INTRODUCTION

Acrylic resins have been widely used in clinical dentistry and medicine\(^1\) due to their ease of use and flexible formability. However, the effects of residual monomers on tissues and organs are far from being negligible. As such, acute allergies\(^2\)–\(^5\) may occur after implantation of acrylic-related resins, and hypotension or circulatory failure has been reported\(^6\)–\(^8\). To address these mounting concerns over cytotoxicity complications and health hazards, biological evaluation of these acrylic resins has been performed to determine the precise risks and benefits of these resins.

To minimize the cytotoxic-induced side effects of acrylic resins\(^9\)–\(^17\), we devised a new acrylic resin\(^18\) that was engineered to reduce cellular damage as much as possible. Therefore, the aim of the present study was to investigate in vitro the cytotoxic effects of this novel resin monomer, and thereby compare the obtained results with several conventional acrylic monomers.

MATERIALS AND METHODS

Preparation of materials

A monomer of the novel acrylic resin, methacryloyloxyethyl methyl succinate (TA), as depicted in Fig. 1 was previously synthesized under Japanese patent specific number, H6-25048. This monomer was synthesized in cooperation with Shofu Incorporated (Kyoto, Japan).

Three reference acrylic monomers were used in this study: Methyl methacrylate (MMA; Wako Pure Chemical Industries Ltd., Osaka, Japan), Ethyl methacrylate (EMA; Sigma-Aldrich Japan KK, Tokyo, Japan), and Lauryl methacrylate (LMA; Inoue Chemical Industry Co., Ltd., Tokyo, Japan).

TA (Methacryloyloxy-ethyl methyl succinate)

\[
\text{H}_3\text{C} \quad \text{C} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{CH}_3
\]

Fig. 1 Monomer of novel resin.

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We have previously synthesized a novel acrylic resin monomer, methacryloyloxyethyl methyl succinate (TA). The aim of this in vitro study, therefore, was to examine its influence on cell viability using L-929 mouse fibroblasts and then compare the results with MMA, EMA, and LMA. Medium containing each monomer was changed every 15 minutes as some monomers were volatile. After one hour of exposure, these mediums were replaced with a normal medium and cells were further incubated for 72 hours. IC\(_{50}\) value for each monomer was determined, and chronological cell viability and cyt morphologic observation were evaluated. Viability was impaired in a dose-dependent manner. All monomers, except TA, tended to correlate between molecular weight and cell viability. On the other hand, TA showed excellent viability and did not impair growth abruptly. These results thus demonstrated that cellular damage by TA was much lower than that by other monomers.

Key words: Monomer, Cell viability, Methyl methacrylate
Sigma-Aldrich Japan KK, Tokyo, Japan). These monomers are depicted in Fig. 2.

**Measurement of monomer volatility**

Monomer solutions were prepared by dissolving MMA, EMA, or TA in 1× phosphate buffered saline (PBS; Wako Pure Chemical Industries Ltd., Osaka, Japan). Concentration of dissolved monomer was such that 1 mmol/l was present in the 10 ml added to the 10-cm polystyrene culture dishes (Becton Dickinson Labware, NJ, USA), which were then incubated at 37°C in 5% CO₂.

Concentration of persistently dissolved monomer was determined by high performance liquid chromatography (reversed phase liquid chromatography, HPLC) every 15 minutes for one hour. As for the measurement of remaining monomer in each solution, it was performed using Agilent ZORBAX ODS column (4.6×250 mm; Agilent Technologies, CA, USA). Mobile phase was a mixture of 70% acetonitrile and 30% distilled water, and flow rate was 0.5 ml/min. With detection wavelength set at 210 nm, HPLC data were recorded with a standard data capture system. Based on the captured data, the amount of each monomer was determined using the linear regression equation obtained from a calibration curve. Volatility was then calculated based on a peak area ratio at each time period in relation to the initial value using the Class-VP data system (Shimadzu Co., Kyoto, Japan).

**Experimental cell line**

L-929 cells (ATCC CCL 1; Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) were maintained in minimum essential medium with Earle’s salts (MEM-Earle; Invitrogen Japan KK, Tokyo, Japan) containing 5% fetal bovine serum (FBS; Invitrogen Japan KK, Tokyo, Japan). Culture medium was changed every two days, and L-929 cells were cultured until subconfluence.

**Calculation of IC₅₀ (50% inhibitory concentration)**

The cell culture schedule is shown in Fig. 3. L-929 cells (2×10⁴ cells/cm² in 120 μl serum-free medium) were seeded into 96-well tissue culture plates and cultured for 24 hours at 37°C in 5% CO₂. Culture medium was then changed to serum-free medium for 6-hour incubation for control of intracellular signaling and cell cycle. Medium was subsequently removed and replaced with fresh medium containing 5% FBS for another six hours.

Monomer solutions were prepared by dissolving and diluting MMA, EMA, LMA, and TA in FBS-free medium to 1, 10, 50, and 100 mmol/l concentrations, and to which the cells were exposed for one hour. All monomer-containing mediums were replaced every 15 minutes. As a negative control, cells were grown in standard FBS-free medium. After exposure, medium was removed, cells were washed with PBS, fresh culture medium including 5% FBS was added, and cells were incubated for another 24 hours.

Cell viability was assessed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). CCK-8 solution (10 μl) was added to each well of the plate and further incubated for one hour. After incubation, optical density at 450 nm (OD₄₅₀) and 600 nm (OD₆₀₀) were measured using a microplate reader. Relative cell viability was calculated as follows: cell viability [%] = (OD₄₅₀ - OD₆₀₀ of control)/(OD₄₅₀ - OD₆₀₀ of treated cells) × 100%. Each experiment was repeated a minimum of five times. Dose-response curves of relative cell viability were plotted to delineate monomer concentrations, as well as the respective 50% inhibitory concentration as
compared with the control (IC₅₀ value).

**Chronological cell viability and cytomorphologic observation**

L-929 cells (2×10⁴ cells/cm² in 10 ml serum-free medium) were seeded into 10-cm polystyrene culture dishes and cultured with the schedule shown in Fig. 3. After monomer exposure at 1 mmol/l, specimens were placed in fresh culture and further incubated for 12, 24, 48, and 72 hours. CCK-8 solution was used to evaluate cell viability after monomer exposure. Treated cells were observed under a phase-contrast microscope.

**Statistical analysis**

Data are presented as means and standard deviations. Statistical analysis was performed using t-test for two independent variables or by ANOVA followed by Bonferroni test. Level of significance was set at p<0.05. Correlation coefficients were calculated for molecular weight and cell viability at 1 mmol/l monomer, except for TA, after 72 hours of incubation.

**RESULTS**

**Monomer volatility**

Fig. 4 shows the monomer volatility data. After only 15 minutes, MMA volatility was calculated to be 32.0%, while that of EMA was 41.4%. MMA volatility reached 50% after 45 minutes, and EMA volatility reached 50% after 35 minutes. On the other hand, TA was not volatile. LMA could not be detected.

**IC₅₀ value**

All monomers impaired L-929 cell viability in a dose-dependent manner. All monomers, except TA, impaired growth abruptly (Fig. 5). Table 1 shows the IC₅₀ values after 24 hours of incubation, as calculated

<table>
<thead>
<tr>
<th>Monomer</th>
<th>IC₅₀ [mmol/l]</th>
</tr>
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<tbody>
<tr>
<td>MMA</td>
<td>6.9</td>
</tr>
<tr>
<td>EMA</td>
<td>12</td>
</tr>
<tr>
<td>LMA</td>
<td>6.9</td>
</tr>
<tr>
<td>TA</td>
<td>72</td>
</tr>
</tbody>
</table>

![Fig. 4 Monomer volatility.](image-url)

![Fig. 5 Dose-response curves of cell viability by each monomer after 24 hours of incubation. Viabilities were normalized against control cultures (n=5).](image-url)
from the dose-response curves of cell viability.

Chronological cell viability and cytomorphologic observation

Time-dependent changes in cell viability after exposure to monomers at 1 mmol/l are shown in Fig. 6, and chronological cytomorphologic observations for each monomer at 1 mmol/l are shown in Fig. 7. MMA was found to abruptly reduce cell viability at 12 hours, but viability increased thereafter. EMA showed a similar trend as MMA, but viability recovered to a lesser degree. Cell shape with MMA and EMA exhibited severe atrophy until 24 hours, but these cells recovered after 48 hours. LMA did not severely impair viability at 12 hours, but viability reduced with time; and after 72 hours, it was less than 50%. As for cell shape with LMA, severe atrophy was exhibited at all time points. At 12 hours, the ranking of cell viability for the three references monomers was LMA > EMA > MMA; but after 48 hours, the ranking changed to MMA > EMA > LMA. TA was found to be the best among the monomers.
with regard to cell viability. It was significantly better than other monomers at all time points, and no negative effects were observed at 1 mmol/l exposure. In summary, cell viability was significantly different among the monomers ($p<0.01$).

**DISCUSSION**

_Establishing experimental conditions_

The objective of the present study was to determine whether TA, a novel compound, would suppress the growth of cultured cells, and thereby evaluate the affinity of TA for cells in relation to several alkyl esters. MMA was selected because substantial experiment data have been accumulated to date. EMA possesses one more carbon atom than MMA, and LMA possesses ten carbon atoms more than MMA and a molecular weight comparable to TA. To ensure comparable experimental conditions without using solvents such as DMSO, monomers were added to culture medium only. With regard to the monomers that were selected as comparators, it was confirmed that volatility varied markedly where the speed and volume of volatilization tended to be greater for low-molecular-weight hydrophobic monomers with a methyl group. The volatility of TA was extremely low, and the volatility of LMA could not be detected. Due to the marked differences in volatility among the monomers, strict experimental conditions were therefore established and implemented for monomer addition. As a result, medium containing each monomer was changed every 15 minutes, and to which cultured L-929 cells were exposed for an hour.

Apart from monomer volatility, water solubility of these monomers was also taken into consideration and these data are shown in Table 2. As each monomer has a different solubility, precise assessment of chronological cell viability (i.e., how cell viability changed with time at 12, 24, 48, and 72 hours) was performed based on exposure to monomers at 1 mmol/l. In addition, cells were seeded with serum-free medium for 24 hours and incubated for six hours whereby the latter was done for control of intracellular signaling and cell cycle. In this manner, cells were made quiescent by 30 hours of incubation in serum-free medium. Under these conditions, it was thus possible to keep to a minimum the differences among the monomers and among the cells — without using solvents such as DMSO.

Comparison of new resin and three alkyl esters

The three reference monomers — MMA, EMA, and LMA — significantly hindered cellular proliferation when compared with TA. In particular, IC$_{50}$ for MMA and LMA ranked the lowest at 6.9 mmol/l. Moreover, LMA hindered cellular proliferation for a long time: even at 72 hours after LMA exposure, phase-contrast microscopy showed clearly cells with a spherical shape. On the other hand, with MMA and EMA, cells of spherical shape recovered at 48 hours after exposure; and at 72 hours after exposure, cells had returned to their original shape. In other words, recovery of cellular proliferation and cell shape were particularly marked for MMA and EMA: while MMA and EMA strongly hindered cells initially, cellular recovery was faster when compared to other monomers.

As some studies have suggested a correlation between cytotoxicity and monomer molecular weight, this issue was also investigated in the present study. Fig. 8 shows the relationship between molecular weight and cellular viability after 72 hours of culturing at a dose of 1 mmol/l. Tendency of correlation between molecular weight and cell viability was seen for all monomers except for TA. These findings thus suggested that continuous cell damage by hydrophobic monomers was proportional to molecular weight. 

**Table 2** Molecular weight and water solubility of the four monomers studied

<table>
<thead>
<tr>
<th>Monomer</th>
<th>Molecular weight</th>
<th>Water solubility ($%$)</th>
<th>[mmol/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMA</td>
<td>100.12</td>
<td>1.72*</td>
<td>172</td>
</tr>
<tr>
<td>EMA</td>
<td>114.14</td>
<td>0.46*</td>
<td>40.3</td>
</tr>
<tr>
<td>LMA</td>
<td>254.41</td>
<td>&lt;0.01*</td>
<td>&lt;0.39</td>
</tr>
<tr>
<td>TA</td>
<td>244.24</td>
<td>0.61*</td>
<td>25</td>
</tr>
</tbody>
</table>

*Water solubility (%) data were reprinted from Mitsubishi Rayon Co. Ltd. (Tokyo, Japan).

†TA was evaluated by high performance liquid chromatography (HPLC).

*Fig. 8 Tendency of correlation between molecular weight and cellular viability after incubation with each monomer at 1 mmol/l for 72 hours. Correlation coefficients were calculated for all monomers except TA. The monomers exhibited a tendency of correlation between molecular weight and viability $r=0.879$, but TA showed no negative effects on viability.
weight. However, while LMA and TA have comparable molecular weights, TA did not induce continuous cell damage probably because its molecular design involved an ester. Cytotoxicity of fatty acids decreases with increasing hydrocarbon chain length\(^2\). However, when polymerized by an increasing number of saturated hydrocarbons, biological toxicity increases\(^3\), as shown in the case of LMA.

In the case of protozoan, one study found that toxicity was reduced by using a carbonyl group instead of a vinyl or methyl group\(^4\). In light of this finding, chemosynthesis of TA monomer was carried out by inserting three ester bonds instead of saturated hydrocarbons. It seemed that this approach served to reduce cytotoxicity, as the cytotoxicity of TA was found to be more favorable than conventional resin monomers\(^5\) in this study. Based on the results of this study, TA seemed well poised as a useful resin monomer for clinical applications in the future.

**ACKNOWLEDGEMENTS**

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**REFERENCES**