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The Effects of Insulin Like Growth Factor -1 (IGF-1) on the Plasticity of Umbilical Cord Blood Derived Mesenchymal Stem Cells Colonies to In Vitro Neurogenic Differentiation

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Abstract
Umbilical cord blood derived mesenchymal stem cells (UCB-MSCs) are pluripotent, easily expanded in culture, and has been much interest in their clinical potential for tissue repair and gene therapy. This study was performed to investigate the possibility of obtaining clonally expanded culture of MSCs derived from human UCB then studying the effect of Insulin like growth factor-1 (IGF-I) on the plasticity of MSCs colonies to their *in vitro* neurogenic differentiation and determining the differentiation pathway using neural marker (nestin). The mononuclear cells (MNCs) were obtained from cord blood after gradient density centrifugation, these cells were cultivated in a culture medium Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% Fetal bovine serum (FBS), then incubated at 37°C and 5% CO_2 for three weeks. In most cases, the cultures of plastic-adherent cells proved to be heterogonous. Both spindle-shaped and round cells were observed. Immunophenotypically, the MSCs were found to be positive for CD71 and negative for CD34. These results indicated that MSCs are not hematopoietic in origin. These cells after passage retained their fibroblast –like morphology. Regardless to the concentration of IGF-I, this growth factor stimulates the differentiation of MSCs –toward the neuronal pathway. So, the MSCs colonies showed the ability to maintain their plasticity to form the specialized cells (neuronal like cells) after treated with IGF-I even in culturing for long time and these cells stained positively to the nestin marker. In conclusion: the IGF-I promoted and maintained the plasticity of UCB-derived MSCs and their colonies to differentiate into neuronal-like cells.
Key words: Mesenchymal stem cells, Umbilical cord blood, Nestin, Neurogenic differentiation, Insulin like growth factor-1.

1. Introduction

Umbilical cord blood (UCB) has several characteristics that make it attractive for use in regenerative medicine. It contains populations of stem cells rather than hematopoeitic stem cells such as endothelial, epithelial, mesenchymal, and pluripotent embryonic-like stem cells (Harris *et a*l. 2007).

Mesenchymal stem cells (MSCs) are pluripotent, self-renewing cells with the potential for tissue regeneration (e.g., the repair of bone, cartilage, tendon, ligament, skeletal muscle, and cardiac muscle). MSCs have also been shown to transdifferentiate into cells of ectodermal origin, such as neurons (Patel *et al*. 2008; Waheed *et al*. 2011). Storch *et al*. (2004) described the generation of neuronal cells from human MSCs. These cells expanded in floating aggregates resembling neurospheres, displayed clonal capacity and were differentiated at the single cell level to both neurons and glial cells.

Mesenchymal stem cells are known to express the surface antigens Stro-1, NGFR, CD44, CD73, CD105, and CD106. However, expression of these antigens is not predictive of the differentiation potential of the cells (Phinney *et al.* 2006). MSCs can be expanded tremendously within a relatively short period of time. This rapid proliferation could result in an expansion of a thousand-fold in two to three weeks time (Pountos *et al.* 2007).

The most important property of MSC populations *in vitro* is their ability to form colonies after low-density plating or single-cell sorting (Javazon *et al*. 2004). As demonstrated by Owen and Friedenstein, (1988) the colonies which derived from colony forming unit-fibroblast (CFU-F) assays are extremely heterogeneous in both appearance (morphology and size) and differentiation.

Many factors affect the differentiation of MSCs; these include soluble growth factors (GFs) and cytokines. Insulin like growth factor – 1 (IGF) are part of a number of GFs and cytokines responsible for burst-like growth of early erythroid progenitor cells *in vitro* and may play a role in the ontogeny of marrow development (Lu *et al*. 2009).

IGFs have a variety of actions. In particular, IGFs promote cell population multiplication in cultures of numerous cell types (e.g. chondrocytes (Hiraki *et al*. 1986), myoblasts (Ewton *et al.* 1987), neuroblastoma cells (Mattson et al. 1986)], etc).

Insulin and IGF-I is a 70 amino acid single chain protein which has many growth-promoting and metabolic functions in the central nervous system (CNS), and correspondingly, their receptors are abundantly

expressed in the brain (Froesch *et al.* 1985). Insulin and IGF-I are neurotropic since they can support neuronal growth, survival, and differentiation in the absence of other GFs, and they promote neurite outgrowth, migration, protein synthesis, neuronal cytoskeletal protein expression, and nascent synapse formation. So, according to this fact, the present study aims to investigate the possibility of obtaining clonally expanded culture of MSCs derived from human UCB and studying the effect of IGF-I on the plasticity of MSCs colonies to their *in vitro* neurogenic differentiation then determining the differentiation by immunocytochemistry procedure by use of specific marker.

2. Materials and Methods

2.1 Cord blood collection

Human umbilical cord blood samples were obtained mainly from the Department of Obstetrics and Gynecology 'Azadi teaching Hospital in Duhok'. All UCB samples were collected freshly after normal deliveries. Immediately after delivery of the baby, the umbilical cord clumped, breaking the link between the baby and placenta. Each cord was washed out with antiseptic solution (70% ethanol, 10% Betadine), then the blood which is withdrawn by syringe. While placental was still *in utero*, the cord blood collected in tubes containing anti-coagulant (Jin *et al.* 2009).

All the experiments of the present study were carried out under sterile conditions and the experimental work of this study was done in Animal Tissue Culture Lab/Scientific Research Center at University of Duhok.

2.2 Isolation of mononuclear cells *(MNCs) derived from human UCB*

Each 10 ml of UCB sample was diluted with an equal volume of 0.9% normal saline (NaCl) and layered over an equal volume of Ficoll-Paque (1.077 g/mL) (US Biological: USA) then centrifugated for 30 min at 3000 rpm at room temperature. After density gradient centrifugation, the resulting mononuclear cells (MNCs) were retrieved from Buffy coat layer by pipetting, and then washed two times with 0.9% NaCl at 3000 rpm for 10 min. (Barkhatov *et al.* 2008).

2.3 Culturing and maintaining of M NCs derived from human UCB in vitro

The MNCs were suspended in 5 ml of Iscove's Modified Dulbecco's Medium (IMDM) (US Biological: USA) supplemented with 10% heat inactivated Fetal bovine serum (FBS) (Sigma: Germany) , 100000 U/L penicillin, and 50mg/L streptomycin, then plated in 25 cm2 plastic cell culture flasks. The cells were maintained at 37°C in 5% CO_2 for three weeks with 50% of media being changed every week. The cultures were inspected continuously by inverted microscope to get hold of developing adherent fibroblastoid cells. When adherent cells reached approximately 80% confluence, they were detached with trypsin- ethylenediaminetetraacetic acid (EDTA) [25% (w/v) powdered trypsin in 0.04% (w/v) EDTA in calcium and magnesium ion free phosphate buffer saline (CMF-PBS), washed twice with PBS, with centrifugation 3000 rpm for 10 minutes, and the supernatant (PBS) was aspirated and the cells were resuspended in 1ml of IMDM and were considered ready for imunophenotyping study and passaging.

In order to verify the nature of the MSCs, the cells were recultured in 96-multi –well tissue culture plates at a density of 1×10^4 cells/well in IMDM supplemented with 10% FBS at 37°C in 5% CO_2. The cells were allowed to grow and expand for nine days to form a monolayer of adherent fibroblast-like cells. Then the medium was aspirated and the multi-well plates washed two times with PBS, then were fixed with 4% formalin diluted in PBS for 10 min, then washed with PBS and they were left to dry, and they were ready for immunophenotypic procedure (Oh *et al*. 2008; Waheed *et al*. 2011)

To analyze cell-surface expression of typical marker proteins, UCB-MSCs were labelled with the specific markers (CD34, and CD71) (US Biological: USA) for immunophenotypic analysis. The immunocytochemistry staining procedure which dependent was according to the manufacturer instructions of the immunohistochemistry detection kit (Mouse IgG, BioAssay : US Biological: USA) (Lorette 1999).

2.4 The effects of Insulin like growth factor -1 (IGF-1) on the plasticity of MSCs colonies

Human UCB-derived adherent MSCs were cultured to form monolayer, then the cells were washed, harvested and seeded in suspension culture by non-adhesive bacteriological Petri dish (to prevent their attachment) for six days, then these suspended cells was used as a source of colony - stimulating activity of MSCs from UCB. Therefore, these cells were divided into three groups (each group contains 3.4×10^4 viable cells) as follow: Group No.1 (control group) treated with culture medium only, Group No.2 treated with 25ng /ml IGF-I (Sigma: USA) in IMDM+10%FBS, and Group No.3 treated with medium containing 50ng/ml IGF-I in IMDM+10%FBS. Then the cells were cultured in 4- multiwell plate and incubated for seven days. In this time the cells were attached and began to form undifferentiated colonies.

In order to evaluate the plasticity of MSCs colonies, the large colonies which were formed in (three groups) were carefully aspirated from culture by a finely drawn out Pasteur pipette attached to mouth-controlled

tube. These colonies were allowed to reculture in 96-multiwell tissue culture plates into three groups: the control, treated groups with 25 ng/ml IGF-I and 50 ng/ml IGF-I, and incubated under the same conditions. Cultures were screened by inverted microscopy to detect the attachment, growth and differentiation of MSCs from these colonies (Waheed & Hammash 2008).

2.5 Immunophenotyping analysis of neural marker for MSCs colonies

To evaluate neural differentiation capacity of MSCs colonies after treatment with IGF-I and in control group, the immunocytochemical staining for neural marker (nestin) (US Biological: USA) was performed to confirm the neural marker expressing cells in cultured of MSCs colonies. So, after treatment and differentiating of MSCs colonies, the medium was aspirated and the attached cells were washed for two times with PBS, then were fixed with 4% formalin diluted in PBS for 10 min, washed with PBS and they were left to dry, and they were ready for immunophenotypic analysis. The immunocytochemistry staining procedure which dependent was according to manufactures instructions of immunohistochemistry detection kit (Mouse IgG, BioAssay : US Biological: USA) (Lorette 1999).

3. Results

3.1 Morphological and cell expansion characteristic of human UCB–derived MSCs

Within two days of culture the morphological changes were observed in most of MNCs, these cells became elongated and some of them appeared with small unipolar processes (Fig. 1A) and on fifth day, most of the adherent cells began to proliferate and form fibroblast-like cells (Fig. 1B) while non adherent cells were discarded through continuous change of medium. As growth of cells continued and by the end of the third week of MNCs-derived MSCs culturing, an almost homogenous confluent layer of fibroblast-like cells occupied the whole plastic surface (Fig. 1C). According to the morphological aspect and growth characterization of these MSCs, they are considered to be fibroblastic F-type cells. After first passage, the culturing cells retained their fibroblast-like morphology and they showed high proliferation in culture.

3.2 Immunophenotyping analysis of MSCs

To determine the phenotypic nature of MSCs, the surface antigen CD34, and CD71, were examined by immunophenotypic staining technique. The results of immunophenotypic analysis showed that the adherent cells were strongly stained with $CD71^+$ and the cells appeared in brown color (DAB stain) (Fig. 2A). But they were negative for $CD34^-$ (Fig. 2 B). These results indicate that these cells are MSCs and not hematopoietic in origin.

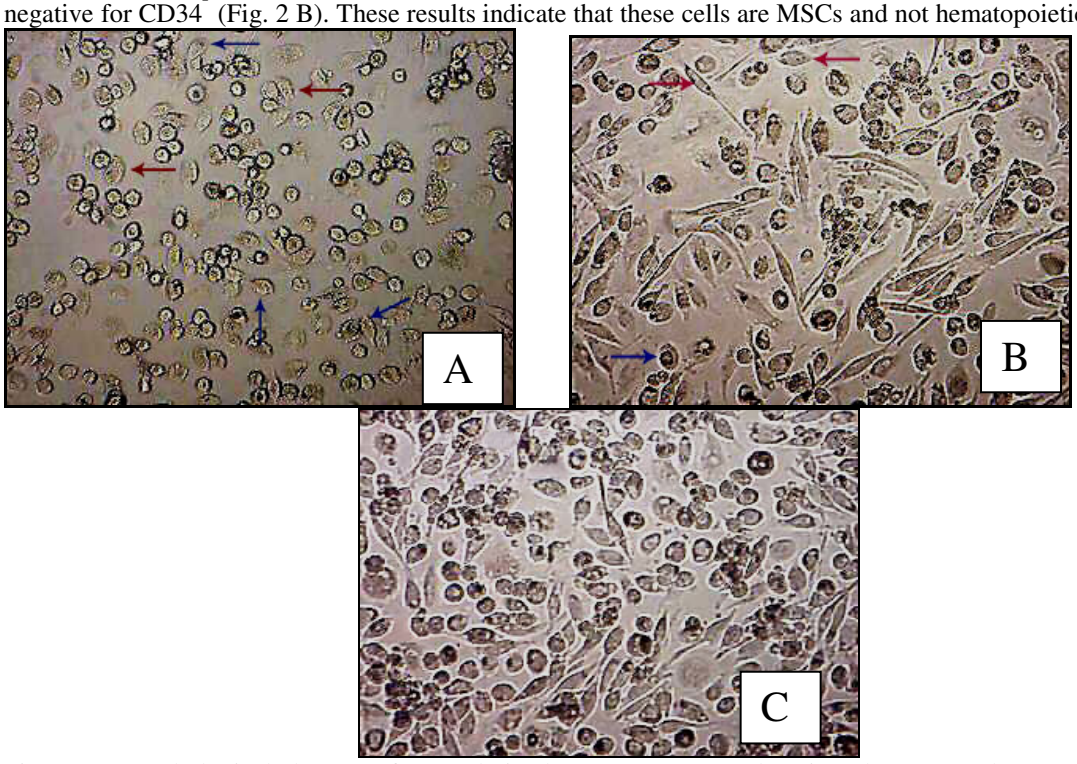

Figure 1: Morphological changes of UCB-derived MNCs, (A) Two days in culture, note that most of MNCs -derived MSCs become oval (red arrows) and other appear with small unipolar processes (blue arrows). (B) Five days in culture, note that the MSCs appeared in spindle shaped with short and long processes (red arrow) as well as some of a small round cell can also be seen (blue arrow). (C) Third week of MNCs-derived MSCs showing

the formation of homogenous layer of fibroblast-like cells and few small round cells. (X 100) (Living tissue).

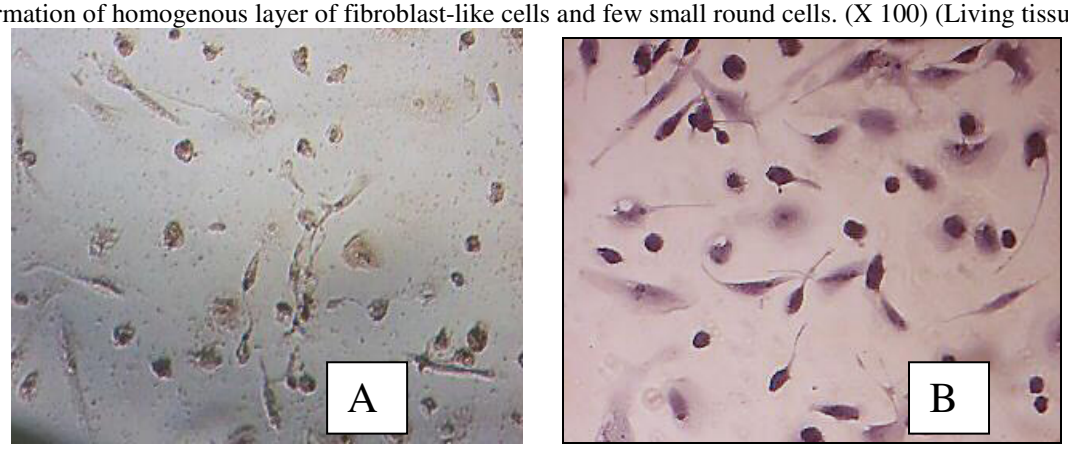

Figure 2: Immunophenotypic analysis of UCB-derived MSCs at the first passage. The positive cells are stain with brown color (DAB stain) while the negative cells are stain with blue color (Hematoxylin stain). (A): MSCs show positive response for CD71 (X100). (B): MSCs show negative response for CD34 (B: X100) (Fixed tissue).

3.3 The effects of Insulin like growth factor -1 (IGF-1) on the plasticity of mesenchymal stem cells colonies

The most characteristic *in vitro* features of MSCs are when these cells seeded at low density are their capacity to generate single-cell derived colonies (Fig. 3). The single precursors cells with colony-forming ability are usually used as an indicator for mesenchymal progenitor potential are also termed CFU-F in case of control group, while in treated groups is called CFU-derived neuronal precursors. The plasticity of MSCs colonies to generate specialized cell types was demonstrated conclusively by the observation that within 48hr from the re-culturing these large individual colonies, (in both control and treated groups) were attached and several cells were formed. Then on the sixth day of culture, these cells began to migrate from these colonies and occupy the surrounding area. The result of the present study showed that's regardless to the concentration of IGF-I, this growth factor stimulates the differentiation of MSCs –toward the neuronal pathway and the type of cells which observed in case of control group was the fibroblast-like cells while in the IGF-I treated groups was neuronal - like cells (Fig 4 A, B).

The result of immunophenotyping analysis to neuronal marker (nestin) showed that in control group these colonies with their cells stained negatively to nestin marker (Fig. 5A), while in both treated groups with IGF-I, stained positively to this marker (Fig. 5B), that means the MSCs with their colonies have the ability to maintain their plasticity to form the specialized cells often in culturing for a long time.

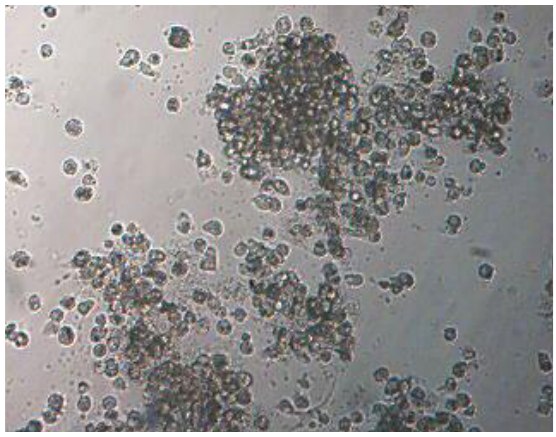

Figure 3: This figures showing the formation of MSCs colony (X100) (Living tissues).

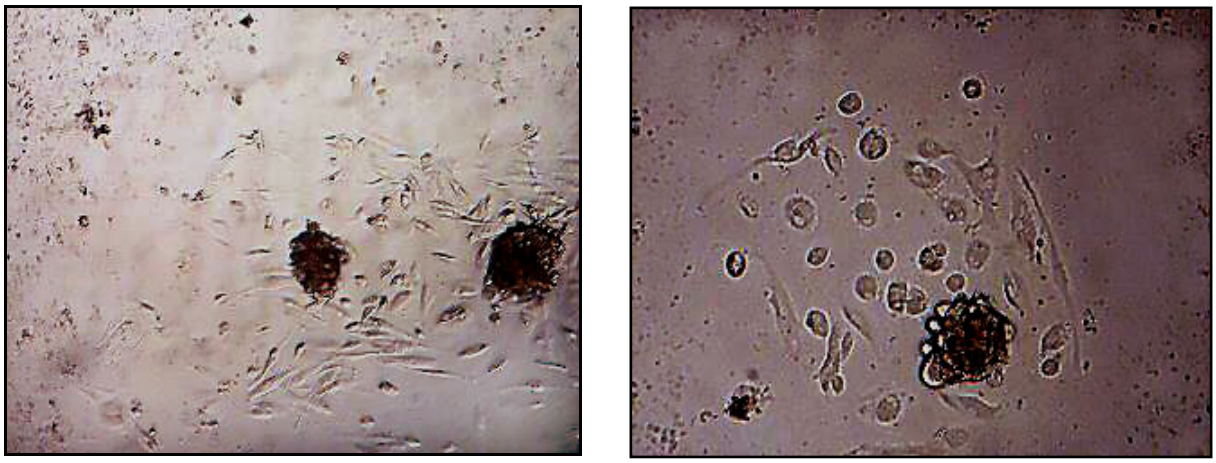

Figure 4: (A) This figure shows the plasticity of aspirated MSCs colonies from control group to generate fibroblastoid MSCs (X50) (Living tissue). (B) This figure shows the plasticity of the MSCs colony in treated group with 50ng/ml IGF-I, that's after re-culturing these colonies, they showed highly proliferating rate and form many neuronal precursor cells which migrate from this colony to the surrounding area (X100) (Living tissue).

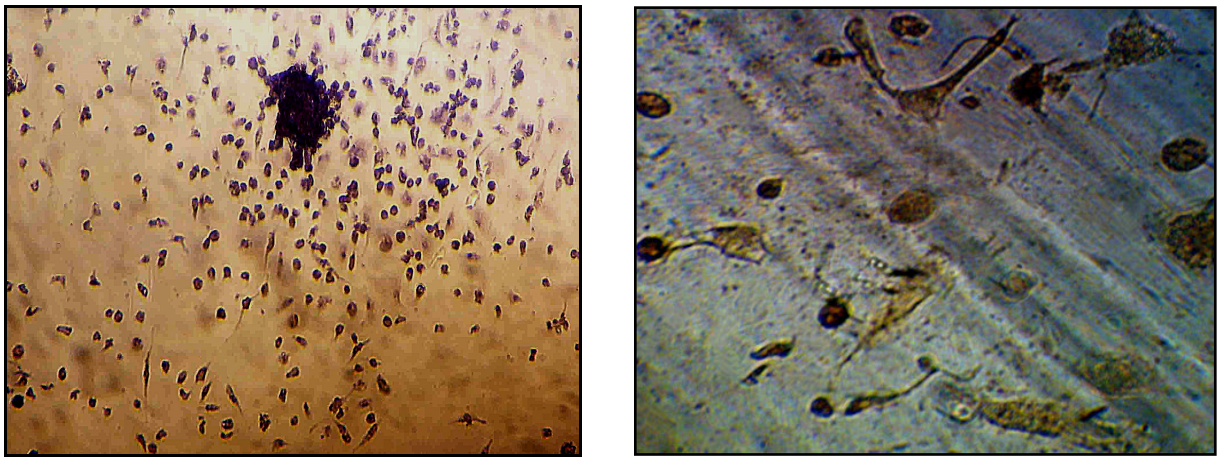

Figure 5: (A) This figure shows that the MSCs colony and their fibroblastoid cells in control group stained negatively to nestin marker and appears in blue color (X50) (Fixed tissue). (B) This figure shows that MSCs colonies and their neuronal –like cells stained positively to nestin marker (neuronal marker) and stained with brown color (X200) (Fixed tissue).

4. Discussion

The general approach to the culture of MSCs involves isolating the MNCs containing MSCs from CB and seeding these cells on tissue culture flask in the presence of IMDM containing FBS. Within the time of culture non adherent hematopoietic stem cells (HSCs) are removed through continuous change of medium, while the adherent cells which have a fibroblastoid morphology and high replicative capacity are cultured and passaged to expand the MSCs population. These results are in agreement with the results described by (Karahuseyinoglu *et al.* 2007, Waheed *et al.* 2011), who demonstrate that under routine culture conditions for UCB- derived MNCs-derived- MSCs can be divided into two major categories: adhering and non adhering cells. Further more MSCs can also be classified according to their morphological aspect and growth characteristic into two groups: epitheloid E-type cell and fibroblastic F-type cell. The fibroblastic F-type cell were considered to originate from mesenchymal tissue and usually appeared late during routine MSCs cultivation. According to this fact and from the results of this study we proposed that fibroblastic F-type cells in UCB and conformed that they are mesenchymal in origin.

In the present study, surface antigen CD34 and CD71 were used, to determine the phenotypic analysis of MSCs by technique of immunocytochemistry. This technique is reliable, simple, easy to do and easy to inter plus more sensitivity and specificity. The resolution of the results was very clear, sharp and strong. The isolated MSCs are spindle-shaped with short and long processes, positive for CD71 and negative for CD34, These results

were also observed by others (Kern *et al* 2006), they demonstrated that the UCB-derived MSCs showed a high percentage of CD71 positive cells by immunostaining and negative for CD34. One of the characteristic *in vitro* features of MSC when seeded in low density is their capacity to generate single-cell derived colonies of adherent cells.

A number of different cytokine combinations have been tested for the ability to expand progenitor cells. A cytokine that has not been extensively studied in this regard is IGF-I. IGF -I is released in the bone marrow (BM) by stromal cells, it can also be released by distant organs into serum/extracellular fluid and thereby function as a systemic regulatory mechanism of hematopoiesis (Shimon & Spielberg 1994). IGF-I has complex regulatory functions on adult human hematopoietic progenitor cells and can stimulate the proliferation of both myeloid and erythroid progenitors (Aron 1992**).** In the present study, we investigated whether IGF-I can be used for *in vitro* expansion of UCB-derived MSCs and their neurogenic differentiation.

Different sources of adult stem cells are currently explored for their potential use to repair degenerated CNS tissue. Due to their high degree of accessibility and plasticity, MSCs might have the potential to be used for this purpose, given that they promoted regeneration and provided neuroprotection and functional recovery after transplantation in the lesioned CNS (Zhang & Chen 2005).

Various GFs cytokines, and neurotrophic factors influence neurogenesis and the differentiation fate of NSCs. IGF-I was of particular interest because it was reported to be capable of inducing adult neural progenitor cells to differentiate into oligodendrocytes. IGF-I is the only potent oligodendrogenic factor described for adult neural progenitor cells (Hsieh *et al.* 2004).

From the results of the present study the differentiation of UCB-derived MSCs into neuronal-like cells in both treated groups of (25 and 50 ng\ml IGF-I) were observed as compared with control groups, and can concluded that the two different concentrations of IGF-I used in this study have the same effect on their potential to stimulate the differentiation of UCB-derived MSCs to neuronal–pathway. This high potential of neural differentiation along with using efficient differentiating stimuli may make these cells promising candidates for experimental treatment of neural injuries.

The actions of IGF-I on the differentiation of UCB-MSCs appeared to be similar to their actions on the neural stem cells (NSCs). Arsenijevic *et al*., (2001) reported that IGF-I is a key factor in the regulation of NSC proliferation in part through distinct intracellular mechanisms. IGF-I receptor family is evolutionarily conserved from invertebrates to mammals, and IGF-I receptors are present throughout the central nervous system during embryogenesis. IGF-I and IGF-II are expressed during brain development and are believed to act on virtually all neural cells. The IGF-I receptors are expressed in germinal regions that co-localize both EGF receptors and the FGF-receptor-1, considering that IGF family members play an important role in telencephalic development (De Pablo & De la Rosa 1995).

The present results of the differentiated UCB-derived MSCs to neural-pathway were confirmed with detection technique by immunocytochemical analysis for neural marker (Nestin) and showed that the detection by nestin marker express positive response of the differentiated cells which accept chromogen DAB stain. Nestin, an intermediate filament protein, is a neural precursor cell marker that has been expressed in rats, mice, and humans (Selander & Edlund 2002) and expressed in undifferentiated cells during the development of brain and is considered as a marker for neuroepithelial stem cells (Rani *et al.* 2006).

5. Conclusion

In conclusion: the IGF-I promoted and maintained the plasticity of UCB-derived MSCs and their colonies to differentiate into neuronal-like cells.

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