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Note

Biocompatible, biodegradable and sterically stabilized phospholipid nanomicelles improve cryopreservation of oral keratinocytes: A preliminary investigation

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Abstract

In this proof-of principle study, we determined whether biocompatible, biodegradable and sterically stabilized phospholipid nanomicelles (SSNMs) improve viability and membrane integrity of cryopreserved oral keratinocytes. Cultured chemically transformed hamster oral keratinocytes were frozen gradually with and stored in liquid nitrogen in the presence of 10% dimethylsulfoxide (DMSO) or SSNMs composed of distearoylphosphatidyl-ethanolamine-*N*-poly(ethylene glycol) 2000 (size, 17 ± 1 nm; 0.1 and 1.0 nmol). Forty-eight hours later, cells were thawed and their viability was determined. Lactate dehydrogenase (LDH) activity in cell lysates and supernatants was quantified as well. SSNMs evoked a significant, concentration-dependant increase in cell viability in comparison to 10% DMSO ($p < 0.05$). There was also a significant decrease in LDH activity in the supernatant of cells cryopreserved with SSNMs in comparison to 10% DMSO ($p < 0.05$). These data indicate that SSNMs improve cryopreservation of oral keratinocyte by promoting cell viability and plasma membrane integrity. We suggest that SSNMs should be further developed as a novel nanocryopreservative for keratinocytes.

Keywords: Nanomedicine; Nanobiotechnology; DSPE-PEG; Dimethylsulfoxide; LDH; Cell viability

Allogeneic keratinocytes are being increasingly used to treat burn wounds, chronic skin ulcers and donor sites (Beele et al., 2005). For this approach to be successful, however, viability and function of implanted cells should be maintained upon thawing from hypothermic storage. Although preservation solutions are commercially available, they were originally developed for organ transplantation and are not designed to meet the metabolic and functional requirements of distinct cells, such as keratinocytes (Pasch et al., 1999; Beele et al., 2005). Hence, there is an ongoing need to develop preservation solutions customized for keratinocytes to improve their preservation efficacy and utilization in clinical practice.

Accordingly, the purpose of this proof-of-principal study was to begin to address this issue by determining whether biocompatible, biodegradable and sterically stabilized phospholipid nanomicelles (SSNMs) composed of US FDA-approved generally regarded as safe materials improve viability and membrane integrity of cryopreserved oral keratinocytes.

SSNMs (size, 17 ± 1 nm as determined by quasi-elastic light scattering) were prepared as previously described by Önyüksel et al. (1999). Briefly, lyophilized poly(ethylene glycol) (mol mass, 2000) covalently linked to distearoyl-phosphatidylethanolamine (1.0 μM; [Avanti Polar Lipids, Alabaster, AL]) was dissolved in 10 mM HEPES buffer, vortexed for 6 min and incubated for 1 h at 25 °C on the day of the experiment.

7,12-Dimethylbenz[a]anthracene-transformed golden Syrian hamster oral epidermoid carcinoma cells were used as previously described by Rubinstein et al. (2001). Cells were seeded in microtiter flat-bottom 96-well cell culture cluster (Costar,

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Cambridge, MA) at a density of 30,000 cells/well in 100 μl DMEM culture medium supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY) and antibiotics (50 U penicillin/ml, 50 μg streptomycin/ml and 2 μg amphotericin B/ml; GIBCO). Cells were maintained in 95% air–5% CO_2 atmosphere at 37 °C. Medium was exchanged the next day and every other day thereafter. Cell viability was always >95% as determined by morphological examination using phase-contrast microscopy (Nikon, Tokyo, Japan), and by 0.1% Trypan blue dye exclusion test using a Hy-Lite counting chamber (Fisher Scientific, ITASCA, IL).

After reaching confluency (5–6 days), cells were trypsinized (0.01% [w/w]), centrifuged (200 g, 10 min) and suspended in DMEM culture medium containing either 10% dimethylsulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO), the standard cryopreservant (Pasch et al., 1999), or 0.1 and 1.0 nmol SSNMs, aliquoted (1.0 ml) into Costar Bio-Freeze vials, frozen gradually (1 °C/min) at −196 °C and stored in liquid nitrogen for 48 h. Thereafter, vials were transferred from hypothermic storage into a 37 °C water bath. Once ice crystals have disappeared, cells were centrifuged, resuspended in the culture medium and their viability determined by 0.1% Trypan blue dye exclusion test. Each experiment was conducted in triplicate.

Cell membrane integrity was assessed by determining lactate dehydrogenase (LDH) activity, a stable cytosolic enzyme released upon an increase in plasma membrane permeability, in cell lysates and supernatants using an ELISA kit (Sigma–Aldrich) and a thermoregulated ELISA microplate reader (SpectraMAX, Molecular Devices, Palo Alto, CA) according to the manufacturer's instructions. Data are expressed as Berger-Broida units/ml and normalized for protein content (μg) using the BCA assay (Pierce, Rockford, IL). Each experiment was conducted in duplicate.

Data are expressed as means ± S.D. Statistical analysis was performed using ANOVA and Tukey's post hoc test. $p < 0.05$ was considered statistically significant.

SSNMs evoked a significant, dose-dependent increase in keratinocyte viability after thawing in comparison to 10% DMSO (Fig. 1; each group, $n = 4$ experiments; $p < 0.05$). Cell viability was 96 ± 1% with 1.0 nmol SSNMs in the preservation solution compared to 74 ± 2% with 10% DMSO (Fig. 1; $p < 0.05$). SSNMs had no significant effects on the standard curve of LDH activity (data not shown; each group, $n = 3$ experiments; $p > 0.5$). After thawing, LDH activity in oral keratinocyte lysates was significantly higher in cells cryopreserved with 1.0 nmol SSNMs (73 ± 9% of baseline) compared to cells cryopreserved with 10% DMSO (15 ± 1% of baseline) (each group, $n = 4$ experiments; $p < 0.05$).

The new finding of this proof-of-principal study is that cryopreservation of oral keratinocytes with biocompatible, biodegradable and sterically stabilized phospholipid nanomicelles significantly improves cell viability and membrane integrity upon thawing. Although phospholipids and liposomes, small-sized in particular, have been shown to cryoprotect spermatozoa and erythrocytes (De Leeuw et al., 1993; Yamaguchi et al., 1993; Wilhelm et al., 1996; Zeron et al., 2002; Kheirolomoom et al., 2005; Ricker et al., 2006), cryopreservation of oral keratinocytes with phospholipid nanomicelles has not been reported. To this end, we used biocompatible and biodegradable SSNMs rather than small liposomes because they are easier to manufacture and can be stored for an extended period of time (Önyüksel et al., 1999; Rubinstein et al., 2001). This, in turn, enables production of large standardized batches that are ready for use (Önyüksel et al., 1999).

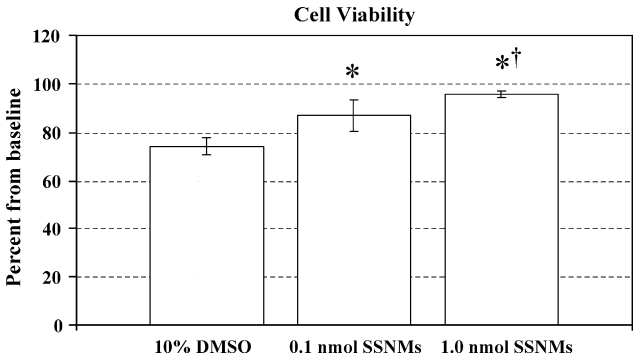

Fig. 1. Viability of oral keratinocytes after cryopreservation in culture medium containing 10% DMSO or 0.1 and 1.0 nmol sterically stabilized phospholipid nanomicelles (SSNMs). Data are means ± S.D. (each group, $n = 4$ experiments; $^{*}p < 0.05$ in comparison to 10% DMSO; $^{\dagger}p < 0.05$ in comparison to 0.1 nmol SSNMs).

The mechanism(s) underlying the protective effects of SSNMs in oral keratinocytes during the freeze-thaw process was not elucidated in this study. Conceivably, SSNMs could self-associate with the cell surface thereby providing a physical barrier to freeze-thaw damage and prevent plasma membrane phase separation by influencing its phospholipid packing without changing phospholipid content (De Leeuw et al., 1993; Zeron et al., 2002; Kheirolomoom et al., 2005; Ricker et al., 2006). To this end, small unilamellar liposomes have be shown to repair transient plasma membrane disruptions formed during hypertonic treatment of human erythrocytes (Yamaguchi et al., 1993). In addition, we found that phospholipid mixed nanomicelles loaded with hydrophobic quantum dots strongly associate with the plasma membrane of cultured human breast cancer cells (Rubinstein et al., 2006).

Alternatively, SSNMs might modify phospholipid composition of oral keratinocyte plasma membrane by replacing lost membrane phospholipids leading to membrane stabilization during the freeze-thaw process (De Leeuw et al., 1993). However, this possibility seems unlikely because Ricker et al. (2006) showed recently that phospholipids added to the cryopreservation solution fail to integrate into the plasma membrane of equine spermatozoa. Clearly, additional studies are warranted to unravel the mechanisms underlying the cryoprotective effects of SSNMs in oral keratinocytes (Ricker et al., 2006; Rubinstein et al., 2006).

In summary, we suggest that biocompatible, biodegradable and sterically stabilized phospholipid nanomicelles should be further developed as a novel nanocryopreservative for keratinocytes.

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