Preservation of microplate-attached human hepatoma cells and their use in cytotoxicity tests

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Abstract

We investigated the feasibility of hypothermic- or cryogenically-preserved human hepatoma Hep G2 cell precultured in 96-well plates in cytotoxicity testings. First, we observed that microplates precoated with both collagen (CN) and pronectin (PN) showed significantly improved living cell adhesion ($71.0 \pm 5.5\%$) after 48 hr of cryopreservation with 10%-DMSO containing culture medium, whereas non-coated surfaces gave very low living cell adhesion ($33.5 \pm 2.1\%$). Hypothermic preservation was most suitable for short-term storage, and cryogenic preservation at -20°C allowed cells to be used within a week of the storage period. Only cryopreservation in a deep freezer (-85°C) gave satisfactory results in much longer period of storage. Second, we evaluated the cytotoxicity of ten chemicals during 48 hr of exposure using hypothermically (4°C for 2 days) or cryogenically (-85°C for 7 days) preserved cells cultured in CN/PN-precoated microplates in comparison with results from freshly inoculated cells. Although almost the same LD_{50} values were obtained, LD_{10} values of relatively hydrophilic chemicals obtained with cryopreserved cell were significantly lowered. These results shown that CN/PN-precoating is effective in keeping cells attached even in recultivation of preserved cells and that the toxicities of relatively hydrophilic chemicals tend to be overestimated when we use preserved cells in that manner.

Introduction

Recently, *in vitro* cytotoxicity tests using cultured mammalian cells have been noted as a viable alternative to animal testing. Although the procedures of such *in vitro* cytotoxicity tests are much simpler than those involving animals, special equipment and skillful operations are necessary to the growth and maintenance of cells and the determination of chemical cytotoxicity. As one means of reducing the labour involved in such procedures, usage of microplate-attached and hypothermically – or cryogenically – preserved cells have potential. If such microplates can be used with cells to obtain the same results on a cytotoxicity evaluation, cells can be inoculated in a large number of microplates simultaneously, stored at low temperature, and supplied to individual laboratories. This would save the laboratories the time it takes to perform this procedure. Moreover, such a system may minimize

inter-laboratory variations in experimental results.

Recently, some investigators have attempted to preserve cells attached in tissue culture dishes (Smith, 1981; Ohno et al., 1991; Sensebe et al., 1992). According to preliminary results, the extracellular matrix is one of the crucial factors for cell survival upon thawing and recultivation (Meredith et al., 1993). The presence of such extracellular matrix proteins was preferable to stabilize cell organelles and plasma membrane against the stress associated with the freeze-thaw process (Pang et al., 1997). Among such extracellular matrices, gelled or surface-deposited collagen (CN) gave good results when the cells were cryopreserved (Koebe et al., 1990). Pronectin (PN) was also reported as a protein useful to sustaining cell adherence upon thawing (Wendland and Subramani, 1993). Thus, inclusion of such extracellular matrix proteins is promising for use with cell that are preserved and recultivated in microplates in cytotoxicity tests.

Notably, several recent reports have examined the possibility of hypothermic storage for surface-adhering cells (Gordon et al., 1982). Because the change of osmosis pressure in preservation without freezing and thawing is much smaller than that in cryopreservation, cell survival is better when the storage time is within 48 hr (Knight et al., 1973).

Few reports have addressed differences in toxicity evaluation between preserved and non-preserved cells. Pang et al. (1997) evaluated the toxicity of allyl alcohols using cryopreserved hepatocytes. They reported that the sensitivity of stored cells against those chemicals were higher than that of non-preserved cells. However, why preserved cells cannot show the same results on evaluation of chemical toxicity as those obtained from non-preserved cells has not yet been elucidated. Thus, it is important to evaluate and compare toxicity using preserved and non-preserved cells, focusing on the characteristics of the chemicals investigated.

In this paper, we have investigated the feasibility of usage human hepatoma Hep G2 cells cryogenically or hypothermically preserved on microplates in cytotoxicity testing after subsequent to recultivation. High cell stability has been obtained using microplates pre-coated with the combination of collagen and Pronectin F. The limitation of high temperature storage without using a deep freezer has been clarified. In addition, the differences in the response characteristics between the cells with and without preservation in cytotoxicity tests have been elucidated using 10 chemicals that have various hydrophobicity/hydrophilicity balances (Log P).

Materials and methods

Materials for cell culture

Ninety-six multi-well plates precoated with type I collagen (Nitta-gelatin, Tokyo, Japan) were used and the concentration was $30 \mu\text{mL}^{-1}$. Some plates were also postcoated with Pronectin F (Protein Polymer Technologies, Inc. CA, US) by using $10 \mu\text{mL}^{-1}$ of coating solution applied by a method described elsewhere (Varani et al., 1993). Fibronectin (FN; Nitta-gelatin, Tokyo, Japan) was also examined at a concentration of $10 \mu\text{mL}^{-1}$.

For 24 hr before storage, Hep G2 cells (human hepatoma cells from a cell line established by Aden et al., 1979) obtained from the Japanese Cancer Research Bank (JCRB) were inoculated in the plates

at $1.0 \times 10^5 \text{ cells}\cdot\text{cm}^{-2}$ and cultured in Dulbecco's modified Eagle's (DME) medium (Nissui Pharm, Tokyo, Japan) supplemented with 20 mM-HEPES (N-2-hydroxyethylpiperazine-N-2-ethansulfonic acid, Dojindo Lab., Kumamoto, Japan), 10%-fetal bovine serum (FBS), 100 units-penicillin $\cdot\text{mL}^{-1}$, 100 $\mu\text{streptomycin}\cdot\text{mL}^{-1}$.

Cryopreservation and hypothermic preservation

The medium was exchanged to one containing 10 vol% dimethylsulfoxide (DMSO) for cryopreservation. The plates were frozen in an expanded polystyrene box tightly bound by packaging tape at about $-1^\circ\text{C}\cdot\text{min}^{-1}$ to -4 , -20 and -85°C by using normal (Medical Freezer MDF-330, Sanyo Co., Osaka, Japan) and deep (ESPEC BFH-112, Tabai Espec Co., Osaka, Japan) freezers also used in previously published report (Watts and Grant, 1996). After preservation, the cells were thawed in an incubator at 37°C , and the medium containing 10%-DMSO was exchanged DMSO-free culture medium immediately after thawing. Hypothermic preservation was performed at 4°C in a normal refrigerator (SR-26VE, Sano Co., Osaka, Japan) in DMSO-free DME medium with Hank's balanced salt solution. The cells were stored for 38 hr up to 6 mo. Cryogenic vials (Cryoware, Nalgene Co., NY, U.S.) were used for storage of cell suspension.

Measurement of cell survival and attachability

We used the acid phosphatase (AP) assay (Connolly et al., 1986) to measure the number of living cells; because this method gives a rapid and easy count (Martin et al., 1993). After the culture medium was removed, the cells were rinsed with $100 \mu\text{L}$ -PBS/well and soaked in sodium acetate buffer (pH 5.5) containing $37 \mu\text{mL}^{-1}$ -p-nitrophenylphosphate (Sigma, St. Louis, U.S.) and $5 \mu\text{mL}^{-1}$ of Triton X-100 (Wako, Osaka, Japan). After 2 hr incubation at 37°C , the absorbance at 405 nm in each well was measured by a microplate reader (MPR A4i, TOSOH Co., Tokyo, Japan). It was confirmed that the absorbance at 405 nm was proportional to the number of living cells ($r^2 = 0.997$). Although the original AP assay (Connolly et al., 1986) used 1N-NaOH to strengthen the absorbance at 405 nm, this was not necessary in our investigation because Hep G2 cells have a sufficient amount of acid phosphatase due to their hepatic origins. A cell-free well in each microplate was used for the determination of blank absorbance, and the blank value was subtracted from the absorbance of each

cell-containing well. Detached cells were counted by haemocytometer using the trypan blue dye exclusion method. In all figures, each point represents the mean of six wells with two independent runs. The number of living cells compared to the cell number before storage was represented as cell survival. For cytotoxicity tests, the medium, prepared from ultra-high purified water (Milli-Q sp UF, Millipore Co., Bedford, MA), was used as the negative control, and the number of living cells in the medium containing chemicals was standardized to the number of living cells in the control, giving the relative number of live cells for the dose response curves shown in Figure 1. All of the chemicals tested were purchased from Wako (Wako, Osaka, Japan).

Calculation of ED_x values and statistical analysis

The logistic curve expression was employed for describing dose response curves. ED₅₀ and ED₁₀ values were also calculated from the equation formulated by their experimental data. The correlation coefficients (R^2) between experimental data and the formulated curve were also calculated and indicated the well value ($R^2 > 0.933$). The curve fitting and the calculation were carried out by the Simplex method with DeltaGraph (ver. 4.0.5, SPSS, U.S.).

The data were statistically analyzed using one-way analysis of variance (StatView 5.0, SAS Inst. Inc., U.S.) followed by Duncan's test. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

Coating protein and cell survival

To maintain cells at high stability after thawing, three typical cell matrix proteins were examined in terms of the efficiency with which they kept cell attached to microplate wells. Table 1 shows cell attachability and survival as a function of the number of the cell inoculated. The cells were preserved for 48 hr at -85°C in plates precoated with the various proteins. Most cells were detached from non-coated plates, even though the cells were alive. This might have occurred as a result of freezing and thawing shock (Hubel et al., 1991), which can denature adherent matrix proteins between the cell layer and the microplate surface. Recently, it has been shown that the presence of collagen alters the interaction of external ice crystals' formation

with the plasma membranes (Allenspach and Kraemer, 1989; Koebe et al., 1990). This may explain the difference in cell survival between bare and collagen-coated surfaces. Therefore, it can be concluded that cryopreservation of the cells attached and spread on culture surfaces should be performed in plates coated with extracellular matrix proteins.

Among 4 kinds of cell matrix proteins and their combination, the cell survival on the surface precoated with both collagen (CN) and Pronectin F (PN) was the highest. In fibronectin (FN) coated plates, cell attachability was lower than that on the other surfaces. On the other hand, cell attachability on PN coated surfaces was moderate, though cell survival was lower than that on the CN coated surface. This may have resulted from free PN having cytotoxicity as reported previously (Wendland and Subramani, 1993).

Cell suspensions were also cryopreserved for 48 hr in a cryovial and inoculated to bare surface. The survival of such cells immediately after thawing was 98.7%. However, after thawing and 24 hr incubating the number of cells alive and attached was 54.2%. The attachability of such cells on non-coated surfaces was lower than that on cryopreserved cell monolayers. Therefore, cryopreservation of surface-attached and spreading cell monolayers using extracellular matrix proteins would be a simpler and more effective strategy than inoculation of cryopreserved cell suspension to microplates.

To optimize the concentration of the coating solution, a comparison was made of cells cryopreserved at -85°C for 48 hr on three types of plates precoated at various concentrations (Figure 1). In CN coated plates, cell survival increased to 64.2% with an increase of CN concentration up to $10\ \mu\text{mL}^{-1}$. In the plate coated with $30\ \mu\text{mL}^{-1}$ of CN and $10\ \mu\text{mL}^{-1}$ of PN, cells were maintained in the best condition throughout the experiments. In subsequent experiments, all of the plates were coated with CN and PN by applying this coating condition.

Cell survival and recovery after storage

To investigate the feasibility of high temperature storage, the relationship between preservation duration and cell survival at various freezing temperatures was examined as shown in Figure 2. Cryopreservation at -85°C maintained the cells in the best condition in terms of long term preservation over 7 days. Although cryopreservation at -4°C or -20°C did not require a liquid nitrogen or deep mechanical freezer, cell sur-

Table 1. Effect of protein coating on cell attachment and survival (% of the number of cells inoculated)

Plate coating	Cell attached		Cell detached	
	Alive	Dead	Alive	Dead
Non-coated	33.5 ± 2.1	2.9 ± 0.3	45.6 ± 3.9	19.8 ± 1.1
FN	56.3 ± 3.9	7.8 ± 1.1	10.1 ± 0.5	21.1 ± 2.6
CN	64.2 ± 3.5	6.7 ± 1.2	24.1 ± 1.8	3.3 ± 0.5
PN	64.1 ± 4.5	9.9 ± 2.1	9.7 ± 1.3	16.3 ± 0.8
CN/PN	71.9 ± 5.5	10.0 ± 1.4	7.4 ± 0.5	10.7 ± 1.0
Cryo vial* (No-coated)	54.2 ± 1.4	1.3 ± 0.4	1.4 ± 1.1	43.6 ± 3.3

The mean values of six wells ± S.D. from two independent experiments excepting the FN results. $P < 0.05$ based on each of the other indexes. Survival and attachability thawing. *Cell suspension was preserved in cryo vial.

Figure 1. Effects of coating protein concentration on cell survival. All plots in the figures were the mean values of six wells from two independent experiments with error bar (S.D.). $P < 0.05$ vs. control (0 $\mu\text{g/ml}$ of coating solution).

vival upon thawing worsened with prolongation of preservation time, particularly after 7 d. In the preservation of 2 d, hypothermic cell preservation performed at 4 °C gave a better result than that from cryopreservation at all freezing temperatures examined. This result agreed well with that of Schulak et al. (1987) on short time cell storage. Thus, simple cell storage at

Figure 2. Relation between preservation time and cell survival. All plots in the figures were the mean values of six wells from two independent experiments with error bar (S.D.). * $P < 0.05$, ** $P < 0.01$ between cells.

–4 °C or –20 °C may be useful only for relatively short times; i.e., less than one week.

Compared with cell survival of cryopreserved rat primary hepatocytes attached and spread on culture dishes such as a 35-mm dish (56.6%, 9d at –80 °C ;

Figure 3. Cell recovery after thawing. All plots in the figures were the mean values of six wells from two independent experiments with error bar (S.D.). * $P < 0.05$, ** $P < 0.01$ between cells.

Ohno et al., 1991), the cell survival of Hep G2 cells attached to the 96-wellplate showed higher attachability. The Hep G2 cell line may have a strong tolerance to freeze-thaw shock (67.7%, 7d at -85°C).

In order to decide how long preserved cells must be cultured before being used for *in vitro* cytotoxicity tests, cell recovery after thawing was examined. Figure 3 shows cell growth kinetics after thawing compared with that of non-preserved cells. Recovery of hypothermically preserved cells showed the best results for short-term storage (Figure 3a). The recovery of the cells preserved at -80°C gave the best growth recovery for a term of over 30 d of preservation. Thus, based on the results of cell survival immediately after thawing (Figure 2) and subsequent cell recovery (Figure 3), it can be said that hypothermic preservation would give good results for short-term preservation within 49 hr. For long-term preservation, cryopreservation should be performed at -85°C . However, for short-term preservation within 7d, cryopreservation at -20°C gave the highest cell survival and recovery. Ideally, cryopreservation should be performed in a specialized programmable freezing unit to provide controlled temperature changes during freezing (Song et al., 1995). However, this facility may not be readily available to many research groups. The ability to recover cell survival in the simple adherent monolayer culture described herein, using a standard freezer (-20°C), would be a distinct advantage.

Dose-response curves determined by preserved cells

In vitro cytotoxicity tests of 10 kinds of chemicals were performed on cryopreserved cells. Cryopreservation was performed at -85°C for 7 d and hypothermic preservation was performed at $+4^{\circ}\text{C}$ for 2 d. The octanol/water partition coefficient (Log P) values of each chemical was taken from the Experimental Log P Database (Environmental Science Center, Syracuse Res. Co., NY, US).

Figure 4 shows dose response curves of the relative cell number when exposed to these chemicals for 48 hr. In the case of 2-mercaptoimidazoline, Paraquat, and acetaldehyde, significant differences in the relative cell number between non-preserved and cryopreserved cells were found. This may be because cryopreserved cells were damaged by osmotic shock induced by freezing and thawing, especially in the cell membrane. Therefore, Paraquat, which has strong cytotoxicity via peroxidative action (Wright et al., 1997), may permeate easily into the target organelle in the cell body. In the same manner, the toxicities of acetaldehyde showed relatively large differences between non-preserved and preserved cells at a low concentration of this chemical. Pang et al. also reported that the sensitivities of cryopreserved cells against allyl alcohols were higher than that of non-preserved cells (Pang et al., 1997). Based on image analysis of rhodamine 123 fluorescence intensity in cytoplasm, they

Figure 4.

Figure 4. Continued. Dose-response curves of normal and preserved cells when administered with various chemicals. (C; concentration (mM)) All plots in the figures were the mean values of six wells from two independent experiments with error bar (S.D.). *P < 0.05, **P < 0.01 between cells.

conclude that allyl alcohol may cause some loss of mitochondrial membrane potential.

If differences in the toxicity evaluation of the three types of the cells were related to the membrane permeability of the chemical, the log P values should be related. LD₁₀ and LD₅₀ values, which are often used as toxicity indices, indicate the lethal concentrations (mM) reducing 10 and 50% of the number of living cells, respectively. In general, there are some positive correlations have been found between Log P and Log LD₅₀ values found herein and those

presented in a previous study investigating comparable chemicals (Seibert et al., 1989). Figure 5 shows the relation between Log P and Log [LD_x cryo-preserved cell.LD_x normal cell] values derived from their respective dose response curves shown in Figure 4. The vertical axis, Log [LD_x (cryo) preserved cell.LD_x normal cell], indicates the differences of toxicity evaluations represented by the LD_x values (x = 10 or 50) between non-preserved and (cryo) preserved cells. When the index equals to zero, there is no difference between preserved and non-preserved

Figure 5. Relation between Log P and Log (LD_x preserved cell/ LD_x normal cell) for ten chemicals. The latter indicates the sensitivity in toxicity evaluations determined from the LD_x values ($x = 10$ or 50) between non-preserved and (cryo) preserved cells. Namely, when the index equals to zero, there is no difference between preserved and non-preserved cells. Lower values of the index mean the larger difference, higher mean small difference.

cells. For example, when $LD_{10\text{cryopreserved cell}} = 1.02 \times 10^{-5}$ (mM), $LD_{10\text{normal cell}} = 9.56 \times 10^{-4}$ (mM) in Paraquat administered, the index, $\text{Log} [LD_x (\text{cryo}) \text{ preserved cell}/LD_x \text{ normal cell}]$, is -1.49 . In fact, the toxicities at a low concentration of acetaldehyde or Paraquat, both of which have low Log P values (hydrophilic) among those of the chemicals tested, showed relatively large differences between non-preserved and cryogenic- and hypothermic-preserved cells, as can be seen in the LD_{10} values. Hydrophilic chemicals like these can permeate the cell membrane because the shock of freezing may break the membrane integrity.

To test this hypothesis, the uptake of a hydrophilic dye, trypan blue, was examined in normal and cryopreserved cells. The difference of the ratio of stained cells to survival cells (measured by AP assay) between preserved and normal cells is shown in Table 2. Notably, the difference of the uptake between normal and preserved cells became significant only during

Table 2. Effect of trypan blue dye (%-the number of the cell inoculated)

Preservation	Cell survival (AP assay)	Stained cell	Stained/Cell survival
-85°C , 7d	71.2 ± 8.1	20.8 ± 5.5	0.292
4°C , 2d	83.0 ± 6.5	4.2 ± 1.0	0.051
Not preserved	98.7 ± 2.1	1.2 ± 0.1	0.012

The mean values of six wells $\pm$ S.D. from two independent experiments excepting the FN results. $P > 0.05$ based on each of the other indexes. Cell survival and numbers of cryopreserved cells were measured by AP assay and cell counting with a microscope, respectively, at 1 hr of incubation after thawing.

the 5 min after dyeing. It is therefore concluded that the membrane integrity was physically broken by the preservation even in hypothermic preserved cells.

Pang et al also conducted the toxicity tests using cryopreserved rat hepatocytes (Pang et al., 1997). They tested three kinds of hepatotoxicant, though they did not examine the dose response relation. In terms of ATP content and urea synthesis, the cryopreserved rat hepatocytes were sensitive for detecting toxicity, and the results agreed with our results. However, the toxicity must be evaluated exactly and needed to agree with the results obtained from normal cells. Thus, careful interpretation must be applied when using preserved cells for cytotoxicity tests of such chemicals.

The toxicities of pentachlorophenol and 2,4,5-trichlorophenol, both of which have high Log P values (hydrophobic), had small differences between normal and preserved cells. In addition to the Log P values, permeability of the chemicals into a cell body might also be subject to the effect of molecular weight. Nonselective enhancement of membrane permeability of non-ionic solutes is strongly subjected to the molecular weight (Albalak et al., 1996). However, there were no significant differences of LD_x values related to the molecular weight, when cells were exposed to ionic or non-ionic chemicals (data not shown). The difference in toxicity evaluation was not due to this factor. Thus, it is concluded that the difference in LD_x values was almost tenfold between preserved and non-preserved cells.

Conclusion

An efficient method of cryopreservation of human hepatic carcinoma cells in microplates was developed

using collagen and Pronectin F as plate coating proteins. By using this surface pretreatment, over 60% cell survival could be attained even for cryopreservation at -85°C for six mo. Hypothermic preservation can be performed at 4°C for use within 48 hr, while cryogenic preservation can be performed at -85°C for use at over 30 d. Cryopreservation at -20°C performed in a mechanical freezer would be useful for short-term preservation within 7 d.

The toxicities estimated by the preserved cells were ten times those estimated by the non-preserved cells, in terms of the LD_{50} ($x = 10$ to 50) values.

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