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 o their concentrations of BaP, BaA, and DBA; a fourth laboratory (Laboratory 4) measured only the BaI 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 tha of BaP. The present concentrations of BaP (29.0- 36.4 μg/g) were allowable according to the EU con trol (< 50 μg/g), but they remained above the allowe value in Japan. As regards sample B, all laborato ries reported similar levels for each compound Sample C contained BaA, BaP, and DBA at concen

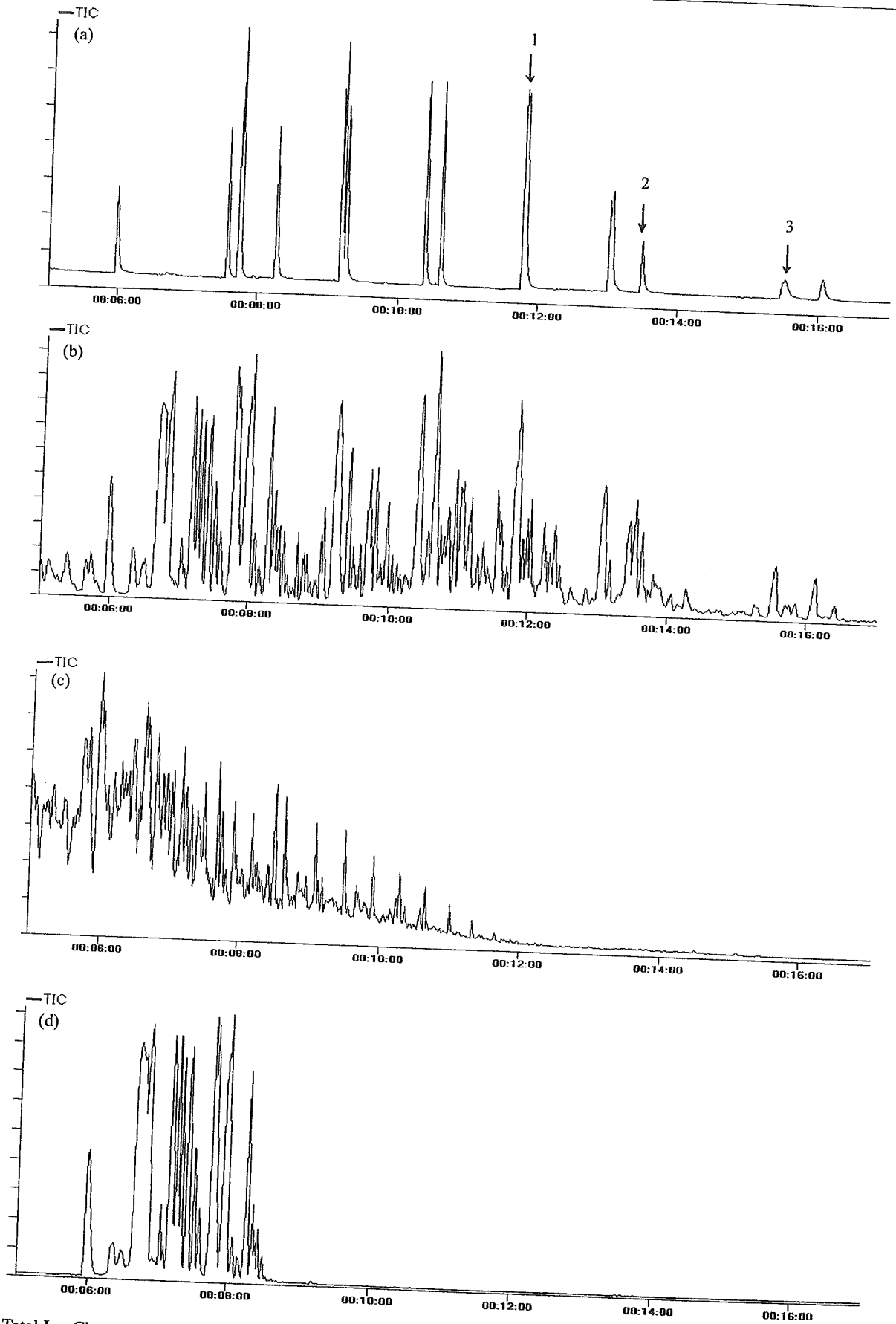

Fig. 1. Total Ion Chromatogram of Creosotes

(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 New	Railway Sleeper New	Railway Sleeper New	Railway Sleeper Used	Railway Sleeper Used	Stake 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.

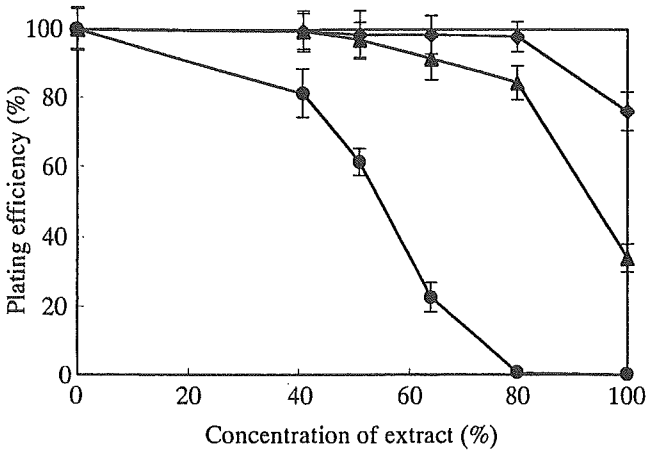

Figure 1. Plating efficiencies of V79 cells treated with medium extracts of samples A (●), B (▲), and C (♦). The samples were extracted with 5% FCS-GMNP for 24 h and the extracts were tested in the colony assay. Values are expressed as means ± SD for eight wells.

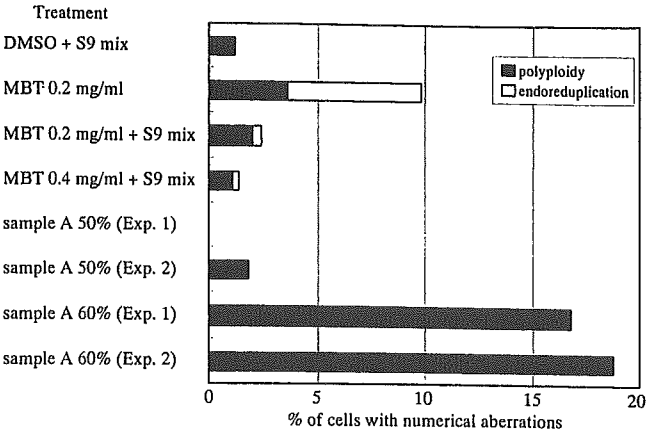

Figure 2. Numerical chromosome aberrations induced by MBT and sample A. Five hundred cells were examined.

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.

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TABLE III
Chromosome Aberration Test of Culture Medium Extracts of Samples

Sample	S9 mix	Conc.[a] (%)	Poly.[b] (%)	Cells with Structural Aberrations (%)							MI[c] (% of Control)
				ctg	ctb	cte	f	csb	cse	total	
A	−	0	1	0	0	1	0	0	0	1	100
		50	1	1	0	8	0	0	0	9	107
		60	20	1	0	10	0	0	0	11	146
		70	26	1	3	7	0	0	0	11	20
	+	0	4	0	0	0	0	1	0	1	100
		10	0	0	0	0	0	0	0	0	84
		20	3	0	0	7	0	0	0	7	114
		30	3	1	3	12	0	0	0	14	139[d]
		40	0	2	6	10	0	0	0	16	93[e]
B	−	0	1	0	0	1	0	0	0	1	100
		60	0	0	0	0	0	0	0	0	92
		80	0	1	0	0	0	0	0	1	91
		100	0	0	0	0	0	0	0	0	101
	+	0	4	0	0	0	0	1	0	1	100
		60	2	1	0	1	0	1	0	3	86
		80	1	1	0	0	0	0	0	1	86
		100	2	0	0	7	0	0	0	7	85
C	−	0	1	0	0	1	0	0	0	1	100
		60	0	0	1	0	0	0	0	1	103
		80	1	0	0	0	0	0	0	0	107
		100	1	1	0	0	0	0	1	2	110
	+	0	4	0	0	0	0	1	0	1	100
		60	1	2	0	0	0	0	0	2	93
		80	1	0	0	0	0	0	1	1	99
		100	3	0	0	0	0	0	0	0	106

[a,b, and c] See the footnote in Table II.
[d]Around 50% cells on the preparation were dead.
[e]Around 90% cells on the preparation were dead.

DISCUSSION

In an earlier study, the IC_{50} for MBT and ZDBC in the cytotoxicity test was 49 and 5.4 μg/mL, respectively.[17] These values are compared with samples A to C in the present study and the lowest toxicity concentration in the CA test (Table IV). Similar cytotoxicity was shown in both tests.

ZDBC, strongly cytotoxic, is weakly positive in the CA test using human lymphocytes[18] and negative in the bacterial reverse mutation assay,[18–20] mouse lymphoma cell mutation assay,[18] and the *in vivo* micronucleus test.[18] In the present study, ZDBC induced structural CAs at relatively low concentrations both in the absence and presence of S9 mix, but at lower concentrations in its absence, indicating that ZDBC does not require S9 mix for activity. Sample C, which was compounded with ZDBC, showed a slight cytotoxicity and was negative in the CA test, perhaps due to its low extraction efficiency by culture medium.[11,18]

TABLE IV
Comparison of Cytotoxicity

	IC_{50}*	LTC**
MBT	49 μg/mL***	400 μg/mL
ZDBC	5.4 μg/mL***	6 μg/mL
Sample A	53.1%	70%
Sample B	94.3%	>100%
Sample C	>100%	>100%

*Inhibition concentration at which the relative plating efficiency is inhibited to 50% of the control value in the cytotoxicity test.
**Lowest toxic concentration in the CA test.
***Nakamura et al.[17]

On the contrary, sample A, which was compounded with MBT, showed strong cytotoxicity and induced structural CAs at lower concentrations in the presence of S9 mix than in its absence, suggesting the existence of promutagenic leachates other than MBT. In the present study, MBT yielded an inconclusive response in structural CA induction in the presence of S9 mix, while it yielded a positive response at similar concentrations in another Chinese hamster cell line, CHO.[21] Four percent cte-type structural CAs, however, suggest, based on our historical database, that MBT may show a biologically positive response in the presence of S9 mix.

Sample A showed an interesting phenomenon in that so many dead cells coincided with well living cells in the presence of S9 mix. In the present study the number of mitotic cells were counted on 1000 live cells. MI was 139% and 93% of control at 30% and 40%

extract of sample A, respectively, in the presence of S9 mix. Although the values of MI indicated no cytotoxicity, the ratio of live cells to total cells on the preparation was around 50% and 10%, respectively. Actually a few remaining live cells on the preparation of 40% extract of sample A were well and the chromosome morphology of the metaphase cells was fine.

Numerical chromosome aberrations consist of polyploidy and aneuploidy. Aneuploidy has been implicated in sterility, abortions, stillbirths, congenital abnormalities, and carcinogenesis.[22,23] The *in vitro* CA test is not routinely used to detect aneuploidy, although it could be,[24] but polyploid induction suggests the possibility of aneuploid induction. In the present study, sample A, which had a high concentration of MBT, induced a high frequency of polyploidy in the presence of S9 mix. MBT alone at the same concentration also induced polyploidy, but with a lower frequency and with accompanying endoreduplication. Thus, the induction of polyploidy by sample A did not seemed to be explained simply by the presence of MBT.

Endoreduplication, which shows an characteristic morphology (diplochromosomes), is an endomitotic chromosome duplication that occurs without mitosis-like events during interphase.[25] A typical endoreduplication event is characterized by two periods of DNA synthesis, S1 and S2, separated by a G period of variable duration.[26] Some chemicals, such as 4NQO, acridine yellow, cytoxan, captan,[27] and rotenone,[28] induce endoreduplication without S9 mix. The frequency of endoreduplication induced by those compounds was similar to the frequency induced by MBT in the present study.

The fact that sample A showed stronger cytotoxicity and induced a higher frequency of polyploidy than was predicted by MBT alone might have been due to the presence of other leachables in the sample. This suggests that sample A may be useful as a positive control for the safety evaluation of biomaterials and that the test might overcome the poor predictive value of individual components of materials.

In the Japanese guidelines for basic biological tests of medical materials and devices, the use of V79 cells is preferred in the cytotoxicity test. In the test the introduction of a metabolic activation system is not required. On the other hand, genotoxicity tests require the use of an exogenous metabolic activation system and of their methods following Japanese guidelines for drugs and chemicals, and OECD guidelines. CHL cells are popular in the CA test in Japan. In the present study each of the cytotoxicity test and the CA test followed the corresponding guideline independently. The difference in the cytotoxicty of sample A between with and without S9 mix suggests that the discussion of the introduction of an exogenous metabolic activation system into the cytotoxicity test may be needed.

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Enhancement of Gap Junctional Intercellular Communication of Normal Human Dermal Fibroblasts Cultured on Polystyrene Dishes Grafted with Poly-*N*-isopropylacrylamide

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ABSTRACT

Technology developed to allow recovery of cells without enzyme treatment, involving a dish grafted with a thermoreactive polymer gel of poly-*N*-isopropylacrylamide (PIPAAm), was found to significantly enhance gap junctional intercellular communication (GJIC) in normal human dermal fibroblasts (NHDF cells). NHDF cells were cultured for 4 days on PIPAAm-grafted dishes irradiated with various doses of electron beams, and GJIC was assayed by the scrape-loading dye transfer method. The area of dye transfer was greater in the PIPAAm-grafted dishes than in the control culture dishes, indicating that the PIPAAm-grafted dishes enhanced the GJIC of NHDF cells. Connexin-43 (Cx43) expression was analyzed because Cx43 is considered to be a main component of the gap junctional channel. PIPAAm-grafted dishes irradiated with 100, 250, or 500 kGy of electron beams showed significantly enhanced expression of Cx43-NP, Cx43-P1, and especially Cx43-P2. Enhanced expression of Cx43-P2, a functional transmembrane protein, may be related to the promotion of GJIC. These results suggest that the PIPAAm-grafted dish not only enables the enzyme-free recovery of a cell monolayer for use in the construction of a three-dimensional artificial tissue, but also significantly contributes to the enhancement of GJIC, which may partly promote tissue strength on the surface of the PIPAAm-grafted dish.

INTRODUCTION

GAP JUNCTIONS exist on the cell membrane and work as intercellular channels that allow the exchange of substances with molecular masses up to 1 kDa, such as ions, sugars, and amino acids, by the function called gap junctional intercellular communication (GJIC).[1–3] Gap junctions are constructed from transmembrane proteins, called connexins,[4,5] that form a hemichannel, called a connexon. GJIC is suggested to be well correlated with passage of metabolites,[6] cell proliferation,[7] and cell differentiation[8]; thus, enhancement of the function of the gap junction is supposed to be important in the differentiation of engineered tissue products, such as those involving heart cells.[9–11] Poly-*N*-isopropylacrylamide (PIPAAm)-grafted dishes, which were originally developed as a thermosensitive scaffold for cell culture, are useful to maintain the GJIC of tissues cultured on them because they do not require enzyme treatment, which destroys connexins.[12–14]

PIPAAm is a thermoresponsive polymer that has a low critical solution temperature of 32°C: hydrated PIPAAm

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has an extended chain conformation below 32°C and dehydrated PIPAAm has a collapsed chain conformation above 32°C.[15–27] This property of PIPAAm has been exploited in intelligent materials for drug delivery systems and chromatography technology.[16–23] The PIPAAm-grafted dish has been found to enable the recovery of cell monolayers easily without enzyme treatment because cells cannot adhere to a hydrophilic surface below 32°C.[24–26] Cell monolayers are the basic units used to construct three-dimensional tissues *in vitro*. Because a cell monolayer recovered without enzyme treatment maintains normal adhesive and junctional proteins, it can easily adhere to the other tissues or cell sheets to construct a three-dimensional artificial tissue.[27–29] Thus, the PIPAAm-grafted dish has the potential to enable the development of new techniques in tissue engineering.

Although the PIPAAm-grafted dish has made a new era in tissue engineering possible, its effects on connexin-43 (Cx43) expression and GJIC have not been studied well. These effects are important because Cx43 plays an important role in cell proliferation and cell differentiation.

In this study, GJIC and expression of Cx43 molecules were examined by scrape-loading dye transfer (SLDT) assay[30] and Western blotting, respectively, using NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams in order to clarify the safety and appropriateness of this material for the culture of artificial cultured tissues.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide monomer (NIPAAm) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Isopropyl alcohol was obtained from Dojindo (Kumamoto, Japan), and Lucifer yellow dye was from Molecular Probes (Eugene, OR).

Cell culture

Normal human dermal fibroblasts (NHDF cells; Sanko Junyaku, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (GIBCO DMEM; Invitrogen, San Diego, CA), supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen) and antibiotics (penicillin [100 units/mL]-streptomycin [100 units/mL]) (Invitrogen) at 37°C. NHDF cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air.

Preparation of PIPAAm-grafted culture dishes

One hundred microliters of 40% NIPAAm dissolved in isopropyl alcohol was added to 35-mm dishes and irradiated with various doses of electron beams (25, 100, 250, or 500 kGy), using an area electron beam-processing system (Nissin High Voltage, Kyoto, Japan). The PIPAAm-grafted dishes were then rinsed three times with ice-cold sterile water (2 ml) for 5 min, sealed, and dried under vacuum.

Cell morphology

NHDF cells were cultured on control and PIPAAm-grafted dishes. Confluent cells (after 4 days of culture) were fixed with formalin solution, stained with 3% Giemsa solution, and observed with an optical microscope.

Protein assay

The protein concentration of cells cultured on control and PIPAAm-grafted dishes was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). Ten-microliter cell samples were

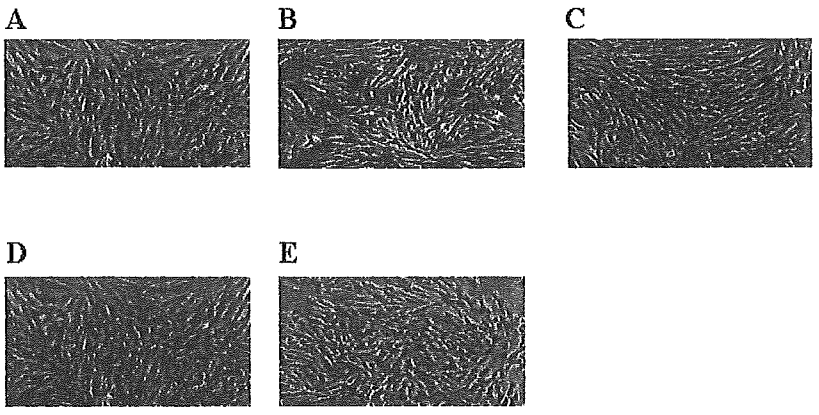

FIG. 1. Optical microscopy images of NHDF cells cultured on PIPAAm-grafted dishes. NHDF cells were cultured for 4 days on PIPAAm-grafted dishes prepared by irradiation with various doses of electron beams (0, 25, 100, 250, or 500 kGy). (**A**) Non-irradiated; (**B**) 25-kGy electron beam; (**C**) 100-kGy electron beam; (**D**) 250-kGy electron beam; (**E**) 500-kGy electron beam.

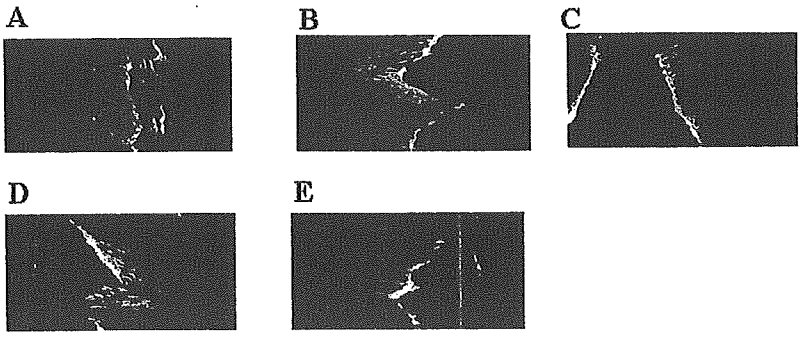

FIG. 2. Fluorescence of NHDF cells by SLDT assay. Transmission of Lucifer yellow into NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams was detected 5 min after scrape-loading. (**A**) Nonirradiated; (**B**) 25-kGy electron beam; (**C**) 100-kGy electron beam; (**D**) 250-kGy electron beam; (**E**) 500-kGy electron beam.

added to 200 μL of the working solution and incubated at 37°C for 30 min in a 96-well plate. Absorbance was then measured at 562 nm in accordance with the manufacturer's protocols.

Scrape-loading dye transfer assay

NHDF cells were seeded on control and PIPAAm-grafted dishes at a density of 1×10^5 cells/mL and cultured for 4 days to form a confluent monolayer. Confluent NHDF cells were washed three times with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} [PBS(+)], and the cell monolayer was scraped with a surgical blade. Fluorescent dye (Lucifer yellow; MW 457.2) at a concentration of 0.1% in PBS(+) was added.[30,31] Cells were exposed to the dye at 37°C for 5 min, and then the dye was discarded and the cells were washed four times with PBS(+). The distance that the dye had migrated was measured under a fluorescence microscope equipped with a type UFX-DXII CCD camera and super high-pressure mercury lamp power supply (Nikon, Tokyo, Japan). The dye migration was measured from the cut edge of the scrape to the edge of the dye front in the cells that were visually detectable.[30]

Western blotting

NHDF cells were cultured for 4 days. After being washed with ice-cold PBS(–) three times, the cells were lysed in 500 μL of lysis buffer (50 mM Tris-HCl [pH 6.8] containing 150 mM NaCl, 5 mM EDTA, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) for 30 min on ice with shaking. The cell lysates were centrifuged (10,000 rpm) at 4°C for 20 min, and the supernatants were collected. The protein concentrations of the lysates were determined by BCA assay.

Equivalent amounts of protein sample were applied to 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and then transferred to a nitrocellulose membrane at 120 V for 60 min. The membrane was blocked with Block Ace (Yukijirusi, Tokyo, Japan) overnight at 4°C. After being washed for 30 min in PBS with 0.05% Tween 20, the membrane was incubated for 2 h with anti-Cx43 polyclonal antibody [diluted 1:1000 in PBS(–) with 0.05% Tween 20; Zymed Laboratories, South San Francisco, CA], followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:5000; Zymed Laboratories). The image was visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences/GE Healthcare, Little Chalfont, UK).

Statistical analysis

Significant differences between groups were evaluated by Student t test. Mean differences were considered significant when $p < 0.05$.

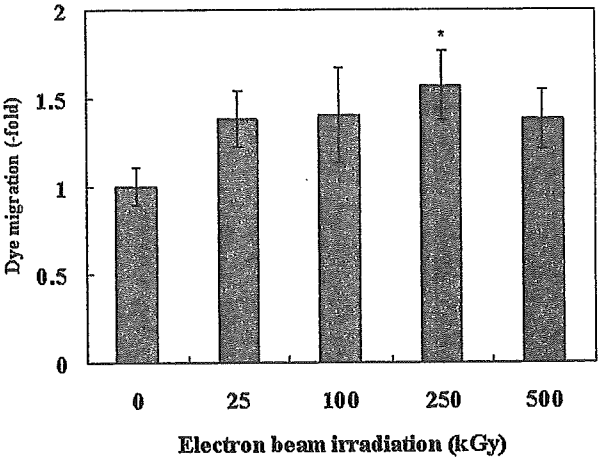

FIG. 3. Positive dye transfer in NHDF cells cultured on PIPAAm-grafted dishes. Transmission of Lucifer yellow was detected 5 min after scrape-loading in NHDF cells cultured on PIPAAm-grafted dishes irradiated with various electron beam doses (0, 25, 100, 250, or 500 kGy). Values represent means ± SD for three dishes. *Significant difference compared with control at $p < 0.05$ by t test.

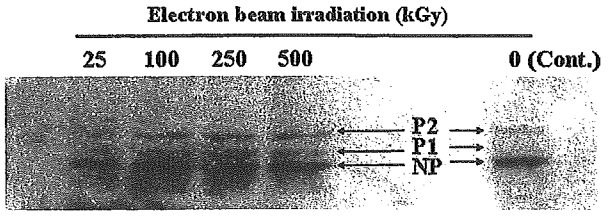

FIG. 4. Western blot of Cx43-NP, Cx43-P1, and Cx43-P2 expression; lysates of NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (0, 25, 100, 250, or 500 kGy) were applied to SDS–polyacrylamide gels. Fractionated proteins in the gels were transferred to nitrocellulose membrane and immunoblotted with anti-Cx43 polyclonal antibody as described in Material and Methods. Images of Cx43 on Western blot were captured with an Image scanner and analyzed with NIH Image software.

RESULTS

The appearance of NHDF cells grown on PIPAAm-grafted dishes irradiated with various doses of electron beams are shown in Fig. 1. No significant differences were observed by optical microscopy analysis between cells grown in dishes irradiated with various doses of electron beams. These results suggest that PIPAAm-grafted dishes are not toxic to NHDF cells.

The SLDT assay showed that dye migration in cells cultured on PIPAAm-grafted dishes irradiated with electron beams (25, 100, or 500 kGy) was enhanced by about 1.4-fold compared with that on control dishes. Interestingly, the dye migration in cells cultured on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam was particularly enhanced, about 1.6 times higher than that on control dishes (Figs. 2 and 3). These results suggested that the GJIC of NHDF cells cultured on PIPAAm-grafted dishes was enhanced and that the GJIC on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam was affected the most.

To further elucidate the effects of the PIPAAm grafting of culture dishes on GJIC, we analyzed the expression of Cx43, a transmembrane protein involved in GJIC. There are three forms of Cx43: Cx43-NP (nonphosphorylated Cx43), Cx43-P1 (monophosphorylated Cx43), and Cx43-P2 (another phosphorylated Cx43); Cx43-P2 is the most important and functional protein involved in GJIC. The results of Western blotting showed that the expression of Cx43-P1 and Cx43-P2 in NHDF cells cultured on PIPAAm-grafted dishes irradiated with 25, 100, 250, or 500 kGy of electron beams was considerably enhanced. Further, NHDF cells cultured on PIPAAm-grafted dishes irradiated with 100, 250, or 500 kGy of electron beams showed enhanced Cx43-NP expression (Figs. 4 and 5A). The Cx43-P2 expression of cells cultured on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam dose showed the highest value, about 46% higher than that of control dishes. Cells cultured on PIPAAm-grafted dishes irradiated with electron beam doses of 25, 100, and 500 kGy were shown to have enhanced total Cx43 expression. Cells cultured on PIPAAm-grafted dishes irradiated with 100- and 250-kGy electron beam doses showed the highest total Cx43 expression, about 36.6% higher than that of control dish (Fig. 5B).

The Cx43-P2 expression of NHDF cells cultured on PIPAAm-grafted dishes irradiated with 25, 100, 250, and 500 kGy correlated well with GJIC ($R^2 = 0.9398$).

DISCUSSION

Thermoresponsive PIPAAm-grafted dishes irradiated with electron beams have been used to culture cell monolayers because the monolayers can be recovered without enzyme treatment, making PIPAAm a useful material for tissue engineering.

It has been reported that junctional proteins, cellular adherence proteins on the cell membrane, interact via

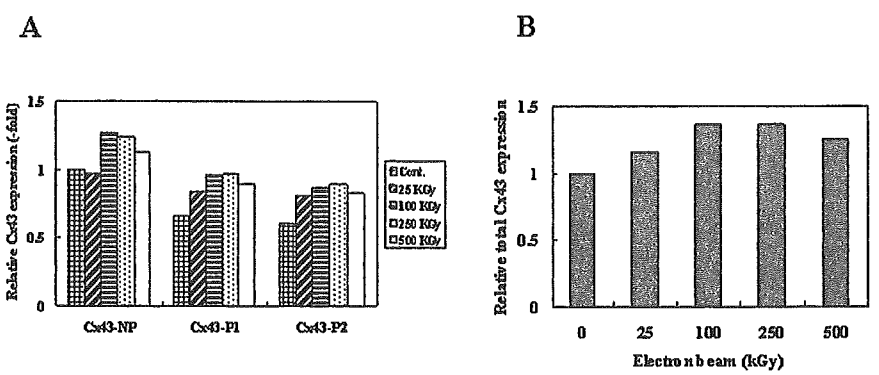

FIG. 5. Relative expression levels of Cx43-NP, Cx43-P1, and Cx43-P2 (**A**) and relative expression levels of total Cx43 (NP+P1+P2) (**B**) of NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (0, 25, 100, 250, or 500 kGy).

GJIC.[31] In this study, an SLDT assay demonstrated that dye migration in cultured NHDF cells was significantly enhanced in all PIPAAm-grafted dishes tested. Therefore, the chemical structure of the PIPAAm surface may stimulate junctional proteins on the cell membrane, and the stimulated junctional proteins may induce the enhancement of GJIC.

Cx43 expression of NHDF cells cultured on PIPAAm-grafted dishes irradiated with a 250-kGy electron beam changed significantly. Structural differences in PIPAAm triggered by the 250-kGy electron beam induced Cx43 protein expression by NHDF cells, probably by affecting the gene expression of NHDF cells. Further, total Cx43 expression was shown to be enhanced in cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (25, 100, 250, or 500 kGy). Differences due to the electron beam dose should be studied further.

Although the mechanism involved was not determined, it has been reported that basic fibroblast growth factor (bFGF) and keratinocyte growth factor (KGF) enhance GJIC activity and the expression of Cx43.[32–35] If bFGF and KGF in FCS are adsorbed onto the PIPAAm surface, cells can efficiently access these growth factors from the PIPAAm surface, and GJIC may be enhanced. It is also reported that bFGF activates protein kinase A (PKA),[36] an important regulator of Cx43, promoting the phosphorylation of Cx43 and enhancing GJIC.[37] Therefore, bFGF adsorbed onto the PIPAAm surface may bind its receptor and induce the activation of PKA, resulting in an enhancement of GJIC on NHDF cells caused by the increase in Cx43-P2 band protein.

In the process of posttranslational change, Cx43-P2 becomes insoluble in Triton X-100.[38] Thus, not all Cx43-P2 may be included in the lysate, and some Cx43-P2 may have been included in the pellet. More Cx43-P2 may have existed than was detected in the present results obtained by Western blotting.

In this study, it was shown that the use of PIPAAm-grafted dishes irradiated with various doses of electron beams enhanced GJIC and Cx43 expression in cultured NHDF cells. This suggests that PIPAAm-grafted dishes may promote efficient tissue regeneration, because GJIC plays an important role in increasing tissue strength.[39]

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