Cytotherapy, 2017; 19: 990–1001

International Society for Cellular Therapy
ISCT®

CrossMark

Carcinogenicity, efficiency and biosafety analysis in xeno-free human amniotic stem cells for regenerative medical therapies

TATSANEE PHERMTHAI[1], SASIPRAPA THONGBOPIT[1], PUTTACHART POKATHIKORN[1], SUPARAT WICHITWIENGRAT[1], SUPHAKDE JULAVIJITPHONG[1,2] & NEDNAPIS TIRAWANCHAI[3]

[1]Stem Cell Research and Development Unit, Department of Obstetrics & Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, [2]Infertility Unit, Department of Obstetrics & Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, and [3]Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Abstract

Background aims. Human amniotic mesenchymal stromal cells (hAMSCs) are a potent and attractive stem cell source for use in regenerative medicine. However, the safe uses of therapeutic-grade MSCs are equally as important as the efficiency of MSCs. To provide efficient, clinic-compliant (safe for therapeutic use) MSCs, hAMSC lines that completely eliminate the use of animal products and have been characterized for carcinogenicity and biosafety are required. *Methods.* Here, we have efficiently generated 10 hAMSC lines under human umbilical cord blood serum (hUCS)-supplemented medium (xeno-free culture) and fetal bovine serum (FBS)-supplemented medium (standard culture) and investigated carcinogenicity and immunosuppressive properties in the resultant hAMSC lines. All hAMSC lines were examined for efficiency (growth kinetics, cryopreservation, telomere length, phenotypic characterization, differentiation potential), carcinogenicity (proto-oncogene and tumor suppressor gene and epigenomic stability) and safety (immunosuppressive properties). *Results.* Stem cell characteristics between the xeno-free hAMSC lines and the cell lines generated using the standard culture system showed no differences. Xeno-free hAMSC lines displayed normal growth proliferation potential, morphological, karyotypic, phenotypic differentiation properties and telomere lengths. Additionally, they retained normal immunosuppressive effects. As a marker of carcinogenicity and biosafety, proto-oncogenes expression levels showed no differences in xeno-free hAMSCs, and we detected no SNP mutations on hotspot codons of the *P53* tumor suppressor gene and stable epigenomic imprinting in xeno-free hAMSC lines. *Conclusions.* Xeno-free hAMSC lines retain essential stem cell characteristics, with a high degree of certainty for meeting biosafety and carcinogenicity standards for a xeno-free system supplemented with allogenic hUCS. The cell lines are suitable and valuable for therapeutic purposes.

Key Words: *amniotic membrane, epigenetics, mesenchymal stromal cells,* P53, *umbilical cord serum, xeno-free*

Introduction

Mesenchymal stromal cells (MSCs) are of particular interest for use in regenerative medicine because they display extensive self-renewal, multi-lineage differentiation, anti-inflammatory properties, low immunogenicity and non-tumorigenicity. Traditionally, MSCs have been isolated from bone marrow (BMSCs) and widely used for clinical therapy for stroke, Alzheimer's disease and tissue damage and inflammation [1–3]. However, the uses of BMSCs are limited by the invasive procedures necessary to obtain them, low stem cell numbers and declining potency with donor age [4]. MSCs from alternative sources are increasingly of interest for therapeutic purposes.

An attractive MSC source is human amniotic membrane, which can be obtained through noninvasive procedures without any ethical conflicts, have a simple isolation process and have a high yield of stem cells [5]. Human amniotic membrane-derived mesenchymal stromal cells (hAMSCs) exhibit typical MSC properties to BMSC with respect to spindle shape morphology, multilineage differentiation capability and expression of common MSC phenotype CD29, CD44, CD73, CD90, CD105 and major histocompatibility (MHC) class I markers [5,6]. Insausti *et al.* found that

Correspondence: **Tatsanee Phermthai**, PhD, Obstetrics & Gynecology Department, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Road, Siriraj, Bangkoknoi, Bangkok 10700, Thailand. E-mails: tatsanee.phe@mahidol.ac.th, bsuteevun_1@yahoo.com

(Received 9 February 2017; accepted 24 April 2017)

http://dx.doi.org/10.1016/j.jcyt.2017.04.004

hAMSCs also express the pluripotency marker Oct-4 [6]. Moreover, hAMSCs appear to be a more efficient source than BMSCs due to their superior proliferation capability and greater long-term growth ability [7]. hAMSCs also elicit a strong immunosuppressive response [8,9] and display anti-inflammatory, anti-angiogenic and non-tumorigenic characteristics [6]. Additionally, hAMSCs have been reported to exhibit superior differentiation and proliferation potential to MSCs from other stem cell sources [10]. hAMSCs exhibit high neuro-regenerative potential and have successfully treated neurological disease [11]. They significantly express various neurotrophic factors: brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), glial cell–derived neurotrophic factor (GDNF) and ciliary neurotrophic factor [12]. hAMSCs represent an alternative therapeutic option for neuro-regeneration [12,13]. It is necessary to provide an approach to isolate, scale up and preserve hAMSC lines that are suitable for clinical use.

Stem cell lines used for "research-use-only" (RUO) do not have the same burden of safety to be met as stem cell lines intended for clinical use. At the clinical level, not only must stem cell lines be efficient, but they must also meet certain safety requirements. Conventionally, *ex vivo* stem cell manipulation has not been able to avoid co-culture with animal-derived products, which carry the risk of animal viral contamination, prion disease, retention of xeno-protein in MSC cytoplasm and potential to trigger immune reactions [14–16]. Alternative culture supplements, human serum (HS), serum-free medium and human umbilical cord blood serum (hUCS), have been considered to avoid these safety concerns. hUCS has been reported to be an efficient supplement in the culture of amniotic fluid stem cells, Wharton's jelly stem cells and fibroblast feeder cells for embryonic stem cells [16–18] and also for expansion of amniotic membrane stem cells [19,20]. This is due to the nourishing growth factors in cord blood, which are delivered *in vivo* from mother to fetus. Previous studies by Kim *et al.* and Ma *et al.* described the successful expansion of hAMSCs cultured with hUCS-supplemented medium [19,20]. They showed that hAMSCs expanded under hUCS have superior growth potential to hAMSCs expanded under fetal bovine serum (FBS)-supplemented medium. The cells also retain MSC markers and differentiation capacity.

However, one more concern about using MSCs for cell-based therapies is safety. All aspects of genomic stability, carcinogenicity and immune suppressive properties of MSCs still need to be clearly understood before use in clinical applications. In this study, we used supplementation with allogenic hUCS to provide xeno-free hAMSC lines. We examined the fidelity of MSC-specific characteristics, including immunophenotypic appearance, proliferation potential, differentiation capability and telomere length. We estimated the risk of carcinogenicity in hAMSCs using proto-oncogenes and tumor suppressor gene expression, tumor suppressor *P53* gene mutation analysis via single nucleotide polymorphisms and epigenetic stability via the expression of imprinted genes (*H19, IGF2, SNRPN*). We also evaluated the biosafety of the uses of MSCs in clinical cell replacement therapies, through examination of immunosuppressive properties.

Materials and methods

Samples

Fresh amnion (n = 10) and cord blood (n = 60) samples were obtained from healthy donors after full-term delivery. The amnion and cord blood samples were screened for infectious diseases: human immunodeficiency virus (HIV), hepatitis B and C virus and sexually transmitted diseases. All donors for amnion and cord blood underwent informed consent procedures in accordance with the requirements of the Ethics Committee of Siriraj Hospital, Mahidol University, Bangkok, Thailand. The Institutional Review Board approved the use of tissues.

Preparation of hUCS

Term human cord blood samples were allowed to undergo blood clotting for 4–6 h at room temperature. The blood was centrifuged at 2800 rpm for 5 min at 20°C. Cord serum was collected and sterilized by passage through a filter membrane with a pore size of 0.22 μm. Twenty serum samples of hUCS were pooled in each serum batch. Cord serum was analyzed for mycoplasma and then kept at –20°C until use.

Establishment of xeno-free hAMSC lines

Ten samples of amnion were used to establish the standard and xeno-free hAMSC lines. Each individual sample was mechanically peeled off from the placenta using sterile technique. The tissue was washed several times in sterile normal saline solution (0.9% weight/volume of sodium chloride) and phosphate-buffered saline (PBS) to remove excessive blood and cellular debris. Amnion was minced into small pieces (approximately 1×1 mm^2) and placed in a tissue culture dish (Corning), containing alpha minimum essential medium (α-MEM; Gibco, Invitrogen), 1% penicillin/streptomycin (Sigma-Aldrich) and either 10% FBS (PAA) or hUCS (in-house preparation) maintained at 37°C, 5% CO_2 in a humidified atmosphere. After 5 days, the medium was changed and cells outgrowing from tissue were observed. When colony outgrowth reached to 80%–90% confluence, cells were harvested and subcultured to the