Research Journal of Biological Sciences 2 (3): 307-310, 2007

Endothelial Differentiation of Rat Mesenchymal Stem Cells

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Abstract: Bone Marrow (BM) derived Mesenchymal Stem Cells (MSCs) are pluripotent cells which can replicate as undifferentiated cells, have potential to differentiate into endothelial, osteogenic, adipogenic and other lineages under determined culture conditions and are considered as a valuable source for the *in vivo* investigation of tumor angiogenesis, cell and gene based therapy. The aim of this study was to optimize *in vitro* expansion of MSCs derived Endothelial Progenitor Cells (EPCs) in a rat model. MSCs were isolated from bone marrow by a combination of gradient density centrifugation and adherence properties. *in vitro* cultures of MSCs were established using the 6 different commercially available Growth Media (GM) including DMEM, DMEM+VEGF, Endopan, MV2 and Panserin, and their potential to differentiate into osteogenic, adipogenic and endothelial cells was shown. By using Endopan MSCs exhibit the highest proliferation rate and the uppermost capacity to EPCs differentiation in comparison with other exploiting GM. Endopan is favorable medium for EPCs preparation. It may be very helpful in the different applications of EPCs in small-animal cell based diagnostics and therapeutic models.

Key words: Bone marrow, MSC, DMEM, GM, EPCs,VEGF

INTRODUCTION

The study of tumor angiogenesis is currently a leading theme in oncology as it plays a critical role in the growth and metastatic spread of tumors (Naumov *et al*., 2006). The specific incorporation of Endothelial Progenitors Cells (EPCs) into the vasculature of xenotransplantated tumors was shown *in vivo* using PET (Tamura *et al*., 2004) and MRI (Arbab *et al*., 2006) and was suggested by the direct histological examinations of sections (Reyers *et al*., 2002). It was shown that infusion of EPCs, but not mature endothelial cells, promoted neovascularization (Urbich *et al*., 2005) and may be promising tools for molecular imaging *in vivo* (Tamura *et al*., 2004; Arbab *et al*., 2006; Reyers *et al*., 2002; Urbich *et al*., 2005). BM derived Mesenchymal Stem Cells (MSCs) have a high differentiation potential (Pittenger and Martin, 2004) including EPCs differentiation under appropriate growth conditions (Oswald *et al*., 2004) and, therefore, present an excellent source for EPCs. In this study we attempted to measure the proliferation properties of MSCs and to evaluate their possibilities to differentiate into EPCs in the presence of different commercially available endothelial growth media.

MATERIALS AND METHODS

Purification and expansion of MSCs: BM was obtained from 6 weeks old male Wistar rats (Charles River Laboratories, Germany) by flushing the medullar cavity of femurs and tibiae with DMEM (Sigma, Germany). The proportion of Mononuclear Cells (MNCs) was enriched by density gradient centrifugation as previously described (Boyum *et al*., 1983). Separated MNCs (1.5×10^4 cells/cm^2) were plated into fibronectin covered plastic flasks (BD Falcon, Germany) and cultured in the different GM including DMEM, DMEM supplemented with 50 ng mL^{-1} of VEGF (Cedarlane Laboratories, USA), Endopan, MV2 (Promocell, Germany), Panserin 401 supplemented with Panexin D (PanBiotech GmbH, Germany) and EBM-2 (Cambrex, USA). All GM were supplemented with 10% of fetal calf serum (FCS, except Panserin) and antibiotics. The adherent cells developed into visible colonies of spindle-shaped MSCs within 6 to 8 days. Nonadherent cells were removed during routine fresh medium replacement every 2 days. MSCs in 2 weeks of culture were characterized by their capacity to differentiate into adipocytes, osteoblasts and EPCs using MSCs Adipogenesis, MSCs Osteogenesis kits

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(Millipore, MA, USA) according to manufacturer's recommendations or by administration of VEGF (50 ng mL^{-1}) to near confluent cell culture as described earlier (Oswald *et al*., 2004).

Flow cytometry: For phenotypic characterization, cells were stained with CD11a, CD11b, CD29, CD31, CD44, CD45, CD71, CD90, CD106 (Serotec, Germany), CD105 (Biozol, Germany) and sheep-anti rat von-Willebrand-factor (vWF, Cedarlane Laboratories, USA) according to manufacturer's recommendations. Cell cycle analysis in long term culture was performed using propidium iodide (PI, Sigma, Germany) and carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) as described earlier (Boyum *et al*., 1983). Cells (2×10^5 per sample) were analyzed by flow cytometry (CyFlow) using the CyFlow software (Partec, Germany).

Microscopy: The cells were analyzed using a fluorescent microscope Axioskop 2 plus (Zeiss, Germany). Slides were stained with FITC-conjugated CD90 or vWF according to manufacture prescriptions. For cytological evaluation, cells were harvested, fixed with 4% paraformaldehyde, dehydrated and stained with Mayer's Hemalum-Eosin (HE) solution (Merck, Germany) according to conventional procedure.

Statistics: The experimental data are expressed as the mean±Standard Deviation (SD) of several independent experiments. The statistical significance of the recorded effects was assessed using Student's t-test assuming two-tailed distributions and unequal variances.

RESULTS

***In vitro* expansion of MSCs:** MSCs were isolated from BM cells by a combination of gradient density centrifugation and adherence properties. Colonies of adherent MSCs demonstrating an elongated fibroblast-like morphology of MSCs appeared in 2 further weeks of culture independent of GM used. However the MSCs number of cells and their proliferation activity were different. A similar percentage (Fig. 1A) of MSCs in S+G_2+M phases was revealed if they grew in DMEM (19±2%), DMEM+VEGF (22±2%), Panserin (17±2%) or EBM-2 (23±1%). Significant higher (in comparison to DMEM) proportion of MSCs in S+G_2+M phases was found in case of MV-2 (25±1%, $p<0.05$) and Endopan (29±2%, $p<0.001$). As a result, a similar number of MSCs was obtained in 2 weeks of *in vitro* culture (Fig. 1B) using DMEM (~$3x10^5$ MSCs/10^6 BM MNCs, set to 100%), DMEM+VEGF (160±32%, $p>0.05$), EBM-2 (104±18%, $p>0.05$) or Panserin (86±25%, $p>0.05$). Significantly higher cell numbers were registered in MSCs *in vitro* culture growing 2 weeks MV-2 (286±37%, $p<0.001$) and the highest in Endopan (434±42%, $p<0.001$).

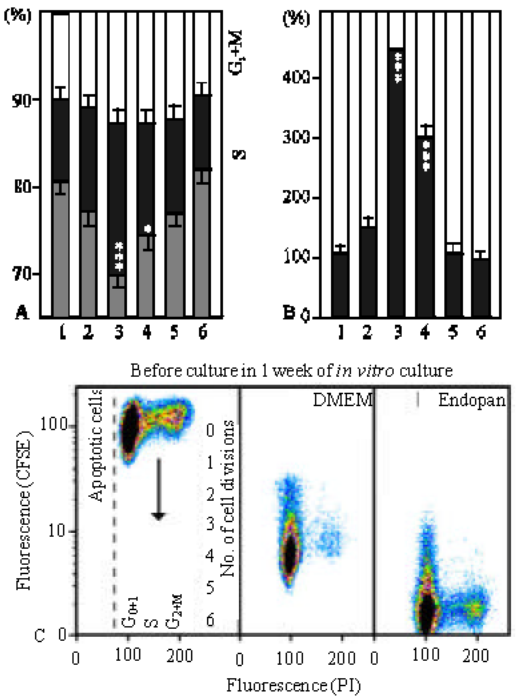

Fig. 1: Effect of growth media on MSCs proliferation. The percentage of MSCs in G_{0+1}, S and G_2+M phases of cell cycle (A) and total number of cells (B) in 2 weeks of culture in different growth media including DMEM (1), DMEM+VEGF (2), Endopan (3), MV-2 (4), Panserin (5) and EBM-2 (6) are shown. Significant differences (*$p<0.05$, ***$p<0.001$) are noted by asterisks. Flow cytometry (CFSE versus PI fluorescence) of MSCs proliferation (C) before (generation "0") and in 1 week of *in vitro* culture (generations „1-6") in DMEM and Endopan is shown. Positions of cells in G_{0+1}, S and G_2+M phases of cell cycle, the area occupied by apoptotic cells and number of cell divisions are noted

Heterogeneous of MSCs divisions: Cycling patterns of MSCs in DMEM and Endopan were compared by using dot plot (CFSE/PI) flow cytometry (Fig. 1C). Before culture, cells had the similar relatively high level of CFSE fluorescence (division "0"). In 1 week, nearly all MSCs had passed through 4-5 rounds of cell divisions (DMEM). Meanwhile, some cells grew slowly and were registered in

the 2nd and 3rd division. Cells proliferated faster in Endopan and mainly underwent 6 divisions. Nevertheless, some delaying cells (from 3 to 5 divisions) were also registered.

Immunophenotype of MSCs: Flow cytometry demonstrated that MSCs in 2 weeks of culture were positive for CD29, CD44, CD71, CD81, CD90, CD105 and 106 and negative for CD4, CD11a, CD11b, CD14, CD31, CD43, CD45, CD172a and vWF independent of GM used.

Differentiation of MSCs: To asses the pluripotent properties of MSC in the culture, their osteogenic and adipogenic differentiation capacities were tested. The majority of MSCs in culture did not stain for either lipids (Fig. 2A) or mineral (Fig. 2B). Meanwhile, independent of the GM utilized, significant changes in MSCs cultures were registered in the appropriate time after adipogenic, osteogenic or endothelial cell differentiation. Proportions of mature adipocytes assessed with Oil Red O staining of lipid vacuoles (Fig. 2C), osteoblasts which phenotype were indicated by Alizarin Red S (Fig. 2D9 or EPCs expressing endothelial-specific markers such as CD31, CD90, CD105, CD106 and vWF were obtained independent of GM used. In last case flow cytometry demonstrated reduction of SSC ($p<0.01$) by EPCs in comparison to MSCs (Fig. 3A, B) and expression of von Willebrand factor (vWF+, Fig. 3C). Fluorescent microscopy revealed specific Weibel-Palade bodies in EPCs (Fig. 3C, inset) but not in MSCs (Fig. 3B, inset). However, during differentiation period cells in near confluent culture significantly loss their proliferation potential, mostly by G_1 block of the cell cycle.

Spontaneous differentiation of MSCs in long-term culture: It was found that MSCs could proliferate extensively *in vitro* in DMEM during a period up to 2 months (12 passages) without any relevant differentiation as revealed by comparison of MSCs of 6, 12 and 24th passages. During this time, MSCs in Endopan were completely differentiated into EPCs. Flow cytometry revealed subpopulation of cells (67±12%) with the same decreasing level of SSC (Fig. 3D) and expression of vWF (vWF+, Fig. 3D) showing Weibel-Palade bodies (Fig. 3D, inset) as it was registered in case of VEGF induces EPCs differentiation (Fig. 3C). EPCs expressed endothelial-specific markers CD31, CD90, CD105, CD106 and vWF, did not express CD11a, CD11b, CD14 and CD45. At the same time, Endopan induced differentiation did not change proliferation activities of EPCs as it was registered after VEGF administration to near confluent culture of MSCs.

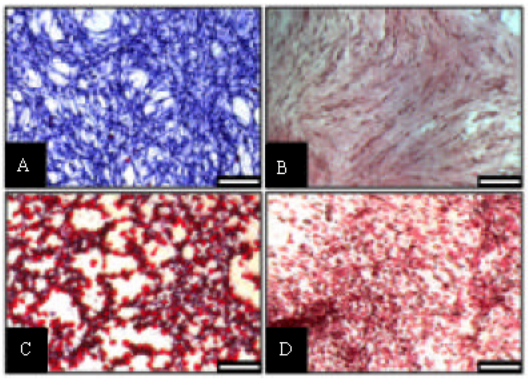

Fig. 2: Osteogenic and adipogenic *in vitro* differentiation of rats MSCs. Culture of MSCs (4 weeks old rats) before (A, B) and after adipogenic (C, E) and osteogenic (D, F) differentiation were evidenced by Oil Red O (red)/HE (blue) (A, C, E) and Alizarin Red (B, D, F) staining. Scale bars correspond to 50 μM

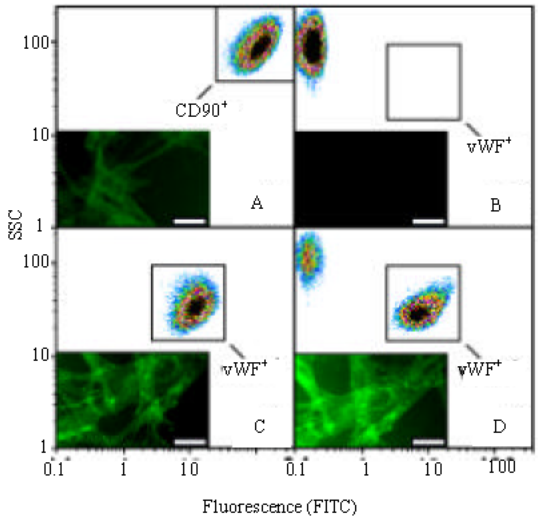

Fig. 3: Effect of growth media on MSCs differentiation. Dot plot histograms (fluorescence of FITC versus SSC) of MSCs growing in DMEM before (A, B), after EPCs differentiation induced by VEGF administration during 1 week (C) or in Endopan (D) stained with FITC conjugated antibody to CD90 (A) or vWF (B-D) are shown. Areas occupied by cells expressing CD90 ($CD90^+$) or vWF (vWF^+) are noted. Insets contain images of cells stained with CD90 (A) or with vWF (B-D). Scale bars correspond to 10 μm

DISCUSSION

The capacity of rat MSCs to osteogenic and adipogenic differentiation under *in vitro* conditions is consistent with that reported for human MSCs (Pittenger and Martin, 2004; Oswald *et al*., 2004; Bonab *et al*., 2004). Evaluation of different culture media for MSCs in our study, in agreement with previous results (Bonab *et al*., 2004) revealed that DMEM consistently supported MSCs growth. Panserin or EBM-2 can be used for MSCs cultivation with the same success as DMEM. Meanwhile, the growth conditions could be improved using Endopan or MV-2 as growth medium. Since the addition of growth factors to MSCs culture media in general tends to induce differentiation and loss of multilineage capacity (Wu *et al*., 2004) we had investigated the impact of VEGF supplementation on MSCs proliferation and differentiation properties. The expression of CD31, CD90, CD105, CD106 and vWF together with the presence of Weibel and Palade bodies (1964) unequivocally identify EPCs differentiation supporting previous results in human MSCs (Oswald *et al*., 2004). It was found that MSCs growing during 2 months in endothelial GM Endopan revealed similar differentiation capacities. The resulting mixed population of MSCs and EPCs may have even advantages in some of therapeutic applications as a fraction of MSCs may act as cytokine factories stimulating vascularization (Ziegelhoeffer *et al*., 2004).

CONCLUSION

Our results suggest that MSCs are able to self-renew and to differentiate in osteogenic, adipogenic and endothelial cells *in vitro*. They exhibit the highest proliferation rate and the EPCs differentiation capacity in Endopan growth medium. Therefore, this medium may be favorable for EPCs application in small-animal cell based diagnostics and therapeutic models.

ACKNOWLEDGMENT

This research and the authors are kindly sponsored by the Bundesministerium für Bildung und Forschung, BMBF Contract 03ZIK042.

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