INTRODUCTION

Recently, the organic base materials such as resin composite, glass polyalkenoate cement, and adhesive resin cements have been widely used in dental materials in response to the demand for better treatment and esthetics. Many functional monomers have been developed to provide dental materials having excellent adhesive properties and high strength. However, it has been pointed out that the residual monomers eluted from organic base materials occasionally cause pulp irritation and allergies. Many reports on the cytotoxicity of bisphenol-A derivatives and other dimethacrylate monomers, such as bisphenol-A diglycidyl methacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA), and urethane dimethacrylate (UDMA) et al. have been published. On the other hand, polybasic acids and poly(alkenoic acid)s in dental cements and the adhesive functional monomers in adhesives play important roles in the hardening reaction and adhesive properties. Since their compounds are water-soluble and are directly applied to the dentin, it is important to evaluate the biocompatibility of their hydrophilic functional monomers and carboxylic acids. For example, the cytotoxicity of 2-hydroxyethyl methacrylate (HEMA), which is a hydrophilic compound, is higher than that of methyl methacrylate (MMA), which is a hydrophobic compound. However, there is hardly any study that has examined the cytotoxicity of hydrophilic functional monomers except for HEMA and the relationship between the toxicity of hydrophilic functional monomers used in dental materials and their structure. Therefore, this study evaluated the cytotoxicity of polybasic acids, poly(alkenoic acid)s, and the functional monomers with carboxyl, phosphoryl, or sulfo group by measuring cell growth, and examined the relationship between the cytotoxicity and molecular structure of their compounds.

MATERIALS AND METHODS

Acidic compounds used in this study

Tables 1 and 2 show the formula, name, and codes of polybasic acids, poly(alkenoic acid)s, and the monomers with various functional groups such as carboxyl, phosphoryl, and sulfo group used in this study. Polybasic acids, poly(alkenoic acid)s (UPA), acrylic acid (AA), and 3-allyloxypropanoic acid were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Mono(2-acryloyloxy ethyl) acid phosphate, 2-acrylamide-2-methyl acid sulfate, and 3-acryloyloxypropanoic acid were obtained from Uni-chemical Co., Ltd. (Osaka, Japan), Nitto chemical industrial Co., Ltd. (Fukuoka, Japan), and Nippon Paint Co., Ltd. (Osaka, Japan), respectively.

Preparation of copolymers and analysis of composition

Copolymers of AA and UPA were prepared by radical polymerization. For example, the copolymer of AA and maleic acid (Ma) was prepared as follows: 3.6 g of AA, 5.8 g of Ma, and 0.47 g (5 mass% for the monomer mixture) of ammonium persulfate (Wako Pure Chemical Industries., Osaka, Japan), which is a hydrophilic compound, was added in a test tube. The test tube was flame-sealed on a burner, and then heated at 80°C for 15 hours. After heating, the tube was opened and the reactant was diluted with water. The mixture was dialyzed with a Spectra/Por 6 membrane (MWCO: 1,000, Spectrum Medical Industries., Inc., Cal., USA) with low molecular weight cutoff of 1,000 in order to remove low molecular weight compounds, and then the dialyzed solution was concentrated and freeze-dried.
copolymer, about 0.47 g of the copolymer was weighed, and dissolved in water to produce 100 mL of the copolymer solution. A volume of 10 mL of the solution was added into a conical beaker which contained 3.0 mL of 0.1 mol/L calcium chloride aqueous solution, and then titrated with 0.05 mol/L sodium hydroxide aqueous solution. The equation for the calculation was as follows:

\[ W = m_1 + m_2 \]
\[ m_1/M_1 + m_2/M_2 = 0.05 \times f \times V \]

From (1) and (2)

\[ m_1 = (0.05 \times f \times V \times M_1 \times M_2 - W \times M_2) / (M_2 - M_1) \]

where, \( W \) is mass (g) of copolymer weighed, \( V \) is the titration value (L) of 0.05 mol/L NaOH solution, \( f \) is factor, and \( m_1 \), \( m_2 \), \( M_1 \), and \( M_2 \) are mass and formula weight of AA and Ma, respectively.

The molecular weight distribution curves of the prepared copolymers were obtained by gel permeation chromatography (LC-6A, apparatus, Shimadzu, Kyoto, Japan), and the average molecular weight values were then calculated from the obtained curves. The chromatographic conditions were as follows: two columns in series (Asahipak GSM 700 and GS 520, Asahi Kasei Corp., Tokyo, Japan); mobile phase was 30 mmol/L potassium chloride aqueous solution at room temperature and flow rate was 1.0 mL/min; refractive index detector; standard specimens were poly(acrylic) acids with molecular weights of 0.7, 3.5, 35, and 100×10⁴ (Nippon Paint, Osaka, Japan). The molar ratios and average molecular weight of each copolymer are shown in Table 1.

<table>
<thead>
<tr>
<th>Chemical formula</th>
<th>Name</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOOC–CH–CH₂–COOH</td>
<td>Malic acid</td>
<td>MAL</td>
</tr>
<tr>
<td>HOOC–CH–CH–COOH</td>
<td>Tartaric acid</td>
<td>TAR</td>
</tr>
<tr>
<td>HOOC–CH₂–C–CH₂–COOH</td>
<td>Citric acid</td>
<td>CIT</td>
</tr>
<tr>
<td>HOOC–CH₂–CH–CH₂–COOH</td>
<td>Tricarballylic acid</td>
<td>TRIC</td>
</tr>
<tr>
<td>HOOC–HC–CH–COOH</td>
<td>Tetrahydrofuran-2,3,4,5-tetracarboxylic acid</td>
<td>TETR</td>
</tr>
<tr>
<td>( \left(\text{CH}_2–\text{CH}\right)_n \text{COOH} )</td>
<td>Poly(acrylic acid) ( \text{MW}=18.9^{*1} )</td>
<td>PAA</td>
</tr>
<tr>
<td>( \left(\text{CH}_2–\text{CH}\right)_m \text{COOH} \quad \left(\text{CH}_2–\text{CH}\right)_n \text{COOH} )</td>
<td>Copoly(acrylic acid /maleic acid) ( \text{m:n}=82:18^{*2}, \text{MW}=9.3 )</td>
<td>AA/Ma</td>
</tr>
<tr>
<td>( \left(\text{CH}_2–\text{CH}\right)_m \text{COOH} \quad \text{C} \quad \left(\text{CH}_2–\text{CH}\right)_n \text{COOH} )</td>
<td>Copoly(acrylic acid /itaconic acid) ( \text{m:n}=85:15, \text{MW}=11.7 )</td>
<td>AA/It</td>
</tr>
<tr>
<td>( \left(\text{CH}_2–\text{CH}\right)_m \text{COOH} \quad \text{C} \quad \text{C} \quad \left(\text{CH}_2–\text{CH}\right)_n \text{COOH} )</td>
<td>Copoly(acrylic acid /aconitic acid) ( \text{m:n}=83:17, \text{MW}=15.7 )</td>
<td>AA/Ac</td>
</tr>
</tbody>
</table>

*1: Obtained by GPC (MW=Average molecular weight /10⁴).
*2: Obtained by titration.
Preparation of medium containing each compound

A little water was added to 5 mmol of each acid, and pH of the solution was adjusted to about 6.5 by 1.0 mol/L sodium hydroxide aqueous solution, and then was diluted with water to 50 mL. The aqueous solution of 0.1 mol/L of each acid was prepared, and was filtrated by a 0.20 µm Millipore filter (Millex®-LG, MA, USA) to sterilize it. The concentration of the copolymer was calculated from the weight mean of the formula weight of each monomer by using the molar ratio obtained by analysis. The Dulbecco’s modified Eagle medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) including antibiotics (penicillin-streptomycin, Invitrogen Corp., New York, USA) and 5% fetal bovine serum (MultiSerTM Foetal Bovine Serum, Thermo Trace Ltd, Melbourne, Australia) was used. Then, 0.5, 1.5, and 2.5 mL of the solution were drawn off in a pipette, and 2, 1, and 0 mL of Dulbecco’s phosphate buffered saline solution (PBS(-) solution, Nissui) were added, respectively. Each solution was adjusted to 2.5 mL in order to fix the quantity of medium for dilution, and was diluted with the medium to 50 mL. The concentration of polybasic acid in each solution became 1, 3, and 5 mmol/L, respectively, and the pH of the solution became at 7.7−8.1, since it was diluted with the medium. The 2.5 mL PBS(-) solution was diluted with medium to 50 mL, and the medium without each acid was used as a comparison (control).

Cytotoxic test

Non-carious human premolars that had been extracted for orthodontics reasons were used in this experiment with informed consent of the patient. The handling and culture of the tooth pulp that was removed from the tooth were carried out according to the procedure developed by Kawase et al.18). The tooth pulp tissue was cut into small pieces, and then pre-cultured at 37°C under 5% CO2−95% air of over 95% humidity to increase the number of cells. The pre-cultured cells were subcultured four times to permit fibroblasts them to outgrow any possible contaminating other cells19-22) and to increase the number of cells, and the cells which were subcultured 5−7 times were used in this study. Cell suspension culture liquid of 200 µL containing 3×10⁴ cells/mL was added to each well of a hydrophobic 96-well culture plate and the cell were cultivated for 2 days. Then, the culture solutions were exchanged culture solution of 200 µL with the medium containing each of the carboxylic acids and monomers mentioned above, respectively, and eight wells of the plate were used for one sample. The medium containing their compounds was changed every 2 days, and the cells were cultivated for 6 days. After cultivation for 6 days, all the 96-well plates used in this study were stored in a freezer at -20°C until analysis. Cell growth was evaluated by DNA quantity of the cells which proliferated on each day of cultivation. And ALPase activity was measured as an indication which the cell was fibroblastic23) and in order to examine whether the cells keep the characteristics of the calcification after cultivation for 6 days.

Determination of DNA

Determination of DNA of the fibroblasts was carried out according to the method of Puzas et al.24). To four empty wells of the plates used in this study, 0, 5, 10, and 20 µL of 0.1 mg/mL standard DNA (Sigma, USA) were added. The plate was left for about 60 min in a dryer at 60°C to evaporate the water in the wells. To each well of the plate, 45 µL of 2 mol/L perchloric acid (Wako) was added, respectively, and the pH of the solution became at 7.7−8.1, since it was diluted with the medium. The 2.5 mL PBS(-) solution was diluted with medium to 50 mL, and the medium without each acid was used as a comparison (control).

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Measurement of ALPase activity

Measurement of ALPase activity was carried out according to the method of Lowry et al. and Puzas and Brand. The plates were taken out of the freezer and were left for 5 min at room temperature. Next, the plates were stored for 10 min in a freezer, and then were left for 5 min at room temperature in order to destroy the cells. To four empty wells of the plate, 0, 10, 25, and 50 µL of 1 µmol/mL p-nitrophenol (Sigma) as a standard material were drawn off in a pipette, and 200, 190, 175, and 150 µL of 1 mol/L sodium hydroxide solution were added, respectively. Next, to all wells of the plate except for the four wells mentioned above, 50 µL of 0.25 mol/L 2-amino-2-methyl-1-propanol buffer solution containing 16 mmol p-nitrophenol phosphates was added. The buffer solution was prepared as follows; 11.15 g of 2-amino-2-methyl-1-propano was dissolved in about 450 mL water, and the pH of the solution was adjusted to about 10 by using concentrated hydrochloric acid, 0.5 mL of 1 mol/L magnesium chloride solution was added, and then this was diluted with water to 500 mL. The plate was stored for about 15 min in an oven at 37°C. After it was taken out of the oven, 150 µL of 1 mol/L sodium hydroxide solution was added to each well in order to stop the reaction. The p-nitrophenol produced in each well was analyzed by a micro-plate visible spectrophotometer (ELISA, BIO-RAD, GA, USA) at 410 nm.

The mean value and the standard deviation of eight values that had been obtained from one experiment were calculated, and the statistical analysis of the results for each group of measurements was conducted using Tukey’s test with the level of statistical significance at p<0.05.

RESULTS

Cytotoxicity of the polybasic acids and poly(alkenoic acid)s

Figures 1 and 2 show the cell growth and ALPase activity of the fibroblasts cultivated by the medium containing various polybasic acids and poly(alkenoic acids). The cell growth of each polybasic acid and the polymer after 6 days was not significantly different compared with that of control (p>0.05). Regarding the ALPase activity in each polybasic acid and the polymer, there was no significant difference compared with that of control (p>0.05).

Cytotoxicity of the monomers with various functional groups

Figure 3 shows the cell growth and ALPase activity of the fibroblasts cultivated in the medium containing the monomers with various functional groups. The cell growth when exposed to AA, ACPA, and AEPO after 6 days decreased remarkably compared with that of control (p<0.05) and those exposed to ALPA and AMPS were not significantly different. The ALPase activity of the fibroblasts cultivated in the medium containing 1 mmol/L of AA was higher than that of control (p<0.05), but was lower in the case of 3 and 5 mmol/L (p<0.05). The ALPase activity of the fibroblasts cultivated in the medium containing 1, 3, and 5 mmol/L of ALPA was slightly lower than that of control (p<0.05), and the activity of the fibroblasts exposed to AMPS was higher than that of control (p<0.05).

Figure 4 shows the photographs of the fibroblasts cultivated in the mediums containing 3 mmol/L of the monomers with various functional groups after 6 days. The fibroblasts cultivated in the medium containing ALPA and AMPS show a densely packed shape the same as control, whereas those exposed to AA were somewhat poorer than control, and those exposed to ACPA were sparse. The fibroblasts exposed to AEPO were spherical and few, and the cells were probably lifeless.

DISCUSSION

Glass ionomer cements and polycarboxylate cements containing polyelectrolytes as cement liquid are clinically widely used in clinical practice, because they are adhesive to tooth and show comparatively high biosafety. Moreover, adhesive resins are also widely used because of their high adhesiveness and mechanical strength. The polybasic acids and poly(acrylic acid)s in the cements and adhesive functional monomers in adhesives play important roles in the adhesiveness and mechanical properties. Various monomers modified with phosphoryl and carboxyl group are used in dental materials, and many new monomers with adhesive properties are being studied. Those monomers are water-soluble, and are directly applied to the dentin and enamel, so it is important to evaluate the biocompatibility of the monomers with various functional groups and the compounds with carboxyl groups.

Cytotoxicity of polybasic acids and poly(alkenoic acid)s

The cell growth of fibroblasts cultivated in the medium containing polybasic acids and poly(alkenoic acid)s was not significantly different compared with that of the control (Figs. 1 and 2). Furthermore, ALPase activity was not significantly different compared with that of the control except for AA/Ac. Their acids were very safe compared with AA (Fig. 3) and the growth was not dependent on the number of the carboxyl group in the molecule.

Meryon et al. compared the in vitro cytotoxicity of two commercial glass-ionomer cements, which contained water-soluble polybasic acid and ionomer, using a model cavity system, and reported that both cements were cytotoxic to fibroblasts and macrophages. Umemo et al. reported that the cytotoxicity appeared when a large amount of poly(acrylic acid) was administered. Stanley reported that the tooth pulp stimulation was caused when the viscosity of PAA was decreased. On the other hand, Imanishi reported that the cytotoxicity was weak, since PAA with high molecular weight did not diffuse easily to the dental tubule and the residual monomer while preparing PAA was strongly related to the cytotoxicity. Results obtained at 5 mmol/L in this study showed that the cell growth of AA/Ma, AA/It, and...
AA/Ac which had many carboxyl groups slightly decreased. It is thought that the reason to which the cytotoxicity of poly(alkenoic acid)s in this study was weak is because the compounds with the low-molecular weight was removed by the dialysis. As for the cytotoxicity of commercial cements, it is necessary to think about not only the influences of polybasic acid and poly(alkenoic acid)s in the cement liquid but also the residual monomer and other compounds, etc.

Cytotoxicity of the monomers with various functional groups

The cell growth of fibroblasts cultivated in the medium containing AA, ACPA, and AEPO was significantly different compared with that of the control (Fig. 3). Almost all the cells exposed to ACPA and AEPO seemed to die out (Fig. 4). On the other hand, the cell growth of the fibroblasts exposed to ALPA and AMPS was not

Fig. 1 Effects of various polybasic acids on cell growth of cultured fibroblasts, and their ALPase activity after cultivation for 6 days. Identical letters indicate that the mean values are not significantly different in each test group (p>0.05), and the letters in ALPase activity show the difference from the control.
significantly different compared with that of the control. In the comparison between ALPA with an allyloxy group and ACPA with an acryloyloxy group, ACPA was very toxic, but ALPA showed low toxicity. It is thought that the cause of the toxicity of ACPA depends on the combination of the acryloyloxy and carboxylato group or the steric structure of the molecule. On the other hand, as the cytotoxicity of saturated fatty acids with carbon number of 3 to 7 is decreased with increase in carbon number, it is considered that the toxicity of ALPA with a comparatively long main chain and without an ester bond was lower.

The cells exposed to AEPO with a phosphoryl group were spherical and few (Fig. 4), and the cells were probably lifeless. However, those exposed to AMPS increased as well as the control. It is considered that the sulfo group of AMPS has comparatively little effect on organism. Regarding, the effect of the difference in
molecular structure between AMPS and AEPO on the cell growth, it may be due to the difference in the number of anions in each acidic group such as univalent or divalent anions and the bonding in the molecule of each acid such as amide or ester.

Up to now, many reports on the cytotoxicity of HEMA that is hydrophilic compound has been published. Watanabe et al.\textsuperscript{16} studied the concentration of 50% cell growth inhibition (GI\textsubscript{50}) of HEMA was about 8.5 mmol/L. And those by Li et al.\textsuperscript{31}, by Walther et al.\textsuperscript{32}, and by our study\textsuperscript{33} were 6.3, 1–5, and 3 mmol/L, respectively. The cytotoxicity of polybasic acids and poly(alkenoic acid)s was remarkably low compared with that of HEMA, and that of the acidic functional monomers such as AA, ACPA, and AEPO in this study was similar to HEMA. It was proven that their acidic functional monomers including HEMA showed the strong cytotoxicity. Generally, the hydrolysis of the ester bonds was promoted.

Fig. 3 Effects of the monomers with various acidic functional groups on cell growth of cultured fibroblasts, and their ALPase activity after cultivation for 6 days. Identical letters indicate that the mean values are not significantly different in each test group ($p>0.05$), and the letters in ALPase activity show the difference from the control.
by the presence of acid and base. Since the pH of the medium containing acidic functional monomers in this study was approximately 7, it was not considered that the monomers were hydrolyzed by it. However, when the ester bonds of the monomers were hydrolyzed by some components which were included in the medium, it was thought that the lower carboxylic acid produced, namely methacrylic acid, influenced the cell growth, because the cytotoxicity of lower carboxylic acid was considerably strong\textsuperscript{33,34}. On the other hand, since the structure of the acidic functional monomers was similar to that of a surfactant, it was considered that the cell growth cultivated with medium containing the monomers decreased. However, the result of the present study could not perfectly clarify the relationship between the structure of these compounds and cell toxicity. In addition, it is necessary to examine the cytotoxicity of various compounds such as monomers with a sulfo group and an acryloyloxy group.

Fig. 4 Photographs of the fibroblasts exposed to the medium containing 3 mmol/L of each monomer after 6 days.
CONCLUSIONS

Within the limitations of this in vitro study, the following conclusions were drawn:

1. Cell growth of the fibroblasts cultivated in the medium containing polybasic acids and poly(alkenoic acid)s up to the concentration to 5 mmol/L was not significantly different compared with that of control without their acids.

2. The cell growth of the fibroblasts cultivated in medium containing 1 mmol/L of 3-acryloyloxy propanoic acid and mono(2-acryloyloxy ethyl acid) phosphate, which had an acryloyloxy and a phosphoryl or carboxyl group, respectively, decreased remarkably compared with that of control and the cells were probably lifeless.

3. That of 3-allyloxy propanoic acid and 2-acrylamide-2-methyl acid sulfate, which had an ether bond and a carboxyl group or an amide bond and a sulfo group, respectively, was not significantly different compared with that of control.

REFERENCES


