.[9] It is therefore not considered important to measure and restrict the content of the phenols in creosotes manufactured in Japan.

The purpose of this study was to improve our previous analytical method[9] for the simultaneous determination of low levels of various PAHs (primarily BaP, BaA, and DBA) in creosotes and creosote-treated woods. Bestari *et al.* investigated the PAH content in wood products and the leaching behavior of creosote-treated wood by high-performance liquid chromatography (HPLC).[13] Anklam *et al.* used a HPLC system equipped with a fluorescence c tector for the determination of the BaP content creosotes.[14] HPLC determination is sensitive PAHs, but the identification of individual PAHs comparison of their retention time is less accur than the use of gas chromatography (GC); furth more, it remains difficult to detect 3 PAHs simul neously when using fluorescence HPLC. A GC s tem equipped with a capillary column and mass lective-ion-monitoring (SIM) is useful for detecti each chemical selectively and provides suffici separation for the quantification of the PAHs i complex.[15–22] Therefore, we used GC-mass sp trometry (GC-MS) for the determination of PA in creosotes. With our previous method, the sen tivity of detection was low with respect to the det mination of 3–10 μg/g of PAHs in creosote and cr sote-treated wood products,[9] and a long period time was required for GC-MS analysis, *i.e.*, m than 30 min per sample. Therefore, we adopted evaporation-concentration step and changed the c umn temperature conditions in order to increase sensitivity of testing and to reduce the amount time needed to perform GC-MS.

MATERIALS AND METHODS

Samples —— Four commercially available cr sotes (Nos. 1–4) and non-creosote type (creosc alternative) oil-based wood preservative paints w purchased from stores in Tokyo Metropolis, and Gumma and Kagawa prefectures. Four creoso (Nos. 5–8) were provided by the Japan Arom Industry Association, Inc. (JAIA). The three t products used for the collaborative study (codes C) were supplied by a manufacturer. Three new cr sote-treated wood products were supplied by Japan Wood Preservers Industry Associat (JWPIA). The JWPIA reported that two of the pr ucts (samples B and C) were Kempas, those w used for railway sleepers. The other sample was Ja nese hemlock, which was used for building foun tions (sample A). Two previously used railway sle ers (samples D and E) that had been treated by cr sote penetration and were imported for use in g dening were also purchased. One of these samp was pine wood. Sample F was a brand-new woo stake made with Japanese cedar, the surface of wh was painted with creosote.

Chemicals —— An EPA PAH mixture contain acenaphthene, acenaphtylene, anthracene, B

benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*ghi*]perylene, BaP, chrysene, DBA, fluoranthene, fluorene, indeno[1,2,3-*cd*]pyrene, naphthalene, phenanthrene, and pyrene at a concentration of 2000 μg/ml in dichloromethane : benzene (1 : 1) (Spelco, U.S.A.) was used as a standard for the PAHs. The PAH standard mixture was diluted with dichloromethane to reach a final concentration of 10 μg/ml. Acenaphthene-d10, chrysene-d12, and phenanthrene-d10 (Spelco, or Wako Pure Chemical Industries, Ltd., Japan) were obtained as an internal standard for the determination of PAH levels. Each internal standard was dissolved in dichloromethane at concentrations of 5–20 μg/ml. All of the other chemicals were purchased from Wako Pure Chemical Industries, Ltd., Japan.

Instruments —— A gas chromatograph (Hewlett-Packard GC5890 Series II Plus) connected to a JEOL JMS-AM II 20 mass spectrometer was used for the analysis of the individual PAHs. System control and data processing were performed using the JEOL Automass II program (JEOL Datum). The column was a PTE™-5 fused silica capillary column (30 m in length and 0.25 mm i.d. coated with a 0.2 μm thickness of 5% diphenyl-95% dimethylpolysiloxane film, Spelco). Sep-Pak Plus silica-cartridges (Part No. WAT020520) for solid phase extraction were obtained from the Waters Corporation (U.S.A.).

Extraction —— For the creosote oils, the weight (g) of a 0.5 ml sample was measured in advance. The Sep-Pak Plus silica-cartridge was conditioned using 10 ml of dichloromethane. Then, 0.5 ml of the sample was loaded onto the cartridge and was eluted with 10 ml of dichloromethane into a round-bottomed flask. The eluate was evaporated to approximately 2 ml under reduced pressure.

For extraction from the wood products, a section of approximately 2 cm in depth from the surface of the sample was removed, and that section was further cut into small pieces of *ca.* 2–3 mm wide and 2 cm long. A portion of the 1.0 g fragment was placed into a screw-capped glass tube. After the addition of 20 ml of dichloromethane, the tube was incubated for 24 hr at 37°C. The extract was filtered off and collected into a round-bottomed flask. The wood sample and tube were washed with a small amount of dichloromethane. The combined eluate was evaporated under reduced pressure. The concentrated eluate was loaded onto a Sep-Pak Plus silica-cartridge and eluted with 10 ml of dichloromethane from the cartridge into a flask. The eluate was then concentrated to a volume of approximately 2 ml by evaporation.

GC-MS Determination —— The total volume of the extract solution was adjusted to 5 ml with dichloromethane. For the determination of a high quantity of PAHs over the range of a calibration curve, a series of diluted sample solutions (1 ×, 20 ×, 100 ×, 500 ×, and 1000 ×) was prepared with dichloromethane. Two ml of each (diluted) extraction solution was obtained and spiked with 0.5 ml of the internal standard solution (*e.g.*, chrysene-d12 20 μg in dichloromethane), and the sample solution (1 μl) was injected into the GC-MS system in a splitless manner. The GC column temperature was programmed as follows: the column was first maintained at 60°C for 2 min and was then heated at a rate of 25°C/min to 300°C, after which it was held at this temperature for 6 min. The injection temperature was maintained at 280°C. The GC-MS transfer line temperature was 280°C, and the ion source temperature was 180°C. The carrier gas was helium and the column flow was maintained at 1.0 ml/min. The MS electron impact ionization energy was 70 eV. Detection was carried out using a total scan, (TIC) *m/z* = 45–500, and SIM. The individual *m/z* values for each compound are shown in Table 1. The PAH standard solutions (*e.g.*, 0.5, 1, 2, 5, and 10 μg/ml) spiked with 0.5 ml of the internal standard solution were injected into the system, and the calibration curves for the ratio of the peak area of each PAH to the internal standard for the respective mass of each ion were established. Compounds in the sample solutions were identified based on their retention times and on the basis of agreement with the mass chromatograms of the PAH standard solutions using a BenchTop/PBM Mass Spectral Identification system (Palisade Co., U.S.A.) with the Wiley Registry of Mass Spectral Data (John Wiley & Sons. Inc., U.S.A.). Each PAH concentration (μg/ml) in an appropriate diluted sample solution was derived from the calibration curves, and then the levels of the PAHs (μg) per 1 g of sample were derived.

RESULTS AND DISCUSSION

GC Conditions

GC-MS is the method of choice for conducting analyses of water and soil in the U.S. EPA.[16] A column with a film of 5% diphenyl-95% dimethylpolysiloxane gives excellent separation of 16 PAHs, so we used a PTE™-5 column of this type. In our previous study, the column was heated at a rate of

Table 1. PAHs Covered in this Study

Peak No.	Compound	CAS No.	Molecular formula	Ion for quantification (m/e)	GC retention time (min)	IARC cancer risk	Determination	Regulation
1	Naphthalene	91-20-3	$C_{10}H_8$	128	5.59			
2	Acenaphtylene	208-96-8	$C_{12}H_8$	152	7.35			
IS1	Acenaphthene-d10		$C_{12}H_{10}$	164	7.44			
3	Acenaphthene	83-32-9	$C_{12}H_{10}$	154	7.45			
4	Fluorene	86-73-7	$C_{13}H_{10}$	166	8.16	3		
IS2	Phenanthrene-d10		$C_{14}H_{10}$	188	9.11			
5	Phenanthrene	85-01-8	$C_{14}H_{10}$	178	9.12	3		
6	Anthracene	120-12-7	$C_{14}H_{10}$	178	9.15	3		
7	Fluoranthene	206-44-0	$C_{16}H_{10}$	202	10.23	3		
8	Pyrene	129-00-0	$C_{16}H_{10}$	202	10.36	3		
9	Benz[*a*]anthracene	56-55-3	$C_{18}H_{12}$	228	11.47	2A	*a*)	*b*)
IS3	Chrysene-d12		$C_{18}H_{12}$	240	11.47			
10	Chrysene	218-01-9	$C_{18}H_{12}$	228	11.49	3	*a*)	
11	Benzo[*b*]fluoranthene	205-99-2	$C_{20}H_{12}$	252	13.02	2B	*a*)	
12	Benzo[*k*]fluoranthene	207-08-9	$C_{20}H_{12}$	252	13.04	2B	*a*)	
13	Benzo[*a*]pyrene	50-32-8	$C_{20}H_{12}$	252	13.30	2A	*a*)	*b*)
14	Indeno[1,2,3-*cd*]pyrene	193-39-5	$C_{22}H_{12}$	276	15.33	2B	*a*)	
15	Dibenz[*a*,*h*]anthracene	53-70-3	$C_{22}H_{14}$	278	15.36	2A	*a*)	*b*)
16	Benzo[*ghi*]perylene	191-24-2	$C_{22}H_{12}$	276	16.06	3	*a*)	

a) Compounds determined in this study. *b*) Concentration of the compounds in creosotes was regulated in Japan.

10°C/min, and the duration of analysis of 16 PAHs exceeded 30 min per sample.[9] In the present study, we changed the temperature rate to 25°C/min in order to reduce the duration of the analysis. The final temperature was set at 300°C, as based on the separation of indeno[1,2,3-*cd*]pyrene, DBA, and benzo[*ghi*]perylene. The retention time and quantitative ion mass of each PAH is shown in Table 1 under the present conditions. The GC-MS analytical time was reduced from > 30 to 17 min.

The calibration curve for the ratio of the peak area of each PAH to the internal standard (*e.g.*, chrysene-d12) for the respective mass of each ion was established, and each PAH concentration was derived from the calibration curve. The linear calibration curve for each PAH was obtained within the range of 0.2 and 5 μg/ml. The correlative coefficients of BaA, BaP, and DBA were 0.9984, 09961, and 0.9983, respectively. The limit of the quantity of these compounds in the injection solution was considered as 0.2 μg/ml, *i.e.*, the lowest concentration used for the linear calibration curve.

Solid Phase Extraction

Column chromatography is generally used to separate PAHs from various other chemicals in creosote.[15,18,21] Here, target PAHs were separated by a solid phase extraction approach using Sep-Pak Plus silica-cartridges. A PAH standard solution (10–20 μg in 0.1 ml) was loaded onto the cartridge, and then was eluted with 10 ml of dichloromethane. The recovery of PAHs in this fraction was 94.4–97.8%. In the next eluate with 10 ml dichloromethane, no PAH was detected. A similar recovery test was carried out using creosote, and a high yield of PAHs was eluted from the cartridge (data not shown). Solid phase extraction with dichloromethane was found to be efficient for separating the PAHs contained in creosote.

Extraction of PAHs

In this study, we first compared the efficiency of the solvents (*i.e.*, dichloromethane, methanol, hexane, and saline) on the extraction of PAHs from creosote-treated woods. A higher concentration of PAHs was detected in the extracts with dichloromethane than in those with hexane, methanol, and saline (Table 2). The dichloromethane extract could be loaded directly onto the Sep-Pak Plus silica cartridges, and was eluted with dichloromethane. Therefore, we found that dichloromethane was a suitable solvent for the extraction of PAHs from creosote-treated wood.

Soxhlet-extraction techniques have generally

Table 2. Comparison of Extraction Solvent on Recovery of PAHs from Creosote-Treated Wood Product

Compound	Amount detected (μg/g)			
	Dichloromethane	Methanol	Hexane	Saline
Naphtalene	295	81	9	0
Acenaphtylene	173	28	11	1
Acenaphtene	4854	1496	639	3
Fluorene	4385	1250	685	4
Phenanthrene	17578	5680	2749	6
Anthracene	4728	1590	1146	2
Fluoranthene	6406	2397	1097	4
Pyrene	4494	1613	762	4
Benz[*a*]anthracene	1019	315	131	4
Chrysene	976	291	131	3
Benzo[*b*]fluoranthene	558	106	38	2
Benzo[*k*]fluoranthene	382	71	26	2
Benzo[*a*]pyrene	380	82	25	0
Indeno[1,2,3-*cd*]pyrene	157	45	17	0
Dibenz[*a,h*]anthracene	134	24	18	0
Benzo[*ghi*]perylene	152	32	9	0

Sample (1.0 g) was extracted with 20 ml of each solvent at 37°C for 24 hr. In the case of saline, the water layer was used, and liquid-liquid extraction was performed by shaking with dichloromethane. The extract was loaded onto a Sep-pak silica cartridge, and was eluted with 10 ml of dichloromethane. The fraction was diluted with dichloromethane to the appropriate concentration, and injected into GC-MS.

been used for the extraction of creosote-treated wood, but such an approach requires long extraction periods and is unsuitable for processing large numbers of samples. Some groups have reported that mechanical shaking and liquid extraction methods are also useful, as is Soxhlet extraction.[13,15,18,21,22] Our group also previously confirmed the usefulness of soak-extraction for the analysis of wood samples.[9] Sonication is thought to enable the rapid extraction of PAHs. Here, we compared the potential usefulness of sonication and soaking for extraction of PAHs from wood samples. Dichloromethane (20 ml) was added to 1 g of creosote-treated wood, and extraction was carried out with sonication for 30 min at room temperature or with incubation for 24 hr at 37°C. After extraction by either soaking or sonication, both samples were once again subjected to extraction by soaking them in 20 ml of dichloromethane for 24 hr at 37°C. The concentrations of 8 PAHs extracted under both sets of conditions are shown in Table 3. The amount of PAH released by the soaking approach to extraction was higher than that detected by sonication extraction. Upon the second cycle of soaking extraction following sonication, a considerable amount of PAH was detected. This finding indicated that a large quantity of PAH remained in the wood, even after sonication; moreover, most of the PAH was released after a single treatment by soaking extraction.

After the samples had been soaked, some amount of dichloromethane containing the PAHs remained in the wood, and thus complete extraction of PAHs was not considered possible. This issue may lead to a reduction in the recovery percentage, especially in cases involving a small quantity of PAHs. However, as noted above, the amount of extracted PAHs did not increase, even upon repetition of the soak extraction, and the PAHs not extracted from the wood under these conditions would not be expected to pose a health risk. We therefore chose the condition under which 1 g of sample would be incubated with 20 ml of dichloromethane for 24 hr at a temperature of 37°C for the extraction of PAHs.

In our previous study, the test solution that was extracted from the sample was not concentrated before injection into the GC-MS.[9] For creosotes, 0.1 g of sample was diluted with 20 ml of dichloromethane, and the extract from 1 g of wood sample was diluted to a total volume of 25 ml. In addition, the elution of 3 target PAHs from the GC column was slow. The limit of determination of each PAH per sample was > 40 μg/g according to that method. In the improved method, the eluate from the Sep-Pak Plus silica-cartridges was concentrated by evaporation before injection into the GC-MS. The eluate from 1 g of wood sample or 0.5 g of creosote was

Table 3. Amount of PAHs Extracted from Wood Product by Each Condition

Compound	Amount determined (μg/g)			
	Condition A		Condition B	
	(1) Incubation	(2) Incubation	(1) Sonication	(2) Incubation
Benz[*a*]anthracene	668	38	310	540
Chrysene	535	25	253	420
Benzo[*b*]fluoranthene	278	17	122	166
Benzo[*k*]fluoranthene	156	7	73	139
Benzo[*a*]pyrene	141	3	53	102
Indeno[1,2,3-*cd*]pyrene	41	ND	29	22
Dibenz[*a*,*h*]anthracene	12	ND	12	5
Benzo[*ghi*]perylene	31	ND	23	16

One gram of wood sample was extracted with 20 ml dichloromethane by incubation for 24 hr at 37°C for conditon A or sonication for 30 min at room temperature for conditon B (1). After obtaining the solution, each sample was again extracted with the same volume of dichloromethane by incubation for 24 hr for condition A or 48 hr for condition B (2). The amount of PAHs in each extract was determined. Data are the average values of 2–3 experiments. ND means < 1.0 μg/g.

Table 4. Analytical Results of Trial Creosote Products Containing Low Concentrations of Regulated Compounds Performed in 4 Laboratories

Compound	Concentration (μg/g, mean ± S.D., n = 2–5)			
	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4
(a) Sample A				
Benz[*a*]anthracene	53.0 ± 6.9	52.0 ± 1.0	50.8 ± 0.6	NT[a)]
Benzo[*a*]pyrene	29.7 ± 5.4	29.0 ± 0.5	30.9 ± 0.3	36.4 ± 0.8
Dibenz[*a*,*h*]anthracene	6.5 ± 0.2	4.0 ± 0.2	5.2 ± 0.7	NT
(b) Sample B				
Benz[*a*]anthracene	18.0 ± 0.9	15.0 ± 0.4	14.5 ± 0.8	NT
Benzo[*a*]pyrene	12.5 ± 1.1	9.0 ± 0.3	11.0 ± 0.5	12.3 ± 1.3
Dibenz[*a*,*h*]anthracene	3.2 ± 1.0	2.3 ± 1.3	1.8 ± 0.2	NT
(c) Sample C				
Benz[*a*]anthracene	5.9 ± 0.2	6.0 ± 0.1	4.8 ± 0.2	NT
Benzo[*a*]pyrene	3.9 ± 0.3	3.0 ± 0.1	2.9 ± 0.4	3.6 ± 0.6
Dibenz[*a*,*h*]anthracene	ND[b)]	ND	1.1 ± 0.3	NT

a) NT = not tested. *b*) ND = not determined, < 1.5 μg/g.

adjusted to a total volume of 5 ml in solution before injection into the GC-MS. The peak height and area of each PAH observed in the GC chromatogram increased due to alteration of the column temperature. Therefore, the limit of determination of PAHs in the injection solution became 0.2 μg/ml. As regards the amount in the creosote and creosote-treated samples, the limit of determination of 3 PAHs was 1 and 2 μg/g, respectively.

Collaborative Study

In order to validate the method developed here, 3 creosotes containing different amounts of regulated chemicals were used, and these samples were analyzed by three different laboratories in terms c their concentrations of BaP, BaA, and DBA; a four laboratory (Laboratory 4) measured only the Ba content of these samples. The experiment was re peated 2–5 times at each laboratory (Table 4 Sample A contained large amounts of BaA, BaP, an DBA in these 3 creosotes. The value of BaA at concentration of 50.8–53.0 μg/g was higher than th of BaP. The present concentrations of BaP (29.0 36.4 μg/g) were allowable according to the EU cor trol (< 50 μg/g), but they remained above the allowe value in Japan. As regards sample B, all laboratc ries reported similar levels for each compounc Sample C contained BaA, BaP, and DBA at concen

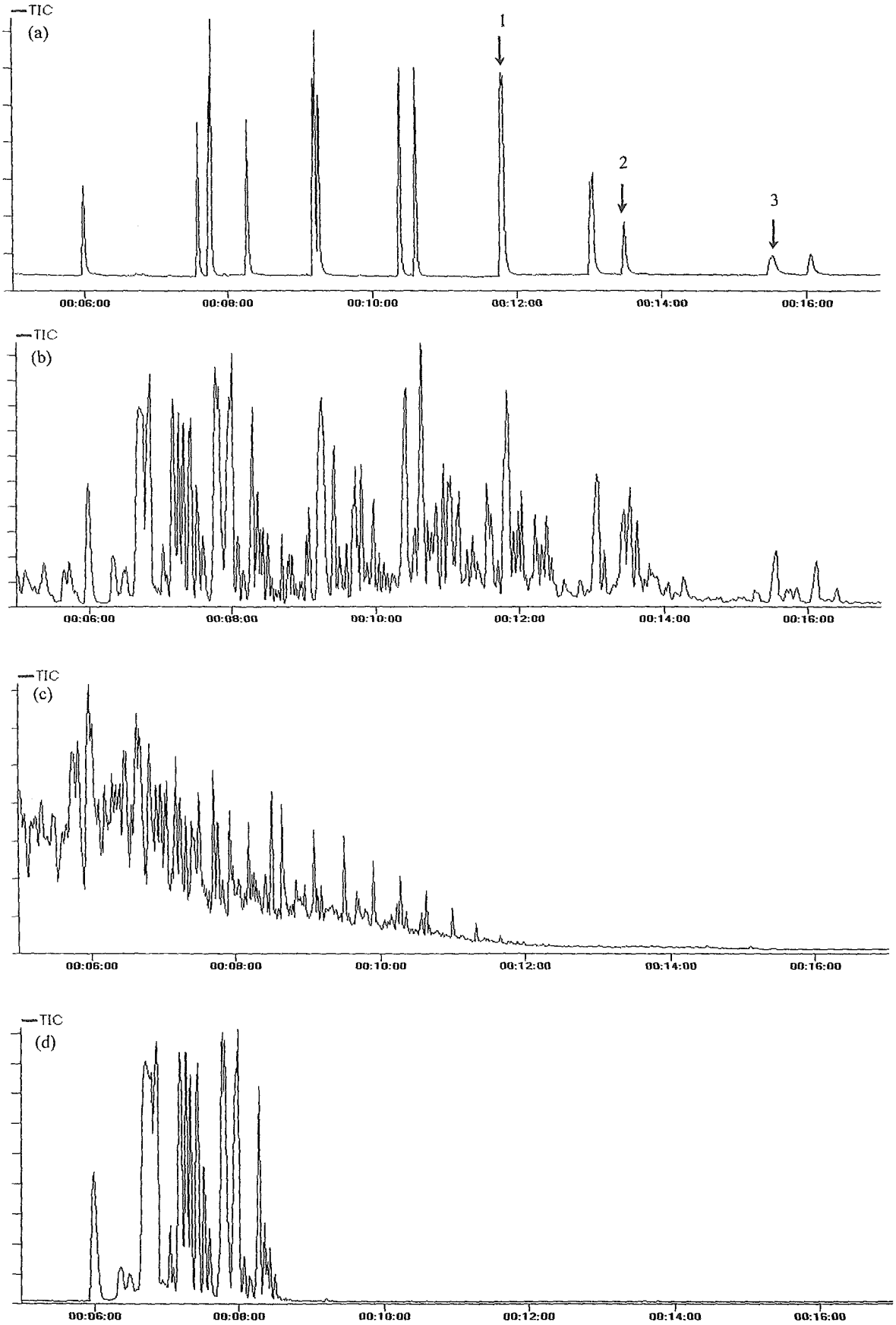

Fig. 1. Total Ion Chromatogram of Creosotes

(a) PAH standard solution (5 μg/ml), (b) sample No. 8, (c) sample No. 9, (d) sample code C. Peak 1: benz[*a*]anthracene, 2: benzo[*a*]pyrene, 3: dibenz[*a*,*h*]anthracene.

Table 6. PAHs Content in Creosote-Treated Wood Products

	Content (μg/g)					
	A	B	C	D	E	F
	Foundation	Railway Sleeper	Railway Sleeper	Railway Sleeper	Railway Sleeper	Stake
	New	New	New	Used	Used	New
Benz[*a*]anthracene	668	193	274	206	455	2
Chrysene	535	172	350	177	562	2
Benzo[*b*]fluoranthene	278	75	230	154	323	1
Benzo[*k*]fluoranthene	156	44	113	103	189	1
Benzo[*a*]pyrene	141	47	126	88	168	ND
Indeno[1,2,3-*cd*]pyrene	41	16	48	20	186	ND
Dibenz[*a*,*h*]anthracene	12	3	18	6	18	ND
Benzo[*ghi*]perylene	31	13	69	14	203	ND

ND means < 1.0 μg/g.

amount in all samples, but still exceeded 10 μg/g in No. 9 and in code C. This finding indicated that some wood preservatives, even those lacking creosote as a constituent, possibly contain high levels of PAHs, *i.e.*, those above the control amount. It is therefore necessary to be aware of the amount of PAHs, not only in cases involving creosote, but also in cases involving oil-type wood preservatives, in which creosote is not listed as a constituent. Sample code A was the only product tested here that conformed to the Japanese regulations.

Sample sections were taken approximately at 2 cm in depth from the surface of wood products manufactured in accord with the Japan agricultural standards (JAS) for the timber of the broadleaf tree. The samples were cut and treated with dichloromethane, and the amount of PAHs contained therein was determined (Table 6). In the commercially available samples A–E, amounts of PAH in excess of 3 μg/g were detected. These findings indicated that creosotes containing more than 10 μg/g each of BaA, BaP, and DBA had been used in the preservation of these samples. A small amount of PAHs per stake material was also detected. Macroscopic studies of sections taken 1 mm from the surface revealed a change in color, which indicated that the creosote had not penetrated to the center of the wood, and instead remained near the surface. Accordingly, almost all of the layers cut from the stake sample contained no creosote, and the total PAH content was low in the test solution prepared from this sample.

In conclusion, we improved upon our previous analytical method for determination of PAHs in creosotes and in creosote-treated woods. We adopted an evaporation-concentration step and changed the column temperature conditions in order to increase the sensitivity of testing and to reduce the amount of time needed to perform GC-MS. The result of a collaborative study indicated that the analytical method developed here appears to be sufficiently stable and can be used for the determination of low levels of BaA, BaP, and DBA. We found that these compounds in high concentrations, thus exceeding the allowed control value, were contained in some creosotes and railway sleepers.

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In vitro induction of polyploidy and chromatid exchanges by culture medium extracts of natural rubbers compounded with 2-mercaptobenzothiazole as a positive control candidate for genotoxicity tests

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Abstract: We tested extracts of custom-made natural rubber samples for cytotoxicity using V79 cells and for chromosome aberration (CA) induction using CHL cells in compliance with the Japanese guidelines for basic biological tests of medical materials and devices. The samples were formulated with a high level of 2-mercaptobenzothiazole (MBT) (A); a low level of MBT (B); or zinc dibutyldithiocarbamate (ZDBC) (C). In the CA test, MBT induced mainly polyploidy, including endoreduplication, and ZDBC induced structural CAs. In the cytotoxicity test, culture medium extracts of A, B, and C suppressed colony formation to 50% of the control value at 53.1%, 94.3%, and >100%, respectively. Culture medium extracts of sample A induced polyploidy and structural CAs in the absence of an exogenous metabolic activation system (S9 mix), but at lower concentrations in its presence, indicating the existence of other leachable promutagens. The extracts of sample B induced structural CAs at the highest concentration and only with S9 mix. Sample C was negative. The facts suggest that sample A may be a candidate for a positive control for genotoxicity tests. The high frequency of polyploidy induced by sample A was not predicted by MBT, suggesting the usefulness of the test for safety evaluation of medical devices. Numerical CAs induced by MBT and sample A are discussed. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 75A: 439–444, 2005

Key words: cytotoxicity; chromosome aberrations; natural rubber; zinc dibutyldithiocarbamate; endoreduplication

INTRODUCTION

Safety evaluation of medical materials is an important step in the production and marketing of medical devices. The Japanese guidelines for basic biological tests of medical materials and devices[1] cover nine assay systems for the initial evaluation. Tests for medical materials are different from those for a single chemical substance in that the samples are extracts of the test material and contain a mixture of chemicals, thus, additive and/or compound effects are expected. We have been testing model materials to search for a positive control for genotoxicity tests because there are no standard positive materials for them.

In the present study, we investigated the culture medium extracts of natural rubber–based materials in the cytotoxicity test using V79 cells and in the *in vitro* chromosomal aberration test using CHL cells in compliance with the Japanese guidelines mentioned above. Rubber materials are widely used for surgical and household gloves and for urinary catheters, although they have induced strong cytotoxicity,[2] severe allergic reactions,[3–5] and urethral strictures.[6–10] The model rubber materials used in this study were originally prepared for sensitization tests.[11] They were custom made with low allergenicity. The only allergenic components are 2-mercaptobenzothiazole and zinc dibutyldithiocarbamate.

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MATERIALS AND METHODS

Cells

We obtained Chinese hamster fibroblast V79 cells (established by Elkind and Sutton[12]) from Japanese Collection of Research Bioresources (JCRB0603, Tokyo) and grew them in

TABLE I
Recipe for Model Materials

Component	Sample A	Sample B	Sample C
Natural rubber	100	100	100
Zinc oxide	5	5	5
Stearic acid	1	1	1
Filler	62	62	62
Black factice	5	5	5
Selected microcrystalline wax	1.3	1.3	1.3
Sulfur	2.0	2.0	2.0
MBT	2.0	0.2	0
ZDBC	0	0	0.5
Sum	178.3	176.5	176.8

Values: dry parts per hundred parts of rubber.

Eagle's minimum essential medium (MEM) (GIBCO 61100-061) supplemented with 10% heat-inactivated fetal calf serum (FCS) in a 37°C humidified atmosphere of 5% CO_2 in air.

We used CHL cells originally established from the lung of a female newborn Chinese hamster by Koyama and colleagues[13] and cloned by Ishidate and Odashima.[14] They were maintained in Eagle's MEM (GIBCO 11095-080) supplemented with 10% heat-inactivated FCS. The doubling time was around 13 h, and the modal chromosome number was 25.

Chemicals and model materials

2-Mercaptobenzothiazole (MBT, CAS No. 149-30-4) from Ouchi Shinko Chemical Industrial Co., Ltd. (Tokyo, Japan) and zinc dibutyldithiocarbamate (ZDBC, CAS No. 136-23-2) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) were dissolved in dimethyl sulfoxide.

Model materials of three rubber sheets (samples A, B, and C; thickness, 1 mm) were prepared with the components shown in Table I by Atom Co., Ltd. (Tokyo) and sterilized with ethylene oxide. Zinc oxide and stearic acid were compounded as vulcanizing accelerator activators. Black factice, selected microcrystalline wax, and sulfur were compounded as a softener, an antioxidant/antiozonant, and a crosslinking agent, respectively. MBT and ZDBC are vulcanizing accelerators. Sample A contained a high level of MBT and the level of MBT in sample B was lower than one tenth of that in sample A. Sample C contained ZDBC instead of MBT.

Cytotoxicity test

Materials were cut into approximately 2 × 15 mm pieces. The pieces (1 g) were put into a centrifuge tube, and 10 mL MEM supplemented with 5% FCS, nonessential amino acids, and 1 m*M* sodium pyruvate (5% FCS-GMNP) was added. After incubation at 37°C in a humidified atmosphere for 24 h, the extract, designated 100%, was decanted and serially diluted with 5% FCS-GMNP to give 80%, 64%, 51%, and 41% extracts.

V79 cells were seeded at 50/well in 24-well plates. After 24-h incubation, the medium was exchanged for 0.5 mL of the serially diluted medium extract or the medium without the extract (for control), and the cells were cultured for 6 days. The colonies formed were fixed with 10% formalin and stained with 5% Giemsa solution. The number of colonies on each well was counted, and the relative plating efficiency was calculated as the ratio of the number of colonies in the treated sample to the number in the control. The cytotoxic potential of the extracts was expressed as the concentration at which the relative plating efficiency was 50% of control (IC_{50}). The IC_{50} value was calculated by the probit method.

Chromosome aberration (CA) test

Materials were cut into approximately 2 × 15 mm pieces. The pieces (1 g) were put into a centrifuge tube, and 10 mL culture medium for CHL cells was added. After incubation at 37°C in a humidified atmosphere for 48 h, the extract, designated 100%, was decanted and diluted with the culture medium.

CHL cells were seeded at 1.5 × 10^5/plate (60 mm in diameter) and incubated for 17 h. They were then treated with extracts for 6 h in the presence or absence of S9 mix followed by expression cultivation with fresh medium for another 18 h. S9 mix was purchased from Kikkoman (Noda, Japan). The S9 fraction[15] was prepared from the livers of Sprague Dawley rats pretreated with phenobarbital and 5,6-benzoflavone. The final concentration of S9 was 5 v/v%. Colcemid (0.2 μg/mL) was added for the last 2 h. Chromosome preparations were made as follows: Cells were trypsinized and incubated in hypotonic KCl solution for 15 min and fixed three times with ice-cold fixative (glacial acetic acid:methanol, 1:3). Two drops of the fixed cell suspension were spread on a clean glass slide, air dried, and stained with Giemsa solution. All slides were coded, and the number of cells with structural or numerical CAs was counted on 100 well-spread metaphases with a modal chromosome number of 25 ± 2. The number of mitotic cells was counted on 1000 live cells and the mitotic index (MI) was used to express the cytotoxic potential of the treatment. The structural CAs were classified into 6 groups: chromatid and chromosome gap (ctg), chromatid break (ctb), chromatid exchange (cte), fragmentation (f), chromosome break (csb), and chromosome exchange (cte, mainly dicentrics and ring chromosomes). The mean and standard deviation (SD) for our historical negative controls of CHL cells are 1.03 ± 1.11 (without S9 mix) and 1.25 ± 1.16 (with S9 mix) for structural aberrations, 0.60 ± 0.93 (without S9 mix) and 0.84 ± 1.02 (with S9 mix) for polyploidy, and 0 for endoreduplication. The experimental groups were judged[16] as negative if the total CA frequency was less than 5.0%, inconclusive if it was 5.0 to up to 10.0%, and positive if it was 10.0% or more. Solvent-treated cells served as the negative control. Experiments were performed at least twice. A representative data from a single experiment are shown, unless otherwise stated.

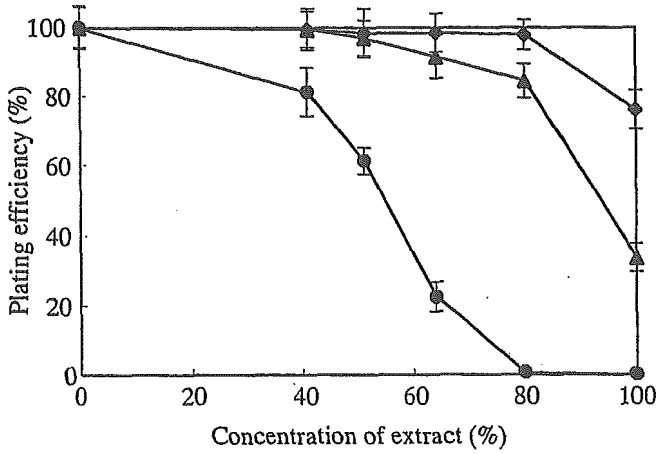

Figure 1. Plating efficiencies of V79 cells treated with medium extracts of samples A (●), B (▲), and C (♦). The samples were extracted with 5% FCS-GMNP for 24 h and the extracts were tested in the colony assay. Values are expressed as means ± SD for eight wells.

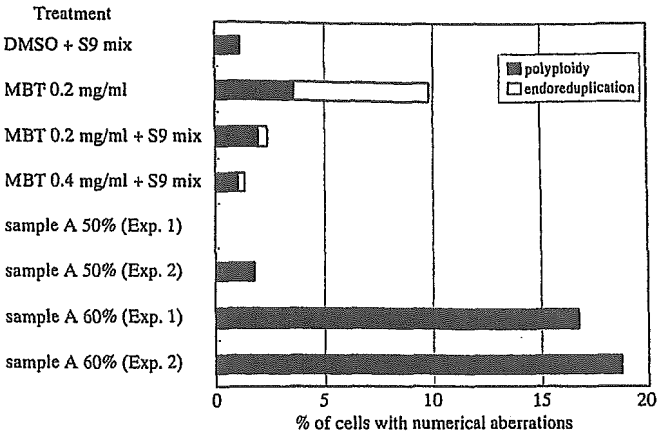

Figure 2. Numerical chromosome aberrations induced by MBT and sample A. Five hundred cells were examined.

RESULTS

In the cytotoxicity test performed with V79 cells, sample A showed the strongest response (Fig. 1). IC_{50} was 53.1%, 94.3%, and more than 100% for samples A, B, and C, respectively.

In the CA test performed with CHL cells, MBT induced polyploidy, including endoreduplication, in the absence and presence of S9 mix (Table II). The number of polyploid cells and endoreduplications was counted on another 500 metaphases for confirmation (Fig. 2). Frequency of polyploid cells and endoreduplications was 3.6% and 6.2% without S9 mix, and 2% and 0.4% with S9 mix, respectively. MBT showed inconclusive response in structural CA induction at 0.4 mg/mL with S9 mix. ZDBC induced structural CAs, mainly cte, in the presence of S9 mix and at lower concentrations in its absence.

Culture medium extracts of sample A induced numerical and structural CAs in the absence of S9 mix and structural CAs in its presence (Table III). The extracts were toxic at the higher concentrations. Structural CAs were induced at lower concentrations in the presence of S9 mix than in its absence. Interestingly, MI of the 40% extract remained high in the presence of S9 mix, although almost all the cells were dead. The numerical CAs were counted on another 500 metaphases for confirmation. The 60% extract induced 16.8% polyploidy and no endoreduplication without S9 mix (Fig. 2, Exp. 1). The 100% of extract of sample B induced 7% CAs in the presence of S9 mix. Sample C did not induce any CAs either with or without S9 mix.

TABLE II
Chromosome Aberration Test of MBT and ZDBC

Chemical	S9 mix	Conc.[a] (mg/mL)	Poly.[b] (%)	Cells with structural aberrations (%)							MI[c] (% of Control)
				ctg	ctb	cte	f	csb	cse	Total	
MBT	−	0	0	2	0	0	0	0	0	2	100
		0.2	8(3)	0	3	1	0	0	0	4	110
		0.4								Tox	12
	+	0	1	0	0	1	0	0	0	1	100
		0.2	5(3)	0	2	0	0	0	0	2	158
		0.4	2	1	2	4	0	0	0	7	87
		0.6								Tox	9
ZDBC	−	0	0	2	0	0	0	0	0	2	100
		0.002	1	0	0	0	0	0	0	0	99
		0.004	0	1	4	11	0	1	0	16	116
		0.006								Tox	10
	+	0	1	0	0	1	0	0	0	1	100
		0.006	0	1	0	2	0	0	0	3	120
		0.008	1	0	2	16	0	0	0	18	104
		0.010	2	2	3	12	0	0	0	16	55

[a]Concentration.
[b]Frequency of polyploidy. Figures in parentheses indicate the number of endoreduplication included.
[c]Mitotic index.