

Stroma-Providing Activity of Adipose Derived Mesenchymal Stromal Cells in Tissue-Related O₂ Microenvironment

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Abstract—This work studied the ability of adipose tissue-derived mesenchymal stromal cells (MSCs) to form stroma for expansion of cord blood hematopoietic cells. We showed that 72-hour interaction of MSCs with cord blood mononuclear cells (MNCs) in vitro at atmospheric (20%) and low (5%) O₂ conditions increased the expression of ICAM-1, HCAM (at the beginning of interaction) on MSCs. Viability of MSCs and MNCs were maintained at high level. Adhesion of MNCs to MSCs was faster at 20% O₂. MSCs promoted the proliferation of adhered MNCs to form the suspension containing great number of hematopoietic colony-forming units, and this effect was more pronounced at 5% O₂. Thus, adipose-derived MSCs supplied sufficient stromal support to cord blood MNCs both at 20% and 5% O₂, providing their adhesion with further expansion of new generation of different hematopoietic lineages.

Keywords—Hematopoietic stem and progenitor cells, mesenchymal stromal cells, tissue-related oxygen.

I. INTRODUCTION

FORMATION of stroma in various tissues particularly hematopoietic is one of the most important physiological function of mesenchymal stromal cells (MSCs). These cells not only provide mechanical structure of tissue but also are able to determine proliferative and differentiation status of hematopoietic progenitor cells by the means of soluble molecules and direct cell-to-cell contact [1], [2]. In vitro research provides wide opportunity for studying such interactions and allows considering certain aspects of functioning and interaction of cells in hematopoietic niche [1], [3]–[5]. In such studies the selection of proper culture conditions are of great practical importance because of the possibility to establish a system for production of hematopoietic progenitors and their subsequent clinical application. Feeder layer of MSCs from human adipose tissue can be considered as an analogue of hematopoietic stroma. They have similar properties with bone marrow MSCs [6] but are more available at the same time. The fraction of cord blood mononuclear cells (MNC) can be used as a source of hematopoietic cells, since it contains significant amount of

hematopoietic progenitor and stem cells.

Hematopoietic niche is characterized by low O₂ concentration (~0–6%). This parameter can significantly affect the number of features of both hematopoietic progenitors [7] and MSCs [8]–[12]. Nevertheless, only a few publications on MSCs - hematopoietic cells interaction have taken this important physiological factor into consideration [5], [13]–[14].

Here we studied the ability of adipose tissue-derived MSCs to maintain hematopoietic cord blood MNCs under standard culture (20%) and tissue-related (5%) O₂ conditions.

II. MATERIALS AND METHODS

A. Isolation and Culture of MSCs

Adipose tissue samples were obtained from 7 donors in “Soyuz” clinic in accordance with the scientific cooperation agreement. MSCs were isolated according to the standard procedure described by Zuk et al. [15] with some modifications [8]. Cells were expanded in α -MEM (Gibco) with 10% FCS (HyClone) under standard culture conditions (20% O₂, 5% CO₂, 37°C) in CO₂-incubator (Sanyo, Japan) and at low O₂ (5% O₂, 5% CO₂, 37°C) in multigas incubator (Sanyo, Japan).

Cultured cells were tested for compliance with the criteria of MSCs: expression of CD73, CD90, CD105, lack of CD45, osteo-, chondro- and adipo-differentiation.

MSCs of 3rd-4th passages were used for experiments.

B. Hematopoietic Cells

The cord blood mononuclear cells (MNCs) fraction was the source of blood-born progenitors and differentiated cells. Cryopreserved MNCs were kindly provided by Cord blood Bank “Cryocenter” (Moscow).

C. Coculture of MSCs and MNCs

2x10⁶ MNC/ml was added to 70–80% MSC monolayer. Cells were cocultured for 72 h in RPMI-1640 (Gibco, USA) with 5% FCS at 20% and 5% O₂. Then nonattached MNCs were removed and used for CFC-test. MSCs with adherent MNCs were further cultured for 4–7 days.

D. Morphological and Quantitative Analysis of MNCs and its Progeny

Cell culture examination was conducted using Nikon Eclipse TiU (Nikon, Japan) microscope equipped with camera DS-Ri1 (Nikon).

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After 24 h randomized view fields of culture dish were photographed and after 72 h the same areas were photographed again. MNCs quantity was analysed using SigmaScan Pro 5.0 Image Analysis Software (SPSS Inc, USA).

After 24 h and 72 h cocultured cells were fixed with methanol, and stained with Giemsa solution (BDH, USA), then morphological analysis of MNCs adhered to MSCs was conducted.

E. Colony-Forming Cell (CFC) Assay

Freshly thawed MNCs and cells after coculture with stromal cells (50×10^3 cells/ml) were cultured in methylcellulose-based medium MetoCult H4534 (StemCells, USA) according to the manufacturer's protocol at 20% and 5% O₂. After 14 days, the number of CFCs was calculated.

F. Immunocytochemical Analysis

After 24 h and 72 h in coculture cells were detached with trypsin-EDTA (Gibco) and stained with monoclonal antibodies against CD90(FITC)/CD45(PE), CD44(FITC), CD54(PE) (Beckman Coulter IO Test, USA). Stained cells were analyzed with flow cytometer Epics XL (Beckman Coulter, USA) using System II software (Beckman Coulter).

Simultaneous staining with anti CD90-PE and CD45-FITC antibodies revealed 2 groups of cells: small CD45⁺/CD90⁻ cells – leukocytes, and rather large CD45⁺/CD90⁺ cells–MSCs. Then we set two gates for cells of different size and analyzed cells in each gate separately.

G. Analysis of Viability

Cells were stained with Annexin - Propidium Iodide (Immunotech, France), and the share of apoptotic, necrotic and viable MNCs and MSCs was analyzed by flow cytometry.

H. Statistical Analysis

Statistical analysis was performed with «Statistica 7.0» software; Mann-Whitney U-test was used. Differences were considered significant at $p < 0.05$.

III. RESULTS

A. Characterization of MSCs

Virtually all cells from stroma-vascular fraction of adipose tissue expressed MSC characteristic markers CD73 ($95.5 \pm 1.3\%$), CD90 (97.3 ± 1.3), CD105 ($99.6 \pm 0.1\%$) and were capable to differentiate in osteo-, chondro- and adipogenic lineages. Only a few cells expressed CD45 ($1.5 \pm 0.5\%$).

B. MSCs Encouraged the MNC Adhesion Elevating CAMs

Interaction with MNCs resulted in significant rise of both CD54⁺ MSCs and the mean fluorescence intensity (MFI) that indicated the increase of ICAM-1 expression on the MSC surface (Fig. 1). MFI grew equally at 20% and 5% O₂ throughout the experiment, 2.1 and 2.8 times respectively comparing to MSC monoculture.

Virtually all MSCs in monoculture and coculture expressed HCAM (CD44). After 24 h the MFI was 1.5-2 times higher on average in coculture than in monoculture, and after 72 h it was

similar in both culture conditions (Fig. 2).

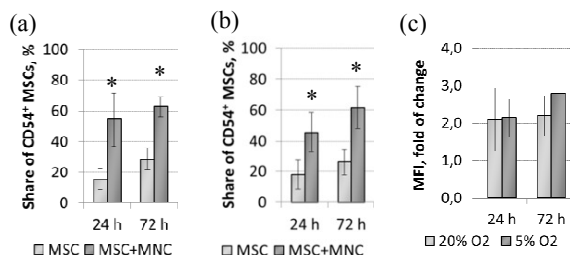


Fig. 1 ICAM1 expression in MSC monoculture (MSC) vs coculture with MNCs (MSC+MNC) at 20% (a) and 5% O₂ (b), * - significant difference between monoculture and coculture ($p < 0.05$); (c) - fold change of CD54 MFI in cocultured MSCs compared with monoculture

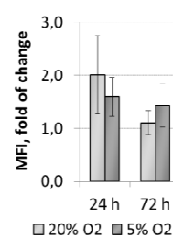


Fig. 2 HCAM expression on cocultured MSCs compared with monoculture

C. Viability of MSCs

After 24 h and 72 h in coculture MSCs showed low rate of cell death and maintained high viability at both O₂ concentrations (Fig. 3).

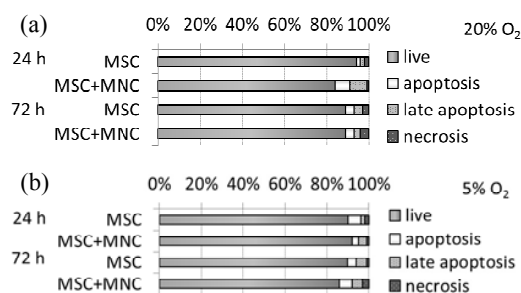


Fig. 3 MSC cell death in monoculture (MSC) and after coculture with MNCs (MSC+MNC) at 20% (a) and 5% O₂ (b)

D. Morphology and Quantity of Attached MNCs

Active adhesion of MNCs to MSCs has been observed on the first day of coculture (Fig. 4). Adhered cells were identified as mature blood elements (lymphocytes, monocytes, erythrocytes) and hematopoietic progenitors of different commitment primarily granulocytic (neutrophilic and eosinophilic myelocytes and metamyelocytes) and also of erythroid (basophilic, polychromatophilic and oxyphilic normocytes).

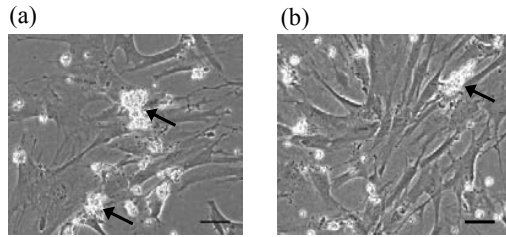


Fig. 4 Adhesion of MNCs (marked with black arrows) to MSCs after 24 h cultivation at 20% O₂ (a) and 5% O₂ (b). Phase contrast, bars 50 µm

After 24 h in coculture the quantity of adhered MNCs at 20% O₂ was 2.5 times higher than at 5% O₂, and it practically did not change during the next 48 h coculture, whereas the number of adhered cells significantly grew at 5% O₂ ($p < 0.05$) (Fig. 5).

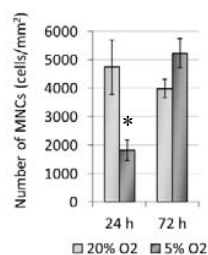


Fig. 5 The dynamics of MNCs adhesion to MSC monolayer at different O₂, * - significant difference between 20% and 5% O₂, $p < 0.05$

E. Viability of MNCs

At both O₂ concentration MSC feeder layer maintained high viability of MNCs whereas this parameter decreased in 72 h in monoculture (Fig. 6).

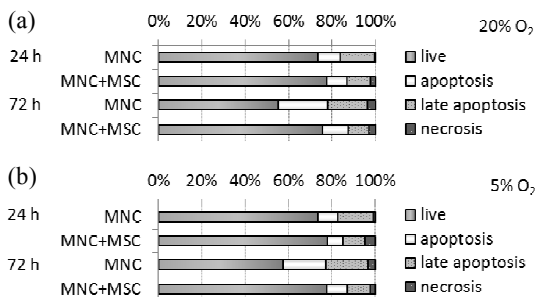


Fig. 6 MNC cell death in monoculture (MNC) and after coculture with MSCs (MNC+MSC) at 20% (a) and 5% O₂ (b)

F. Colony Forming Cell (CFC) Evaluation

To characterize the ability of MSCs to maintain hematopoietic progenitors, the number of CFCs was assessed in the initial cord blood MNCs, in nonattached fraction of these cells after 72 h co-culture with MSCs and in newly grown nonattached fraction after expansion of adherent MNCs during 4-7 days. Initial MNCs formed 25% more colonies at 5% O₂ (Fig. 7(a)). Following 72 h of coculture with MSCs,

MNCs retained the CF-potential, which was again more pronounced after coculture at 5% O₂ (Fig. 7(b)).

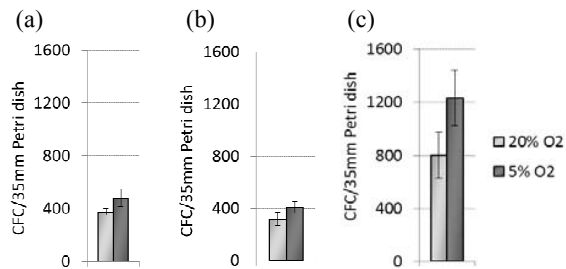


Fig. 7 Colony-formation efficiency of MNCs in MetoCult H4534. (a) Initial MNCs; (b) Non-attached MNCs after 72 h of coculture with MSCs; (c) Non-attached MNCs after 7 days of coculture of adhered MNCs with MSCs

G. MNC Hematopoietic Progenitors Attached to the MSCs

The part of MNCs remained adhered to the stromal cells after 72 h of coculture (Fig. 8). During the ensuing cultivation, these cells were able to form colonies of undifferentiated progenitors on the MSC layer (Fig. 9), to migrate under the MSCs forming “cobble-stone” areas (Fig. 10) and give rise to new population of unattached cells (Fig. 11) containing large number of CFCs (Fig. 7(c)).

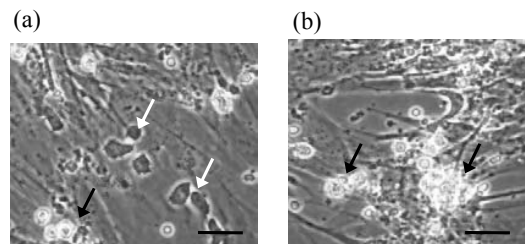


Fig. 8 MNCs adhesion on MSC after 72 hours of coculture at 20% (a) and 5% O₂ (b). Adhered CBMCs look as shiny rounded cells (marked with black arrows), some of them are well spreaded on MSCs' surface (white arrows). Phase contrast, bars=25 µm

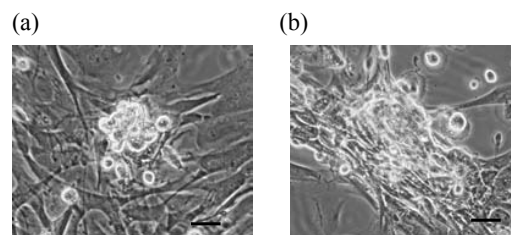


Fig. 9 Colonies of MNCs after long term coculture at 20% (a) and 5% O₂. Phase contrast, bars=25 µm

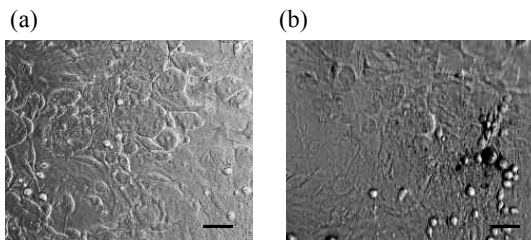


Fig. 10 “Cobble-stone” areas formed by low differentiated attached MNCs after 14 days of coculture with MSCs at 20% (a) and 5% O₂ (b). NAMC, bars=50µm

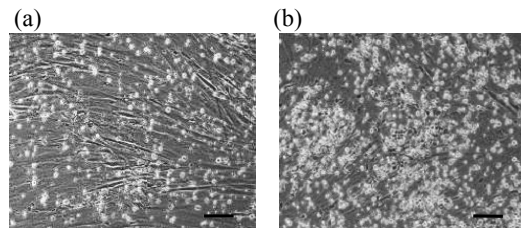


Fig. 11 Suspension of newly grown hematopoietic progenitors arising from low differentiated attached MNCs at 20% (a) and 5% O₂ (b). Phase contrast, bars=100 µm

IV. DISCUSSION

This paper analyzed the stroma-supportive properties of adipose tissue-derived MSCs and the impact of O₂ in local milieu. It was shown the adhesion of a part of MNCs to MSCs within 72 h with adhered MNCs presented by cells of different commitment. The adhesion of MNCs to stromal monolayer was more rapid at atmospheric O₂ concentration, than at low O₂. The interaction with MNCs resulted in increase of ICAM-1(CD54)⁺ MSCs that suggests a role of this adhesion molecule in establishing of specialized intercellular contacts. ICAM-1 functions as a receptor mediating adhesion of leukocytes to endothelial cells [16]–[18], and also plays a crucial role in adhesion of hematopoietic progenitors to MSCs, in particular through ICAM-1/LFA-1 interaction [19]. HCAM (CD44) is another important protein of contact interaction in hematopoietic niche. It is involved in important functions such as cell adhesion to substrate, cell aggregation [20], and also in interaction of hematopoietic progenitors with MSCs [21], as its blockage results in hematopoiesis interruption [8]. We showed that virtually all MSCs expressed this molecule independently of culture conditions. These data are in accordance with previous findings that suggested higher expression of CD44 antigen on MSCs from adipose tissue compared to other origins [22].

Current study showed that MSCs effectively maintained high viability of non-adhered MNCs.

We found that adhered MNCs formed colonies at the surface of MSCs and also migrated under the MSC layer forming there “cobble-stone” areas. Moreover, further cultivation after the removal of non-adhered MNCs resulted in appearance of cell suspension with great number of hematopoietic colony-forming progenitors. These cells are

assumed to be the adherent MNCs’ progeny that switched to suspension after the division of parent cell. Earlier Jing et al. [4] showed the replenishment of non-adherent hematopoietic cells due to the proliferation of hematopoietic cells adhered to MSC.

The concentration of 5% O₂ is corresponded to the most oxygenated sites of bone marrow hematopoietic niche, where self-renewal, proliferation, differentiation and maturation of hematopoietic cells occur [7], [23]. According to our data 5% O₂ more effectively than 20% O₂ maintained hematopoietic progenitors capable to give rise to hematopoietic colonies. Furthermore, the replenishment of colony-forming units in suspension due to proliferation of adhered MNCs was more active at low O₂.

V. CONCLUSION

To sum up, adipose-derived MSCs supplied sufficient stromal support to cord blood MNCs both at 20% and 5% O₂, providing attachment and retention of low differentiated hematopoietic progenitors, formation of “cobble-stone” areas and expansion of new generation of different hematopoietic lineages.

Obtained data may be of great interest for further investigation and development of coculture systems for stromal and hematopoietic cells and for better understanding processes that implicate MSCs in hematopoietic niche and during interaction with allogeneic hematopoietic cells, which should be taken into consideration in the case of MSC allotransplantation.

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