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Morphological Characteristics of Ovine Fetal Liver Cells

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Abstract: In recent years, research in the field of stem cells is rapidly expanding and the published data are of great promise for the future of the regenerative medicine. Mesenchymal stem cells (MSC) are a relatively new direction in these studies. Their ability to differentiate to a number of cell types indicates that they have the potential to be used as therapy for a variety of medical conditions. Along with the aforementioned, in general public a discussion was forwarded regarding the moral standards in science and the use of human embryos and foetuses for stem cells research. Therefore, our investigations are aimed at isolation and culture of the adherent fraction of ovine fetal liver cells and their introduction as an experimental *in vitro* model. Our results showed that the isolation of the cells is comparatively easy and that they are capable of long-term culture without differentiation. In addition, the adherent ovine fetal liver-derived cells have high proliferative capacity and exhibited similar morphological characteristics to MSC. According to these findings we suggest, they could be successfully used instead of human material in fundamental research, in order to avoid ethical restrictions.

Key words: Fetal liver · Mesenchymal stem cells · Morphology · Electron microscopy

INTRODUCTION

After the discovery of stem cells and the enunciation of one of their main properties-their potential for self-renewal by McCulloch and Till [1, 2], began the development of the regenerative medicine, which still holds great promises. There are many therapies based on stem cells that have been elaborated until the present moment. Most of them are still in experimental stage and have not been introduced in clinical practise, with the exception of bone marrow and umbilical cord blood transplantations.

Stem cells can be isolated from pre-implantation embryos and from different tissues of foetuses and adult organisms [3]. Fetal stem cells have certain advantages, compared to adult stem cells. They are less immunogenic, at lower differentiation stage and have a higher potential for repopulation and migration [4]. Data exists that they possessed longer telomeres and stronger telomerase activity [5]. On the other hand, the fetal cells have a certain immunomodulating effect when transplanted and express a unique set of cytokines and growth factors, which can stimulate the regeneration of the recipient tissue [6].

Fetal liver is a rich source of stem cells as it is a main haematopoietic organ during the embryo development [7]. Some studies indicate that when cultured, the adhering fraction cells possess the characteristics of mesenchymal stem cells (MSC) [8]. Thus the fetal liver becomes a promising subject for investigation and in the recent years many research papers on transplantation of fetal liver cells (FLC) have been published [6, 9, 10]. When introduced in immunodeficient mice, FLC expressed a repopulation potential ten times higher, compared to cells, isolated from bone marrow of adult organisms [11]. It has been shown that after transplantation in healthy mice, these cells exhibited homing effect in different organs: liver, brain, kidneys [12], which leads to the conclusion, that they may substitute injured cells in many tissues. Results from clinical trials including patients with moderate forms of liver cirrhosis and hepatitis indicated that the transplantation of FLC improves their treatment [13]. Another prospect in which the cells can be potentially used is the creation of systems for generating a bioartificial liver [14-16].

However, despite the data published so far, there is a need for further investigations to ensure the safety of transplantations of fetal tissue and stem cells in clinical

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practice. Along with the advance of the study in the field of stem cells, there is an ongoing dispute in public, about the ethical aspects of using human embryos and fetal tissues for scientific research. To avoid the above restrictions, we used cells isolated from ovine fetal liver as experimental model.

MATERIALS AND METHODS

The experiments have been carried out in September 2007-May 2008, on the ground of the Institute of Biology and Immunology of Reproduction-Bulgarian Academy of Science and with the permission of the Ethics Committee.

Animals: Seven ewes of the Romanoff breed have been artificially inseminated and the pregnancies have been confirmed by ultrasound examination.

Isolation of the Cell Culture: The ewes were subjected to Cesarean section between the 45th-52nd days of gestation. The foetuses were separated from the amniotic sac, the fetal livers were detached and flushed in medium DMEM/Ham's F12 (Cambrex Bioscience, Verviers, Belgium) in sterile conditions (Fig.1). The livers were divided into small pieces and the fragments repeatedly run through a 20G needle. In some cases enzyme dispersion of the cells with 0.25% trypsin (Cambrex Bioscience, Verviers, Belgium) was used, or a combination of the two methods. After that the cell suspension was centrifuged twice at 300 g for 10 min, the pellet was resuspended and seeded in DMEM/Ham's F12 medium, supplemented with 10% fetal bovine serum (FBS) (Cambrex Bioscience, Verviers, Belgium) and penicillin/streptomycin (Cambrex Bioscience, Verviers, Belgium). The cells have been cultured at 37°C, 5% CO_2, in 24-well plates and/or flasks.

Cryopreservation: The cryopreservation of the cells has been carried out by a standard slow freezing protocol. The cells have been detached by 0.25% trypsin and centrifuged. The pellet has been suspended and cooled to 4°C. An equal amount of cooled medium, containing 20% dimethyl sulfoxide (DMSO) (AppliChem BioChemica, Germany) and 40% FBS has been added to the cell suspension (so that the final concentration of DMSO was 10%).Then the cells have been transferred to 2 ml cryovials, which have been held consecutively at 4°C (to allow the equilibration of the cryoprotective agent), at-20°C and nitrogen vapours for 20 min and afterwards were immersed into liquid nitrogen. The thawing of the samples was performed in water bath on 37°C and the cryoprotective agent was removed by a gradual dilution with fresh complete culture medium and centrifugation.

Morphological Examination: The obtained monolayer culture was stained by the method of May Grunwald-Giemsa and with haematoxylin-eosin. After the removal of the culture medium, the cells were flushed three times with phosphate-buffered saline (PBS, pH 7.2) and fixed in a solution of glacial acetic acid (3%) in methanol (97%) for 2 min. For the haematoxylin-eosin staining, the fixed monolayers were processed with Harris haematoxylin for about 1 min, differentiated with distilled water (20 min) and stained with 1% eosin. For May Grunwald-Giemsa staining the cells were fixed and treated at first with the May Grunwald solution for 150 sec, immersed in diluted Giemsa solution for 30 min, washed in tab water and air-dried.

Electron Microscopy: The preparations for electron microscopy have been performed on the 10th passage of the cultures, when the monolayer confluence was about 90%. Initially, the cells were washed three times with PBS

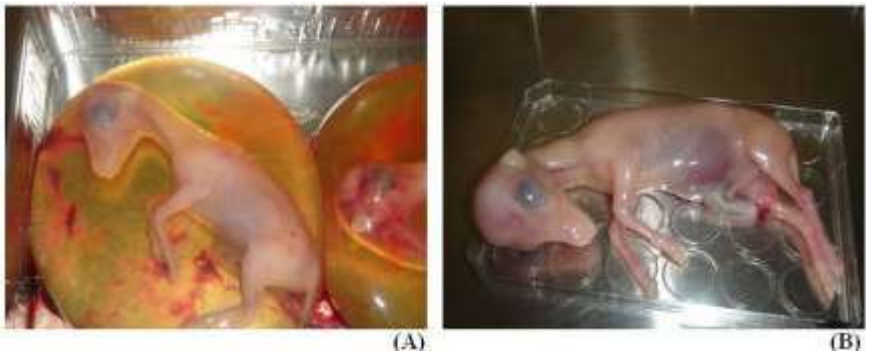

Fig. 1: Ovine foetuses: (A) foetuses in the amniotic sacs; (B) foetus separated from the amniotic sac

and then fixed with 2.5% glutaraldehide in 0.1% cacodylate buffer (pH 7.3), followed by postfixation with 1% OsO_4. The cells were scrapped from the surface of the culture dish and centrifuged. The pellet was dehydrated in ascending ethanol series and Durcupan embedded. Afterwards ultrathin sections were prepared and the samples were observed under JEM 1200 EX electron microscope.

RESULTS

Culture Conditions: After fragmentation of the fetal liver and seeding of the obtained pieces and cells in culture dishes, it was observed that in the period of 24 hours most of them have adhered to the surface. The fetal cells showed good proliferative activity and the isolated primary culture reached confluence approximately between 7 to 10 days after seeding. After subculturing, the growth of the cells intensified and compact monolayer was observed approximately on the 6th day. The cultured cells were also characterized by formation of wave-like layers (Fig. 2). The fetal cells displayed similar morphological appearance after multiple passages and did not tend to differentiate during long-term culture.

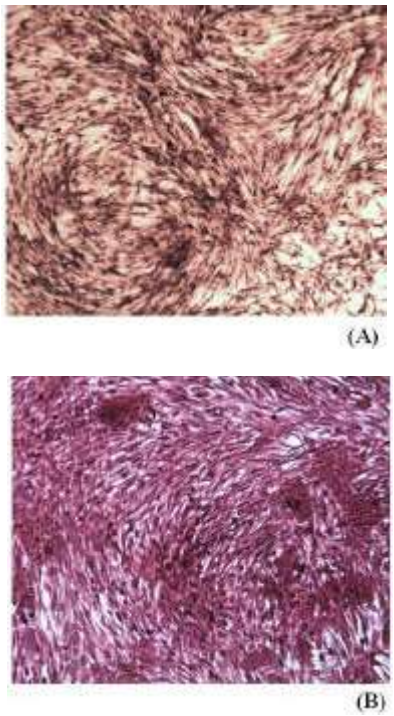

Fig. 2: Typical wave-like layers formed in culture of fetal liver cells (4X, MGG)

Cryopreservation: At every passage part of the cells were cryopreserved in order to provide enough material for research. They showed very good cryotolerance as approximately 75% of them retained their viability after thawing (Trypan Blue exclusion test).

Morphological Analysis: After staining, elongated cells without clearly expressed cell-to-cell contacts were observed. Characteristic features were the relatively large nuclei and the compact cytoplasm. The nuclei were oval, with one to three nucleoli. Polygonal cells with relatively large nuclei were also present (Fig. 3).

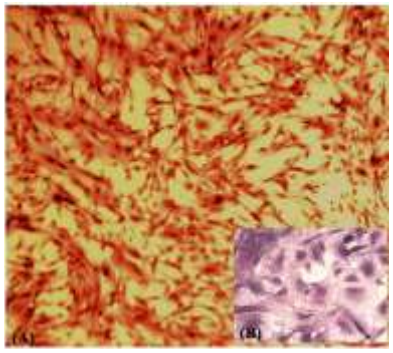

Fig. 3: Monolayer of fetal liver cells: (A) HE staining, 4X; (B) MGG staining, 40X. Polygonal cells with relatively large nuclei could be observed in the cell culture

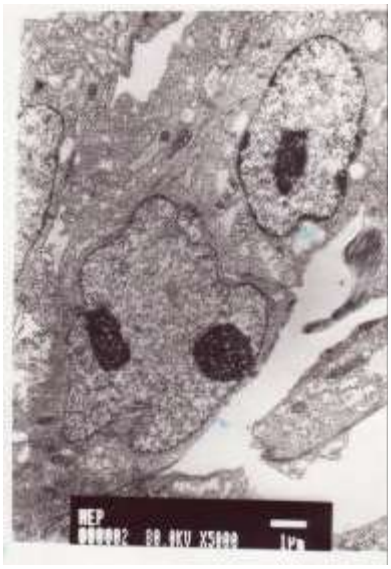

Fig. 4: Micrograph of fetal liver cells. Relatively large nuclei, rich in euchromatin can be observed, with prominent nucleoli and concentrations of heterochromatin located close to the nuclear membrane

Electron Microscopy: Electron microscopy revealed that the cells were bipolar, with strongly elongated shape. The cytoplasm was moderately dense, filled with glycogen granules and abundant with non-assembled ribosomes and fagosomes. Both smooth and granulated endoplasmic reticulum could be distinguished, in certain regions with enlarged cisterns and mitochondria with dense material and well-defined cristae. The nuclei of the cells were rich in euchromatin, with prominent nucleoli, in some cases located close to the nuclear membrane. On some preparations it can be seen that there were concentrations of heterochromatin along the nuclear membranes (Fig. 4).

DISCUSSION

To the present moment, methods of isolation of FLC from humans [8, 17, 18, 19], mice [20], rats [21], sheep [22, 23] have been published. The protocol we preferred was with mechanical dispersion of the fetal livers after the removal of connective and adipose tissues. We concluded that this method for isolation of the cell culture is appropriate, because of its simple performance and results comparable to those, obtained after following the more complicated protocols. Moreover, the enzyme treatment can lead to the induction of non-specific differentiation of the investigated cells. Some cells adhere to the surface of the culture dishes 24 hours after seeding. During the first days of culture, it appeared necessary to wash the monolayers with complete culture medium every day to remove most of the non-adherent cells and fragments.

Our findings confirmed the statement of Matsunaga *et al*. [24] that the FLC are defined by good proliferative activity and capacity for long-term culture. Similar characteristics have been shown by the earlier obtained from us human FLC [25]. Usually the latter reached confluent monolayer on the 6^{th}-7^{th} day of primary culture, while after passaging this period shortens to 5 to 6 days. Another phenomenon, characteristic for adherent fetal cells, is the formation of wave-like layers. The same has been observed in MSC as well [26].

In the early stages of its development, the fetal liver consisted of hepatoblasts, which are precursors of hepatocytes and billiary cells [27, 28] and haematopoietic cells, mostly erythroid progenitors [29, 30]. Our studies on the adherent fraction of human FLC showed that they express mesenchymal stem cells markers and morphology, quite similar to the ovine FLC (unpublished data).

The light microscpic appearance of the isolated ovine cells indicated that they have high nucleo-cytoplasmic ratio, which is characteristic to low-differentiated progenitor cells. This data is confirmed from the studies of Dan *et al*. [31] and Campanioli *et al*. [8] on human cells. The infrequently observed polygonal cells most likely are at a higher stage of differentiation, probably towards Kupffer cells.

In order to further characterize the obtained cell culture, electron microscopy investigations have been carried out. The results show once again that the cells are bipolar, elongated, with no clearly visible contact regions. Although these characteristics are attributed to fibroblasts, the investigated cells differ from them with their relatively smooth cytoplasmic membrane, without papillary projections and a cytoplasm relatively poor in organels. The nuclei are with large dimensions, rich in euchromatin. The nucleoli are prominent, usually more than one. In the majority of cases, they are located closely to the nuclear membrane, where also concentrations of heterochromatin can be observed, which is a morphological evidence for an intense biosintetic activity.

In conclusion, the growth properties of the ovine fetal liver-derived cells and the morphological findings suggested that MSC are predominant in the obtained adherent culture. The isolation protocol is relatively simple and the cells are easy to maintain *in vitro*. Nevertheless, a small portion of cells with features of more advanced stages of differentiation were found in all passages, which suggested the use of ovine fetuses at earlier gestational stage in future research work.

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