Proliferation and differentiation potential of cryopreserved human skin-derived precursors

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Abstract

Objectives: Skin-derived precursors are recognized to be a potentially autologous and accessible source of neural precursor cells for drug screening or cell-based treatments, in many neurological disorders. Thus, it is necessary to investigate appropriate methods for cryopreservation of such human skin-derived precursors (hSKPs). The aim of this study was to evaluate different cryopreservation techniques for retention of hSKPs to discover an optimized protocol.

Materials and methods: We cryopreserved hSKPs treated with 0%, 10%, 20%, 30% and 40% foetal bovine serum (FBS) and three concentrations of dimethylsulphoxide (DMSO) 5%, 10% and 15%, with two different storage periods in liquid nitrogen (2 days: short-term storage; and 2 months: long-term storage). Then, we assessed survival and proliferation levels of the cells after freeze–thaw processes, by viability measurement and colony-forming assay. For detecting hSKPs, we used immunocytochemistry and RT-PCR assessments.

Results: Our findings indicated that hSKPs cryopreserved in 5% DMSO without FBS, had better survival and proliferation potentials compared to other working formulations. With various concentrations of cryoprotectants over different time periods, hSKPs retained their differentiation potentiality and were able to differentiate into neurons (NFM and β III tubulin-positive), glial cells (GFAP-positive) and smooth muscle cells (SMA-positive).

Conclusions: Results revealed that in only 5% DMSO, hSKPs could be cryopreserved for long-term storage with considerable survival and proliferation levels, without losing multipotency.

Introduction

One of the most intensively investigated subjects in medical sciences is the stem-cell biology. This mostly is due to increasing awareness of its potential uses in the therapeutic setting (1). For example, substitution of lost or damaged neurons by neural stem cells or neural progenitor cells (NPCs) could be of great promise for clinical treatment of many neurodegenerative diseases (2). However, therapeutic potential of neural stem cells is considerable, a number of important problems still exist (3).

Human foetal tissue and embryonic stem cells are two current tissue sources of human neural stem cells. However, derivation and use of these are limited due to ethical debates and heterologous transplantation (4,5). Use of adult stem cells such as skin-derived precursors (SKPs) would bypass these problems (6,7). SKPs are multipotent stem cells initially described by Toma *et al.* (4). They display multi-lineage differentiation potential, producing both neural and mesodermal progeny *in vitro*. Different research studies have reported that SKPs mainly differentiate into glial cells, neurons, smooth muscle cells and adipocytes, although extent of their multipotency has yet to be determined (8–12).

SKPs form a novel precursor cell type distinct from other known precursors derived from the skin, such as epidermal stem cells and melanocytic stem cells (9). SKPs have additional benefits over many other adult stem cells by being easily expandable in culture. Thus, large numbers of cells can be obtained from only small pieces of skin, an easily accessed tissue source compared to brain, bone marrow or muscle (13,14). On the other hand, SKPs have potential therapeutic applications for autologous

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cell-replacement strategies with regard to their differentiation ability into further different functional cell types (15,16). Aging, however, decreases human skin-derived precursors (hSKPs) profusion and differentiation potential. Their isolation, expansion and differentiation are extremely difficult when obtained from the elderly. Thus, preserving hSKPs in cell banks early in life could be beneficial for use in clinical protocols in the aging population (17,18).

Cryopreservation is an important method for cell storage for their future longevity (19–21). Thus, there are prerequisites for developing appropriate cryopreservation techniques. Preservation and successful long-term storage of NPCs are important and mandatory for their potential therapeutic applications in regenerative approaches and autologous cell-replacement therapy (22,23).

We realize that hSKPs may be an accessible source for autologous NPC transplantation and will ultimately be used clinically in future (9). On the one hand, prospective use of various therapeutic applications of hSKPs has led to an increased demand for improving cryobiological techniques for storing these cells. Yet until now, reliable studies have not been reported on roles of different concentrations of current cryoprotectant agents for hSKP freezing. Although dimethylsulphoxide (DMSO) is the most commonly used cryoprotectant for NPCs, it is known to be toxic and has differentiation-promoting effects in addition to side-effects, after prolonged exposure (24,25). Moreover, many contradictory results have been reported about the use of serum additives for NPCs cryopreservation (26,27). Thus, it is necessary to investigate an appropriate cryopreservation method for hSKPs. In this study, we have isolated and cultured hSKPs and differentiated them to neural and mesodermal lineages. We used several concentrations of DMSO and foetal bovine serum (FBS) additives to choose best cryoprotectant mixture for hSKP cryopreservation, with the aim of establishing cell banks in the future. This enables investigators to produce large numbers of NPCs from small pieces of human skin (foreskin, for example, early in life) and freeze these for later use. Moreover, ability to cryopreserve hSKPs greatly simplifies use of this cell-culture system.

Materials and methods

hSKP isolation and culture

Small pieces (2–3 cm²) of human foreskin (20 samples) were obtained from voluntary circumcisions of children 4 weeks to 5 years of age. The parents received full information concerning the project and signed letters of consent in advance. The study was conducted in compliance

with our local ethical guidelines and was approved by the Ethics Committee of Kermanshah University of Medical Sciences, Iran. Skin samples were washed in Hanks' balanced salt solution (HBSS; Sigma, St. Louis, MO, USA) and cut into smaller rectangular pieces (2 × 3 mm). Then they were incubated in thermolysin solution (250 µg/ml in HBSS; Sigma) overnight at 4 °C. Epidermis was carefully removed from each tissue piece using fine forceps, and dermis was cut into smaller pieces. For tissue dissociation, samples were incubated in collagenase H (1 mg/ml; Roche, Mannheim, Germany) for 45 min at 37 °C, with agitation. Mixtures were treated with DNAase (0.1% w/v; Sigma) for 5 min, then filtered and centrifuged at 700 *g* for 5 min. Pellets were resuspended in proliferation medium composed of DMEM-F12 (Gibco, Breda, the Netherlands), 3:1, FGF2 40 ng/ml (Axxora, Lörrach, Germany), EGF 20 ng/ml (Axxora), B-27 supplement minus vitamin A 2% (Invitrogen, Carlsbad, CA, USA), LIF 10 ng/ml (Sigma), penicillin G 100 U/ml (Gibco), gentamycin 25 µg/ml (Gibco, Netherlands) and fungizone 1 µg/ml (Sigma). Dissociated cells were counted and seeded at high density (0.8–1.2 × 10⁶ cells/cm²) in 25 cm² culture flasks and cultured in 5% CO₂ at 37 °C. Proliferation medium was added three times a week. Every 7 days of culture, cells floating freely in spheres, were passaged. Medium containing hSKPs growing in suspension was centrifuged at 700 *g* for 5 min and two-thirds supernatant removed. Pellets were resuspended in the remaining medium using fire-polished Pasteur pipettes. Spheres were dissociated by repeat pipetting. Proliferation medium was added, and cell suspensions were split into two 25 cm² flasks (8).

Neural differentiation

To induce neuronal differentiation, spheres were mechanically dissociated by pipetting using a fire-polished Pasteur pipette and resuspended in differentiation medium: DMEM/F12 in 3:1 ratio containing FBS 5%, B-27 serum-free supplement 2% (Invitrogen), NGF 10 ng/ml (Axxora), penicillin G 100 U/ml, gentamycin 25 µg/ml and fungizone 1 µg/ml. Cells were plated at 5 × 10⁵ cells/cm² on to poly-D-lysine (Sigma)-coated culture dishes. Half the medium content was replaced by fresh differentiation medium three times a week (8).

Cryopreservation and thawing

For cryopreservation of hSKPs, cells were prepared as described above. After three passages of spheres, samples were resuspended in specific cryoprotectant. Freezing jars were filled with isopropanol and all vials were transferred into these. Jars were left at –70 °C for 24 h to allow slow

and reproducible reduction in temperature ($-1\text{ }^{\circ}\text{C}/\text{min}$). Samples were then transferred to liquid nitrogen ($-196\text{ }^{\circ}\text{C}$). Here, five different concentrations of FBS (0%, 10%, 20%, 30% and 40%) and 3 different concentrations of DMSO (5%, 10% and 15%; Sigma) were used. After freezing period of between 2 days to 2 months, cells were removed from liquid nitrogen, thawed rapidly in $37\text{ }^{\circ}\text{C}$ water bath with continuous agitation and diluted with culture medium to 10 volumes. Freezing medium was then replaced with serum-free expansion medium and spheres were maintained as described above.

Measurement of cell viability

To measure cell viability, trypan blue (Gibco) exclusion assay was used (26). Fresh or freeze-thawed hSKPs were incubated in triplicate in a 0.25% (w/v) stain solution. Total cell number and number of trypan blue positive cells were determined by counting at least 200 cells per sample, using a haemocytometer. Cell viability (percentage) was the ratio of trypan blue-impermeable cells to total cell count.

Clonogenic survival assay

Human SKP survival after exposure to the freezing process with different concentrations of cryoprotectant, was measured using the colony-forming assay as described by Davis and Temple (26). Clonogenicity was determined by measuring colony formation as described by Lu and Wong (27). After thawing and washing hSKPs, cells were adjusted to final density of 5×10^4 cells/ml in culture medium. They were then seeded in triplicate into 96-well plates as single-cell suspensions in defined proliferation medium. Survival of proliferating cells was determined by their ability to form neurosphere colonies. Numbers of neurospheres were analysed quantitatively on days 4, 7, 10 and 14. Relative survival value was calculated by assessing ratio of colony-forming units (CFUs) of secondary spheres.

At first, hSKPs were treated with different cryopreservation techniques and thawed as mentioned above. Thawed cells were then exposed to fresh culture medium for 2 weeks at $37\text{ }^{\circ}\text{C}$ in humidified 5% CO_2 . Secondary spheres were scored by using an inverted microscope and applying standard criteria for their identification (22).

Immunocytochemistry

In this experiment, we examined expression of nestin, vimentin and fibronectin in spheres of cells, as main criteria for identification of hSKPs (9). Expression of differentiation markers was examined in separate experiments.

After each treatment, cells were fixed in 4% paraformaldehyde (Sigma) for 15 min at room temperature and washed three times in phosphate-buffered saline (PBS). Cells were permeabilized by 1% Triton X-100 (Sigma) for 15 min and washed three times in PBS. Non-specific binding was blocked by 1-h treatment in 6% normal goat serum (Gibco) and 0.1% (w/v) BSA (Gibco). Cells were incubated in primary antibodies at $4\text{ }^{\circ}\text{C}$ overnight. Sources of primary antibodies were as follows: mouse anti-nestin (R&D, Minneapolis, USA), mouse anti-vimentin (R&D), mouse anti-fibronectin (R&D), mouse anti-neurofilament M (anti-NFM; Axxora), mouse anti-glial fibrillary acid protein (anti-GFAP; R&D), mouse anti- β III tubulin (Axxora) and mouse anti-smooth muscle actin (R&D). Cells were washed three times in PBS, and incubated in secondary antibody (goat anti-mouse IgG conjugated to FITC) containing 3% normal goat serum, for 1 h at room temperature. Cells were washed three times in PBS, stained with Hoechst 33258 (Sigma), and visualized against controls (PBS in the place of primary antibodies) using an optical immunofluorescence microscope (TS100; Nikon, Tokyo, Japan) with appropriate fluorescence filters, and images were captured using a Nikon camera (ELWD 0.3/OD75; Nikon).

Reverse transcription–polymerase chain reaction

RNA was extracted from samples using RNXPlus (Sinagen, Tehran, Iran), and cDNA was generated using reverse transcription kit (RT; Qiagen, Hilden, Germany) as directed by the manufacturer. For all cDNA synthesis, minus RT control was performed. Polymerase chain reaction (PCR) was carried out as follows: activation of enzyme $92\text{ }^{\circ}\text{C}$ for 2 min, 30–35 cycles of denaturation at $94\text{ }^{\circ}\text{C}$ for 60 s, gene-specific annealing temperature for 60 s, and extension at $72\text{ }^{\circ}\text{C}$ for 60 s. PCR primers used were as follows: for p75NTR, tgggccaggaaggtgcgatgaa and aaaggggccccagaaacacaca; for pax3, catccggcctgcgtcatctc and tggccttctctcgttctctctg; for snail, tggcctgtctgcgtgggttttgt and cctgggctcggggcatctca; for slug, catcttggggcgagtgagtgcc and cccccgtgtgagttctaagtgtc (6).

Statistical analysis

All analyses were performed using statistical SPSS software 16.0 (Illinois, Chicago, USA) and data are presented as mean $\pm$ SD. Tukey's test was used to determine statistical differences between groups. Data were evaluated by one-way analysis of variance (ANOVA) to assess effects of average percentage differences between experimental groups. Results were considered significant when P -values were <0.01 . All experiments were performed in triplicate unless otherwise noted.

Results

Isolation, culture, expansion and characterization of neuronal precursors from human foreskin

At 1-day post extraction, a heterogeneous population consisting of blood cells, dermal fibroblasts and skin-derived precursors as single cells, was observed (Fig. 1a). By 3 days culture, cells had begun to form small floating spheres, although many dead single cells were present, that had resulted from enzymatic treatment (Fig. 1b). After 7 days, spheres became larger and some spheres seemed to merge together (Fig. 1c). One day after first passage, new spheres formed although some single cells remained. By the seventh day, spheres had grown in size to larger volumes. Such spheres could be cultured,

expanded and passed in proliferation medium for more than 10 passages (at least 4 months). At later passages after 5th passage (Fig. 1d), spheres became very large and their dissociation into single cells became difficult. After the third passage, spheres were dissociated and plated on to poly-D-lysine coated flasks in differentiation medium. Single cells were adherent to culture dishes and developed morphological changes over the following days. Seven days of culture under neural differentiation conditions, cells with elongated cytoplasmic processes were observed (Fig. 1e). By day 14, these cytoplasmic elongations became longer and formed detectable networks (Fig. 1f).

Immunocytochemical analyses revealed that hSKPs expressed nestin (a marker of neural stem cells), vimentin and fibronectin (Fig. 2a). Similar results were obtained from all samples whether cells analysed were single cells

Figure 1. Culture, expansion and differentiation of human skin-derived precursors. Phase contrast micrographs of different culture stages of spheres in proliferating medium, and after differentiation. First day after extraction, a population of mixed single cells was observed (a). Three days after extraction, first small spheres were observed (b). Seven days after extraction, larger spheres were detected (c). After fifth passage, spheres became larger (d). Dissociated cells from spheres (third passage) were plated on to poly-D-lysine coated flasks in differentiation medium. Seven days of culture in neural differentiation conditions (e). 14 days after culture, in differentiation medium (f).

Figure 2. Characterization of human cryopreserved-thawed skin neuronal precursors cultured as floating spheres. Fluorescence photomicrograph of spheres before passaging, immunostained for vimentin, nestin and fibronectin (a). Reverse transcription–polymerase chain reaction analysis for expression of mRNAs for Slug, Snail, p75NTR, Pax3, and as control, β -actin, in two human SKP preparations that had been passaged three and four times (b).

or from spheres. We also used RT-PCR to analyse a subset of transcription factors, Slug, Snail, p75NTR and Pax3. Results indicated that hSKPs express these genes for these proteins (Fig. 2b).

Culture and differentiation of cryopreserved hSKPs

During the first week post-thawing, SKPs formed spheres similar to those isolated from fresh tissue (Fig. 3a). To perform immunocytochemical analysis after neuronal differentiation, dissociated cells from cryopreserved–thawed spheres were plated on to poly-D-lysine coated 96-well plates and cultured in differentiation medium (Fig. 3b). At different times of maturation, cells were fixed and processed for immunocytochemical analysis. Our assess-

ments revealed that, β III tubulin and NFM (both are neuronal markers) were expressed by a number of differentiated cells (Fig. 4a).

SKPs generated glial cells and potential smooth muscle cells additionally. A subpopulation of bipolar cells expressed S100, a marker of myelinating glia, including oligodendrocytes and Schwann cells, and GFAP, expressed in Schwann cells and astrocytes (Fig. 4b). Moreover, we observed a subpopulation of cells with morphology of smooth muscle cells or myofibroblasts, SMA-positive (Fig. 4c).

These analyses revealed that cryopreserved hSKPs in all our experimental groups were able to generate morphologically complex, β III tubulin and NFM-positive neurons (Fig. 4a), as well as bipolar glial cells expressing

Figure 3. Phase contrast photomicrograph of cryopreserved-thawed hSKPs immediately before passaging (a). 14 days after differentiation (b). Note characteristic morphology, which is similar to results of fresh tissue.

Figure 4. Immunofluorescence staining of differentiated neurons. NFM and β III-tubulin positive cells (a), differentiated glial cells; S100 and GFAP positive cells (b) and immunoperoxidase staining of a subset of differentiated cells expressing smooth muscle cell marker SMA positive cells (c) from the cryopreserved SKPs.

S100 and GFAP (Fig. 4b), and SMA-positive smooth muscle cells or myofibroblasts (Fig. 4c). Thus, hSKPs retained their ability to differentiate appropriately, even when cryopreserved over long or short periods of time, or maintained for substantial periods in culture. Our analysis indicated that $17.7 \pm 0.6\%$ of the cells in fresh tissue and $17.2 \pm 0.9\%$ after freeze–thaw differentiated into neurons (β III tubulin and NFM-positive cells). Furthermore, differentiated glial cells (S100 and GFAP positive) and smooth muscle cells (SMA-positive cells) in fresh tissue were $23 \pm 0.3\%$ and $4.3 \pm 0.5\%$, respectively compared to $22 \pm 0.9\%$ and $4.4 \pm 0.1\%$ after cryopreservation. Collectively, these findings revealed that percentages of differentiated neurons, glial cells and smooth muscle cells in the experimental groups after freeze–

thaw, with fresh untreated one, had no statistically significant differences.

Viability of cryopreserved–thawed hSKPs

Immediately after thawing and washing out cryoprotectant agents, trypan blue exclusion assay was performed to measure cell viability. Results showed that dead cells were below 23% in all cases (Fig. 5a). Samples frozen for 2 days or 2 months showed no differences in viability (Fig. 5a,b). Viability assessment in study groups indicated that viability in the groups of cells treated with different concentrations of DMSO only, were better than the others and highest viability was seen after 5% DMSO only (without FBS).

Figure 5. Analysis of cell viability by trypan blue exclusion test of cryopreserved-thawed hSKPs. Percentage of dead cells $\pm$ standard error of the mean, seventh day after thawing, presented in the histogram (frozen for 2 days) (a). Percentage of dead cells $\pm$ standard error of the mean seventh day after thawing, presented in the histogram (frozen for 2 months) (b) (** $P < 0.01$; * $P < 0.05$).

Effects of cryopreservation on colony formation

Recovery of cryopreserved-thawed hSKPs was determined by colony formation assay. Viable single cells were grown and colonies were generated in separate wells. Values for total CFUs generated within 4 weeks were calculated as averages from three independent experiments. This assessment was performed for all freeze-thawed samples separately against fresh controls. Results are expressed as percentages (Fig. 6). Recovery in the 16 study groups showed that colony forming in groups treated with different concentrations of DMSO only, was better than other forms of treatment and best colonogenicity was seen at 5% DMSO without FBS.

Discussion

In the present study, we addressed cryopreservation of human skin-derived cell precursors after deep freezing

(-196°C) using different concentrations of DMSO and FBS to discover best protocols for low cell damage. Skin-derived precursors are an accessible (and can be autologous) source of NPCs that their clinical applications are potentially profound (4,6–12). Results demonstrated that rodent SKPs were more abundant in neonatal than adult skin, and numbers of hSKPs are age-dependent (7,17). So far, we used human foreskin tissue with age of donors ranging from 4 weeks to 5 years, to isolate and characterize hSKPs. In a preliminary study on hSKPs, we found that cryopreservation with low concentrations of DMSO and FBS lead to higher survival and proliferation levels. Accordingly, we used a stepwise method of solutions with increasing DMSO and FBS concentrations to achieve appropriate equilibration.

Previously, it has been reported that human SKPs become depleted in an age-dependent manner. These data support the need to cryopreserve such cells from young donors and importance of establishing master cell banks

Figure 6. Analysis of proliferation level of cryopreserved-thawed hSKPs explored by colony formation assay. Percentage of clonogenic survival $\pm$ standard error of the mean in frozen-thawed spheres is presented in the histogram (** $P < 0.01$; * $P < 0.05$).

from young subjects, for putative future use in cell therapy protocols when degenerative diseases arise. Cryopreservation is a process where cells or whole tissues are preserved by cooling to low temperatures, such as -196°C , at which temperatures biological activity (including biochemical reactions that would lead to cell death) are effectively prevented (28–32). However, cells being preserved are often damaged due to freezing and/or thawing. Factors causing damage to cells include solution effects, intracellular and extracellular ice formation and dehydration (30–32). There are several reports concerning various freezing methods for cryopreservation of NPCs derived from different sources (19–23,33). However, there is no report regarding SKPs NPC cryopreservation methods. A previous studies by Xue *et al.* (22) have reported cryopreservation of olfactory neuroepithelium NPCs treated with six different cryoprotectants: DMSO, ethylene glycol (EG) and glycerol, each with or without 10% FBS. In their study, no significant differences were observed between these different cryoprotectants.

DMSO is a conventional cryoprotectant that has been used for decades by cryobiologists, to reduce ice formation in tissues that are cold-preserved in liquid nitrogen. On the other hand, it is known to be toxic (24,34,35) and it has been reported that small molecules of DMSO are known to have neuronal differentiation-promoting effects (36). In this study, we used DMSO as cryoprotective agent with regard to its general use in NPC cryopreservation and its advantages. It is known that mixtures of cryoprotectants have less toxicity and are more effective than single-agent cryoprotectants. Therefore, we used FBS due to its general use in NPC cryopreservation, availability and low cost. As serum contains many unknown growth factors or cytokines that can induce differentiation of stem

cells, we used low concentrations of FBS and investigated its deletion (34,37,38). Thus, we investigated whether serum-free cryoprotectant would be effective for functional cryopreservation of sphere cultures of hSKPs. Our results indicated that SKPs could be cryopreserved for long periods without reduction in their differentiation potential. Collectively, we conclude that elimination of FBS, besides decrease in DMSO concentration, showed progressive improvement on cell viability and proliferation level. Moreover, the results revealed that cryopreservation with 5% DMSO only, retained as much as 89% cell viability, and had highest proliferation levels (~56%). Similarly, with cryopreservation yields of 50–60% for human stem cells, it is being widely used clinically (39,40). Our study has demonstrated that deep-freezing at -196°C may allow banking of hSKPs, with minimum of 50% recovered with preserved functionality. Actually, these results could be explained by the toxic effect of DMSO and differentiation effect of FBS. It seems that DMSO decreases cell viability in high concentrations and probably reduces proliferation rate. It is probably regard to their effect on inducing differentiation of stem cells (34,37,38).

Conclusion

Our results demonstrated that cryopreservation of human SKPs in freezing medium containing 5% DMSO without FBS was the best formulation for short- or long-term storage of the cells in comparison with other tested cryopreservation formulations. Cryopreserved SKPs in medium containing 5% DMSO had higher cell viability and proliferation level and maintained differentiation potential in neural and mesodermal cell lineages considerably.

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