TABLE I
Transformation Assay of PU- and PLLA-Induced Transformants and Parental Controls

Cell Line	Cloning Efficiency (% of Control)	No. of Foci/Plate[a]
A31-1-1	100	0.3 ± 0.48
A5	106.3	2.3 ± 1.23
A6	110.8	47.0 ± 6.28
L11	144.8	114.1 ± 13.32
L21	126.2	84.5 ± 5.10
A31-1-1 + MC[b]	2.2	30.0 ± 5.15

[a]Average with standard deviation of 15 plates.
[b]0.5 μg/mL (positive control).

Two transformants induced by PU (A5 and A6) and two induced by PLLA (L11 and L21) were thawed and cultured for confirmation of transformation and for DNA microarray analysis, which were conducted in parallel. For the positive control assay, cells were treated with 3-methylcholanthrene (MC, 0.5 μg/mL) 24 h after seeding, washed with fresh medium 72 h later, and incubated in normal medium up to 6 weeks. The number of transformed foci per plate served as malignancy of transformants.

DNA microarray analysis

At least 10^7 cells were harvested and frozen in liquid nitrogen. Total RNA was extracted, purified, assessed for yield and purity, and cDNA probes were synthesized with the Atlas™ Pure Total RNA Labeling System (Clontech) according to the manufacturer's instructions. Hybridization of the ^{33}P-labeled probes to the Atlas Array of Mouse Cancer 1.2 k Array (Clontec 7858-1), on which 1176 cDNAs of cancer-related genes were spotted, were performed with Atlas™ cDNA Expression Arrays according to the manufacturer's instructions. The phosphor images of hybridized arrays were analyzed with AtlasImage™ (Clontech). Genes that were up- or down-regulated more than fivefold relative to the negative controls are discussed.

RESULTS

The number of foci per dish produced by the selected transformants increased in the order A5 < A6 < L21 < L11 (Table I). The transformants isolated from PLLA were more malignant than those isolated from PU. A31-1-1 cells treated with MC induced 30 foci per plate, as expected. Figure 1 shows the actual foci features. The A5 and A6 foci resembled those on the MC-treated dishes. The extracellular matrix appeared lyzed in transformants L11 and L21.

Fourteen genes increased expression more than fivefold in at least one transformant (Fig. 2, Table II). The three most markedly up-regulated genes were c-*fos* protooncogene, FBJ osteosarcoma oncogene B, and Jun oncogene; all increased most in L11. The only

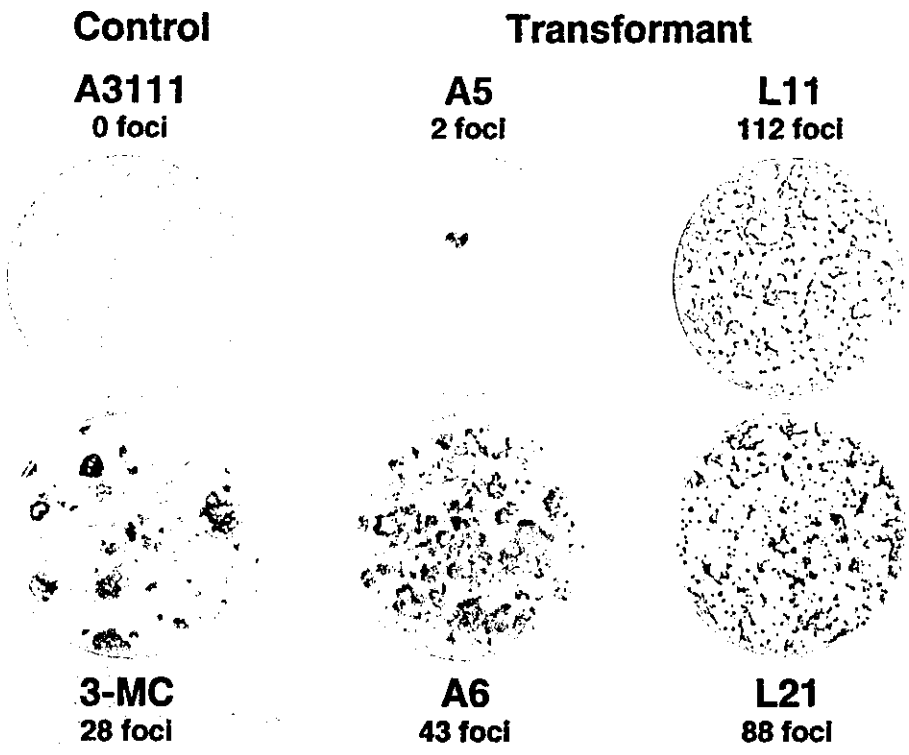

Figure 1. Photographs of dishes (6 cm in diameter) with Giemsa-stained foci in the confirmative transformation assay. The number of foci is shown in the representative plate of the control and each transformant. The control cells grew in monolayers and stained pink. The transformed cells stained blue were observed in the other plates, and the extracellular matrix of L11 and L21 plates appeared lyzed.

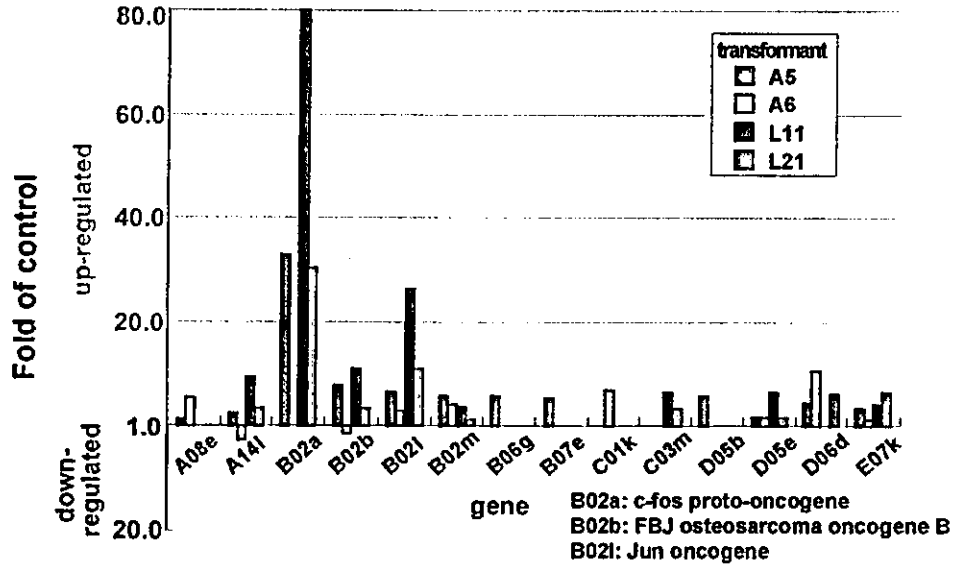

Figure 2. Expression profile of transformant genes whose expression increased more than fivefold in at least one transformant (see Table II for name key).

transformant that did not show any appreciable change in gene expression was A6.

Twenty-five genes decreased expression more than fivefold in at least one transformant (Fig. 3, Table III). The four most markedly down-regulated genes were pleiotrophin (PTN), histidine triad nucleotide-binding protein, protein kinase C iota, and large multifunctional protease 7; all except for large multifunctional protease 7, decreased most in L11. Transformant A6 showed a 20-fold decrease in the expression of large multifunctional protease 7.

Figure 4 shows the expression profiles of 30 oncogenes and tumor suppressor genes. *c-fos* Protooncogene, FBJ osteosarcoma oncogene B, and Jun oncogene were up-regulated markedly in transformants A5, L11, and L21. The expression levels of ras, src, raf, mitogen-activated protein kinases, MEK, and p53 were similar in parental cells and transformants within an approximate twofold increase or decrease.

Among the extracellular matrix-related genes, HSP60, HSP65, HSPD1, mitochondrial matrix protein P1 precursor, 60-kDa chaperonin, GroEL protein, and matrix metalloproteinase 9 were markedly down-regulated, especially in transformant L11 (Fig. 5).

Expression of transforming growth factor (TGF) β1 and 2 and 8 connexin-related genes did not change significantly in any transformants (data not shown).

Table IV lists the genes that were up- or down-regulated more than fivefold relative to parental controls. Large multifunctional protease 7 was down-regulated more than fivefold, in all transformants. The gene expression profile of A6 was unique among the

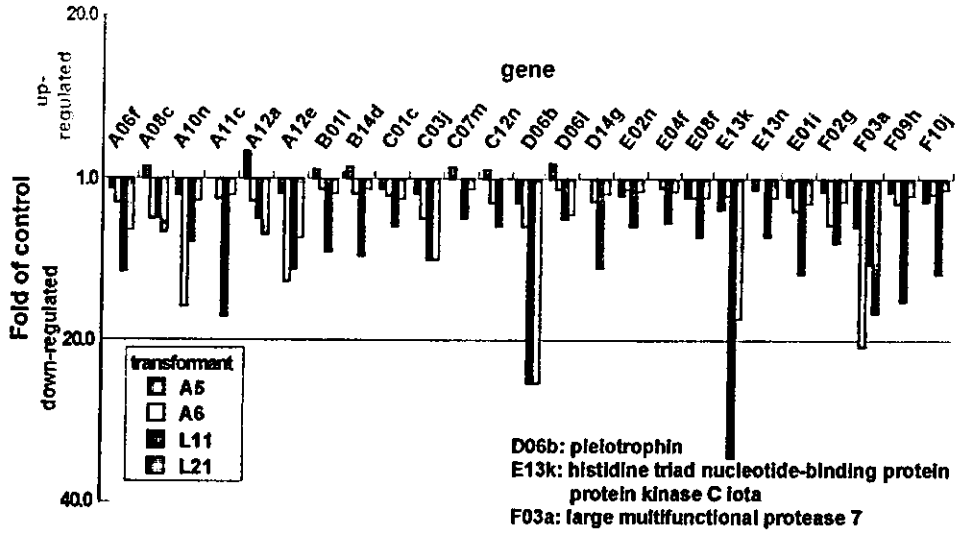

Figure 3. Expression profile of transformant genes whose expression decreased more than fivefold in at least one transformant (see Table III for name key).

TABLE II
Transformant Genes Whose Expression Increased More Than Fivefold

Code	Gene
A08e	Integrin β 7
A14l	Insulin-like growth factor binding protein 10
B02a	c-*fos* protooncogene
B02b	FBJ osteosarcoma oncogene B
B02l	Jun oncogene
B02m	*jun*B protooncogene
B06g	HSP27, HSP25, HSPB1, growth-related 25-kDa protein
B07e	N-oxide forming dimethylaniline monooxygenase 1, hepatic flavin-containing monooxygenase 1, dimethylaniline oxidase 1
C01k	Retinoic acid receptor β, nuclear receptor subfamily 1 group B member 2
C03m	Caspase-activated DNase, DNase inhibited by DNA fragmentation factor
D05b	Insulin-like growth factor II precursor, multiplication-stimulating polypeptide
D05e	Leukemia inhibitory factor, cholinergic differentiation factor
D06d	Proliferin
E07k	Nonreceptor type 16 protein tyrosine phosphatase

four transformants in that proliferin was up-regulated more than 10-fold, thrombospondin 1 (TS-1) was down-regulated more than 10-fold, and large multifunctional protease 7 was down-regulated more than 20-fold. L11 showed the most appreciable changes in expression intensity and in the number of genes down-regulated. In that transformant, c-*fos* protooncogene and Jun oncogene were markedly up-regulated whereas PTN, histidine triad nucleotide-binding protein, and protein kinase C iota were markedly down-regulated. L21 showed marked up-regulation in the expression of c-*fos* protooncogene and Jun oncogene and marked down-regulation in the expression of PTN, histidine triad nucleotide-binding protein, protein kinase C iota, and large multifunctional protease 7.

The only significant association we observed between changes in gene expression and malignancy were for cellular tumor antigen p53, procollagen VI alpha 3 subunit, and connexin 43. The relationship was inverse and was observed when the decrease in expression was less than fivefold (data not shown).

DISCUSSION

DNA microarray analysis of two transformants (A5 and A6) induced on PU film and two (L11 and L21) induced on PLLA film showed L11 to be the most malignant and the one that underwent the most appreciable changes in gene expression levels.

Both c-*fos* and Jun were up-regulated in all transformants except A6. c-*fos*, a protooncogene, is the cellular homolog of v-*fos*, which was originally isolated from a murine osteosarcoma. Fos protein is a major component of the AP-1 transcription factor complex, which includes the jun family. In the present study, the genes involved in bone formation, namely c-*fos*, FBJ osteosarcoma oncogene B, Jun, PTN, ADAM-TS, and MMP9, were among those that changed expression levels (Table IV). They were up- or down-regulated markedly in L21 and even more so in L11. Wang et al.[3] demonstrated in transgenic and chimeric mice that overexpression of c-*fos* affects bone, cartilage, and hematopoietic cell development. Wang et al.[4] also showed that mice lacking c-*fos* are growth retarded, develop osteopetrosis with deficiencies in bone remodeling and tooth eruption, and have altered hematopoiesis. Onyia et al.,[5] investigating gene expression in rat osteoblast-like osteosarcoma cells (ROS 17/2.8) cultured *in vivo*, demonstrated that at 56 days, c-*fos* expression increased up to fivefold, c-*jun* expression increased 2.1-fold, and MMP-9 expression decreased to undetectable levels. Those findings are consistent with the present finding in L11, that is, that *jun*, *fos*,

TABLE III
Transformant Genes Whose Expression Decreased More Than Fivefold

Code	Gene
A06f	Cdk 6 inhibitor, Cdk 4 inhibitor C, Cdk inhibitor 2C
A08c	Fat tumor suppressor homolog (*Drosophila*)
A10n	Thrombospondin 1
A11c	VCAM-1 precursor
A12a	Cysteine-rich intestinal protein
A12e	Delta-like homolog 1, preadipocyte factor 1, SCP 1, FA1, ZOG
B01l	EB1 APC-binding protein
B14d	HSP60, HSP65, HSPD1, mitochondrial matrix protein P1 precursor, 60-kDa chaperonin, GroEL protein
C01c	Apoptosis inhibitor 1
C03j	Clusterin precursor, clustrin, apolipoprotein J, sulfated glycoprotein 2
C07m	Platelet-derived growth factor receptor α precursor
C12n	Hek2 murine homolog, Mdk5 mouse developmental kinase, Eph-related tyrosine-protein kinase receptor
D06b	PTN
D06l	Small inducible cytokine A9
D14g	Avian sarcoma virus CT10 (v-*crk*) oncogene homolog
E02n	Nonreceptor type 11 protein tyrosine phosphatase, phosphotyrosine phosphatase
E04f	Cdk7, CDC2-related kinase 4, Cdk-activating kinase, 39-kDa protein kinase, MO15, MPK7
E08f	Serum-inducible kinase
E13k	Histidine triad nucelotide-binding protein, protein kinase C iota
E13n	Menage a trois 1
F01i	A disintegrin-like and metalloprotease with thrombospondin type 1 motif protein 1
F02g	Matrix metalloproteinase 9
F03a	Large multifunctional protease 7
F09h	Developmentally d neural precursor cell expressed
F10j	Tubulin cofactor a

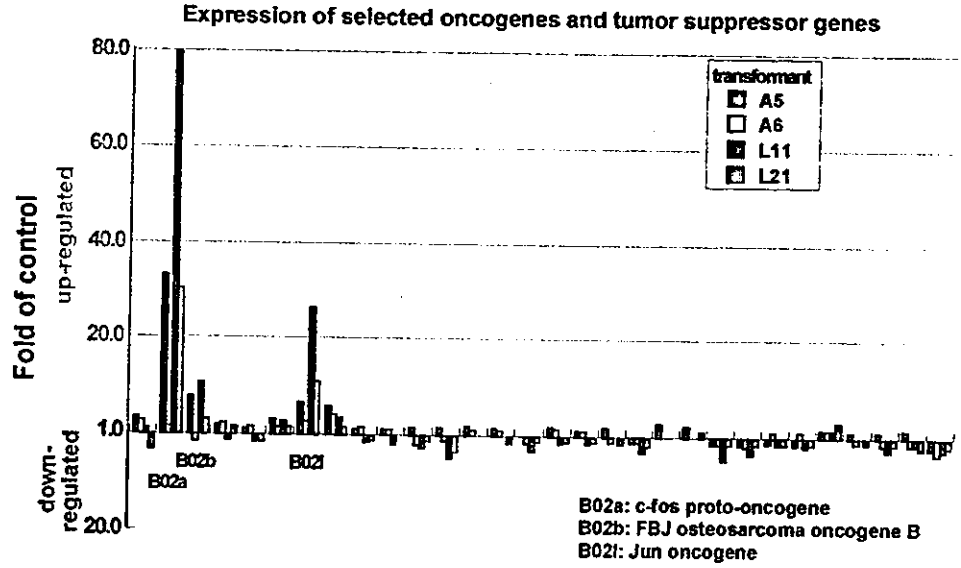

Figure 4. Expression profile of transformant oncogenes and tumor suppressor genes. Thirty-nine spots of oncogene- and tumor suppressor gene-related genes were on the DNA tip and 30 of them were analyzable, but not in all transformants. Only those genes whose expression level changed at least fivefold are named.

and osteosarcoma genes were up-regulated whereas MMP9 was down-regulated, suggesting that PLLA stimulated BALB/3T3 cells to express genes related to osteogenesis.

Nakamura et al.[6] observed bone formation in 6 of 22 tumors induced in rats by a 2-year PLLA subcutaneous implantation. Tumor incidence was 44% (22/50) with PLLA[6] and 38% (11/29) with PU,[1] which correlates well with the *in vitro* malignancy incidence data in the present study. Isama and Tsuchiya,[7] and Ikarashi et al.[8] reported that low-molecular-weight PLLA increases alkaline phosphatase activity and stimulates calcification of mouse osteoblast-like MC3T3-E1 cells.

PTN, a heparin-binding protein that can function as a neurite-promoting factor[9] or a mitogenic factor for fibroblasts,[10] contains two β-sheet domains that correspond to TS-1 repeats.[11] The expression of PTN is increased in various human tumors, suggesting it as a tumor marker and a target for tumor therapy. PTN was shown to regulate bone morphogenetic protein-induced ectopic osteogenesis in rats.[12]

A disintegrin-like and metalloprotease with TS-1 motif protein 1 (ADAM-TS) is a family of zinc-dependent proteases that has an important role in a variety of normal and pathological conditions such as arthritis and cancer. They consist of a signal sequence, a propeptide, a metalloproteinase domain, a disintegrin-like domain, a cysteine-rich region, and a variable number of TS-1 repeats. High levels of their tran-

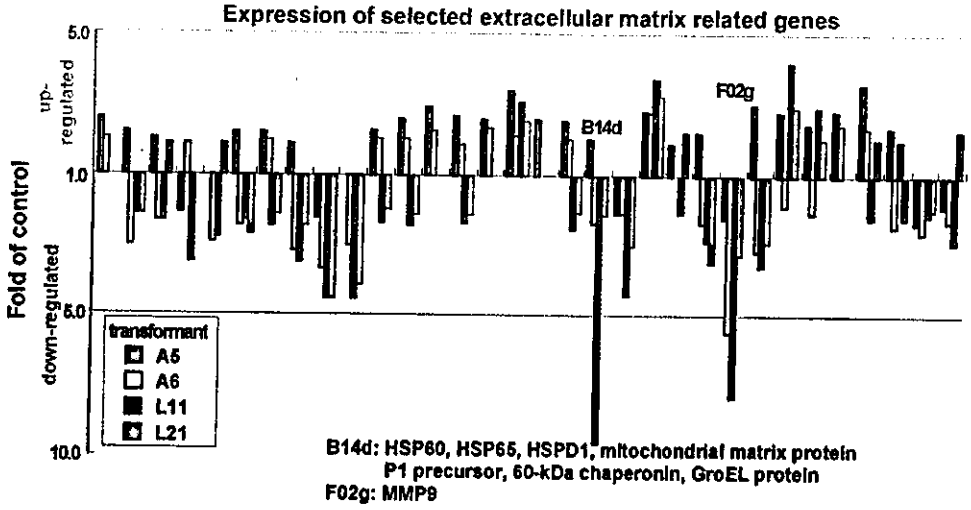

Figure 5. Gene expression profile of extracellular matrix-related genes. Thirty-two extracellular matrix-related genes were analyzable but data were not available in some transformants even in them. The code and name of genes whose expression changed fivefold or less are not shown. L11 showed clear down-regulation in expression of genes of codes B14d and F02g.

TABLE IV
Transformant Genes Whose Expression Increased or Decreased More Than Fivefold of Controls[a]

Transformant	Up-Regulated	Down-Regulated
A5	**c-*fos* protooncogene (33.0)** FBJ osteosarcoma oncogene B Jun oncogene *jun*B protooncogene HSP27, HSP25, HSPB1, growth-related 25-kDa protein N-oxide forming dimethylaniline monooxygenase 1, hepatic flavin-containing monooxygenase 1, dimethylaniline oxidase 1 Insulin-like growth factor II precursor, multiplication-stimulating polypeptide	Large multifunctional protease 7
A6	Integrin β 7 Retinoic acid receptor β, nuclear receptor subfamily 1 group B member 2 **Proliferin (10.7)**	Fat tumor suppressor homolog (*Drosophila*) **Thrombospondin 1 (15.8)** **Delta-like homolog 1, preadipocyte factor 1, SCP 1, FA1, ZOG (12.7)** Clusterin precursor, clustrin, apolipoprotein J, sulfated glycoprotein 2 PTN Matrix metalloproteinase 9 **Large multifunctional protease 7 (20.7)**
L11	Insulin-like growth factor binding protein 10 **c-*fos* protooncogene (79.8)** **FBJ osteosarcoma oncogene B (10.9)** **Jun oncogene (26.3)** Caspase-activated DNase, DNase inhibited by DNA fragmentation factor Leukemia inhibitory factor, cholinergic differentiation factor	**Cdk 6 inhibitor, Cdk 4 inhibitor C, Cdk inhibitor 2C (11.5)** Fat tumor suppressor homolog (*Drosophila*) Thrombospondin 1 **VCAM-1 precursor (17.0)** **Delta-like homolog 1, preadipocyte factor 1, SCP 1, FA1, ZOG (11.2)** EB1 APC-binding protein HSP60, HSP65, HSPD1, mitochondrial matrix protein P1 precursor, 60-kDa chaperonin, GroEL protein Apoptosis inhibitor 1 **Clusterin precursor, clustrin, apolipoprotein J, sulfated glycoprotein 2 (10.0)** Platelet-derived growth factor receptor α precursor Hek2 murine homolog, Mdk5 mouse developmental kinase, Eph-related tyrosine-protein kinase receptor **PTN (25.2)** Small inducible cytokine A9 **Avian sarcoma virus CT10 (v-*crk*) oncogene homolog (11.0)** Nonreceptor type 11 protein tyrosine phosphatase, phosphotyrosine phosphatase Cdk7, CDC2-related kinase 4, Cdk-activating kinase, 39-kDa protein kinase, MO15, MPK7 Serum-inducible kinase **Histidine triad nucelotide-binding protein, protein kinase C iota (34.4)** Menage a trois 1 **A disintegrin-like and metalloprotease with thrombospondin type 1 motif protein 1 (11.8)** Matrix metalloproteinase 9 **Large multifunctional protease 7 (10.4)** **Developmentally d neural precursor cell expressed (15.0)** **Tubulin cofactor a (11.8)**
L21	**c-*fos* protooncogene (30.3)** **Jun oncogene (11.1)** Proliferin Nonreceptor type 16 protein tyrosine phosphatase	Cdk 6 inhibitor, Cdk 4 inhibitor C, Cdk inhibitor 2C Fat tumor suppressor homolog (*Drosophila*) Cysteine-rich intestinal protein Delta-like homolog 1, preadipocyte factor 1, SCP 1, FA1, ZOG **Clusterin precursor, clustrin, apolipoprotein J, sulfated glycoprotein 2 (10.0)** **PTN (25.2)** **Histidine triad nucleotide-binding protein, protein kinase C iota (17.2)** **Large multifunctional protease 7 (16.6)**

[a]Genes in regular and bold text were up- or down-regulated between 5- and 10-fold, and more than 10-fold, respectively. Figures in parentheses indicate fold-increase or -decrease in gene expression compared with parental cells.

scripts are observed in some tumor biopsies and cell lines, including osteosarcomas, melanoma, and colon carcinoma cells.[13,14]

Vascular cell adhesion molecule 1 (VCAM-1) is inducible by inflammatory cytokines and lipopolysaccharides such as interleukin 1, tumor necrosis factor α, interferon γ, and interleukin 4. It functions by binding with integrin $\alpha_4\beta_1$. Kawaguchi and Ueda[15] reported that VCAM-1 was not expressed in the seven osteosarcoma specimens tested in a study on the distribution of integrins and their matrix ligands in osteogenic sarcomas. Those results agree with the finding in the present study that osteosarcoma-like gene expression was down-regulated in L11.

BALB/3T3 cells are sensitive to transformation and must be handled carefully. Repeated subculture and overgrowth are not advised. We cultured the isolated foci under constant conditions to investigate the difference in gene expression. Because DNA microarray analysis was done only once, we discussed only genes that showed clear differences in expression from the controls. Based on these preliminary data, further studies are needed to confirm bone formation by PU and PLLA.

CONCLUSIONS

The gene that showed the greatest change in expression after cell culture on PU was *c-fos* protooncogene. Osteogenesis was a common function of proteins encoded by genes that underwent a marked change in expression. Although the changes in gene expression induced by PU and PLLA differed in intensity, the results were consistent with previously reported findings of *in vivo* tumor formation. PLLA had a greater effect than PU on the expression levels of genes related to bone formation. In the transformants, both up-regulation of oncogenes and down-regulation of other kinds of genes were induced, and the latter appeared to be more related to the malignancy of transformants than the former.

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Risk assessment of di(2-ethylhexyl)phthalate released from PVC blood circuits during hemodialysis and pump–oxygenation therapy

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Abstract

This study deals with in vitro investigation of the release of di(2-ethylhexyl)phthalate (DEHP) during hemodialysis and pump–oxygenation therapy using medical grade PVC tubing. High resolution GC–MS analysis showed that the release of DEHP was time-dependently increased by circulation of bovine blood into a major system for the hemodialysis that is used in Japan, and the amount of DEHP released into the blood had reached 7.3 mg by 4 h of circulation. No significant difference was observed in the release patterns of DEHP under the conditions with and without fluid removal treatment during hemodialysis, indicating that the treatment seems not to be effective for eliminating DEHP from the blood through the hemodialysis membrane. Mono(2-ethylhexyl)phthalate (MEHP) analysis revealed that a small amount of DEHP (3–4%) was converted to MEHP by hydrolysis during the circulation of blood. A considerable amount of DEHP was also released from the PVC circuit mimicking the pump–oxygenation system, and 7.5–12.1 mg of DEHP had migrated into bovine blood from the circuit by 6 h. It was noticed, however, that the release was obviously suppressed by covalently coating the inner surface of the PVC tubing with heparin, though this effect was not observed with ionic bond type-heparin coating. Covalent bond type-heparin coating of PVC tubing seems to offer the advantage of decreasing the amount of DEHP exposure to patients during treatment using a PVC circuit.

Keywords: DEHP; MEHP; Hemodialysis; Pump–oxygenator; PVC tubing; Blood circuit

1. Introduction

Many kinds of phthalate esters have been reported to have weak estrogenic activity in vitro, though there has been no evidence of uterotrophic activity by esters such as di(2-ethylhexyl)phthalate (DEHP) and di-*n*-butyl phthalate (Zacharewski et al., 1998). Phthalate esters do not have a structure likely to bind to estrogen receptors, and they are therefore not considered estrogenic compounds in vivo (Koizumi et al., 2000). However, some of them are considered to be toxic compounds exhibiting effects similar to those of endocrine disruptors in rodents, characterized in male rats by antiandrogenic effects on the development of the reproductive system and production of normal sperm (Poon et al., 1997; Lamb et al., 1987; Tyl et al., 1988), and in female rats by decrease of 17β-estradiol level in the blood (Davis et al., 1994).

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It has been reported that orally administrated DEHP may be absorbed from the gut as a monoester derivative, mono(2-ethylhexyl)phthalate (MEHP), after enzymatic hydrolysis in the intestine (Lake et al., 1977). Recent in vitro studies found that MEHP inhibits FSH stimulated c-AMP accumulation in cultured Sertoli cells and induced apoptosis of germ cells in coculture with Sertoli cells (Heindel and Chapin, 1989; Grasso et al., 1993; Richburg and Boekelheide, 1996; Lee et al., 1999; Richburg et al., 2000), in addition to reducing 17β-estradiol production and aromatase mRNA expression (Davis et al., 1994; Lovekamp and Davis, 2001). These results indicate that MEHP is an active metabolite of DEHP, and suggest that any toxic effects of orally ingested DEHP are more likely governed by the properties of the corresponding monoester rather than by intact DEHP.

Phthalate esters, and DEHP in particular, have been extensively used as plasticizers as a result of the increased flexibility of polyvinyl chloride (PVC), a plastic polymer used in a wide array of products including medical devices such as tubings, intravenous bags, blood containers, and catheters. DEHP is easily released from PVC products not only into foods but also into pharmaceuticals and body fluids that come in contact with the plastic, and the migrated DEHP is directly and/or indirectly introduced into human body (Allwod, 1986; Loff et al., 2000; Tickner et al., 2001). The general toxicity of DEHP was well evaluated in the latter half of the 20th century, and so far the results of risk assessment to human health had indicated that this compound is relatively safe to humans. It has recently been considered, however, that precautions should be taken to limit human exposure to DEHP, particularly that of high risk patients such as male neonates, male fetuses, and peripubertal males, based on findings that DEHP has the potency to cause adverse effects in young rodents.

In consideration of these issues, several agencies and official organizations around the world, including the Japanese Ministry of Health, Labor and Welfare (JMHLW), individually evaluated the safety of DEHP released from PVC products. The US Food and Drug Administration has calculated the ratio of tolerable daily intake (TDI) value to the exposure amount of DEHP in medical treatments with various PVC medical devices utilizing data on toxicity and the release profile of DEHP obtained in reports on various devices (Center for Devices and Radiological Health, 2001). Such data may be very useful for evaluating the safety of these devices for patients.

It is essential that the exposure amount be precisely determined in order to evaluate its significance as an integral part of the assessment of the risk of DEHP to human health. Although several studies on the release of DEHP from PVC medical devices have been reported in Japan (Muramatsu et al., 2000; Hanawa et al., 2000; Tanaka et al., 2001), the JMHLW recently decided to re-estimate the exact amount of DEHP exposure to patients during medical treatments with the major PVC devices used in Japan. In the present study, which was one of the JMHLW projects, in order to clarify safety and evaluate risk assessment, we examined the release level of DEHP from hemodialysis and pump–oxygenation systems using a PVC blood circuit, and also estimated the exposure amount to MEHP that is an active metabolite of DEHP.

Analytical method having high sensitivity, precision, selectivity of quantitative ions, and low background are required to determine DEHP and MEHP for clinical assessment, and hence most of the conventional methods developed up to the present are not available in this point. Column-switching LC–MS method recently developed must be very useful for direct analysis of DEHP released from PVC medical devices because of the high-throughput and low-contamination (Inoue et al., 2003a,b). In addition, LC–MS/MS and high resolution GC–MS analytical techniques having high sensitivity and selectivity of target ions may be also available for the analysis. From these potential methods, we chose high resolution GC–MS technique that has the highest resolution and selectivity of quantitative ions, as the method for determination of the phthalates.

2. Materials and methods

2.1. Chemicals and utensils

The standards, including DEHP, DEHP-d_4, MEHP, MEHP-d_4 and fluoranthene-d_{10} (F-d_{10}), were purchased from Kanto Chemical Co. (Tokyo, Japan) or Hayashi Chemical Co. (Osaka, Japan). Hexane, methanol, anhydrous sodium sulfate, sodium chloride

of DEHP analytical grade, dioxin analytical grade diethyl ether, and HPLC analytical grade distilled water were used in this study. Fresh bovine blood containing heparin (10,000 U/l) purchased from DARD Co. (Tokyo, Japan) was used as a solvent to be circulated into the hemodialysis and pump–oxygenation systems. All utensils were made of glass, metal, or Teflon, and were heated at 250 °C for more than 16 h before use.

2.2. Blood circuits

The hemodialysis system consisted of medical grade PVC tubing (i.d. 3.5 mm), a major product in Japan, provided by company A, and a hemodialyzer composed of a combination of polycarbonate casing and polyethersulfone hollow-fiber provided by company B. The inner volume of the blood circuit and the total area of the inner surface of PVC products including connectors were approximately 140 ml and 950 cm^2, respectively. Pooled bovine blood (5000 ml, Htc. 30%, TP 5.7–5.9 g/dl) containing heparin was circulated into the circuit via a thermoregulator (37 °C) for 4 h at 200 ml/min employing a widely used pump system (JMS GC100), under the respective conditions with and without fluid removal treatment (15 ml/min) during hemodialysis. Physiological saline was used as a dialysate at a flow rate of 500 ml/min, and saline was added to the pooled blood at the same ratio (15 ml/min) for adjusting the Htc. value under the condition of fluid removal treatment. During blood circulation, the blood samples were collected in increments of 10 ml at 10, 30, 60, 120, and 240 min, and stored at −30 °C.

Four kinds of medical grade PVC tubings were used to construct blood circuits mimicking the pump–oxygenation system. Two identical tubings (i.d. 6 mm, length 3 m) were provided by company C, and the inner surface of one was covalently coated with heparin. The remaining two identical tubings (i.d. 9 mm, length 3 m) were provided by company D, and one was coated with an ionic bond type-heparin. A thermoregulator (37 °C) was set in the middle portion of each tubing, and pooled bovine blood (500 ml, Htc. 36 ± 3%) was circulated into each PVC circuit at a flow rate of 1.5 l/min by a pump system (Sarns 8000) typically used for pump–oxygenation treatment. During blood circulation, blood samples were collected in 10 ml increments at 0, 1, 3, and 6 h, and stored at −30 °C.

All investigations of extraction of DEHP from these circuits were repeated in triplicate.

2.3. Extraction of phthalate esters from bovine blood

For DEHP analysis, samples of bovine blood circulated into hemodialysis system (100 μl) and pump–oxygenation system (20 μl) were transferred into screw-capped glass tubes, which were filled up to the level of 1 ml by distilled water. DEHP-d_4 (50 ng) and sodium chloride (10 mg) were added to the sample, which was then mixed well and incubated for 30 min at room temperature. Hexane (2 ml) was added to the sample, which was then shacked for 20 min at room temperature. After centrifugation, the organic phase was collected and dehydrated with anhydrous sodium sulfate followed by GC-MS analysis described below.

For MEHP analysis, 0.01 M HCl (800 μl), MEHP-d_4 (50 ng), and sodium chloride (10 mg) were added to the blood sample (200 μl). After incubation, MEHP was extracted with diethyl ether (2 ml) followed by dehydration, carboxyl-methylation with diazomethane, and GC–MS analysis.

Recovery of DEHP and MEHP from bovine blood was estimated using F-d_{10} as a spike substance and the blood containing DEHP-d_4 or MEHP-d_4, according to the methods described above.

2.4. Measurement of phthalate esters

DEHP and the carboxyl methylated MEHP (MEHP-Me) contents in each sample were measured by GC–MS analysis using a JEOL JMS-700 instrument equipped with a BPX-5 fused silica capillary column (0.22 mm × 25 m, SGE) under the temperature conditions of initial temperature to 120 °C for 2 min and then increasing to 300 °C at 10 °C/min. The electron impact (EI)-mass spectrum was recorded at 70 eV for qualitative analysis, and the ions of *m/z* 149.0240 (DEHP), 153.0492 (DEHP-d_4), 163.0395 (MEHP-Me), 167.0647 (MEHP-d_4-Me), and 212.1410 (F-d_{10}) were selected as the quantitative ions in selected-ion mode (SIM) analysis (resolution = 5000) using the lock and check method of calibrating standard ions (*m/z* 168.9888 of PFK).

Quantitative analysis of each sample was repeated six times for calibration lines and three times for the other samples. Preparation of calibration curves and calculation of quantitative data were performed by the computer software TOCO, Version 2.0 (Total Optimization of Chemical Operations), practicing the function of mutual information (FUMI) theory (Hayashi and Matsuda, 1994; Hayashi et al., 1996, 2002; Haishima et al., 2001).

3. Results and discussion

3.1. Precision of quantitative analysis and recovery of phthalate ester

The precision of the quantitative analysis, which is described as the R.S.D. or S.D. of the measurements, is very important to evaluation of other analytical characteristics such as specificity, linearity, range, accuracy LOD (limit of detection), LOQ (limit of quantitation), and robustness, which parameters are proposed by the ICH guidelines (ICH Guidelines, 1996). Although the exact precision is not easy to estimate in practice, FUMI theory can provide the measured S.D. of every calibration sample without repeated measurements (Hayashi and Matsuda, 1994).

Each calibration line to quantify the concentration and recovery of DEHP and MEHP was prepared by using DEHP-d_4, MEHP-d_4, and F-d_{10} as internal standards. All calibration lines had good linearity ($r = 0.999$) in the low (0–25 ppb) and high (25–200 ppb) concentration ranges tested in GC–MS analysis. The 95% confidence intervals of the calibration lines, which represent the error between the calibration lines obtained under the same experimental conditions, were very narrow, indicating that the precision was sufficiently high. Instrumental LOD and LOQ predicted by FUMI theory from data of DEHP-d_4/F-d_{10} and MEHP-d_4/F-d_{10} standard curves were 0.0204 and 0.6748 ppb for DEHP, and 0.0380 and 0.1266 ppb for MEHP, respectively. Background analyses of DEHP and MEHP originating from each reagent and GC–MS instrument showed that 1.2 ± 0.27 ppb of DEHP and 0.08 ± 0.023 ppb of MEHP were detected as background contamination when 50 ng each of the internal standards (DEHP-d_4 and MEHP-d_4) were used in the quantitative analyses. From these results, the experimental LOD and LOQ were calculated as 2.01 and 3.90 ppb for DEHP, and 0.149 and 0.31 ppb for MEHP, respectively, and the quantitative data described below were corrected by these background values.

Recovery rates of DEHP and MEHP extracted from bovine blood in this investigation were 90.1 ± 6.8 and $72.4 \pm 2.47\%$, respectively.

3.2. Identification of DEHP and MEHP

SIM chromatograms in DEHP and MEHP analyses of bovine blood circulated into the pump–oxygenation systems described below are shown in Fig. 1. A peak detected at 16.7 min in DEHP analysis (Fig. 1A) was identified as DEHP by scan-mode EI-mass spectrometry in which characteristic fragment ions were observed at *m/z* 70, 83, 104, 112, 149, 167, and 279. In MEHP analysis (Fig. 1B), MEHP-Me was detected at 12.4 min, and typical fragment ions such as *m/z* 70, 83, 104, 112, 149, 164, and 181 were observed in the EI-mass spectrometry. The retention times and EI-mass spectra were the same as those of the authentic DEHP and MEHP standards.

3.3. DEHP release from hemodialysis system

Release test of DEHP from the hemodialysis circuit was performed by using the major system in current use in Japan. The release profile of DEHP from the system is shown in Fig. 2. Bovine blood used in this experiment contained 248.9 ± 123.6 ppb of DEHP as the background. Under the condition of fluid removal treatment, the concentration of DEHP in the blood time-dependently increased by circulating the blood through the hemodialysis circuit. The concentration of DEHP after blood circulation for 30, 60, 120, and 240 min was shown in Table 1. A similar amount of DEHP was released from the hemodialysis system under the condition without fluid removal treatment. In this test, the concentration of DEHP had reached 1741.8 ± 65.1 ppb at 4 h of circulation (Fig. 2 and Table 1).

Hemodialyzers are very utile devices often employed in the medical field in treatment for renal failure. Precise evaluation of DEHP exposure is very

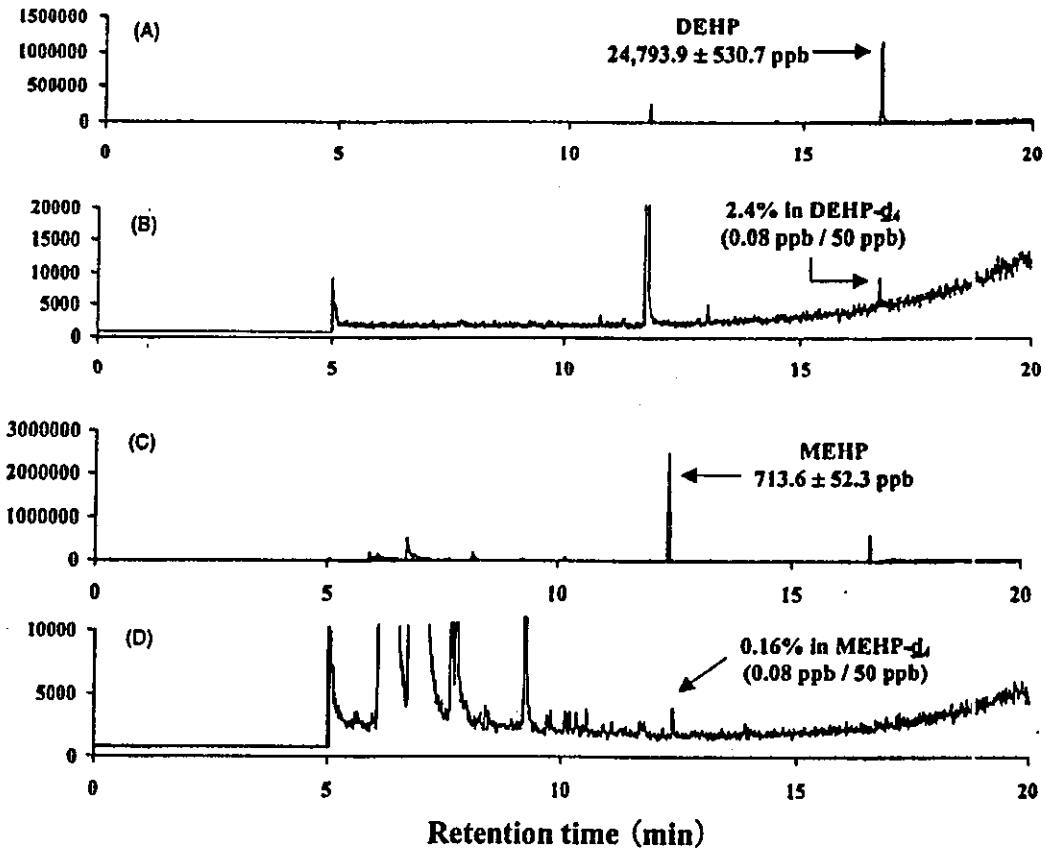

Fig. 1. SIM chromatograms in GC–MS analysis of DEHP and MEHP extracted from bovine blood circulated for 6 h through a pump–oxygenator system consisting of non-coated tubing produced by company D; (A) DEHP analysis; (B) background in DEHP analysis; (C) MEHP analysis; (D) background in MEHP analysis.

important for hemodialysis patients due to the frequent necessity of long-term therapy. In vivo and in vitro studies have reported on the release of DEHP into circulated blood during hemodialysis; DEHP in a range of 3.23–360 mg was extracted from the hemodialysis circuits during a single 4 h dialysis session (Kambia et al., 2001; Faouzi et al., 1999; Flaminio et al., 1988; Pollack et al., 1985; Lewis et al., 1978). US-FDA

Table 1
Amounts of DEHP and MEHP detected from bovine blood circulated into hemodialysis system

Circulation time (min)	Concentration (ppb)			
	DEHP		MEHP	
	With fluid removal treatment	Without fluid removal treatment	With fluid removal treatment	Without fluid removal treatment
0	248.9 ± 123.6	261.6 ± 147.6	13.3 ± 6.9	14.0 ± 6.4
30	441.4 ± 55.5	473.4 ± 124.9	35.6 ± 4.0	30.3 ± 4.6
60	606.2 ± 28.4	657.2 ± 91.8	48.1 ± 5.8	42.9 ± 7.0
120	949.9 ± 85.3	979.6 ± 42.7	57.2 ± 7.5	54.6 ± 2.5
240	1717.8 ± 147.4	1741.8 ± 65.1	78.1 ± 9.2	81.5 ± 6.0

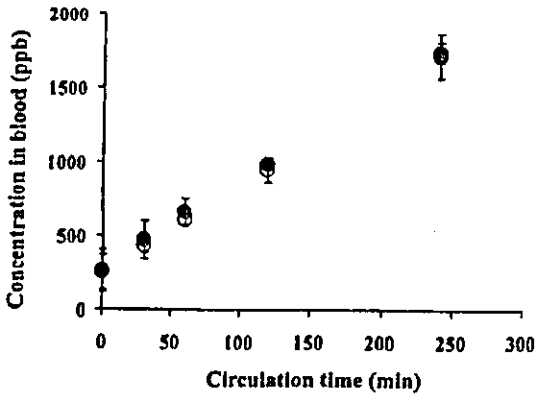

Fig. 2. Release profile of DEHP in a hemodialysis system under condition (○) with and condition (●) without fluid removal treatment.

calculated DEHP exposure amounts of hemodialysis patients as 0.02–0.36 mg/kg per day (4 h dialysis, three times a week; body weight 70 kg) (Center for Devices and Radiological Health, 2001). Our in vitro study revealed that 7.3 mg of DEHP was released from the hemodialysis system over 4 h of blood circulation, and the total amount of DEHP was corrected as 7.8 mg by adding the amount converted to MEHP from DEHP during the 4 h period. The amount of DEHP exposure was calculated from the corrected value as 0.067 mg/kg per day (4 h dialysis, three times a week; body weight 50 kg) if the total amount of DEHP released from the circuit was absorbed into the body. This value was remarkably lower than the TDI value (0.6 mg/kg per day) for intravenous injection to humans proposed by the FDA (Center for Devices and Radiological Health, 2001), but slightly higher than the lower limit of TDI value (0.04–0.14 mg/kg per day) for oral administration to humans estimated by the JMHLW.

In this investigation, fluid removal treatment during hemodialysis session seemed not to be effective for removal of DEHP and MEHP from the circulated blood. However, since DEHP introduced into the body is rapidly excreted as gluconide and other metabolites (Rhodes et al., 1986; Woodward, 1988), a portion of the hydrophilic metabolites may be eliminated during in vivo blood circulation through the dialyzer if sufficient fluid removal treatment is performed during the session.

3.4. DEHP release from the pump–oxygenation system

The blood circuit mimicking the pump–oxygenation system consisted of a pump, a thermoregulator, and four kinds of PVC tubing in medical treatment in Japan. The amounts of DEHP released from these circuits (i.e. the same setup with the four different kinds of tubing) were evaluated. The background content of DEHP in bovine blood used as a circulation solvent was 503.3 ± 69.2 ppb. In the release test using non-coated PVC tubing provided by company C, a significant amount of DEHP was time-dependently released from the circuit as shown Fig. 3, and the concentration of DEHP at each time of circulation is shown in Table 2. On the other hand, DEHP release from the circuit employing covalently bond type of heparin-coated PVC tubing produced by the same company was obviously suppressed; DEHP content in the blood after 6 h circulation was 7480.3 ± 376.2 ppb (Fig. 3 and Table 2). A relatively large amount of DEHP was released from the circuit using non-coated tubing provided by company D, and the concentration of DEHP in the circulation blood reached 24792.9 ± 530.7 ppb after 6 h circulation (Fig. 3 and Table 2). It was noticed that the ionic bond type heparin coating on the inner surface of the PVC tubing (company D), in comparison with the covalent bond type heparin coating, did not greatly effect the release of DEHP.

Several in vivo investigations have been reported on the release of DEHP during extracorporeal membrane oxygenation (ECMO) therapy, which is used mainly for neonates in respiratory failure (Karle et al., 1997). In this investigation, a considerable amount of DEHP was also released from blood circuits mimicking pump–oxygenation therapy for pediatric patients. The total amounts of DEHP, including the amount of MEHP converted from DEHP, during 6 h of circulation were calculated to be 7.8 mg (company C non-coat tubing), 3.7 mg (company C heparin-coat tubing), 12.6 mg (company D non-coat tubing), and 10.6 mg (company D heparin-coat tubing), respectively. The DEHP exposure amount calculated from these value was 0.708–0.721 mg/kg per day when non-coated tubings were used and 0.334–0.606 mg/kg per day for the circuits using heparin-coated tubings (6 h circulation, one time; body weight 11 kg). Exposure amounts of DEHP for the adult patient (body weight 50 kg)

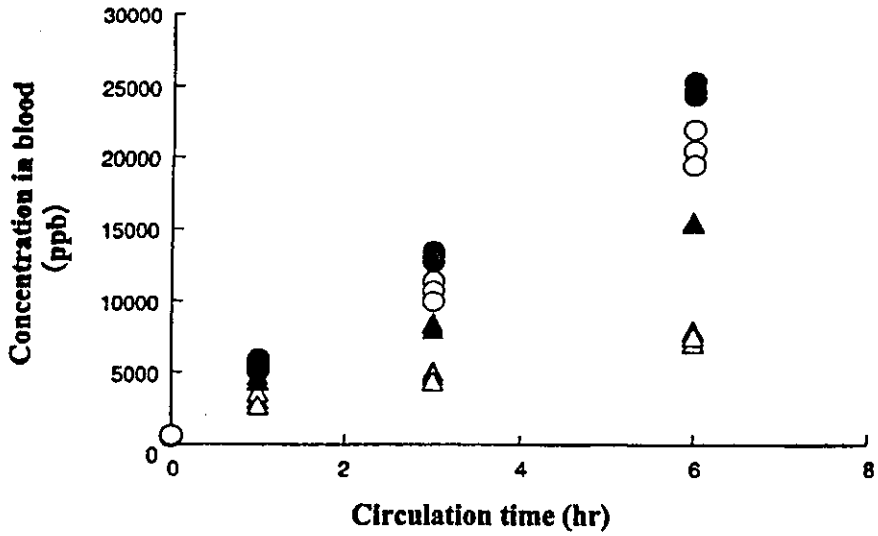

Fig. 3. Release profile of DEHP in PVC blood circuits of the pump–oxygenator. Non-coated tubing produced by companies C (▲) and D (●), and covalent bond (Δ) and ionic bond (○) types of heparin-coated tubing provided by companies C and D, respectively.

predicted by hypothetically adjusting the size of the tubing (i.d. 10 mm, length 4 m) were calculated as 0.346–0.352 mg/kg per day (non-coated tubings) and 0.163–0.296 mg/kg per day (heparin-coated tubings). All of these values were higher than the upper limit of TDI value estimated by the JMHLW.

It has been reported that heparin coating of the inner surface of PVC tubing is very effective for suppressing the release of DEHP from the tubing (Karle et al., 1997; Mejak et al., 2000; Lamba et al., 2000). It was shown in this study that DEHP release was decreased to approximately 50% that of the control tubing by the use of covalent bond-type heparin coating, indicating that this coating may be useful to suppress patients' exposure to DEHP.

3.5. MEHP analysis

MEHP contents in bovine blood circulated into hemodialysis and pump–oxygenation systems were measured in order to determine the conversion ratio of DEHP to MEHP in the blood. As shown in Fig. 4, similar profiles of MEHP detected from the blood circulated through a hemodialysis system were obtained irrespective of the condition of fluid removal treatment (i.e. with or without). Amounts of MEHP in circulated blood originally containing 13.3 ± 6.9 ppb of MEHP as a background were time-dependently increased; after circulation for 240 min, the contents in the blood had reached 78.1 ± 9.2 ppb under the condition of fluid removal treatment and 81.5 ± 6.0 ppb without the treatment (Fig. 4 and Table 1).

MEHP was also detected in the blood circulated into circuits mimicking a pump–oxygenation system, as shown in Fig. 5. The blood used in this

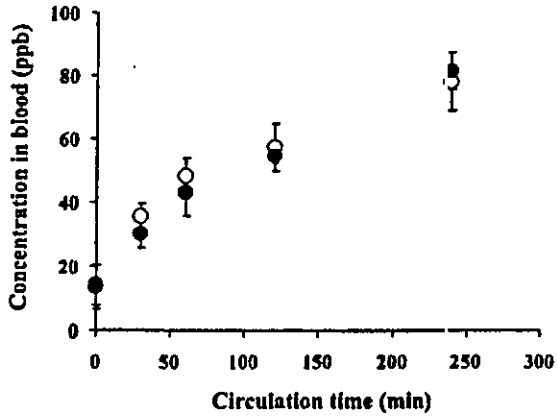

Fig. 4. Amounts of MEHP detected in bovine blood circulated through the hemodialysis system under condition (○) with and condition (●) without fluid removal treatment during hemodialysis session.

Table 2
Amounts of DEHP and MEHP detected from bovine blood circulated into pump–oxygenation system

Circulation time (h)	Concentration (ppb)							
	DEHP				MEHP			
	Company C		Company D		Company C		Company D	
	Non-coat	Heparin-coat	Non-coat	Heparin-coat	Non-coat	Heparin-coat	Non-coat	Heparin-coat
1	4528.4 ± 194.0	3140.4 ± 429.7	5626.2 ± 266.3	5216.0 ± 185.3	221.3 ± 31.4	142.8 ± 19.0	303.4 ± 27.9	479.6 ± 5.2
3	8240.1 ± 193.0	4734.0 ± 365.1	13062.6 ± 335.2	10676.1 ± 687.0	302.0 ± 22.6	199.6 ± 13.1	497.7 ± 37.6	626.1 ± 10.7
6	15503.0 ± 88.5	7480.3 ± 376.2	24792.9 ± 530.7	20683.2 ± 1212.2	416.1 ± 26.3	258.5 ± 24.3	713.6 ± 52.3	696.5 ± 21.7

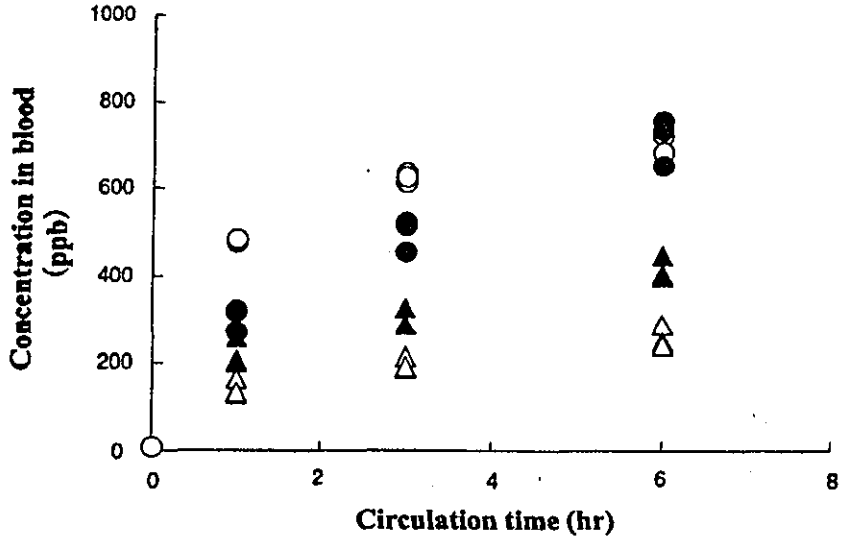

Fig. 5. Amounts of MEHP detected in bovine blood circulated through PVC circuits of the pump–oxygenator. Non-coated tubing produced by companies C (▲) and D (●), and covalent bond (△) and ionic bond (○) types of heparin-coated tubing provided by companies C and D, respectively.

investigation contained 9.1 ± 1.0 ppb of MEHP as a background. As shown in Table 2, 221.3 ± 31.4, 302.0 ± 22.6, and 416.1 ± 26.3 ppb of MEHP were detected in blood circulated for 1, 3, and 6 h in the circuit using the non-coated PVC tubing produced by company C. However, the detectable amount was obviously decreased by use of the heparin-coated PVC tubing (Fig. 5 and Table 2). On the other hand, no significant difference with regard to the profile of MEHP detection was observed between the PVC circuits with non-coated tubing and those with ionic bond type heparin-coated tubing produced by company D. MEHP of 713.6 ± 52.3 and 696.5 ± 21.7 ppb was detected in the blood circulated for 6 h through the non-coated-tubing circuit and the coated-tubing circuit, respectively (Fig. 5 and Table 2).

MEHP is an active metabolite of DEHP, and therefore, given that a portion of DEHP is converted to MEHP in stocked blood, plasma, and transfusion blood, evaluation of patient exposure to MEHP is very important. In this experiment, it was shown that 3–4% of DEHP is also converted to the monoester during blood circulation for hemodialysis and in pump–oxygenator circuits at 37 °C, probably as a result of esterase in the blood.

Thus, present study showed the risk that patients are exposed to considerable amount of DEHP during hemodialysis and pump–oxygenation treatments. However, benefit of medical devices used for the treatments is obviously over than the risk factor, because these devices are essential to save patients' life. Although David et al. (1999) demonstrated that DEHP promoted the proliferation and hepatomegaly associated with hepatocellular tumorigenesis, it has been clearly shown that the toxic mechanism is characteristic in rodents and no tumorigenesis activity is observed to human (Doull et al., 1999). Pharmacokinetics assay showed that metabolic rate of DEHP is relatively fast, and 62–76% of DEHP taken into body is excreted by 24 h after orally administration to marmosets (Rhodes et al., 1986). Furthermore, Japan Plasticizer Industry Association recently reported the results on risk assessment of DEHP against primates, on their web site (http://www.kasozai.gr.jp/) in January 2003, that DEHP administrated to marmosets was not accumulated in testis and did not exert any testicular toxicity such as testicular atrophy different from the case of rodents, suggesting that species specificity regarding appearance of the toxicity may exist between rodents and primates. In fact, no clin-

ical adverse events originated from DEHP exposure to human have been reported up to the present. In consideration of these issues, PVC medical devices used for hemodialysis and pump–oxygenation treatments seem to be relatively safe to patients, in addition to the great benefit factor to patients. However, since the influence of DEHP on humans is not fully understood, precautions should be taken to limit human exposure to DEHP, at least that of high risk patients, originating from use of PVC medical devices.

4. Conclusion

We evaluated the release of DEHP from hemodialysis and pump–oxygenator circuits comprised of PVC tubings. The amount of DEHP exposure for adult patients in hemodialysis therapy did not appear to be remarkably high, though use of normal PVC tubing perhaps should be reconsidered if this treatment is to be applied to patient groups having a high sensitivity to DEHP and/or facing the likelihood of long term therapy. A considerable amount of DEHP (well over the TDI value) was released from the PVC circuits for the pump–oxygenator currently in wide used in surgery for heart and/or lung failure patients in Japan. Although the oxygenator is mainly used for adult patients receiving therapy different from ECMO therapy, and for whom the incidence of use is relatively low in the life of a patient, non-coated PVC tubing for the circuit may also be exchanged for alternative (i.e. coated) tubing if the treatment is to be applied to a high risk patient group even if no significant adverse events have been associated with therapy, based on the finding that the amount of exposure to DEHP by the therapy is over the upper limit of TDI value as estimated by the JMHLW. One current alternative, covalent bond type heparin-coated PVC tubing, may be useful for suppressing the release of DEHP from PVC tubing.

Regardless whether an investigation is in vivo or in vitro, the release test of DEHP is time-consuming and labor-intensive. Consequently, the development of a simple method for predicting the amount of DEHP released from PVC medical devices is now in progress in our laboratory.

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The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte complexes

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Abstract

Polyelectrolyte complexes (PEC) were prepared from chitosan as the polycation and several synthesized functional anion polysaccharides, and their effects on cell attachment, morphology, proliferation and differentiation were estimated using normal human osteoblasts (NHOst). After a 1-week incubation, PEC made from polysaccharides having carboxyl groups as polyanions showed low viability of NHOst on it although the NHOst on it showed an enhancement in their differentiation level. On the other hand, NHOst on PEC made from sulfated or phosphated polysaccharides showed similar attachment and morphology to those on the collagen-coated dish. When the number of NHOst was estimated after 1 week, the number on the PEC was ranged from 70% to 130% of those on the collagen-coated dish, indicating few effects of these PEC on cell proliferation. In addition, NHOst on PEC films made from sulfated polysaccharides differentiated to a level very similar to that observed on the collagen-coated dish, indicating that these PEC films maintain the normal potential of NHOst to both proliferate and differentiate. Measurement of gap junctional intercellular communication of NHOst on PEC revealed that PEC did not inhibit communication, suggesting that PEC films have few effects on cell homeostasis. Thus, PEC made from the sulfated polysaccharide may be a useful material as a new scaffold for bone regeneration.

Keywords: Polyelectrolyte complex; Normal human osteoblasts; Cell proliferation; Cell differentiation; Gap junctional intercellular communication

1. Introduction

The extracellular matrix (ECM) provides an essential three-dimensional (3D) environment for cells to construct several kinds of tissues. The ECM, consisting of numerous kinds of molecules such as proteins, polysaccharides and proteoglycans regulates the behavior of surrounding cells to form tissues and organs precisely [1,2]. For tissue regeneration trials using in vitro techniques, therefore, it is indispensable to develop a synthetic ECM scaffold that functions similarly to the native ECM. For more than a decade, engineering of new tissues by using selective cell transplantation on polymer scaffolds as an artificial ECM instead of tissue transplantation to other living bodies has been studied [3,4]. Recently, many studies on developing a scaffold for tissue regeneration have been done using ECM proteins such as collagen and gelatin [5–7], biodegradable synthetic polymers [8–10] and polysaccharides [11,12]. Because proteins derived from human tissues have many problems such as antigenicity or potential for infection, a biocompatible synthetic polymer or polysaccharide may be preferable for tissue regeneration.

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A polyelectrolyte complex (PEC) is a compound made from an electrically neutralized molecular complex of polyanions and polycations [13]. PEC can be prepared in various forms such as a film (2D) and a hydrogel, a microcapsule or a sponge (3D), which can be used as a scaffold in tissue regeneration studies. The effects of PEC films composed of polysaccharides on cell behavior have been studied, and we have already reported that PEC can stimulate differentiation of osteoblasts and periodontal ligament fibroblasts [14–16]. These studies suggest that PEC can be used as a biomaterial for repairing or regenerating tissues. In addition, because the PEC are composed of polysaccharides, PEC is expected not to elicit immune responses against it and to have better biocompatibility with the human body, although this is yet to be proved. Therefore, it is necessary to study the interactions between PEC and cells, especially human-derived, to clarify the usefulness of PEC as a biomaterial.

In this study, normal human osteoblasts (NHOst) were cultured on various PEC prepared on a tissue culture plate from chitosan as the polycation and modified chitins or hyaluronan as the polyanion. It should be generally agreed that estimating not only functional advantages but also safety and biocompatibility of biomaterials is important to develop them for clinical use, but the latter is not always studied. Therefore, we measured changes in gap junctional intercellular communication (GJIC) as well as the cell number and differentiation. GJIC is very important function for almost all cells to maintain their homeostasis [17]. During this decade, we have studied the effects of model biomaterials on the GJIC of cells cultured on them and suggested a possibility that changes in the GJIC can be used as an index of biocompatibility of biomaterials [18–21]. Therefore, we measured changes in GJIC of NHOst on PEC in order to estimate the biocompatibility of PEC from their effects on these cell functions.

2. Materials and methods

2.1. Chemicals

Fig. 1 shows the chemical structures of the polyanions and the polycation. Chitosan as the cationic polysaccharide and carboxymethylated chitin [CM-Chitin: degree of substitution (DS) = 1.0 (1.0 anionic site/saccharide ring)] were purchased from Katokichi Co., Ltd. (Kagawa, Japan). Sulfated chitin (S-Chitin: DS = 1.5), phosphated chitin (P-Chitin: DS = 1.6), hyaluronan (HA), and sulfated hyaluronan (SHA: DS = 1.05) were prepared as previously reported [14–16,22].

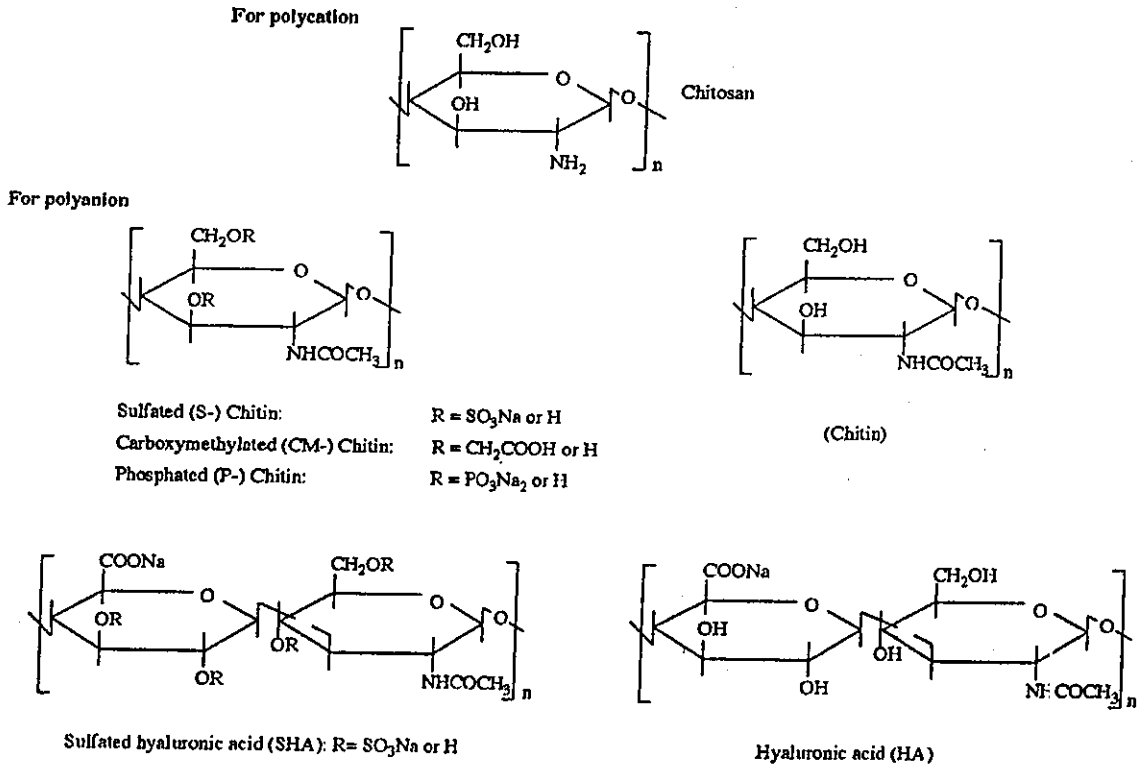

Fig. 1. Polymers for polyelectrolyte complex (PEC) in this study.

2.2. Preparation of PEC and PEC-coated dishes

Polyanions were dissolved individually in distilled water (final concentration = 5×10^{-4} mol of ionic sites/l), and the pH of the solutions was adjusted to 7.4 by adding aqueous HCl or NaOH. Chitosan was dissolved in aqueous 0.5% acetic acid solution and the pH adjusted to 6.0. The ratio of the solutions of polyanions and polycation was adjusted in each combination to neutralize the charge balance of PEC. This mixed solution (1 ml/35 mm tissue culture dish) was allowed to stand overnight at room temperature. After removing the supernatant solution, the dish was dried and annealed at 65 °C in an oven. Then, the dishes were washed with distilled water and oven-dried again to form the PEC-coated dish. This dish was sterilized for 3 min in a microwave oven. Water contact angles of PEC films were measured with the sessile drop method [23], and their zeta potentials were measured by Otsuka Electronics Co., Ltd. (Osaka, Japan).

2.3. Cell culture

NHOst were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NHOst was performed using alpha minimum essential medium (Gibco, Grand Island, NY) containing 20% fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo Japan). The cells were maintained in incubators under standard conditions (37 °C, 5%-CO_2–95%-air, saturated humidity). All assays were performed using alpha minimum essential medium containing 20% FCS, supplemented with 10 mM beta-glycerophosphate. NHOst cells (1×10^5 cells/dish/2.5 ml medium) were cultured on PEC-coated dishes to evaluate the effects of their interaction with PEC. In each experiment, the medium was changed three times before GJIC of the cells was measured and their differentiation level was evaluated after a 1-week incubation.

2.4. Estimation of differentiation level of NHOst cultured on PEC films

The proliferation of NHOst cells cultured on PEC films was estimated by Tetracolor One assay (Seikagaku Co., Tokyo, Japan), which incorporates an oxidation-reduction indicator based on detection of metabolic activity. After a 1-week incubation, 20 μl of Tetracolor One solution was added to each test dish, followed by a further 2 h incubation. The absorbance of the supernatant at 450 nm was estimated by μQuant spectrophotometer (Bio-tek Instruments, Inc., Winooski, VT). Estimation of alkaline phosphatase (ALP) activity was performed according to an original procedure by Ohyama et al. [24]. After estimating the proliferation of the NHOst cells cultured on PEC films, the cells were washed by phosphate-buffered saline (PBS(−)), followed by addition of 1 ml of 0.1 M glycine buffer (pH 10.5) containing 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 4 mM *p*-nitrophenylphosphate sodium salt. After incubating the cells at room temperature for 7 min, the absorbance of the glycine buffer was detected at 405 nm using μQuant to evaluate the ALP activity of the test cells. The amounts of calcium deposited by the cell during a 1-week incubation were evaluated as follows: after fixing the cells in PBS(−) containing 3% formaldehyde and washing the cells with PBS(−), 0.5 ml of 0.1 M HCl was added to each well. The amounts of calcium dissolved in HCl were estimated using a calcium detecting kit (Calcium-C test Wako, Wako, Osaka, Japan) according to manufacturer's instruction.

2.5. Measurements of GJIC activity

NHOst cultured on PEC films were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these films on the GJIC. FRAP analysis was carried out according to the procedure of Wade et al. [25] with some modifications [21]. Briefly, NHOst were plated on PEC-coated dishes and incubated for 1 or 7 days. The cells were incubated for 5 min at room temperature in PBS(−) containing Ca^{2+} and Mg^{2+} (PBS(+)) and a fluorescent dye, 5,6-carboxyfluorescein diacetate. After washing off excess extracellular dye with PBS(+), the cells in PBS(+) contacting at least two other cells were subjected to FRAP analysis under a Ultima-Z confocal microscope (Meridian Instruments, Okemos, MI) with a 10 × objective lens at room temperature. The cells were photobleached with a 488 nm beam, and recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total of 4 min. The data obtained from more than seven independent cells were expressed as the average ratio of the fluorescence recovery rate to the rate obtained from NHOst cultured on a collagen-coated dish.

2.6. Statistic analysis

All data were expressed as mean values ± standard deviation of the obtained data. The Fisher–Tukey criterion was used to control for multiple comparisons and to compute the least significant difference between means.

3. Results and discussion

When NHOst were cultured on five kinds of PEC films, their morphology and attachment to the film differed with the composition of the PEC. Fig. 2 shows the morphologies of the NHOst adhering to PEC films.