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CFU-GM Like Colonies Derived from Embryonic Stem Cells Cultured on the Bone Marrow Stromal Cells

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ABSTRACT

The aim of this study was to isolate mouse embryonic stem cells from late blastocyst stage embryos and to use them as a model system for the study of hematopoietic induction outside the embryo by coculturing of embryonic stem cells with bone marrow stromal cells. Blastocyst stage embryos from pregnant NMRI mice were obtained and cultured for 1-2 days in DMEM medium. The inner cell masses formed colonies 4-6 days after embryo hatching they were collected and trypsinized. Several subcultures were prepared in the medium containing 0.1 mM 2 mercaptoethanol, 1000 U/ml leukemia inhibitory factor and 10% fetal bovine serum. The embryonic stem cells were recognized by alkaline phosphatase histochemistry. Embryonic stem cells were cultured on inactivated mouse bone marrow stromal cells for 3 weeks at 37ºC and 33ºC. The colony assay was performed on semisolid medium containing murine IL-3 and IL-6 to determine the differentiation of embryonic stem cells to hematopoietic progenitor cells *in vitro*. Our results showed that with the effect of bone marrow stromal cells, a highly pluripotent stem cell which is derived from blastocyst stage embryo was able to primarily differentiated into the hematopoietic progenitor cells. CFU-GM like colonies were recognized according to their morphology after culturing the embryonic stem cells on the condition medium supplemented with IL-3 and IL-6. *Iran. Biomed. J. 8 (1): 1-5, 2004*

Keywords: Embryonic stem cells, Hematopoietic stem cells, Bone marrow stromal cells, CFU-GM colony

INTRODUCTION

Totipotent embryonic stem (ES) cells can be maintained in the undifferentiated state *in vitro* using leukemia inhibitory factor [1]. They have the ability to differentiate into several types of somatic cells such as neurons, muscles, endothelial and hematopoietic cells and germ line cells [2, 3]. Several methods have been applied successfully to the establishment of ES cell population. They were mainly obtained from embryo at the blastocyst stages [4-9]. Those cells have some unique characteristics such as high prolifereative capacity and developmental potential and have normal karyotype [7-9]. The ES cells are similar to embryonic cells and show comparable expression of stage specific embryonic antigens [10]. These cells grow as compact colonies of small cells with minimal amount of cytoplasm and prominent nucleoli [5].

ES cells have been used as a model for gene targeting, production of transgenic animals, cell therapy and studying of mechanisms and factors involved in the development of cells and tissue [10].

In vitro or *in vivo* ES cell differentiation has been studied by some investigators [1, 2]. After primary differentiation of ES cells *in vitro,* the embryoid bodies were formed, which could be differentiated under influence of cytokine, growth factor and selected stromal cells as feeder layer [2]. One alternative approach to study the early hematopoietic development is using the model system based on the differentation of ES cells in culture media. Recently, some investigators have showed differentiation of ES cells *in vitro* to all hematopoietic lineages such as erythroid, myeloid, lymphocyte and megakaryocyte [12-18]. Nakano *et al.* [12] reported that by growing and coculturing of ES cells on the feeder layer, which was derived from bone marrow stroma of the OP/OP

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(osteoperotic) mutant the hematopoietic cells were obtained. Li *et al.* [13] have used the mouse S17 stromal cells supplemented with cytokines for differentiation of rhesus monkey ES cells to hematopoietic linage. Palacios *et al.* [14] showed that after disaggregation of embryoid bodies into single cells and culturing them in methyl cellulose medium containing various cytokines, pure population of various hematopoietic lineage can be generated. Hematopoietic stem cells (HSC) are multipotent progenitors that proliferate and repopulate the hematopoietic system for extended periods during primitive and definitive hematopoietic activity. At first, hematopoiesis appears in the blood islands of the extraembryonic yolk sac and then shifts to the fetal liver, bone marrow and spleen [19]. Little is known about the cytokines and stromal cell interactions that regulate the hematopoiesis and the genesis of HSC in utero. Embryonic and fetal hematopoietic development appears to be reflected through *in vitro* differentiation of ES cells.

Here we report the isolation of ES cells from mouse blastocyst stage and after that we use these ES cells as a model system for studying of hematopoietic induction outside of the embryo by coculturing of ES with bone marrow stromal cells and a combination of cytokines.

MATERIALS AND METHODS

Collection and culture of embryo. All animals were cared according to the guide for the care and use of laboratory animals at Tarbiat Modarres University. Blastocyst embryos were collected by flushing the uterine horns of adult NMRI mouse 4 days after ovarian hyperstimulation, using human menoposal gonadotropin and human chorionic gonadotropin injection and natural mating. The embryos were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) for 1-2 days to allow hatching.

Isolation of inner cell mass (ICM) colonies and identification of ES cells. The hatched blastocysts were cultured for formation of inner cell mass colonies and attachment to the floor of plate. The ICM colonies were collected using the blunt heat sealed end of Pasteur pipette, transferred to a drop of 0.25% trypsin/0.02% EDTA under mineral oil and incubated at 37ºC for 3-4 min, 5% CO_2 and 95% air atmosphere. Then, the cells were separated using very fine Pasteur pipette filled with DMEM medium and washed several times with fresh medium.

The isolated cells were transferred to 96-well dishes containing DMEM supplemented with 10% FBS and 10% newborn calf serum, 0.1 mM β mercaptoethanol and 1000 U/ml leukemia inhibitory factor (LIF) [20]. Cells were cultured and incubated for two weeks at 37ºC in water saturated atmosphere and 5% CO_2. Medium was renewed after two or three days. Each well was daily examined for 14 days. The colonies with typical stem cell morphology were subcultured was similar to the previous procedure.

Histochemical staining for alkaline phosphatase (ALP) was carried out by azo coupling technique as described by Donovan *et al.* [21]. The cytospine cell population was fixed with acetone formaldehyde solution (1 min), then washed with deionized water and incubated with 1 mg/ml Fast Red TR salt and 40 µl/ml Naphtol As-Mx phosphate at a pH of 8.4 for 5 min. After washing and counterstaining using hematoxylin solution (2 min), the samples were examined under light microscope.

Mouse bone marrow cell collection and culture. After cervical dislocation of adult female NMRI mice, its femurs were cut transversely. The bone marrow cells were collected by flushing bone cavity several times. Cell suspension was centrifuged at 800 ×g for 5 min. After removing the supernatant and adding fresh medium, cell count was performed. The cells (2×10^6) were transferred to each well of 48-well Petri dishes, which contained 1 ml of DMEM supplemented with 25% BSA and 10^{-7} M hydrocortisone and then cultured in a 5% CO_2 humidified atmosphere at 37ºC for 3 weeks. The medium was renewed according to its colour each 3 or 4 days. After 3 weeks, bone marrow stromal cells were confluent and mitotically inactivated using 5 µg/ml mitomycin C [20].

Coculture of ES cells with bone marrow stromal cells and colony assay. The ES cells prepared earlier were co-cultured with inactivated bone marrow stromal cells for one week at 37ºC followed by one week at 33ºC and 5% CO_2. The medium for this step was the same as medium used for culturing the bone marrow stromal cells.

The culture media employed for the detection of hematopoietic progenitor cells were DMEM supplemented with 20% FBS, 10 ng/ml IL-3, 5 ng/ml IL-6, 1% agar (final concentration 0.03%) with 10% conditioned medium (lung conditioned media). This medium is specific for CFU-GM.

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After trypsinization and removal of the ES cells, which cocultured with bone marrow stromal cells, 10^4 to 10^5 isolated cells were transferred to the plate, filled with the medium described earlier. After culturing at 37ºC and 5% CO_2 up to four weeks the colonies were recognized and studied.

RESULTS

After embryo hatching, there were two morphologically distinct cell types: inner cell mass colonies and trophectoderm cells. The latter attached to surface of plate as a monolayer. The ICM cell colonies were small cells with a large nucleus and minimal amount of cytoplasm (Fig. 1) and were tightly packed. The morphology of ICM colonies in the first, second and third subculture was similar. However, after the fourth subculture, they formed a monolayer on the plate. The hatched embryo (89%) formed ICM colonies and only 10% of these colonies could produce ES cells.

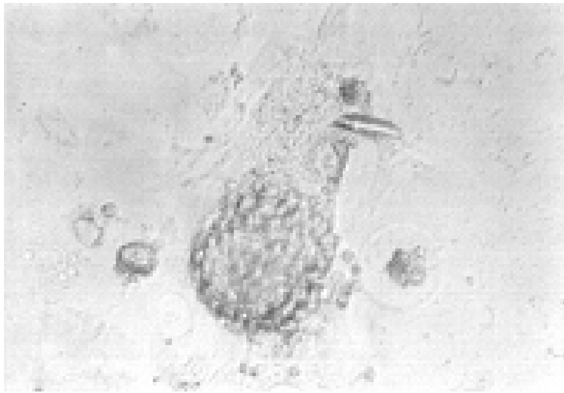

Fig. 1. Morphology of an ICM colony was illustrated 72 h after culturing of hatched blastocyst. Magnification × 100.

The undifferentiated stem cells had strong ALP reaction and the trophoblast cells had not ALP reaction at this stage (preimplantation period). Thus, ALP histochemistry was used for identification of ES cells. After ALP reaction red staining of cells indicated a positive enzyme reaction (Fig. 2). The enzymes were localized on the surface and cytoplasm of ES cells.

After transferring and culturing the ES cells on the inactivated bone marrow stromal cells for two weeks, the embryoid bodies were formed.

After colony assay on the semisolid medium containing IL-3, IL-6 and lung conditioned medium, the colonies were recognized morphologically as CFU-GM colonies (Fig. 3).

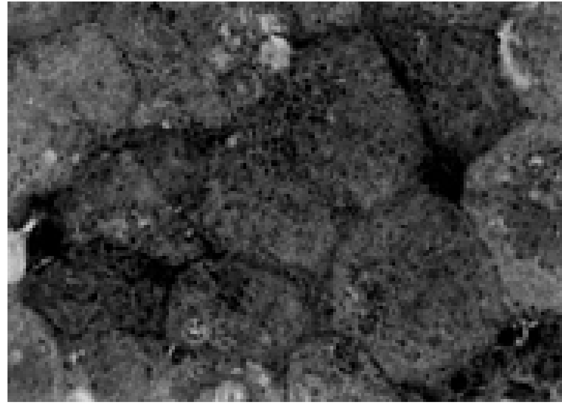

Fig. 2. Alkaline phosphatase histochemistry of ES cells, red staining of cells indicated a positive enzyme reaction. Magnification × 400.

DISCUSSION

The main purpose of this study was to establish the isolation and production of mouse ES cells from NMRI mice and then their differentiation to hematopoietic cells in controlled condition.

Our results showed that the ICM colonies obtained from early to late mouse blastocyst could produce ES cells. Some investigators reported that the proliferation rate of ES cells was rapid [1, 5, 23] but we observed that ES cell colonies were formed in a slow rate. These differences may be due to laboratory culture condition and strain of mouse [5, 23]. However, these cells had a high level of ALP reaction.

Our results demonstrated that LIF in the absence of feeder layer is efficient to prevent the differentiation of ES cells. Also, Pease *et al.* [24] demonstrated that ES cell lines could be isolated from murine 129/Sv He blastocysts in the absence of feeder cells in the culture medium supplemented with recombinant leukaemia inhibitory factor. In contrast, Anderson [25] had unsuccessful experiments in application of murine and human LIF assisting isolation of ES cells. Therefore, it appears that the action of LIF on ICM cells may be species and strain dependent [23, 26].

We showed that ES cells cocultured on a monolayer of irradiated bone marrow stromal cells could primarily differentiate into the embryoid bodies and after colony assay with condition media (Lung Condition Media), IL-3 and IL-6 they secondarily differentiated again to myeloid

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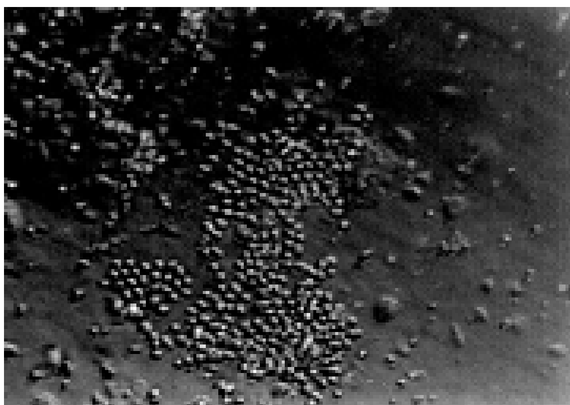

Fig. 3. CFU-GM like colony was formed after primary differentiation in the semisolid medium. Magnification × 100.

progenitor cells (CFU-GM like colonies). Our results indicated that adult mouse bone marrow stromal cells after long term culturing could prepare a suitable micro-environment which induce primary differentiation of ES cells. The nurse stromal cells secret some soluble factors, which mediate proliferation and differentiation of stem cells *in vitro* in a similar manner to *in vivo* condition.

The observation of other investigators showed the derivation of multilineage hematopoietic progenitors from ES cell lines *in vitro* [12-18]. They reported the use of stromal cell lines and conditioned media to enhance ES derived hematopoiesis. Nakano *et al.* [12, 27] have introduced a differentiation induction system for ES cells to hematopoietic cells using coculturing of ES cells on a novel stromal cell line OP9. They showed that ES cells gave rise to erythrocyte, myeloid and B lineage cells and concluded that OP9 system should facilitate the induction and development of hematopoietic cell differentiation. Kaufman *et al.* [28] showed that by coculturing murine bone marrow cell line S17 or yolk sac endothelial cell line with human ES cells, myeloid, erythroid and megakaryocyte colonies were derived. These cells expressed the cell surface antigen CD34 and the hematopoietic transcription factor TAL-1, LMO-2 and GATA-2. In this manner, Ling and Neben [29] reported that the addition of exogenous Flt-3L to the ES/OP coculturing system the mature and functional B lymphocytes were generated.

Our results showed that through the effect of bone marrow stromal cells a highly pluripotent stem cells derived from NMRI blastocyst stage embryo were able to primarily differentiated into CFU-GM like colonies. This technique provides an opportunity to understand hematopoiesis *in vitro*.

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