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Isolation of stem cells from adult rat kidneys

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ABSTRACT: The kidney has an inherent ability for recovery and regeneration following acute damage. However, there has been much contention as to the source of regenerating renal cells. The aim of this study was to isolate and characterize these cells. Normal rat kidneys were minced and cells were isolated with collagenase I and were cultured in an expansion medium. Adherent cells were isolated and expanded for more than 120 days *in vitro*. These cells had the potential of trans-lineage differentiation into neural cells, adipocytes and osteocytes. These cells also expressed Nucleostemin, Cyclin D1, Notch1 and Survivin which are commonly expressed in stem cells. The results of the current work show that the adult kidney contains a population of multipotent stem cells.

Introduction

The suboptimal current treatments for debilitating disorders such as acute and chronic renal failure have led to the search for enhanced therapeutic options. Stem cell based approaches seems to be promising and the potential of bone morrow stem cells and embryonic stem cells for the treatment of renal injury and kidney tissue engineering has been vastly investigated (Lazzeri *et al.,* 2007; Guillot *et al.,* 2008; Morigi *et al.,* 2004; Herrera *et al.,* 2004).

Tissue specific stem cells have been found in different organs and there are several proofs for the existence of such cells in the kidney. Renal cells have the potential for proliferation and differentiation after injury. These proliferating cells express vimentin, a mesenchymal marker, and Pax-2, a transcription factor essential for embryonic development of the kidney, which suggests their immature phenotype (Maeshima *et al.,* 2002; Lin *et al.,* 2005). Considering the slow cycling property of stem cells, Maeshima *et al*. used a bromodeoxyuridine retaining method, to show that adult kidneys possess progenitor cells (Maeshima *et al.,* 2003). The renal papilla was suggested to be a niche for these cells (Oliver *et al.,* 2004).

Renal stem cells are probably the main source of healing after ischemia (Lin *et al.,* 2005; Duffield *et al.,* 2005). Therefore, they are a promising target for designing new therapeutic approaches. Different studies have shown the presence of stem cells in kidneys (Chen *et al.,* 2008; Plotkin and Goligorsky, 2006; da Silva Meirelles *et al.,* 2006). However, primary methods for isolating these cells and studying their characteristics should be further developed.

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The purpose of this work was to isolate and characterize adult rat kidney stem cells. We developed a method for isolation of these cells and evaluated their differentiation potential and transcription profile.

Materials and methods

Animals

Normal female Wistar rats weighting 180-230 g were purchased from Pasteur Institute of Iran. Animal care and experiments were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Isolation of renal cells

Rats were anesthetized with intraperitoneal injection of Ketamin (80 mg/kg) and Xylazine (8 mg/kg). Kidneys were harvested and the rats were euthanized. After removal of perinephric fat and renal capsule, the kidneys were minced and incubated in collagenase I at 37ºC for 45 minutes. The dispersed cells were then collected by centrifugation.

Cell Culture

The isolated cells were disseminated in Dulbecco's modified Eagle medium (DMEM) (GIBCO-BRL, Grand Island, NY, USA) containing 20% fetal bovine serum (GIBCO-BRL), stem cell factor (20 ng/ml) (R&D Systems, Inc. Minneapolis, Minn., USA), basic fibroblastic growth factor (bFGF) (25 ng/ml) (PeproTec, Rocky Hill, New Jersey, USA) as well as penicillin and streptomycin (GIBCO-BRL). After 24 hours non-adherent cells and debris were discarded and fresh medium was added to the adherent cells. Cultures were maintained at 37ºC and 5% CO2.

Differentiation

Cells were deposited in 24-well plates with DMEM culture medium containing 20% FBS. Neural differentiation was obtained in the presence of 3-isobutyl-1-methylxanthine (isobutylmethylxanthine, 0.5 mM) (Sigma), dibutyryl cyclic AMP (1mM) (Sigma) and retinoic acid 10^{-6} M. Immunostaining with neuron-specific enolase antibody (DAKO, Copenhagen, Denmark) was used to evaluate neural differentiation. For osteocyte differentiation, the cells were treated with 10 mM beta-glycero-phosphate (Merck, Darmstadt, Germany), 50 g/ml ascorbic acid 2-phosphate, and 10^{-7} M dexamethasone (Sigma). The presence of calcium deposits was assessed by staining with Alizarin Red S (Sigma) for 10 min at 4ºC, after fixing the cells with paraformaldehyde 4%. Adipocyte differentiation was achieved in the presence of dexamethasone (10^{-7} M) and isobutylmethylxanthine (0.5 mM). Oil Red O staining was used to assay the accumulation of oil droplets in the vacuoles.

Immunocytochemistry

Immunostaining was performed on the cells fixed in 4% paraformaldehyde for 15 minutes and permeabilized with 0.4% triton X-100 for 20 minutes at room temperature. After inactivation of endogenous peroxidase with 0.1% H_2O_2 for 20 minutes, the cells were preincubated with 1% bovine serum albumin in phosphate-buffered saline (pH: 7.4) for 30 minutes. The cells were incubated with HRP-labeled anti neuron-specific enolase antibody at 4°C overnight, then washed with PBS-Tween 0.1% three times and incubated with diaminobenzidine solution (Sigma) for 10 minutes.

Gene Expression Analysis

Total RNA was isolated and random hexamer primed cDNA synthesis was carried out using revert aid first strand cDNA synthesis kit (Fermentas, Ontario, Canada). For PCR amplification, primer sequences were as follows: Survivin forward: TCTACACCTTCAAGAACTGGC; Survivin reverse: TTCTTCCACCTGCTT CTTGAC; Cyclin D1 forward: ATGTTCGTGGCCTCTAAGATG; CyclinD1 reverse: TGCGGATGATCTGCTTGTTC; Nucleostemin forward: TCCGAAGTCCAGCAAGTA TTG; Nucleostemin reverse: AATGAGGCACCTGTCCACTC; Notch1 forward: AGAT GCTCCCAGCCAAGTG; Notch1 reverse: CCATGGTCCACAACATAGCAC; Oct4 forward: AAGCTGCTGAAACAGAAGAGG; Oct4 reverse: ACACGGTTCTCAATGC TAGTC; Beta 2 microglobin forward: CCGTGATCTTTCTGGTGCTT; Beta 2 microglobin reverse: TTT TGGGCTCCTTCAGAGTG. The reaction mixture was heated at 94ºC for 2 minutes and then subjected to 30 cycles of denaturation (94ºC, 30 sec), annealing (55ºC, 40 sec) and extension (72ºC, 40 sec), followed by one cycle of 72ºC, 4 minutes. PCR products were separated on a 2% agarose gel and visualized by staining the gel on ethidium bromide.

Isolation and characterization of bone marrow mesenchymal stem cells

Rat bone marrow mesenchymal stem cells were isolated and characterized as previously described (Gheisari *et al.,* 2008).

Results

Isolation of adherent fibroblast-like cells from adult kidney

About 3-5 days after isolation of renal cells, colonies displaying adherent fibroblast-like morphology were visible. Fresh medium was added to the cells after 3 days. Non-adherent cells and debris were discarded after 1 week by exchanging the medium completely. Adherent cells proliferated and formed a monolayer of about %80 confluence after about 1 month (Fig. 1). These adherent cells could be readily expanded by successive cycles of trypsinization, seeding and culture for more than 120 days *in vitro*.

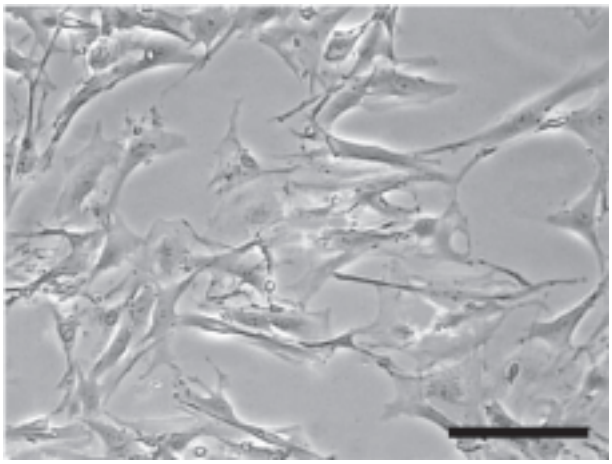

FIGURE 1. Fibroblast like morphology of the isolated cells from adult rat kidney. Scale bar: 200 µm.

Differentiation potential of isolated Cells

To evaluate the differentiation potential of the isolated and expanded cells from adult rat kidney, the cells were seeded at a density of about $1\infty10^3$ cells per well of a 24-well plate and treated with osteogenic, adipogenic, and neurogenic media for two weeks. In the case of neural differentiation, morphologically long and thin processes were apparent after about 7-10 days. Staining with HRP-labeled anti neuron-specific enolase after two weeks suggested differentiation to neural lineage (Fig. 2a). However, we acknowledge that, functional tests are required to further confirm differentiation to neurons. In the case of adipogenic differentiation, lipid vacuoles appeared in the cytoplasm and began to develop and coalesced over time. Oil red O staining in-

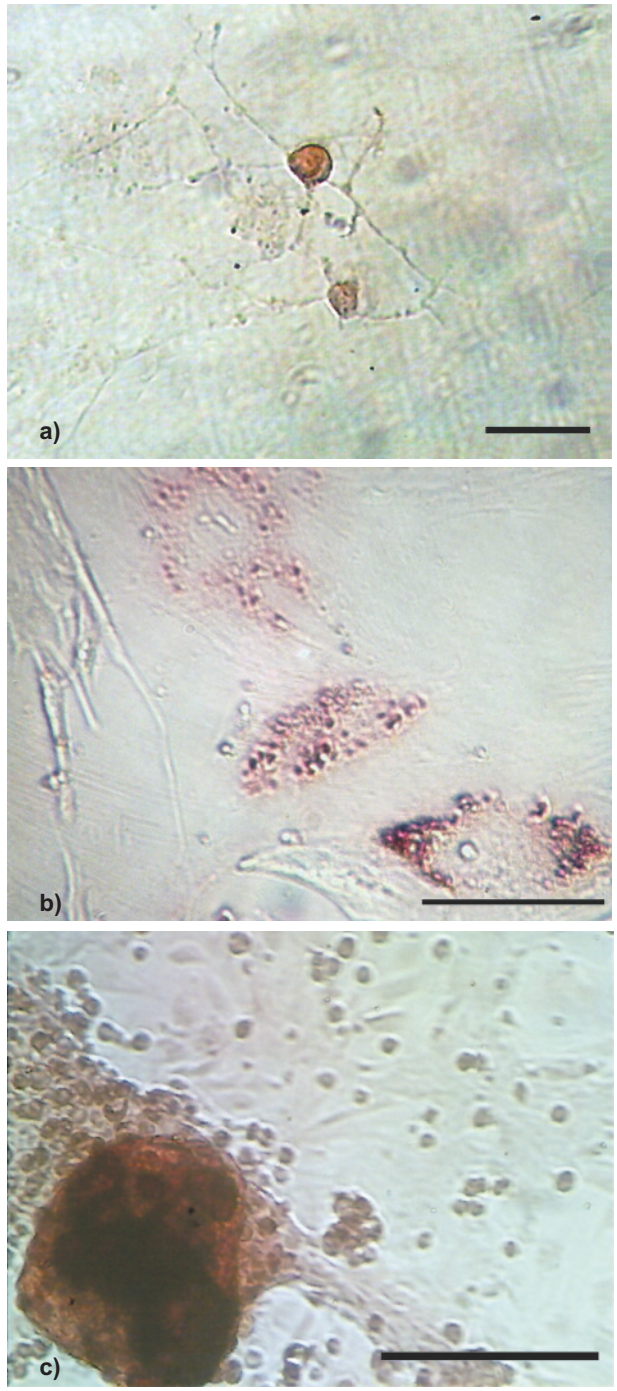

FIGURE 2. Differentiation potential of renal stem cells. a) Immunostaining with HRP-labeled neuron-specific enolase for detection of neural differentiation. b) Oil Red O staining for adipocyte differentiation. c) Alizarin Red S Staining for osteocyte differentiation. Scale bars: 200 µm.

dicated adipocyte differentiation in these cells (Fig. 2b). These cells also underwent osteogenic differentiation. Calcium deposits, were stained with Alizarin red S (Fig. 2c).

Gene Expression Patterns

For a more detailed study of the isolated cells, RT-PCR analysis was performed for some of the genes which are commonly expressed in stem cells. Our results showed that these cells expressed notch1, cyclin D1, nucleostemin and survivin; oct-4 was not detectable. The same analysis was performed for rat bone marrow mesenchymal stem cells. The expression pattern of the evaluated genes was similar in these two cell populations (Fig. 3). The bone marrow mesenchymal stem cells population that was used in this study had been previously characterized in our lab (Gheisari *et al.,* 2008).

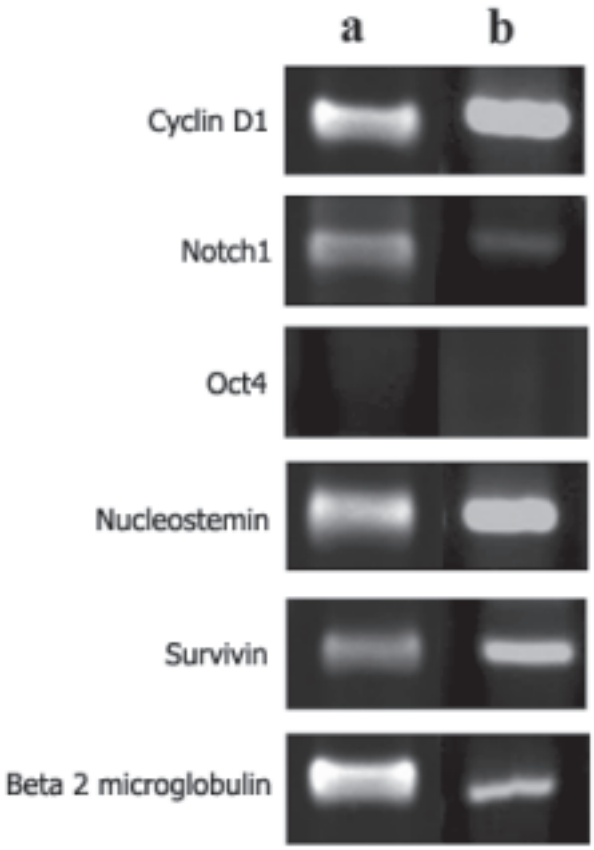

FIGURE 3. RT-PCR analysis for cyclin D1, notch1, oct-4, nucleostemin and survivin in the rat renal stem cells (a) and bone marrow mesenchymal stem cells (b). Beta 2 microglobulin was used as an internal control.

Discussion

The results of the current study indicate that there exists a population of stem cells in the kidney that show some similarities to bone marrow mesenchymal stem cells. This finding is in line with a previous report showing that mesenchymal stem cells exist in most adult organs (da Silva Meirelles *et al.,* 2006). In addition, similar to our study, some other investigators have shown the presence of stem cells in kidneys (Bussolati *et al.,* 2005; Sagrinati *et al.,* 2006; Dekel *et al.,* 2006). Further studies are required to compare renal stem cells isolated by different methods.

The expression of some genes implicated in the proliferation and self renewal of stem cells was studied by RT-PCR assay. The cells expressed Survivin, Nucleostemin, Notch1 and Cyclin D1. Survivin is a 17 kD Protein with dual role both in promoting cell proliferation and inhibition of apoptosis. This protein is expressed during embryonal development but is absent in most normal terminally differentiated cells. Its overexpression is seen in most cancer cells (Zangemeister-Wittke and Simon, 2004; Johnson and Howerth, 2004; Wheatley and McNeish, 2005). CD34+ hematopoietic stem cells also express this protein (Fukuda *et al.,* 2002; Fukuda *et al.,* 2004). Nucleostemin is a nucleolar protein recently cloned and described by Tsai and McKay (Tsai and McKay 2002). It is a P53 binding protein commonly found in the nucleoli of cancer cells, embryonic and neural stem cells and placenta tissue, but not in terminally differentiated cells. It is also assumed to have a central role in the self renewal and control of cell cycle in these cells (Liu *et al.,* 2004; Sijin *et al.,* 2004; Tsai and McKay, 2005; Kafienah *et al.,* 2006). Notch is a conserved signaling system that regulates variety of cell-fate decisions, stem cell maintenance and initiation of differentiation in embryonic and postnatal tissues (Grego-Bessa *et al.,* 2004; Kojika and Griffin, 2001; Greenwald, 1998). Notch 1 signaling is involved in the development of many organs including renal proximal tubules and podocytes (Cheng and Kopan, 2005). Cyclin D1, a common marker of highly dividing cells including stem cells and cancer cells (Yaghoobi *et al.,* 2005; Fu *et al.,* 2004; Rao and Stice, 2004), is essential for progression of the G1-S phase of the cell cycle. Taken together, the expression of these genes in the isolated renal cells implies a potential role for them in controlling proliferation, survival and self renewal. To compare this expression pattern with the profile of bone marrow stem cells, the same RT-PCR analysis was performed for these cells. Interestingly the

pattern of expression of the evaluated genes was the same in the isolated renal stem cells and bone marrow stem cells. Although the number of analyzed genes is inadequate for an absolute assessment, it could be hypothesized that similar pathways are used by the isolated renal cells and bone marrow stem cells to control the properties related to "stemness".

These cells have a gene expression pattern similar to undifferentiated stem cells *in vitro*. However we don't know if these cells are in a similar undifferentiated state *in vivo*. It has been suggested that the role of stem cells in the kidney could be played by some of the differentiated cells (Vogetseder *et al.,* 2005).

Tissue specific stem cells are commonly expected to differentiate into cell types of the organ of origin, but our results show that renal stem cells are also capable of differentiating into cells that are not commonly found in the kidney such as osteocytes. This implies that these cells probably have high plasticity.

One of the pitfalls of our stem cell isolation protocol is that it is a simple method based on the adherence property of the cells. Therefore, it is possible that the isolated cells are heterogeneous and the above-mentioned features could not be necessarily attributed to the whole cell population. In addition, it is possible that there exist other kinds of stem cells in the kidney that we were not able to detect by this method. The kidney is histologicaly a complex organ with at least 26 different types of cells. It seems unlikely that a single adult stem cell may be capable of forming all these cells (Zerbini *et al.,* 2006; Lin, 2006). We hypothesize that there exists more than one type of stem cells in the kidney and different methods of isolation could result in different cell types.

Gupta *et al.* have developed a method for isolation of renal stem cells named multipotent renal progenitor cells (Gupta *et al.,* 2006). Their method is similar to our method, except that they used different cytokines and mediators in the process of cell isolation and expansion. It seems that multipotent renal progenitor cells and the cells isolated in this study are in some way similar; however, the effect of these different combinations of cytokines on the type of isolated cells remains to be studied.

The adult kidney has the ability to regenerate following injury. The potential sources of regenerating cells include surviving epithelial cells, bone marrow cells and renal stem cells (Lin *et al.,* 2006). It has been shown that 28 day after injury, the majority of remaining cells are descendants of either surviving epithelial cells or renal stem cells, suggesting that intra renal cells are the main source of recovery following kidney injury (Lin *et al.,* 2005). Similarly, Duffield *et al*. (2005) used three different methods for cell labeling to evaluate the participation of bone marrow cells in renal injury. They concluded that these cells do not make a significant contribution to epithelial repair. Instead intrinsic tubular cell proliferation accounts for the replacement of most renal tubular cells following ischemia (Duffield *et al.,* 2005). The characteristics of these intrinsic renal cells that are capable of regeneration has not been yet clearly defined.

Successful isolation of renal progenitor cells would be essential for replacement and regeneration of damaged cells. The renal stem cells are potentially clinically useful because these cells, at variance with bone marrow stem cells are probably more committed to renal cells and they have similar properties to differentiated renal cells.

In conclusion the results of the current study indicate that there is a population of stem cells in the adult kidney. These cells are probably one of the sources of regeneration after kidney injury. They could be valuable in cell based therapeutic approaches for the treatment of renal failure.

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