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Research Article

An *In Vitro* Study of Long-Term Cryopreserved Umbilical Cord Expression of Vascular Endothelial Growth Factor (VEGF)

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Abstract

VEGF-A-expressing cells were observed throughout the entire cross-section of the UC. The results of this study, in which VEGF-A was expressed, suggest that UCs may be able to contribute to angiogenesis even after long-term cryopreservation for 7 years. Cryopreservation affects tissue activity. Based on our results, the authors believe that UC can be a useful biomaterial in the clinical application of regenerative medicine.

ABBREVIATIONS

VEGF: Vascular Endothelial Growth Factor; MSCs: Mesenchymal Stem Cells; UC: Umbilical Cord; UCB: Umbilical Cord Blood; OCT: Compound: Optimal Cutting Temperature Compound; PBS: Phosphate-Buffered Saline; DAPI: 4',6-diamidino-2-phenylindole; HE: Hematoxylin and Eosin; EPCs: Endothelial Progenitor Cells; UC-MSCs: Umbilical Cord derived Mesenchymal Stem Cells

INTRODUCTION

Recent studies [1-4] revealed the presence of mesenchymal stem cells (MSCs) in various tissues. The umbilical cord (UC) is also one of the tissues where MSCs are present [5,6]. The UC, whose abundant tissue volume is noninvasively available from the neonate at birth, has good clinical applicability for autologous use if allowed to be used as autologous tissue, causing fewer ethical, medical, and safety issues. It has been reported that MSCs secrete Vascular Endothelial Growth Factor (VEGF) [7]. To the best of our knowledge, some studies on growth factors contained in UCB [8,9] have been found, but there have been no studies on UC cryopreserved for a long period. UC collected at birth needs to be cryopreserved until the time of use. In this study, we focused on the cryopreserved UC. The present study is preliminary and unique in that the authors determined the expression of VEGF in UC cryopreserved for a long period of time.

MATERIALS AND METHODS

Ethical Consideration

This study was approved by the ethics committee at Kitasato University (approval number: B-07-13) after obtaining written informed consent from all enrolled pregnant women. The collected samples of UC were numbered in order not to allow individual identification.

Preparation of UC

UC was collected from full-term neonates who had not presented with any problems during pregnancy and delivery. Figure 1 shows an outline of the experiment. The UC was cut into approximately 10-cm sections and washed with phosphate-buffered saline (PBS; Wako Pure Chemical Industries Co., Ltd., Osaka, Japan) until no attached blood remained. The UC was cut into approximately 2-cm sections. Subsequently, the UC sections were suspended in a solution for tissue cryopreservation: LaboBanker2™ (TOSC, Tokyo, Japan) and stored at -80°C until the time of use. UCs, which had been cryopreserved for 7 years or longer, were thawed at room temperature [Figure 2a,b].

Histological and immunohistochemical assessments

Specimens removed from LaboBanker2™ (TOSC, Tokyo, Japan) were cut into 5 mm-sized pieces after washing. Cryosections were prepared by encapsulating the sections in Optimal Cutting

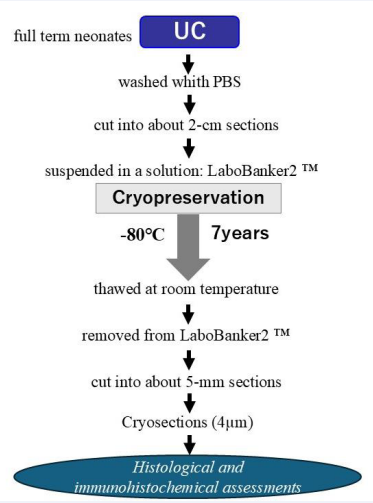

Figure 1 Overview of the study. Umbilical cord; UC

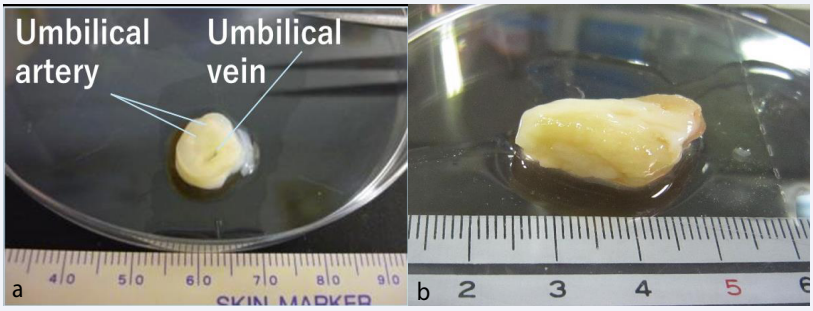

Figure 2 Pretreatment of the umbilical cord.
a) Cross section of the UC: Finding before cryopreservation
The umbilical cord was washed up to the moment when no attached blood remained also in the vascular lumen. The cross section identified the umbilical vein and arteries. Figure cited from the author's previous research report: ref. 17
b) Cross section of the UC: Finding after cryopreservation;
UC, which had been cryopreserved for 7 years or longer, were thawed at room temperature.

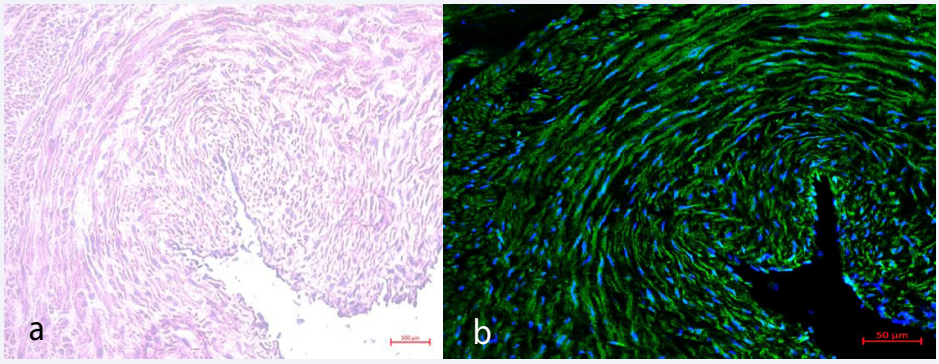

Figure 3 Histopathological and immunohistochemical findings
a) Hematoxylin and eosin (HE) staining: HE staining showing spindle-shaped cells. The solid arrow indicates the umbilical vein. Bar: 500 μm.
b) Immunohistochemical staining: Anti-VEGF-A antibody/DAPI
VEGF-A-expressing cells were observed throughout the entire cross-section of the UC. Bar: 50 μm

Temperature (OCT) compound. In accordance with a previous report [10], thin sections (4μm) were prepared by Kawamoto's method. Briefly, the OCT compound of the specimen was removed, and the specimen was washed three times with PBS. For histological assessment, the unfixed sections were stained with H&E and observed with an optical microscope (Axioskop 2 plus; Carl Zeiss, Jena, Germany). For immunohistochemical assessments, the unfixed sections were blocked with Blocking One™ (NACALAI TESQUE, INC. Kyoto Japan) at room temperature for 30 min. Subsequently, the Anti-VEGF-A antibody (#ab46154 Abcam, Cambridge, UK) was used as the primary antibody and incubated overnight at 4°C. After washing with PBS 3 times, Alexa Fluor 488 Goat anti-rabbit IgG (#A11008, Invitrogen, California, US) was used as the fluorescent secondary antibody. Sections were stained with DAPI (DAPI Solution #62248, Invitrogen, California US) and sealed in Fluoromount™ (Diagnostic BioSystem Hague, Netherlands). Images were captured using a confocal scanning laser microscope (LSM710; Carl Zeiss, Jena, Germany).

RESULTS AND DISCUSSION

VEGF-A-expressing cells were observed throughout the entire cross-section of the UC [Figure 3a,b]. VEGF-A binds to VEGFR-2, which is specifically expressed on vascular endothelial cells. Its functions include the regulation of gene expression of angiogenesis-related factors, the proliferation and migration of vascular endothelial cells, and vascular permeability [11,12]. The presence of EPCs, which express VEGF, has been reported not only in early embryonic stages but also in late embryonic stages and beyond [13]. The expression of VEGF and the reception of its signals during the embryonic period are thought to be regulated [11]. Therefore, it has not been determined whether the expression of VEGF-A identified in this study was due to MSCs, EPCs, or other cell types. Regardless of its origin, the results of this study, in which VEGF-A was expressed, suggest that UCs may be able to contribute to angiogenesis even after long-term cryopreservation for 7 years.

In recent years, there have been reports of the clinical application of UC-MSCs as allogeneic regenerative medical materials [14]. However, there have been few reports of the clinical application of UC-MSCs as autologous tissue. Some reports have examined the expression of VEGF in UC-MSCs to diagnose the condition of the mother and fetus [15]. However, this study investigated the expression of VEGF in UC-MSCs for use in treatment, not for diagnosis. When intending to apply MSCs clinically, the authors consider autologous tissues preferable from the viewpoints of safety and ethics. The UC is usable as autologous tissue and can be collected noninvasively and easily at birth. UC collected at birth needs to be cryopreserved until use. Cryopreservation affects tissue activity [16]. Based on our results, the authors believe that UC can be a useful biomaterial in the clinical application of regenerative medicine.

LIMITATIONS

In this study, we could not confirm the expression of VEGF-A in the umbilical cord prior to freezing. The number and volume of tissue samples were limited because they were obtained in clinical settings.

CONCLUSION

The authors determined the expression of VEGF in UC that had been cryopreserved for 7 years. Thus, the potential for angiogenesis in UC cryopreserved for 7 years was suggested. UC can be a useful biomaterial in the clinical application of regenerative medicine.

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