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Human breastmilk as a source of progenitor/stem cells

Mleko kobiece jako źródło komórek progenitorowych/macierzystych

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Summary

Introduction. Human breastmilk is a complex fluid adapted to satisfy the nutritional requirements of an infant. Some studies indicate the presence of progenitor/stem cells in the human breastmilk.

Aim. The aim of this study was to obtain *in vitro* culture of the breast milk-derived cells and identify expression of pluripotency genes in the cells.

Material and methods. Mature breastmilk (20-30 ml) samples were obtained from health breastfeeding women in very early range of lactation in a aseptic way. The isolation procedure was based on the protocols described by Hassiotou et al. with modifications. The cells were cultured *in vitro* under standard conditions and characterized on molecular level.

Results. The presence of the cells of various origin was detected in human breastmilk. The isolated breast-milk-derived cells were adherent to the plates. We found the expression of pluripotency genes: OCT4, SOX2 and NANOG in the isolated breastmilk cells by real-time PCR and present it in contrary to human bone-marrow derived stem cells.

Conclusions. Human breastmilk contains a variety of cells. A population of progenitor/stem cells express pluripotency genes. Human breastmilk could be therefore a non-invasive source of progenitor cells for regenerative and personal medicine in the future.

Streszczenie

Wstęp. Właściwości odżywcze mleka kobiecego są znane od dawna, ale obecnie pokarm kobiecy jest traktowany jako element terapii. Badania wskazują na obecność w nim komórek progenitorowych/macierzystych.

Cel pracy. Celem pracy było potwierdzenie obecności komórek macierzystych w mleku kobiecym oraz weryfikacja ich pluripotencjalności.

Materiał i metody. Próbki mleka (20-30 ml) uzyskane były od zdrowych kobiet w 3. dobie po naturalnym porodzie. Izolacja komórek progenitorowych i macierzystych została przeprowadzona z zastosowaniem metody opisanej przez Hassiotou i wsp. z modyfikacjami. Wyizolowane komórki były hodowane w warunkach *in vitro* i charakteryzowane na poziomie molekularnym.

Wyniki. W hodowli *in vitro* otrzymano kilka typów komórek. Komórki macierzyste zidentyfikowane w mleku wykazywały ekspresję genów pluripotencjalności: OCT4, SOX2, NANOG, w odróżnieniu od komórek kontrolnych.

Wnioski. Mleko kobiece zawiera heterogenną populację komórek. Może być ono łatwodostępnym, nieinwazyjnym źródłem komórek progenitorowych i macierzystych o wzmożonej ekspresji genów pluripotencji.

Conflict of interest
Konflikt interesów
None
Brak konfliktu interesów

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INTRODUCTION

Stem cell biology has become an intriguing field. Several types of human stem cells have been isolated and identified *in vivo* and *in vitro*. The presence of stem/progenitor cells, called human breastmilk-derived stem cells (hBSCs) has been found in mother's milk in recent years (1, 2). The cell population in the breastmilk derives from various sites of the mammary gland. Blood-derived leukocytes are the best-known, luminal epithelial cells and myoepithelial cells residing in the

breast are described (3), whereas the hBSCs are the most extensively studied recently. New data revealed that leukocytes constitute only a small minority (< 2%) of the cells in a mature milk of healthy mother (4). Luminal and myoepithelial cells and their precursors represent nearly 80-98% of the non-immune cell types found in human milk in healthy condition (5). Numbers of breastmilk stem cells with multilineage properties is estimated at 5-10% of cell population in colostrum (2, 6). According to the recent evidence, breastmilk stem/progenitor cells are scarce in the resting breast; however, they are activated during pregnancy and lactation, undergoing a controlled program of proliferation, differentiation and apoptosis stimulated by hormonally-driven cues. However, breastmilk cell complexity is very individual and it subject to various factors such as the stage of lactation, the degree of breast fullness, infant feeding, the health status of the breastfeeding dyad, and others.

Term "stem cells" may refer to various types of cells which (a) are unspecialized and can generate one or more cell lineage types of the three germ layers, (b) have the ability to replenish their own population (feature of self-renewal). Expression of the three transcription factors, OCT4, SOX2 and NANOG, is essential for the major properties of stem cells: self-renewal and pluripotency (7). In this study we evaluated the expression of these genes in the breastmilk-derived cells. OCT-4 (octamer-binding transcription factor 4) is a pluripotency regulator that controls lineage commitment of embryonic stem cells (8). SOX 2 is a member of the SRY-related HMG-box (SOX) transcription factor family with a diverse role in stem cell potency and maintenance, embryonic development and cancer (9). OCT4 together with SOX2 stimulate the expression of NANOG (10). The presence of pluripotent stem cells in human milk generates numerous questions and implications for breastfeeding, newborn and maternal health, but also opens a new perspective of future potential applications of these cells in the personal and regenerative medicine.

AIM

The objectives of the current study were to identify the cellular constituents of human breastmilk by phenotypic characterization of the cells and expression of the pluripotent markers.

MATERIAL AND METHODS

Collection of breastmilk samples

All procedures were approved by the 2nd Local Ethical Committee at the Medical University of Warsaw (Decision No. KB/239/2016). The breastmilk samples were obtained between 1st and 4th day post-delivery from two healthy volunteers in the morning. Breastmilk (20-30 ml) samples were transported to the laboratory immediately under aseptic condition.

Breastmilk cell isolation and culture

The isolation procedure was based on the protocols described by Hassiotou et al. with modifications. 20 ml breastmilk was diluted with equal volume of sterile phosphate buffer saline (PBS) and centrifuged at 805 g for 20 minutes at 20°C. The cell pellet was washed three times in PBS with antibiotic-antimycotic mixture and resuspended in low-glucose DMEM supplemented with 20% fetal bovine serum (FBS). The total cell concentration and viability of each sample were determined in a Burker chamber and trypan blue exclusion. The cells were seeded in standard, polystyrene culture plates as well as low adherent plates (Corning Matrigel® hESC-qualified Matrix, Catalog #354277). Basic culture medium based on low-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic-antimycotic mixture containing 10,000 U of penicillin (base), 10 mg of streptomycin (base), and 25 μg of amphotericin B/mL (Fungizone® Antimycotic, Gibco®) and Mammo-Cult medium (Stemcell™ technologies) supplemented with 1% antibiotic-antimycotic mixture were used respectively. Subsequently, the cells were cultured under standard conditions (at 37°C in 5% CO_2, humidified atmosphere) for 7 days. Media changes were performed every 2 days. Morphology of the cell cultures was visualized using an inverted microscope (Nikon, Eclipse TE 2000-U) after 7 days in culture.

Real-time PCR

Total RNA was extracted from cells using RNeasy kit (Qiagen) according to the manufacturer's protocol and RNA concentration was assessed using a Nanodrop spectrofotometr (Thermo Scientific). cDNA was subsequently prepared from 250 ng of RNA template by reverse transcription kit (Applied Biosystems). The expression of OCT4, SOX2, NANOG was determined by real time PCR (Assays IDs: Hs04260367_gH, Hs01053049_s1, Hs02387400_g1 Applied Biosystems) using a 7500Fast Real Time PCR System (Applied Biosystems). Each sample was analyzed in triplicate. BM-MSCS served as a negative control. The relative expression of the target genes was normalized to the reference gene GAPDH (Hs99999905_m1) and the data was analyzed using the $\Delta\Delta$Ct method.

RESULTS

The number of cells obtained from 20 ml of fresh breastmilk was found to range between 3-5 million/ml (fig. 1a). The presence of the cells of various origins was detected in the samples of the human breastmilk (fig. 1a-f). Isolated breastmilk cells were adherent to the culture plates. The number of adherent cells after 24 h was found to be 2 to 4 million/ml. Lactocytes (fig. 1e) and fibroblast-like myoepithelial cells (fig. 1f) appeared as single cells adhering to culture plastic. Cells from milk samples, seeded in low adherent plates and Mammo-Cult medium, were found to grow in mammospheres (fig. 1b, c).

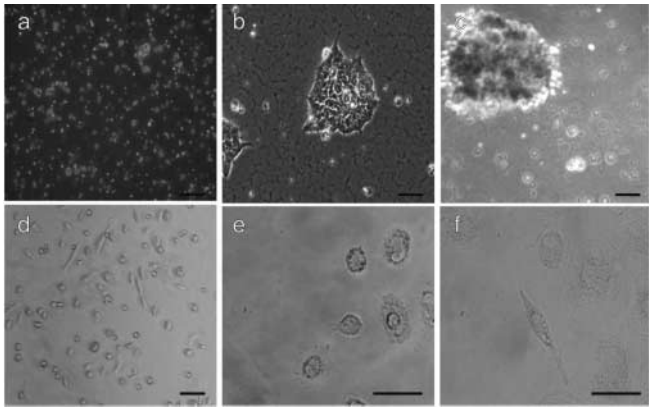

Fig. 1a-f. The morphology of human breastmilk cells in *in vitro* culture: a – fresh cell culture; b, c – mammosphers in Mammo-Cult medium after 7 days of culture; d, e, f – cells adherent to the culture dish: lactocytes and fibroblast-like myoepithelial cells after 7 days of culture

We analyzed OCT4, SOX2 and NANOG mRNA levels by real-time PCR to estimate the expression of the pluripotency genes in the isolated breastmilk cells. Data was compared with a negative control, that is obtained in bone marrow mesenchymal stem cells, as a rule negative for OCT4/SOX2/NANOG expression. Relative mRNA expression of OCT4, SOX2, NANOG was 1.6 to 2.8 more expressed than in BM-MSCs and the differences were significant (fig. 2). Our results indicate that breastmilk cells from different donors display a variable expression of pluripotency markers which are found in human embryonic stem cells.

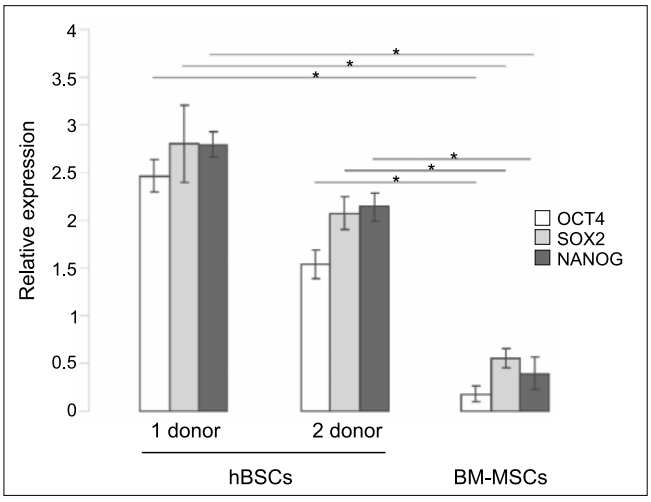

Fig. 2. Quantitative real-time PCR assay for expression of OCT4, SOX2 and NANOG by human breastmilk cells and in comparison with bone marrow stem cells. Bars represent the mean ± SEM

DISCUSSION

Recent breakthroughs are indicating that breastmilk cellular composition is more heterogeneous as previously regarded. It contains not only various somatic cell types, but also stem cells. For many years, leukocytes were considered the most abundant breastmilk cells, but recently the presence of other breastmilk cells has been demonstrated. Besides leukocytes, it contains lactocytes (milk-secretory cells), myoepithelial cells (from the ducts and alveoli of the mammary gland) and a hierarchy of progenitor and stem cells. This arouses the brand new meaning of breastfeeding. The benefits of breastfeeding are well documented. These are, for the child, infection and allergy control, prevention of necrotizing enterocolitis, obesity, chronic conditions, such as type I diabetes, celiac disease, Crohn's disease, and even faster psychomotor development (11-13). The benefits for the mother are associated with a decreased risk of breast and ovarian cancers (14).

Our study confirms the presence of numerous, adherent, non-immune cells types in the breastmilk samples. The natural presence of stem cells in breastmilk raises questions of the origin and a role of these cells in infant development. It has been suggested that presence of non-immune cells in milk is an effect of voiding dead cells which are no longer necessary in the mammary gland. However, studies based on trypan blue exclusion and *in vitro* cultures have shown that more than 90% of these milk cells remain viable (2). What is more, maternal stem cells were shown to pass through the newborn gut epithelia, in the animal model, in an unknown way and enter the tissues of the newborn (15). Breastmilk is therefore far more than just a nutrients supply ensuring the appropriate growth of the child and these unique properties are associated with the cellular composition of the human milk.

Without a doubt, mother's milk is a quite promising source of stem cells with the therapeutic implication deriving from them. We demonstrate the presence of stem cell markers OCT4, SOX2, NANOG in the human breastmilk cells at mRNA level. It is likely that pregnancy/lactation-associated hormonal cues activate this cell population via up-regulation of pluripotent genes, normally found in human embryonic stem cells (hESCs). SOX2 and NANOG were expressed at higher levels among the breastmilk samples tested than OCT4, what is compatible with results of Hassiotou et al. (1). It is necessary to investigate the role of pluripotency genes in the somatic stem cells. It has been speculated that the same set of transcription factors plays an important role in the maintenance of multipotency and self-renewal of stem cells, but also the genes are re-expressed during the process of the tumor cell differentiation (16). The OCT-4 expression may be, for example, dramatically increased in cancer cells (17).

Nowadays, we still do not know and do not understand the relationship between milk macronutrients and somatic cells content, and their health implication. Further studies are required to understand the precise nature of breastmilk stem/progenitor cells and to explore their potential clinical applications.

CONCLUSIONS

Human breastmilk contains heterogeneous cell populations. Cells isolated from human breastmilk and cultured *in vitro* express pluripotent stem cells markers: OCT4, SOX2, NANOG. Human breastmilk can be therefore an non-invasive source of human stem/progenitor cells for future regenerative and personal medicine purposes.

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