

Preservation of chondrogenic potential of mesenchymal stem cells isolated from osteoarthritic patients during proliferation in response to platelet-derived growth factor (PDGF)

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Abstract

Mesenchymal stem cells (MSC) exert unique ability to differentiate into several cell lineages including: chondrocytes, osteoblasts, adipocytes and myocytes. These properties place MSC as a very promising source of cells for regenerative medicine. However, the optimal conditions for MSC propagation in vitro that could preserve their ability to differentiate into these lineages have to be elucidated. In the present study we tested the hypotheses that: (i) low oxygen concentration, and (ii) the presence of select growth factors, facilitates MSC proliferation in vitro without affecting their chondrogenic potential. MSC cultured in reduced oxygen (5%) proliferated more rapidly than in normal oxygen concentration (21%). Although all tested cytokines: PDGF-BB, FGF, IL-1 β , IL-15 and TNF- α stimulated MSC proliferation in reduced oxygen level, the best effect was observed in the presence of PDGF-BB. MSC cultured in low oxygen tension and in the presence of PDGF-BB retain their ability to differentiate into chondrocytes. Cultured in chondrogenic medium containing ITS, dexamethasone, TGF- β and ascorbic acid, MSC started to synthesize mRNA encoding chondrocyte specific genes: aggrecan and collagen II. Therefore, culture in low oxygen tension in combination with PDGF BB can be used to propagate MSC in vitro without affecting their chondrogenic potential.

Key words: mesenchymal stem cells, cytokines in vitro culture

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Introduction

Mesenchymal stem cells (MSC) are present in adult bone marrow and several other tissues [1]. Upon specific stimulation/culture conditions these cells differentiate into specialized cells capable to form tissues like bone [2], cartilage [3], muscle [4], tendon [5], fat [6] or hematopoiesis supporting marrow stroma [7]. Due to these properties, there is increasing interest in MSC as a potential source of cells/tissues that could be used for tissue regeneration.

However, there are reports indicating that multi-lineage potential of MSCs can be affected by conditions used for their propagation in vitro [8,9]. Although some progress has been made, the optimal conditions for MSC propagation have to be elucidated. In the present study we tested the hypotheses that (i) low oxygen concentration, and (ii) the presence of select growth factor(s), facilitates MSC proliferation in vitro without affecting their chondrogenic potential. We report that MSC cultured in low oxygen and

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the presence of growth factors proliferate and retain their ability to produce chondrocyte-specific proteins.

Materials and methods

Bone marrow aspirates were isolated from bone marrow of osteoarthritis (OA) patients (n=5; age 52±8 years) undergoing joint replacement. Nucleated cells were separated using standard density gradient centrifugation on Gradisol G (Aqua Medica, Lodz, Poland), washed with phosphate buffered saline (PBS, Lublin, Poland) and cultured for 5 days in DMEM culture medium (Gibco, USA) supplemented with 15% fetal bovine serum (FBS, Biochrom, Berlin, Germany). Next, non adherent cells were discarded and adherent cells after trypsinisation (Trypsin-EDTA solution 10x, Sigma) were harvested for: (i) the analysis of the expression of surface markers characteristic for MSC, and (ii) further culture in tested conditions in DMEM medium as stated above.

Expression of MSC characteristic surface molecules CD105 (Serotec), and CD166 (Pharmingen), or lack of monocytes/macrophages specific CD14 (Pharmingen), and leukocyte specific CD45 (Pharmingen) markers on cultured cells were analyzed using fluorochrome conjugated respective antibodies and flow cytometry (FACScalibur, Becton Dickinson, USA).

To estimate the influence of oxygen tension on MSC proliferation, washed adherent cells (10^4 /well) were cultured in DMEM supplemented with 10% fetal bovine serum at 5% (nitrogen was used as a gas to replace oxygen) or 21% oxygen concentration for 5 days, followed by addition of [3 H]-TdR (Amersham, UK, 1 μ Ci/well) for 24 hours, cells harvesting and counting DNA incorporated radioactivity using Rackbeta liquid scintillation counter (Pharmacia, Sweden).

The effects of select cytokines on MSC proliferation were estimated based also on [3 H]-TdR incorporation after 7 days of culture in the presence of: FGF (R&D, USA, 5 ng/ml), PDGF (ICN, 5 ng/ml); IL-1 β (R&D, 1 ng/ml), IL-15 (R&D, 25 ng/ml), TNF α (R&D, 10 ng/ml).

To confirm chondrogenic potential of propagated in vitro MSC, cells were harvested using trypsin/EDTA solution (Sigma, USA, 0.125 trypsin, 0.05 mg/ml EDTA), washed and resuspended in Matrigel (Becton Dickinson, USA) at density 5×10^6 /ml, and cultured in DMEM medium containing insulin, transferin, selenite (ITS) (Becton Dickinson, USA), dexamethasone (Sigma, USA, 10^{-7} M), TGF β (R&D, USA, 10 mg/ml), ascorbic acid (Sigma, USA, 50 mg/ml). After 7 days in culture, cells were harvested and total RNA was isolated using TRISOL reagent (Invitrogen, USA) according to the manufacturer protocol. The expression of mRNA encoding chondrocyte specific genes: aggrecan and collagen IIA, and house keeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) were analyzed using semiquantitative RT-PCR.

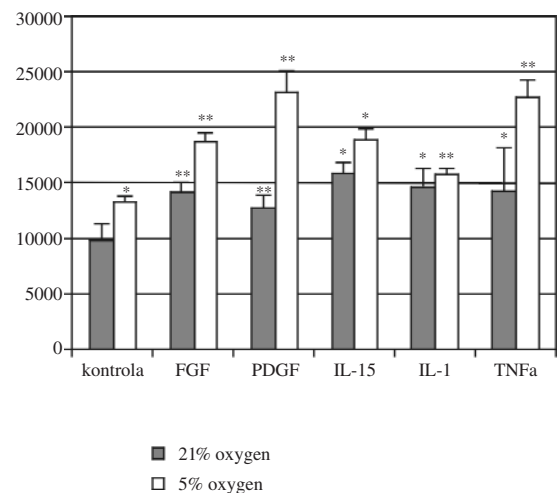


Fig. 1. The effect of cytokines on proliferation of MSC isolated from OA patient (*p<0.05; **p<0.005). Data from one representative experiment is shown

Results

MSC cultured in reduced oxygen (5%) proliferated more rapidly than in normal oxygen concentration (21%) (Fig. 1). Although all tested cytokines stimulated MSC proliferation in low oxygen tension, the best effect was observed in the presence of PDGF-BB (Fig. 1). Based on these results, in further experiments cells were cultured either in the presence of PDGF-BB or medium (control).

Neither oxygen concentration, nor used cytokines exerted any visible effect on cell morphology (Fig. 2). Cells cultured in tested conditions, i.e. in different oxygen tension, and/or in the presence of PDGF-BB, expressed surface markers characteristic for mesenchymal stem cells, i.e. the presence of CD105 and CD166 (Fig. 3). In addition, cultured cells lacked markers characteristic for: (i) monocytes/macrophages - CD14, and (ii) pan-leukocyte CD45 (Fig. 3). It is likely that present in bone marrow monocytes/macrophages do not survive culture in our conditions for more than one passage. These results confirm the identity of cultured cells as MSC. Similar data were obtained using other tested cytokines (data not shown).

To test whether MSC cultured in vitro in low oxygen tension and PDGF-BB retain their chondrogenic potential cells were cultured for 7 days in the presence of factors known to trigger MSC differentiation to chondrocytes or media alone (control), and the expression of mRNA encoding chondrocyte specific genes (i.e. collagen II and aggrecan) were analyzed by RT-PCR. Indeed, chondrogenic medium triggers both collagen II and aggrecan mRNA expression in cultured MSC (Fig. 4). Similar results were obtained for all tested MSC lines, indicating that our optimized culture conditions preserve the ability of MSC isolated from osteoarthritis patients to differentiate into chondrocytes.

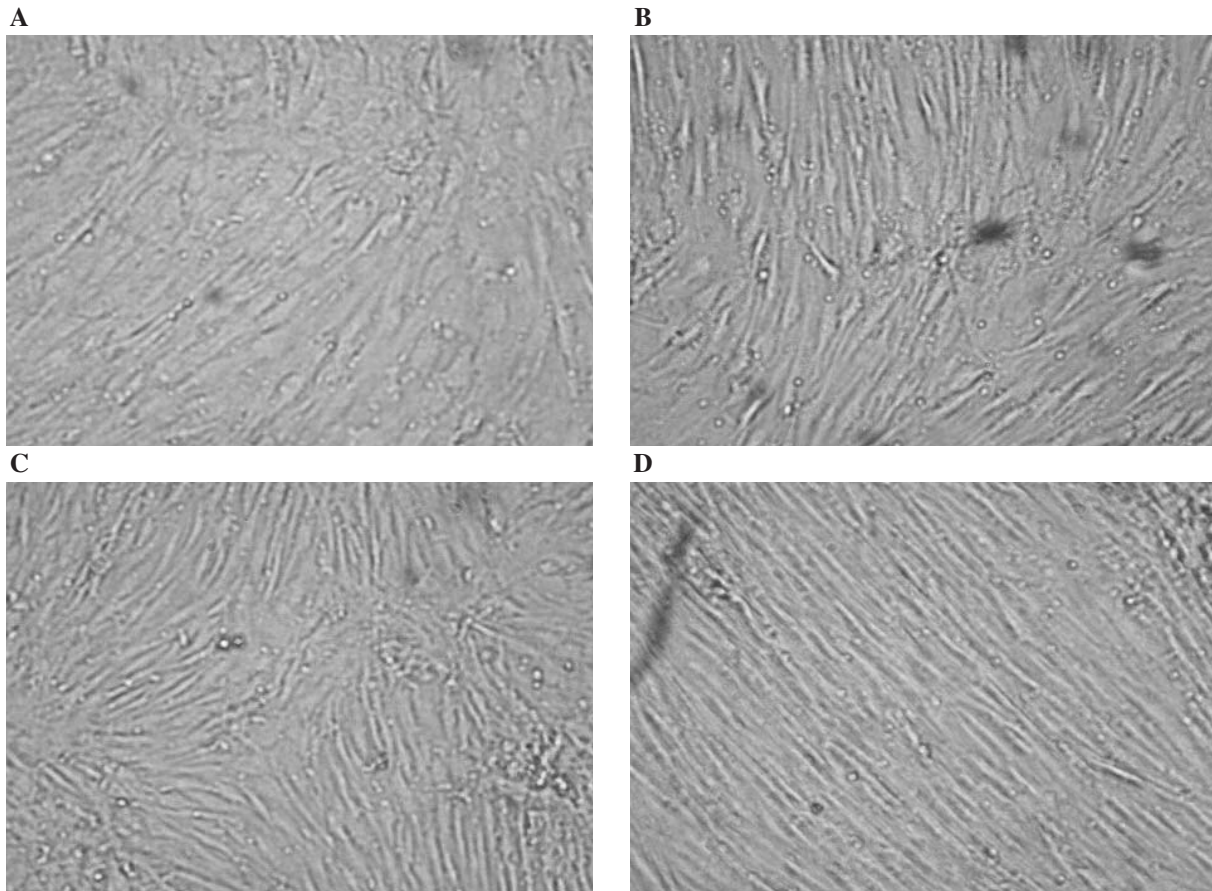


Fig. 2. Morphology of cells cultured in vitro in: A) 21% of oxygen; B) 21% of oxygen in presence of PDGF BB; C) 5% of oxygen; D) 5% of oxygen in presence of PDGF BB

Discussion

Given the limited ability of articular cartilage for self regeneration, its defects caused by disease or trauma often leads to joint destruction and disability [10]. The scale of the problem is enormous. It is estimated that the most common form of arthritis, osteoarthritis affects 12% of people over the age 25 and 50% over the age 65 [11]. At certain stage of joint destruction the only effective treatment is the total joint replacement with endoprosthesis. In the USA about 500 000 joint replacement surgeries are performed each year. However, although this final stage procedure helps many people, this procedure is not suitable for everyone and brings some additional problems related to half life of the endoprosthesis and necessity for revision surgeries. It is clear that there is an urgent need for earlier and possibly more effective treatment of damaged cartilage.

Research has focused on several directions, with the main emphasis on the regeneration of the damaged cartilage. In one of these approaches, fragments of destroyed cartilage have been replaced with autologous cartilage. However, although transplanted tissue is accepted and start to synthesize cartilage characteristic proteins,

transplanted fragments do not integrate well with the original cartilage and may wear off. It has been suggested that lack of integration may be caused by final stage of transplanted in the cartilage chondrocytes that, although able to synthesize matrix proteins, are not flexible enough to form necessary fine links between transplanted and remaining original cartilage. To solve these problems, isolated and propagated in vitro chondrocytes have been used either as a cell suspension or seeded on biodegradable scaffolds to regenerate degraded cartilage. There are reports from several laboratories that this procedure results in a partial cartilage regeneration [12]. However, these procedures also have their limitations and were used for relatively young patients with trauma, but not disease (OA or RA) caused destruction. Not knowing these diseases pathogenesis, it has been feared that chondrocytes derived from patients inherit some defects that may hamper cartilage regeneration. In addition, it has been documented that cultured in vitro chondrocytes undergo dedifferentiation and no longer synthesize chondrocyte specific proteins. Although we have recently shown that select culture conditions trigger dedifferentiated chondrocytes to

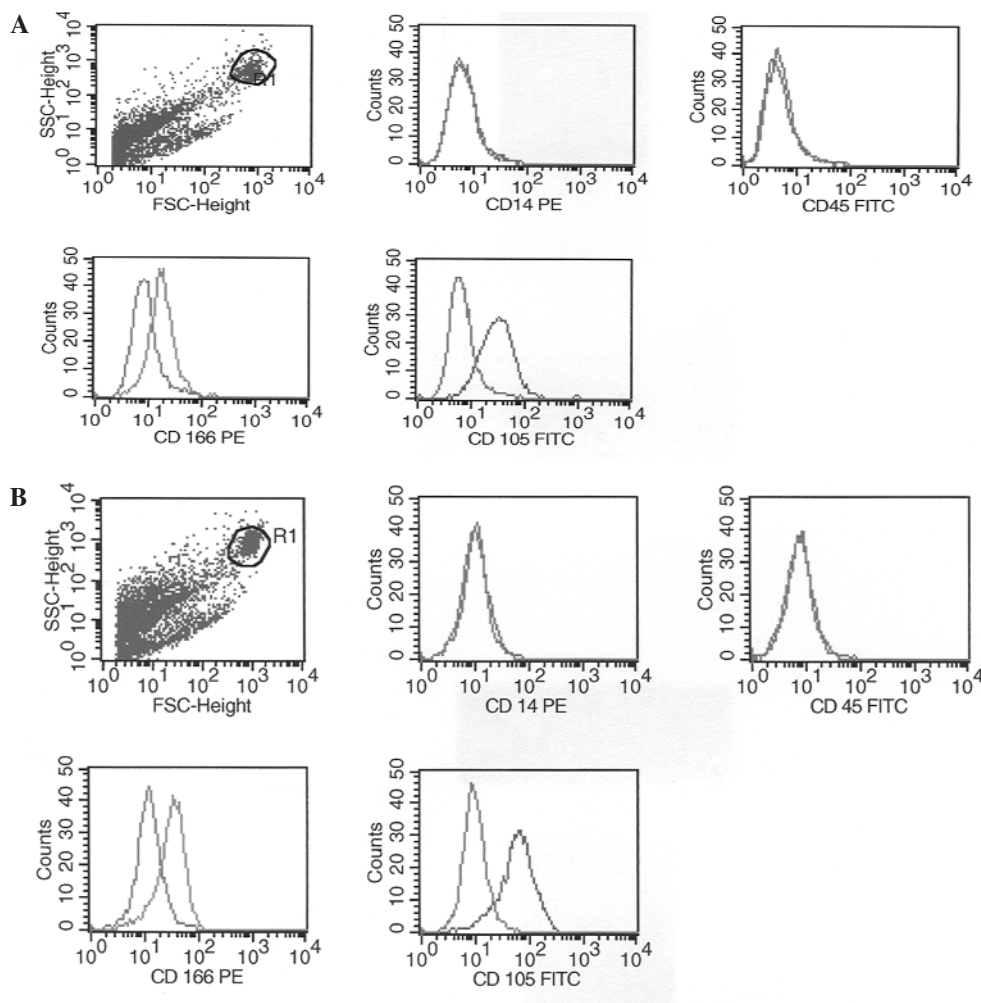


Fig. 3. Expression of cell surface proteins on MSCs cultured in presence of PDGF BB in: A) 5% of oxygen; B) 21% of oxygen. Cells were stained with specific for CD14, CD45, CD105 and CD166 antibodies

synthesize again collagen II and aggrecan, we can not rule out the possibility that these cells are not flexible enough to fully integrate into original cartilage.

Another natural and promising type of cells for cartilage regeneration are MSC. These undifferentiated cells may be triggered in vitro/in vivo for differentiation toward chondrocytes. At the same time, just because they have to undergo full process of differentiation, they might be more flexible than adult chondrocytes to integrate into the remaining mature cartilage.

However, present methods of efficient in vitro propagation of MSC are not optimized. In addition, considering reports that self-renewal and proliferative capacity of MSCs are limited and may decrease with age [13, 14], there is a need to better understand processes that regulate propagation and differentiation of these cells.

In the present study we hypothesized that propagation and differentiation of MSC toward chondrocytes should be

carried out in conditions more closely remaining natural process of cartilage formation. Recent results confirmed that MSC functioned optimally in an atmosphere of reduced oxygen that more closely approximates documented in vivo oxygen tension [15, 16]. Therefore, we carried out series of experiments where cells isolated from patients with OA and undergoing hip replacement, were cultured in the presence of low and normal oxygen tension. We have chosen OA as the model disease of joint destruction because: (i) OA affects more people, than any other disease, (ii) there is no current cure of patients with OA other than joint replacement, and (iii) the demand for new technology in this disease is most urgent. As previously reported [3] these cells spontaneously proliferate in normal oxygen level (Fig. 1). In addition, as expected when cells were stimulated with several growth factors they proliferated even faster in both 5% and 21% of oxygen (Fig. 1). The best result was obtained in the presence of PDGF-BB.

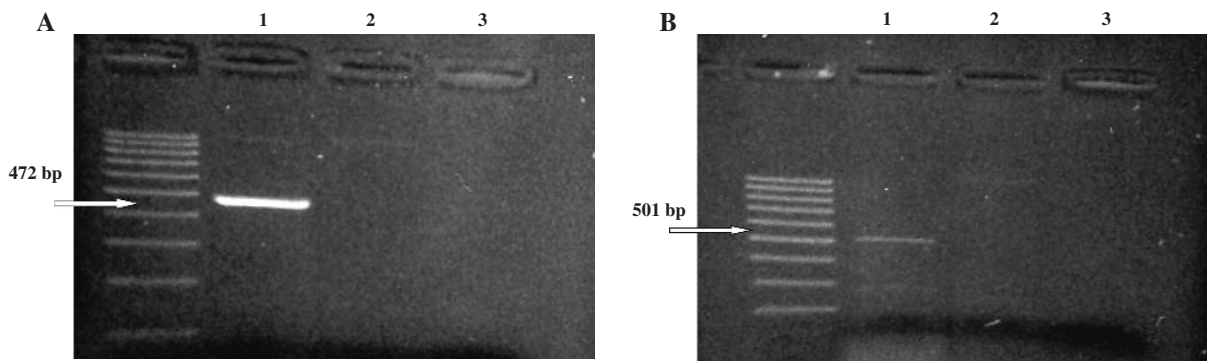


Fig. 4. **A)** expression of collagen IIA mRNA by: 1) MSC cultured in Matrigel and chondrogenic medium; 2) MSC cultured in monolayer and standard medium; 3) control – cDNA; **B)** expression of aggrecan mRNA by: 1) MSC cultured in Matrigel and chondrogenic medium; 2) MSC cultured in monolayer and standard medium; 3) control – cDNA

The most important tests were carried out to answer whether propagated in vitro MSC still retain their ability to differentiate toward chondrocytes. Results reported in our study prove that indeed, proliferating in vitro MSC, even at low oxygen concentration and in the presence of PDGF, are able to further differentiate into cells capable to synthesize collagen and aggrecan when triggered by chondrogenic medium (Fig. 4). These results expand recent findings that although chondrogenic potential of MSC is unstable throughout mitotic divisions in vitro, as compared to the osteogenic and adipogenic potential [17], it can be retained provided specific cell stimuli by growth factors.

Therefore our study is a proof of concept that one can optimize in vitro conditions that preserve proliferating cells potential to differentiate into chondrocytes. However, we must stress that reported in this paper conditions are far from the final conditions that can be used for tests in vivo. Although it was not a goal of the present report, our preliminary data indicate that MSC cultured in reported here conditions and additionally seeded as pellets or on scaffolds, further differentiate into chondrocyte/cartilage like tissue. However, at present these preliminary data just indicate directions that should be explored in the near future.

In conclusion, we present data suggesting that MSC could be propagated in vitro at low oxygen tension and the presence of growth factors, and that these conditions do not hamper their chondrogenic potential.

Acknowledgements

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