Cryobiology 74 (2017) 61–67

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

The effect of cryopreservation on anti-cancer activity of human amniotic membrane

Khashayar Modaresifar [a, c, 1], Sara Azizian [a, c, 1], Maryam Zolghadr [b, 1], Hamideh Moravvej [d], Abolhassan Ahmadiani [a], Hassan Niknejad [a, b, *]

[a] Department of Pharmacology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Nanomedicine and Tissue Engineering Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Department of Biomaterials, Faculty of Biomedical Engineering, Amirkabir University of Technology, Tehran, Iran
[d] Skin Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 4 September 2016
Received in revised form
7 November 2016
Accepted 5 December 2016
Available online 9 December 2016

Keywords:
Amniotic membrane
Cryopreservation
Apoptosis
Angiogenesis
Cancer
Epithelial cell

ABSTRACT

Human amniotic membrane (AM) is an appropriate candidate for treatment of cancer due to special properties, such as inhibition of angiogenesis and secretion of pro-apoptotic factors. This research was designed to evaluate the impact of cryopreservation on cancer cell death induction and anti-angiogenic properties of the AM. Cancer cells were treated with fresh and cryopreserved amniotic condition medium during 24 h and cancer cell viability was determined by MTT assay. To evaluate angiogenesis, the rat aorta ring assay was performed for both fresh and cryopreserved AM within 7 days. In addition, four anti-angiogenic factors Tissue Inhibitor of Matrix Metalloproteinase-1 and 2 (TIMP-1 and TIMP-2), Thrombospondin, and Endostatin were measured by ELISA assay before and after cryopreservation. The results showed that the viability of cultured cancer cells dose-dependently decreased after treatment with condition medium of fresh and cryopreserved tissue and no significant difference was observed between the fresh and cryopreserved AM. The results revealed that the amniotic epithelial stem cells inhibit the penetration of fibroblast-like cells and angiogenesis. Moreover, the penetration of fibroblast-like cells in both epithelial and mesenchymal sides of fresh and cryopreserved AM was observed after removing of epithelial cells. The cryopreservation procedure significantly decreased anti-angiogenic factors TIMP-1, TIMP-2, Thrombospondin, and Endostatin which shows that angio-modulatory property is not fully dependent on proteomic and metabolomic profiles of the AM. These promising results demonstrate that cancer cell death induction and anti-angiogenic properties of the AM were maintained within cryopreservation; a procedure which can circumvent limitations of the fresh AM.

1. Introduction

Amniotic membrane (AM) is the innermost layer of fetal membranes. From the chorion side, the AM is composed of a spongy layer, a fibroblast layer, a compact layer, a basement membrane, and a layer of epithelial cells. The latter cells secrete collagen type III and IV, and also non-collagenous glycoproteins such as laminin, fibronectin, and nidogen. The collagens of the fibroblast layer (type I and III) secreted by amniotic mesenchymal cells are highly compacted. The AM has no blood vessels and nerves and obtains its nutrients from the chorion or from amniotic fluid by diffusion mechanism [5,6].

The human amniotic membrane has successfully been used in different clinical fields during the past century, especially in surgeries [26], skin transplantation [3,22], healing skin burns and wounds [14], treatment of Stevens-Johnsons syndrome [13], and reconstruction of cornea [15]. In addition to antibacterial properties [29] and preventing the loss of water and electrolytes, the AM promotes the rate of re-epithelialization of skin in burns and reduces the risk of infection [1]. Moreover, the AM's potential to be used in vascular tissue engineering has been recently evaluated [9,24].

Some special properties, such as inhibition of angiogenesis, secretion of pro-apoptotic factors, and modulation of the immune system have made the AM an appropriate candidate for cancer

* Corresponding author. Department of Pharmacology, School of Medicine, Koodakyar St, Deneshjoo Blvd, Velenjak, P.O. Box 1985711151, Tehran, Iran.
E-mail address: niknejad@sbmu.ac.ir (H. Niknejad).
[1] The first, second and third authors have equally contributed to this study.

http://dx.doi.org/10.1016/j.cryobiol.2016.12.001
0011-2240/

treatment [10,16,17,23,28]. We have shown in previous studies that the AM reduces the viability of cancer cells via apoptosis induction [17,20]. Apoptosis is divided into two pathways including the extrinsic and intrinsic pathways. The extrinsic pathway relies on the receptor and initiates apoptosis by activating caspase-8, while the intrinsic pathway occurs through the release of cytochrome *c* from mitochondria and commences the apoptosis by activating caspase-3 [20]. It has been shown that the condition medium of the AM activates both intrinsic and extrinsic pathways of apoptosis. We have also shown previously that the AM is capable of inhibition of angiogenesis which is a crucial factor in tumor growth [21,31]. Among the angiostatic factors, Angiostatin and Thrombospondin are two natural factors in the amniotic membrane, which the former is a proteolytic fragment of plasminogen that has an intense anti-proliferative effect on cancer cells and usually prevents the growing of the early tumors in mice [30].

Up to date, most of the studies on anti-cancer properties have been carried out using fresh amniotic membrane [17], whereas the preservation of the AM is necessary to keep it safe from the risk of transferring pathogens, especially because of the window period between infection and seroconversion. Moreover, the fresh AM is not always available to be used. Due to these facts, various methods have been suggested for the preservation of the AM. A common method for long-term storage is cryopreservation in which the AM is put in a cryoprotectant solution and kept at −80 °C or below. A growing number of efforts have been made to optimize cryopreservation of the AM. Cell viability of stromal and epithelial layers of the AM processed into allograft pieces of different sizes was recently evaluated before and after cryopreservation [25]. Moreover, the effects of cryopreservation have been assessed on the activity of AM's biological signals and the native architecture of its extracellular matrix [2]. However, the process of cryopreservation can affect the AM's cells and extracellular matrix. These effects may change the anti-cancer properties of the AM. Therefore, the anti-cancer properties of the cryopreserved amniotic membrane, including the reduction of viability of cancer cells and its effect on angiogenesis, were evaluated in this study.

2. Materials and methods

2.1. Preparation of amniotic membrane

To prepare the AM, the placenta (n = 28) was collected from mothers having elective caesarean at 36–38 weeks of gestational age. All experiments were approved by The Ethical Committee of Shahid Beheshti University of Medical Sciences and the proper informed consent was obtained from the parents. The results for syphilis, HCV, HBV, and HIV tests were negative. The placenta was delivered to the lab in a sterile bag containing phosphate-buffered saline (PBS) at 4 °C. The AM was separated from the chorion by peeling and washed with cold PBS three times to remove blood.

2.2. Cryopreservation of the AM in Me_2SO

As described previously [19], the AM sections were placed epithelial side up on nitrocellulose membranes and put in sterile vials containing 70% PBS, 10% fetal bovine serum (FBS) (Sigma-Aldrich), 10% Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich) and 10% Me_2SO (Sigma-Aldrich), and were kept at −80 °C. After 6 months, the AM was thawed at room temperature and then was rinsed several times with PBS (at 4 °C) in order to remove the cryoprotectant solution completely.

2.3. Microscopic and histological evaluation of cryopreserved AM

The surface of cryopreserved AM was evaluated using scanning electron microscopy (SEM), as described previously [4]. For histological analysis, the samples were fixed with 10% formalin and subsequently embedded in paraffin. Then the samples were cut into sections with the thickness of 4 μm. After staining with hematoxylin and eosin (H&E), the samples were evaluated with light microscope with magnification 400× (Zeiss, Germany) [31].

2.4. Cell culture

The effect of cryopreserved AM on viability and proliferation of cancer cells was evaluated using human cervical cancer cell line HeLa and breast cancer cell line MDA-MB-231 (Pasteur Institute of Iran). The cells were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma-Aldrich), and incubated in humidified air containing 5% CO2 at 37 °C. After reaching 75% confluence, the cancer cells were detached using trypsin-EDTA (Sigma-Aldrich) and sub-cultured in 24-well plates (5×10^4 cell/well) for 24 h in 5% CO_2 at 37 °C overnight.

2.5. The effects of fresh and cryopreserved AM on viability of HeLa and MDA-MB-231 cancer cells

The fresh and cryopreserved AM were cut into 2×2 cm^2 pieces and put in 12 well plates so that the epithelial side was upward. Each well was supplied with 1 ml of DMEM containing 10% FBS and 1% penicillin-streptomycin. After 24 h of incubation at 37 °C and 5% CO_2, the supernatant of each well was collected and filtered by 0.22 μm filters. The cancer cells were treated with 200, 400, 600 and 800 μL of the supernatant, respectively. An untreated group of cancer cells was defined as control group. The viability of cancer cells treated with the AM supernatant was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24 h. For this purpose, the 0.5% MTT solution (5 mg of MTT per 1 ml of distilled water) was sterilized by 0.22 μm filter and was added to cancer cells. Then the cell culture plates were incubated at 37 °C for 4 h. Afterward, the formazan crystals were dissolved in Me_2SO and the optical absorption of dissolved formazan was measured at the wavelength of 570 nm, using spectrophotometer (Cecil, CE 7500, UK) in order to determine the percentage of live cells.

2.6. Evaluation of angiogenesis by rat aorta ring assay

The fresh and cryopreserved AM were divided into two sections after being rinsed with PBS. One section was kept intact (with epithelial cells) and cut into 2×2 cm^2 pieces. The other section was put in 0.03% trypsin-EDTA solution for 10 min in order to eliminate the amniotic epithelial cells. The process of removing cells was completed using a cell scraper. Then this section was also cut into 2×2 cm^2 pieces. Both sections (with and without amniotic epithelial cells) were put at the bottom of 12 well plates in two positions: epithelial side up and mesenchymal side up. Each well was supplied with 2 ml of DMEM containing 15% FBS, 10 ng/ml bFGF (Sigma-Aldrich) and 1% penicillin-streptomycin. The plates were incubated at 37 °C and 5% CO_2.

To evaluate angiogenesis *in vitro*, the rat aorta ring assay was applied. The Ethical Committee of Shahid Beheshti University of Medical Sciences approved all animal experimental procedures. Aorta rings were dissected from the left descending thoracic aorta of 12 weeks old rats weighing 150–200 g. The dissected aorta was rinsed with PBS containing penicillin-streptomycin and then was cut into circular sections with the thickness of 2 mm. These sections