Introduction

The component substances of biomaterials and their metabolites contain many chemical substances for which the teratogenicity has not yet been determined in human beings. Since numerous biomaterials are currently being developed, a method for testing embryotoxicity in vitro in a speedy, effective, and precise manner is needed. Braun et al. (1979) reported an in vitro embryotoxicity test for the first time. Thereafter, Yoneda et al. (1981) and Trosko et al. (1985) also reported testing protocols. Flint (1983) developed the micromass culture method. However, in all of these in vitro test methods, they usually did not go beyond the bounds of the initial pilot study, even though numerous test systems that are closer to in vivo studies have been developed, including one that involves whole embryo cultures (Fantel, 1982; Hurst et al., 1993; Zhao et al., 1997). At the same time, utilization of embryonic stem (ES) cells has been researched extensively, leading to the creation of experimental animals such as knockout mice, and also breakthroughs in technological developments in the study of embryotoxicity. Spielmann et al. (1997) developed the embryonic stem cell test (EST), which is an in vitro embryotoxicity test method that can be used to estimate the risk of embryotoxicity of chemical substances relatively quickly compared to the conventional methods that involve animal experiments. In a recent validation study in Europe, the EST was found to be reproducible, demonstrating an overall accuracy of 80% and 100% correct prediction of strong embryotoxicity for chemicals studied under blind conditions.
Plastic materials are frequently used in dental restorations, in the severe environmental conditions of the oral cavity. Continued improvement of monomers, single units of polymerized materials, has been made to obtain better clinical performance. In this study, we mainly examined newly synthesized monomers (Matsukawa et al., 1994; Hayakawa et al., 1998) using the EST method for assessment of embryotoxicity.

**Materials and methods**

### 1. Cells

We used embryonic stem cells from the D3 mouse cell line (ES-D3 cells; Fig. 1) and clone A31 from BALB/c 3T3 cells (3T3 cells) from mice. We obtained the ES-D3 cells from Prof. Rolf Kemler (Max Planck Institute, Freiburg, Germany). The 3T3 cells were purchased from American Type Culture Collection (ATCC; Cat. No. CCL-163).

### 2. Culture mediums

For ES-D3 cells, preheat treated 20% (v/v) fetal calf serum (FCS) (HyClone, USA) was added to 1% (v/v) nonessential amino acids (Gibco, USA), 0.1 mM β-mercaptoethanol (Sigma, USA), 2 mM L-glutamine (Gibco), and Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with penicillin/streptomycin (Gibco). Except during tests, 1,000 U/mL of mouse leukemic inhibitory factor (mLIF) was added to inhibit natural cell differentiation. For 3T3 cells, a culture solution was prepared by adding 10% (v/v) FCS to DMEM with 4 mM L-glutamine and penicillin/streptomycin.

### 3. Preparation for test solutions

Eighteen dental monomers were used: 2.0-EPDMA, 3.0-EPDMA, 4.0-EPDMA, 1.6-ADMA, 1.8-ADMA, 1.10-ADMA, 6-HHMA, Bis-GMA (6F), MEPC, Phosmer M, BPE-1300, BSNa, EDMABA, GAM, GMR, MTOA, PTSNa, and QTX. Each structural formula is shown in Fig. 2. For each of the monomers, 50 mg was dissolved into 200 μL DMSO (Sigma, USA) in a sterilized short glass test tube and each test tube was shaken for two hours. Culture medium (9.8 mL at 37°C) was added to the test tube and vigorously shaken by hand. It was then diluted serially in culture medium to obtain each test solution. For the negative control group, only DMSO was added.

### 4. Differentiation assay

ES-D3 cells were diluted in each test solution to a final concentration of 3.75 x 10⁴ cells/mL using a hemacytometer, and a 20 μL cell suspension was dropped 60-70 times onto the inside of the lid of a 10 cm diameter Petri dish using a micropipette. Each drop of the cell suspension contained approximately 750 cells. Five mL of sterilized phosphate buffered saline (PBS) was poured into the Petri dish, and the lid was quickly reversed and placed on the dish before the cell suspension on the lid could flow down. Suspenion culture was carried out for three days in a CO₂ incubator (5% CO₂ and 95% air; 37°C) (Sanyo, Japan). Drops of the cell suspension on the inside of the Petri dish lid were then collected into a 6 cm diameter Petri dish for germiculture. The test solutions were replaced with new ones using a pipette and each test solution was subjected to reaction for two days in the above listed incubator. Subsequently, two 24-well multidishe were used for every test solution. Each of the one embryoid bodies (EBs) (Fig. 3) formed were placed in the well with a micropipet, and the EBs were cultured statically for five days (Fig. 4). The presence of beating myocardial cells in each well was examined under an inverted phase difference microscope (IX-70, Olympus, Japan). The number of wells in which beating cells were observed at each concentration was examined, and the ID₅₀ was calculated from the ratio of the number of the above wells to the number of wells in which EBs were successfully disseminated.
5. Cell viability assay
In order to examine the effects of the test solutions on the two kinds of cells, i.e., 3T3 cells and ES cells, cell suspensions of $1 \times 10^4$ cells/mL were prepared in the absence of mLIF, inoculated into a 96-well multidish, and test solutions were added to each well after two hours. Finally the MTT assay was performed on day 10 of the culture. The absorbance was read on an ELISA reader (Spectra Max Plus, Molecular Device, USA) at 570 nm.

6. Statistical analysis
The results are expressed as the mean and the confidence interval of 95%. The data represented four independent experiments where three independent wells were used for each experiment. P values less than 0.05 were regarded as significant.
Results

1. Differentiation assay

Fig. 5 shows the ID_{50} values. MTYA showed the lowest value, 0.44 μg/mL, followed in ascending order of values by GMR, BPE-1300, Bis-GMA (6F), 6-HHMA, 1.6-ADMA, 1.10-ADMA, Phosmer M, 1.8-ADMA, QTX, EDMABA, 2.0-EPDMA, MEPC, PTSNa, GAM, 3.0-EPDMA, 4.0-EPDMA, and BSNa. For ADMA, 1.6-ADMA, 1.8-ADMA, and 1.10-ADMA values were all similar, 29.6 μg/mL, 34.5 μg/mL and 30.4 μg/mL, respectively. On the other hand, for EPDMA, there were great differences seen between the values of 2.0-EPDMA, 3.0-EPDMA, and 4.0-EPDMA, 161.0 μg/mL, 655.2 μg/mL and 953.6 μg/mL, respectively.

2. Cell viability assay

Fig. 6 shows IC_{50} values for the ES-D3 cells. MTYA showed the lowest value, 1.80 μg/mL. It was followed in an ascending order of values by GMR, Bis-GMA (6F), BPE-1300, 6-HHMA, 1.6-ADMA, 1.10-ADMA, Phosmer M, QTX, 1.8-ADMA, EDMABA, 2.0-EPDMA, MEPC, GAM, PTSNa, BSNa, 3.0-EPDMA, and 4.0-EPDMA. This order differed from that seen for ID_{50}.

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Fig. 5 The half-inhibition concentration for differentiation of ES-D3 cells by dental monomers. The longitudinal fine line represents the confidence interval of 95%.

Fig. 6 The half-inhibition concentration for cytotoxicity of ES-D3 cells by dental monomers. The longitudinal fine line represents the confidence interval of 95%.
Fig. 7 shows the IC$_{50}$ values for the 3T3 cells. GMR showed the lowest value, 4.1 pg/mL. It was followed in an ascending order of values by 1.10-ADMA, Bis-GMA (6F), MTYA, QTX, 1.8-ADMA, 6-HHMA, 1.6-ADMA, Phosmer M, BPE-1300, EDMABA, 2.0-EPDMA, MEPC, GAM, 3.0-EPDMA, PTSNa, BSNa, and 4.0-EPDMA.

**Discussion**

In the case of the EST protocol, three parameters, the ID$_{50}$ and IC$_{50}$ of ES-D3 cells and the IC$_{50}$ of 3T3 cells. There were substituted into the equations for I, II and III of the prediction model that is shown in Table 1 in order to classify the findings into three embryotoxicity levels. As a result, Bis-GMA (6F) and MTYA were categorized as class 2, i.e., showing weak embryotoxicity. The other monomers were categorized as being class 1, i.e., no embryotoxicity. (Table 2)

Of the eighteen dental monomers tested, Bis-GMA (6F) and MTYA were classified as weakly embryotoxic, and 2.0-EPDMA, 3.0-EPDMA, 4.0-EPDMA, 1.6-ADMA, 1.8-ADMA, 1.10-ADMA, 6-HHMA, MEPC, Phosmer M, BPE-1300, BSNa, EDMABA, GAM, GMR, PTSNa, and QTX as nonembryotoxic. However, none of the monomers tested were found to be strong.

![Fig. 7](image)

**Fig. 7** The half-inhibition concentration for cytotoxicity of 3T3 cells by dental monomers. The longitudinal fine line represents the confidence interval of 95%.

**Table 1** Prediction model for the embryotoxic potential using the EST.

<table>
<thead>
<tr>
<th>Improved prediction model</th>
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<tbody>
<tr>
<td>Endpoints : IC$_{50}$ T3</td>
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<tr>
<td>Variables : lg (IC$_{50}$ T3)</td>
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<tr>
<td>lg (IC$_{50}$ D3)</td>
<td></td>
</tr>
<tr>
<td>ID$_{50}$</td>
<td></td>
</tr>
<tr>
<td>$\frac{IC_{50} T3 - ID_{50}}{IC_{50} T3}$</td>
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<tr>
<th>Linear discriminate functions I, II and III</th>
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<tr>
<td>I : $5.9157 lg (IC_{50} T3) + 3.590 lg (IC_{50} D3) - 5.307 \frac{IC_{50} T3 - ID_{50}}{IC_{50} T3} - 15.72$</td>
</tr>
<tr>
<td>II : $3.651 lg (IC_{50} T3) + 2.304 lg (IC_{50} D3) - 2.036 \frac{IC_{50} T3 - ID_{50}}{IC_{50} T3} - 6.85$</td>
</tr>
<tr>
<td>III : $-0.125 lg (IC_{50} T3) - 1.917 lg (IC_{50} D3) + 1.500 \frac{IC_{50} T3 - ID_{50}}{IC_{50} T3} - 2.67$</td>
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</table>

**Classification criteria :**

- **Class 1**
  - non embryotoxic
  - if I > II and I > III

- **Class 2**
  - weak embryotoxic
  - if II > I and II > III

- **Class 3**
  - strong embryotoxic
  - if III > I and III > II
The monomer group that strongly influenced the ID50 included 1,6-ADMA, 1,8-ADMA, 1,10-ADMA, 6-HHMA, Bis-GMA (6F), Phosmer M, BPE-1300, GMR, and MTYA. However, there were no common features in the monomers' chemical structures. MTYA has amino radicals in its structure and in the future it will be necessary to examine other monomers with similar structures.

The other group of the monomers that strongly influenced the ID50 included 2.0-EPDMA, MEPC, EDMABA, and QTX. However, the monomer group with 3.0-EPDMA, 4.0-EPDMA, BSNA, and GAM had almost no influence on the ID50 at 500 μg/mL or higher.

The results of the cell viability assay of ES-D3 cells showed the same monomer group as ID50 of ES-D3 cells. The monomer group that strongly influenced the IC50 of ES-D3 cells included 1,6-ADMA, 1,8-ADMA, 1,10-ADMA, 6-HHMA, Bis-GMA (6F), Phosmer M, BPE-1300, GMR, and MTYA. As a reference, the result of IC50 of these in vitro and in vivo was not found. The chemical structure of Bis-GMA (6F) has the H2 end group replaced by F2 and exhibits the same embryotoxicity level (Imai et al., 2001). There was no monomer with a remarkable difference in IC50 of ES-D3 cells and 3T3 cells.

There are few reports on the embryotoxicity of monomers, although Andersen et al. (1982), Korhonen et al. (1982), Kitchin et al. (1984), Butterworth et al. (1992) and Fucic et al. (1994) have published studies on this phenomenon. We have previously reported that the typical composite resin monomers, Bis-GMA, UDMA, Bis-MPEPP, and TEGDMA, which are used as dental filling materials, exhibit weak embryotoxicity (Imai et al., 2001). However, we have found no other reports on other dental monomers.

All eighteen dental monomers tested, new synthetic monomers used as dental materials, except for Bis-GMA (6F) and QTX. Both of these monomers are already contained in commercial materials. EPDMA are expected to be applied for dental hard resin materials and ADMA and 6-HHMA were developed for use as dentin adhesion monomers. Moreover, other monomers are expected as basic raw materials. However, more examination is required for clinical use of MTYA and Bis-GMA (6F).

The dental monomers examined in this study are used for denture base resin, restorative resin, resin cement, resin inlay, resin for crowns and bridges, opaque resin, direct filling resin, pit and fissure sealant, dentin bonding agent, adhesive composite resin, denture adhesive, and artificial resin teeth. These monomers are used in the mouth as polymers after heat-curing, self-curing, or light-curing. Some resin cements available on the market are of the dual-curing type. However, in many cases 100% polymerization is almost impossible. In particular, it is known that the degree of polymerization is lower in self-curing or light-curing than in heat-curing compounds (Nomoto et al., 1992; Marais et al., 1999). As light-curing has increasingly been used recently, there is concern that residual monomers will invade body tissues. It is known that monomers are more toxic in general than polymers (Geurtsen et al., 1998). Another issue is the possibility that powder generated by occlusion and wear could enter into the body, resulting in adverse effects (Sakai et al., 2002).

In recent years, the Quantitative Structure-Activity Relationship (QSAR) analysis has been used in drug design to examine the relationship between the structure and cell toxicity of chemical substances. Based on the data from these

<table>
<thead>
<tr>
<th>Embryotoxicity level</th>
<th>Monomers</th>
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<tbody>
<tr>
<td>strong (I)</td>
<td>None</td>
</tr>
<tr>
<td>weak (II)</td>
<td>Bis-GMA (6F), MTYA</td>
</tr>
<tr>
<td>None (I)</td>
<td>2.0-EPDMA, 3.0-EPDMA, 4.0-EPDMA, 1,6-ADMA, 1,8-ADMA, 1,10-ADMA, 6-HHMA, MEPC, Phosmer M, BPE-1300, BSNa, EDMABA, GAM, GMR, PTSNa, QTX.</td>
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studies, it is known that chemical substances having chemical structures with excellent cell membrane lipid permeability are apt to bond with serum protein. The mechanism by which these chemical substances are taken into the cells is believed to be quite complicated (Klopman et al., 1993). However, the test data that has been accumulated cannot always be applied to the QSAR analysis of embryotoxicity. This is because the amount of data available for use is insufficient for application of the QSAR analysis of embryotoxicity. In the future, through active use of the EST method, data from QSAR analysis of dental monomers can be accumulated and analyses undertaken to determine the embryotoxicities.

There are many processes involved in the mechanisms through which chemical substances can enter into a mother’s body and affect the fetus. These processes include the kinetics of absorption, distribution, metabolism and discharge of chemical substances, movement into and metabolism at the placenta, and movement into and metabolism in the fetus by chemical substances, all of which play complicated roles in fetal effects (Tanimura, 1986a; Tanimura, 1986b; Iguchi et al., 1987). As for the ease of permeating the placenta, in addition to the role of the blood stream, it has been reported that chemical substances with molecular weights up to 600 can easily permeate the placenta while chemical substances with molecular weights over 1,000 do not permeate it. In addition, the higher the liposolubility and partition ratio are, the higher the permeability is (Iguchi et al., 1987).

The EST has been evaluated in a formal validation study funded by the ECVAM in which two other in vitro embryotoxicity tests (micromass test, postimplantation rat embryo culture test) were validated against a set of test chemicals characterized by high levels of in vivo embryotoxicity data in laboratory animals and humans. In this study, the three in vitro embryotoxicity tests provided an overall accuracy of about 80% in predicting the three classes of embryotoxicity, i.e., strong, weak and nonembryotoxic (Spielmann et al., 2002). Thus embryotoxicity data for monomers needs to be made available through testing in vivo or in vitro. In addition, further accumulation of data is needed in order to apply the in vitro ES cell test. In the future, we need to establish a “nonembryotoxicity” principle before new monomers are used in patients. Moreover, a structure-activity correlation needs to be established with regard to absorption in the living body and the relationship to chemical structure modifications.

The test results do not always agree with in vivo data, which involve complex factors. Clinically, there have been no reports indicating that dental monomers directly affect the fetus. However, judging from the fact that Bis-GMA (6F) and MTA6 have been shown to exhibit some embryotoxicity, appropriate consideration needs to be taken when developing products using these monomers. Use of the EST method may become a new source of information for the effects of biomaterials on teratogenicity in humans, which has previously drawn little attention or notice in development of new compounds.

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References

Braun AG, Emerson DJ, Nichinson BB (1979) Tera


