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Cryopreservation of human endothelial cells for vascular tissue engineering ☆

Karla Lehle [a,*], Markus Hoenicka [a], Volker R. Jacobs [b], Franz X. Schmid [a], Dietrich E. Birnbaum [a]

[a] *Clinic of Cardiothoracic Surgery, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93042 Regensburg, Germany*
[b] *Frauenklinik, Technical University Munich, Ismaninger Strasse 22, 81675 Munich, Germany*

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Abstract

To investigate the influence of cryopreservation on endothelial cell growth, morphology, and function human umbilical vein endothelial cells (HUVECs) were frozen following a standard protocol. Cell suspensions were exposed to 10% dimethyl sulfoxide in a high-potassium solution, cooled to −80 °C at 1 °C/min and stored in liquid nitrogen for 7–36 days. Samples were thawed in a 37 °C water bath and the cryoprotectant was removed by serial dilution. The growth of cell suspensions was assayed by culturing 7300 cells/cm^2 for 3–5 days in order to determine the cell multiplication factor. Fresh and cryopreserved/thawed cells were analyzed for their growth, and their anti-inflammatory and anti-coagulant function by using cellular ELISA. Cryopreservation resulted in a retrieval of 66 ± 5% and a viability of 79 ± 3%. Cryopreserved/thawed and fresh cells showed identical doubling times and identical cell counts in the confluent monolayers. However, the lag phase of thawed HUVECs was approximately 36 h longer, resulting in significant differences in the cell multiplication factor at 3 and 5 days after seeding. After expansion to a sufficient cell count the lag phases were identical. Fresh and cryopreserved/thawed cells showed comparable anti-inflammatory and anti-coagulant activity, as judged by the basal and TNF-induced VCAM-1, ICAM-1, E-selectin, and thrombomodulin expression. Cryopreserved/thawed and recultivated endothelial cells are suitable for endothelialization of autologous allograft veins. Such tissue-engineered grafts will offer the necessary clinical safety for those patients who lack autologous material.

Keywords: Cryopreservation; Dimethyl sulfoxide; Endothelial cells; Growth; Function; Tissue engineering

The implantation of arteries or veins is particularly useful in reconstructive surgery (e.g. aortocoronary bypass grafting, peripheral vascular surgery). Limited availability of autologous bypass material and an increasing frequency of

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* Corresponding author. Fax: +49 941 944 9902.
E-mail address: Karla.Lehle@klinik.uni-regensburg.de (K. Lehle).

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reoperations often necessitate the use of less satisfactory autologous graft material. The search for an ideal small-caliber graft has been compared to the search for the holy grail [5]. Synthetic materials such as polytetrafluorethylene (PTFE) have been used successfully in peripheral vascular reconstruction but failed nonetheless when used for aortocoronary revascularization [5]. Reendothelialization of prosthetic surfaces showed only limited success due to technical complexity (stabilized EC adhesions to the substrate under flow conditions, phenotype, and function of implanted cells) [2,19]. Lamm et al. [12] modified the method of Zilla et al. [19] for autologous reendothelialization of deendothelialized cryopreserved allograft veins (CAV) and used 15 grafts for clinical implantation in otherwise inoperable patients. Endothelialized CAVs showed normal connective tissue wall structure and a tight endothelial monolayer. Long-term implantation of heterografts and homografts was accompanied by mechanical fatigue due to lack of stability of collagen [8] followed by immunologic mechanisms [7]. Human umbilical cord cells will be a new cell source for cardiovascular [10] or liver [17] tissue engineering. These juvenile cells represent an attractive and readily available autologous cell source avoiding the invasive harvesting of intact vascular structures [11].

Preservation of the homograft and the endothelial cells of the same individual for prospective use in old age could prevent thrombosis, degeneration, and graft rejection. Human umbilical cord is a readily available tissue material and the use is ethically unproblematic. Cryopreservation of vessels resulted in freezing injury where the endothelial cells were lost [1]. Endothelial cells isolated from cryopreserved umbilical cords were reported to be incapable of forming a monolayer and to show a degenerate morphology as well as a decreased prostacyclin formation [4]. The instability of endothelial cells depended on the influence of cryoprotectants during freezing and thawing [16]. Based on these findings, Pegg [16] developed a cryopreservation protocol for an immortalized endothelial cell line, ECV304. The osmotic changes of cell volume during the introduction of 10% w/w dimethyl sulfoxide at 2–4 °C and during removal at 22 °C were designed not to exceed 30%. In the present study, we used this improved cryopreservation protocol to compare human primary endothelial cells isolated from umbilical veins in terms of growth and function before and after cryopreservation.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were prepared from umbilical cord veins by collagenase digestion [9]. HUVECs were cultivated in Medium 199 (Cambrex, Verviers, Belgium) supplemented with 12% fetal calf serum (FCS), 0.4% heparin, 0.1 ng/ml epidermal growth factor, 1 ng/ml hydrocortisone, 0.05 mg/ml gentamycin sulfate, 0.05 μg/ml amphotericin B (Promocell, Heidelberg, Germany) (growth medium), and seeded into gelatine-coated (0.1%; Merck, Darmstadt, Germany) T25 culture flasks. After 6.9 ± 1.7 days in culture all cells were transferred in T175 culture flasks for 4–7 days. Half of the cells was used for cryopreservation (see below), the remaining cells were expanded and cultivated as fresh control cells. The total number of cells was determined by using an automated cell counter (CASY1, Schaerfe Systems, Reutlingen, Germany) or an improved Neubauer hemocytometer. The percentage of viable cells was calculated by trypan blue exclusion. HUVECs were identified as endothelial origin by their cobblestone appearance under phase contrast microscopy and by the expression of CD31 as shown by FACS analysis. Informed consent was obtained from each patient, and the protocol for isolation of endothelial cells was approved by the Institutional Review Boards of the Technical University of Munich and of the University Hospital of Regensburg.

Cryopreservation/thawing

The cells were harvested by exposure to 0.05% trypsin and 0.02% EDTA (Gibco-BRL, Life Technologies), followed by neutralization with growth medium and equilibration with high-potassium solution (CPTes: 30 mM sodium bicarbonate,