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Age-related Changes in Adhesive Phenotype of Bone Marrow-derived Mesenchymal Stem Cells on Extracellular Matrix Proteins

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ABSTRACT: Mesenchymal stem cells (MSCs) are a promising cell source for cell-based therapies because of their self-renewal and multi-lineage differentiation potential. Unlike embryonic stem cells adult stem cells are subject of aging processes and the concomitant decline in their function. Age-related changes in MSCs have to be well understood in order to develop clinical techniques and therapeutics based on these cells. In this work we have studied the effect of aging on adhesive behaviour of bone marrow-derived MSC and MG-63 osteoblastic cells onto three extracellular matrix proteins: fibronectin (FN), vitronectin (VN) and collagen I (Coll I). The results revealed substantial differences in adhesive behaviour of both cell types during 21 days in culture. Bone-marrow derived MSCs decreased significantly their adhesive affinity to all studied proteins after 7th day in culture with further incubation. In contrast, MG-63 cells, demonstrated a stable cell adhesive phenotype with high affinity to FN and Coll I and low affinity to vitronectin over the whole culture period. These data suggest that adhesive behaviour of MSCs to matrix proteins is affected by aging processes unlike MG-63 cells and the age-related changes have to be considered when expanding adult stem cells for clinical applications.

Keywords *– cell morphology, cell attachment and spreading, fibronectin, vitronectin, collagen I*

1. Introduction

Over the past two decades mesenchymal stem cells (MSCs) have provoked great interest in the scientific community. They have been extensively investigated as a promising cell source for tissue engineering and regenerative medicine (Ghasroldasht et al. 2014, Kuo et al. 2008, Nakamizo et al. 2005, Tuan et al. 2003). Despite the wide distribution of MSCs in the body, the bone marrow remains the principal source for most of the MSC-based pre-clinical and clinical studies. The attractiveness of bone marrow-derived MSCs (BM-MSCs) is based on their ability to be easy extracted and expanded *in vitro* and their remarkable capacity to differentiate into lineages of bone, cartilage, fat, ligament, muscle, and other connective tissues (Bianco et al. 2001, Murdoch et al. 2007, Peister et al. 2004, Verfaillie,

2002). Subsequently, these cells can be implanted in the original donor without risk of immunological rejection (Lynch and Pei, 2014).

The major obstacle for a wider implementation of BM-MSCs in tissue engineering and regenerative medicine is their limited yield. The frequency of MSCs in bone marrow is extremely low, ranging from 1/10 000 to 1/100 000 mononuclear cells (D'Ippolito et al. 1999) while the number of cells required for one transplantation procedure is approximately 10^8 cells or 1×10^6 cells per kg recipient weight (Miura, 2016). To obtain such enormous therapeutically useful number of cells MSCs have to be expanded *ex vivo* (Mauney et al. 2004). During *in vitro* propagation MSCs are a subject of aging, characterized by a shortened lifespan, an increasing loss of proliferation capacity, progressive reduction of differentiation potential, increased senescent cell number and elevated apoptosis (Banfi et al. 2000, Jones and McGonagle, 2008, Sethe et al. 2006). Furthermore, late passages MSCs tend to lose properties of stemness and homing capacity (Hwang et al. 2016). Transplantation of such cells would not produce the desired results and may even be harmful to the recipient. It must be clarified how many population doublings or how long MSCs can be cultured without losing their characteristics of stem cells and which are the exact criteria determining their adequacy for transplantation. Such a criterion could be cell adhesive phenotype because MSC are adherent cells and their fate and functions are controlled by adhesive interactions with the extracellular matrix (ECM) (Kim et al. 2012, 2 Meunier et al. 1971). Cell adhesion is a functional criterion therefore the alteration in cell adhesive phenotype will point deterioration in the quality of MSC. Therefore, it is equally important to investigate the impact of *in vitro* aging of multipotent progenitor cells on their adhesive affinity to ECM proteins. ECM is an important part of the physiological microenvironment of MSCs which provides a structural framework for cell attachment and determines cellular morphology (Wagers, 2012). Moreover, ECM contains biological cues and signals necessary for growth, differentiation, and migration of stem/progenitor cells (D'Ippolito et al. 1999) and regulates the activities of nearby cells directly, through its physical properties, or indirectly, though soluble factors (Li and Xie, 2005). Several studies have shown that mechanical properties and protein composition of ECM change with age (Docheva et al. 2007, Gershlak et al. 2013, Tottey et al. 2011). The overall synthesis of ECM was found to decrease with age, though this is not true for all ECM components (Antia et al. 2008) and during senescence the ECM became soluble and proteolytically digestible (Sell and Monnier, 1989). These changes are thought to be a result from the formation of age-related intermolecular cross-links. Because it seems clear that the ECM changes with age it is possible these changes to affect MSC adhesive affinity to matrix proteins. Studies however on alteration in adhesive behaviour of bone marrow-derived MSC on ECM proteins with aging are still missing.

In the present paper we have investigated adhesive behaviour of bone marrow-derived MSC and MG-63 osteoblastic cells onto three adhesive ECM proteins: fibronectin, vitronectin and collagen I during a 21-days culture period aiming to understand how adhesive phenotype of MSCs alters with aging and how long MSC could keep their attachment affinity to different ECM proteins. We assumed that after reaching the confluence and with further incubation in rat BM-MSCs aging processes are triggered that allow studying the effect of aging on stem cell affinity to ECM proteins. MG-63 cells were used as a model for cells not affected from aging because of their unrepressed replicative activity and long lifespan. The results of this study will contribute to better understanding of the processes of aging in mesenchymal stem cells, in general and of processes of their expanding, in particular.

2. Material and Methods

2.1. Cells and cell culture conditions

MSC were isolated from the bone marrow of femurs and tibias of 4-week-old male rats according to the centrifugation method of Dobson KD et al, 1999 as previously described (Keremidarska et al. 2015). All protocols concerning the use of animals were approved by the Institutional Ethics Committee. The cells of second-passage at sub-confluence were used for all of the experiments.

MG-63 osteoblast-like cells, originally isolated from a human osteosarcoma were purchased from the American Tissue Culture Collection. Both cell types were maintained in DMEM, supplemented with 10% foetal calf serum, 1% antibiotic/antimicotic mixture (Sigma, Germany), 2 mM L-glutamine (Roche Diagnostics, Germany) and 1mM sodium pyruvate (Gibco BRL, Scotland). The cells were incubated at 37°C in a fully humidified atmosphere at 5% CO_2 in air. Media changes were performed twice a week. For cell experiments, the cells were detached with Trypsin-EDTA (Lonza, Belgium), counted in Neubauer haemocytometer and plated at a density of $2x10^4$ cell/cm^2. At the days 1, 3, 7, 14 and 21, cultures were assayed as described below.

2.2. Adhesion assay

Short-term, serum-free cell adhesion assays were performed using purified ECM proteins with optimal concentrations. At indicated time points the cells were harvested and seeded onto sterile glass coverslips (ø15 mm), placed in 24-well plates and pre-coated with collagen I (100 µg/ml, Cytonet, Germany), fibronectin (20 µg/ml, Roche Diagnostics, Germany) and vitronectin (2 µg/ml, Sigma, Germany). Collagen I (Coll I) solution was prepared in 10 mM chloridic acid, while the other protein solutions were prepared in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}. As negative controls plain (uncoated) glass coverslips were used. Protein coating was performed for 30 min at room temperature, followed by double wash with PBS and seeding of the cells with concentration $2x10^4$ cells per sample. To visualize overall morphology of vital, attached cells after 2 hours of incubation in serum-free medium, the cells were stained for 2 min with 0,001% fluorescein diacetate (FDA) (Sigma, Germany) dissolved in acetone, rinsed several times with PBS and then representative pictures of the cells were taken using a fluorescent microscope (Zeiss, Axiovert 25, Germany), equipped with a digital camera. Furthermore, cell number and spreading area of the adhered cells was evaluated by special Motic Image 2.0 software, using FDA pictures. At least six pictures per sample were evaluated.

Additionally, phase-contrast pictures of the cells were taken at each time point of the experiments. These pictures were representative for the morphology, proliferation rates and confluence of the culture at *in vitro* aging conditions.

2.3. Statistical Analysis

All experiments were carried out in triplicates and the data points are average. The error bars indicate standard deviations. Statistical significance between groups was calculated using ANOVA test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results and Discussion

The main objective of the present study was to elucidate how the adhesive phenotype of bone marrow mesenchymal stem cells alters with aging. We have defined the term "adhesive phenotype" as a transitory ability of the cells to interact with different ECM proteins and made efforts to visualize and quantify cell adhesive behaviour during 21 days in culture. In order to gain more information about the dynamics of cell adhesion as well as the effect of aging on the cell adhesive phenotype as a function of proliferative lifespan we compared rat BM-MSCs to MG-63 osteoblastic cells. BM-MSCs are normal somatic cells capable of maximal 30–40 population doublings *in vitro* (Banfi et al. 2000, Keremidarska et al. 2015) before entering a stage of non-dividing state, referred to as replicative senescence (Baxter et al. 2004). Their limited lifespan in culture is a result of telomeres loosening with each cell division. Contrary, MG-63 is an immortal osteosarcoma cell line having an unrepressed replicative activity suggesting that MG-63 cells are not influenced by aging. These two types of cells share the expression of integrin's chains, such as α1-, α2-, α5- and β1-chains (Roncoroni et al. 2013).Thus, comparing the adhesive phenotype of cells with different proliferative capacity and same integrin's profile we can understand and characterize better the dynamics of their interaction with some of the surrounding proteins during transition to senescence.

Phase-contrast pictures (Fig. 1) showed that both cell types have similar fibroblast-like morphology but differ in size: rat BM-MSCs are much larger than MG-63 cells. Fig. 1 confirmed also the differences in proliferation rate among both cell types. As can be seen rat BM-MSCs reached confluence on the day 3 after seeding whereas at the same seeding density MG-63 cells were almost confluent on the next day (day 1) of seeding.

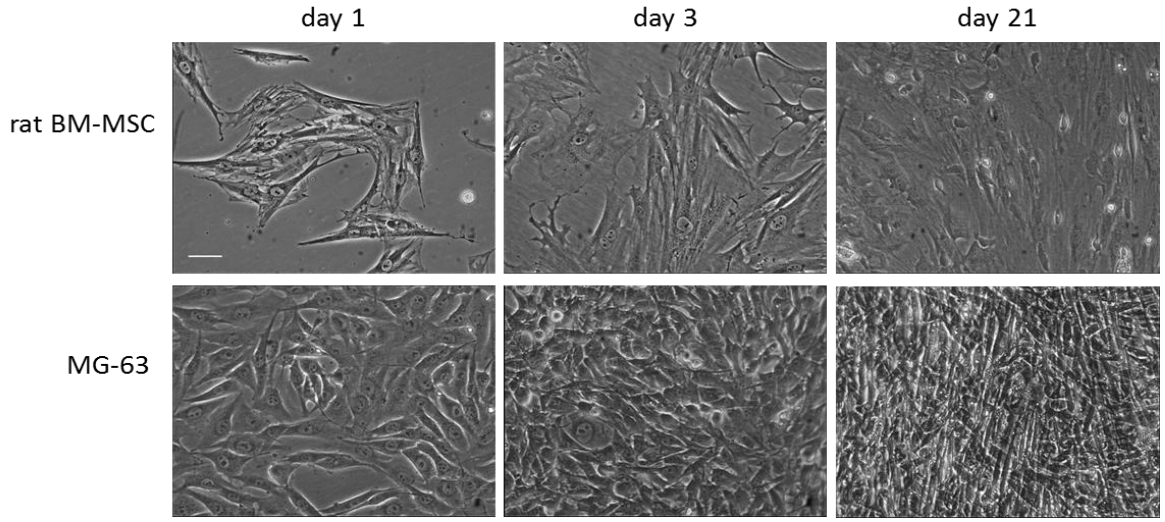

Fig. 1: Phase-contrast pictures of rat BM-MSCs and MG-63 cells showing cell morphology and proliferation rates at different stages of aging process. Bar is 100 μm

Contrary to the findings of Wagner and co-authors, who have observed that with long-term cultivation the cells became much larger with irregular and flat shape (Wagner et al. 2008) we did not find a significant alteration in the overall morphology of stem cells with aging. However, when we studied cell affinity to different ECM proteins we observed substantial differences in cell morphology depending on the type of proteins on which the cells attach and on the incubation time. Rat BM-MSCs demonstrated a highly dynamical adhesive

phenotype in the first week after plating (Fig. 2-A). Initially, on day 1 rat BM-MSCs adhered in the greatest degree onto VN as can be concluded form the high number of cells with polygonal shape. On Coll I the cells were largest in size but cell morphology was rather rounded or motile with many protrusions which are a sign for weaker cell affinity. The poorest cell adhesion was observed on FN where only single cells with rounded morphology were attached. On the day 3 rat BM-MSCs decreased their affinity to VN in comparison to day 1 but increased significantly their adhesion to Coll I and FN because of the high number of attached cells (Fig 2–B).

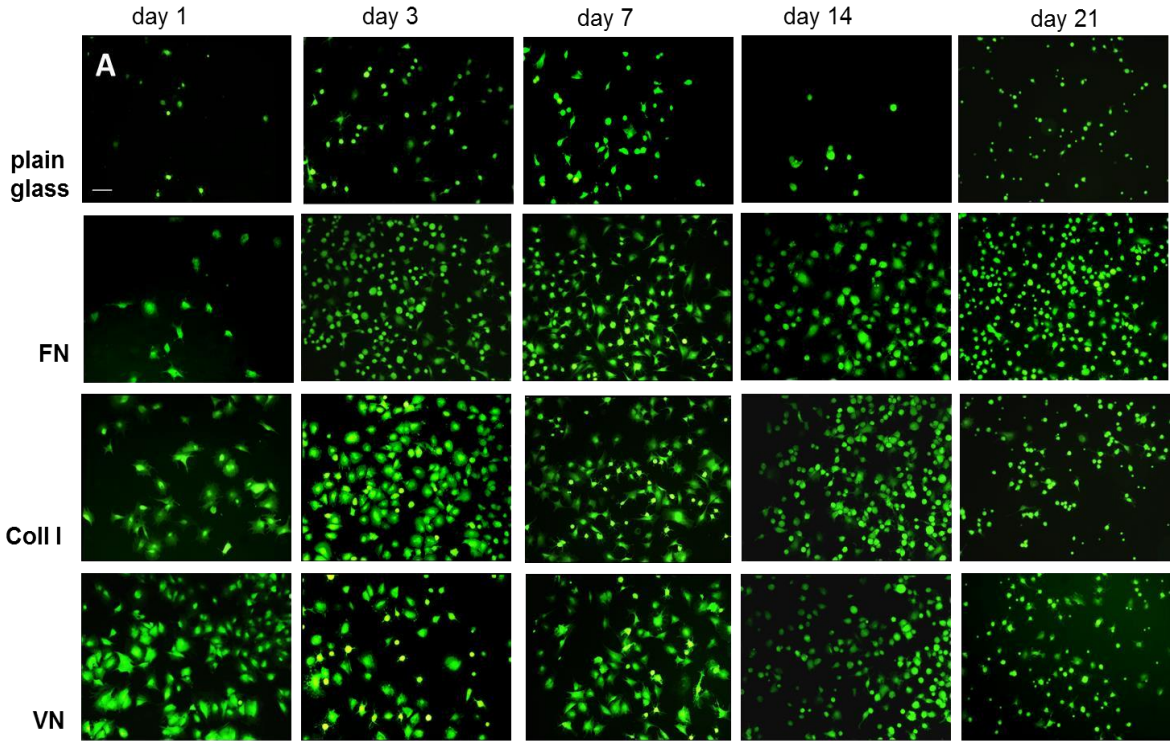

Fig 2-A: Overall morphology of rat BM-MSCs adhering for 2 h on plain glass, FN, Coll I and VN-coated coverslips harvested at different time points. (FDA staining; images taken on inverted fluorescent microscope, bar 100 μm)

The cells on both proteins differed however in their morphology: on Coll I most of the cells were flattened and rather polygonal while on FN were rounded (Fig. 2-A). On day 7 rat BM-MSCs on all studied proteins displayed almost equal adhesiveness though varying shape – from rounded to spindle-like. Based on the overall cell morphology it seems that after reaching confluence (at the day 3) and in the beginning of aging (at the day 7) the affinity of rat BM-MSCs to FN, Coll I and VN became similar. Results from quantitative estimation of the number of attached cells (Fig 2-B) and cell spreading area (Fig 2-C) showed however that more cells attach to FN and Coll I but the cell spread better on VN. With the further incubation (with aging) the cell morphology becomes rounded on all proteins and the cell size decreased indicating progressively diminished cell spreading. Similar adhesive behaviour was observed in tendon fibroblasts from old mice which also adhere less efficiently with aging (Arnesen and Lawson, 2006). In general these results were confirmed by the quantitative measurement of cell spreading (Fig 2-C) showing statistically significant ($p<0.05$) decrease of the cell surface area with ageing, though some variations between the proteins was also observed.

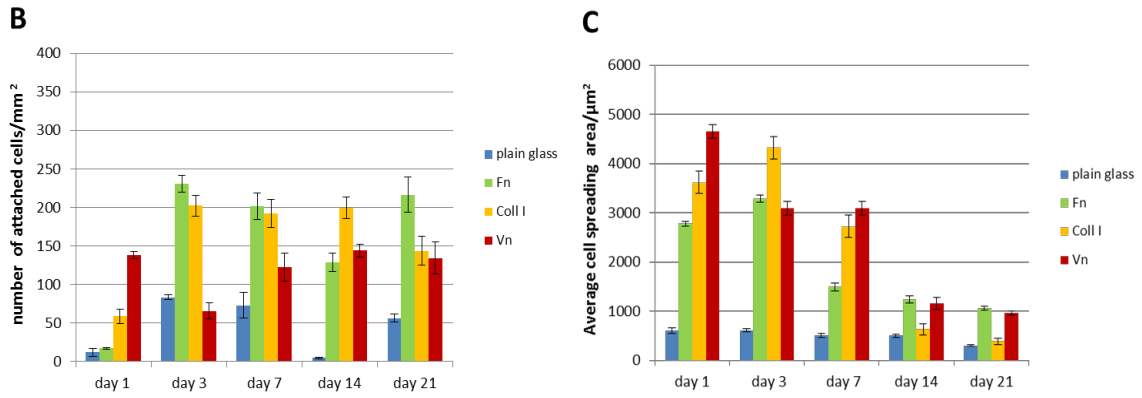

Fig. 2 – B, C: Cell attachment (B) and spreading (C) of rat BM-MSC cells after 2 h of incubation in serum-free medium on plain glass, FN, Coll I and VN at different aging stage.

On the day 1 the spreading of rat BM-MSCs was the highest on VN, followed by Coll I and FN, while the peak in the cell spreading activity on Coll I and FN was observed on day 3. The data for cell adhesion however did not corroborate with the spreading as the number of attached cells was not affected (Fig 2B), suggesting that the physical interaction with the substratum associated proteins is not affected. Conversely, the decreased spreading activity of rat BM-MSCs to FN, Coll I and VN with culture aging seems to correspond well with aging-related deficiency in integrin-mediated signaling, reported for other cell systems. For example, Goldstein and his team have explored the role of α5β1 integrin, the main FN receptor, in cellular aging (Hu et al. 1996) and have found that senescent normal and Werner syndrome fibroblasts exhibited reduced amounts of α_5 polypeptides on their membrane compared to normal young cells. The other integrin polypeptide subunit, β_1, was also found to be processed slower which may cause some immature heterodimer complex formation resulting in a reduced binding affinity to FN. Flickinger et al have found a loss in surface availability of the α_2 subunit of the main collagen receptor $\alpha_2\beta_1$ in late-passages of fibroblasts (Flickinger et al. 1992). Other authors have demonstrated decreased synthesis of β_1 polypeptide with aging in muscles (Larrick et al. 2016). Another reason would be that integrin receptors become less accessible to their ligands as a consequence of glycation and rigidification of ECM (Gao et al. 2008) or "uncoupling" of integrin transmission pathway due to improper protein folding of some of the proteins participating in integrin signaling (focal adhesion kinase, paxillin, and talin) (Arnesen et al. 2006, Hwang et al. 2009). That results in lower efficiency of cell adhesive machinery and increasing harmful effects for the cells. Since FN, VN and Coll I are known as proteins promoting cell proliferation the diminished affinity of rat BM-MSCs to all mentioned above proteins correspond also with the reduced proliferation of the cells with aging (Somaiah et al. 2015).

Conversely, the results obtained for MG-63 cells demonstrated that they had enhanced binding to FN and Coll I over the whole culture period. On FN the majority of the cells acquired their typical polygonal morphology during 21-days of culture with the best spreading on day 1 (Fig. 3-A). On Coll I the spread cells are more elongated suggesting advanced cell polarization. On day 7 a drop in adhesive affinity of MG-63 cells to Coll I was noticed because of the smaller cell size and round-up cell morphology. On VN MG-63 cells seems to spread worst, morphologically they are smallest in size and comparable with the cells attached on the negative control, plain glass. With aging cells became even smaller and rounder suggesting a decrease in MG-63 affinity to VN after day 3 of culture.

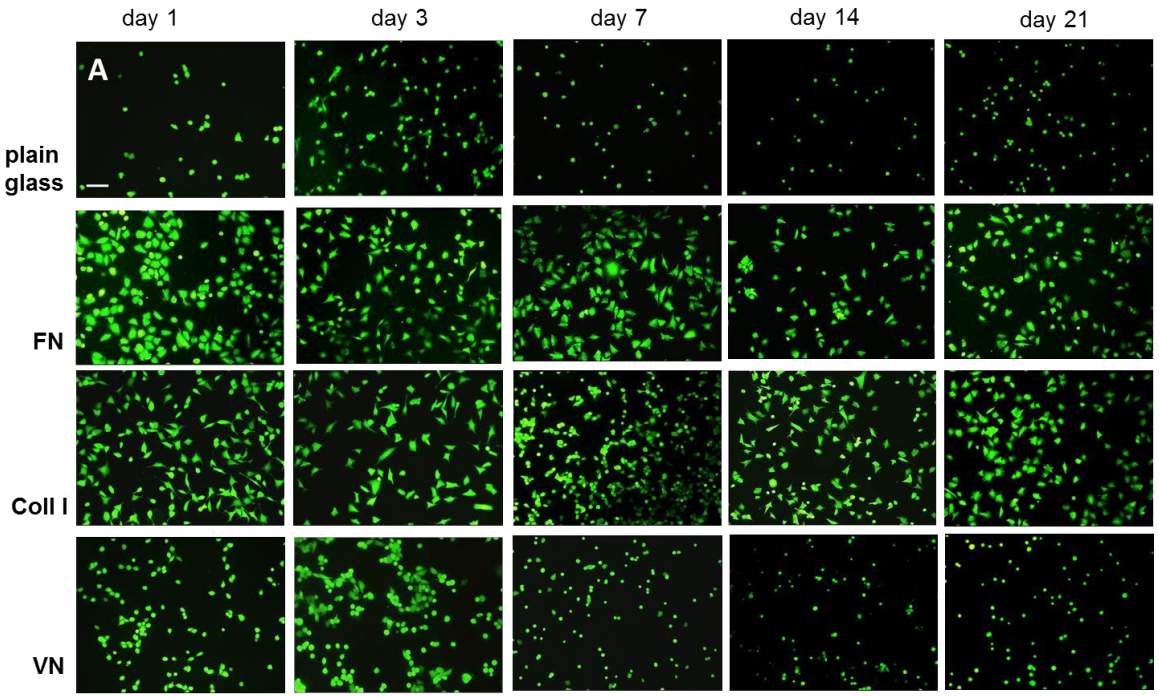

Fig 3-A: Overall morphology of MG-63 cells adhering for 2 h on plain glass, FN, Coll I and VN-coated coverslips after various time of cultivation. (FDA staining; images taken on inverted fluorescent microscope, bar 100 µm)

Quantitative data indicated that in contrast to rat BM-MSCs the number of attached MG-63 cells vary between different proteins (Fig. 3-B) and tend to diminished with aging while the cell spreading (Fig. 3-C) is almost unaffected, though some decrease was found on FN and Coll I at day 7 and 14, but which reverse at day 21.

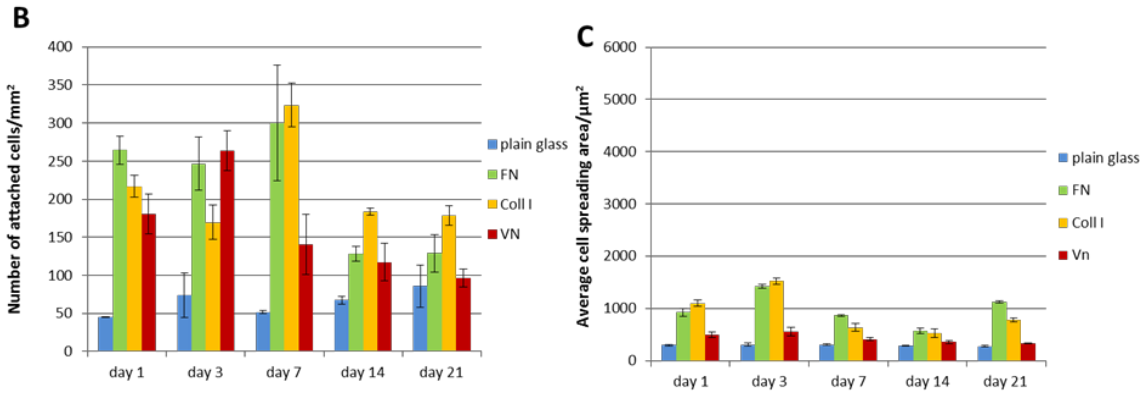

Fig. 3 – B, C: Cell attachment (B) and spreading (C) of MG-63 cells after 2 h of incubation in serum-free medium on plain glass, FN, Coll I and VN at different aging stage.

The high affinity of MG-63 to FN and Coll I was expectable, because FN and Coll I are the main proteins in the bone matrix. It might result from the higher expression of α1, α2, α3, α5, and βl integrin subunits in MG-63 cells detected by Clover and Gowen, (Clover and Gowen, 1994). Moreover, MG-63 cells have shown to produce fibronectin and type I collagen which is in accordance with our own data on viewing of FN matrix over a 21-days cultured period (not published data) and corresponds well to their phenotypic characteristics of early pre-osteobalstic cells, capable of abundant ECM synthesis. Furthermore, there is evidence in the literature about the functional role of FN and Coll I in

the early stages of osteogenesis. Moursi et al (Moursi et al. 1997) reported that the expression of FN mRNA and its subsequent translation and accumulation in the matrix are all high during the early stages of osteoblast differentiation and are reduced during cell maturation (Stein et al. 1990, Winnard et al. 1995). As undifferentiated cells stem cells did not deposit FN during the first days after plating therefore their low affinity to FN was also expected.

4. Conclusion

In general, our results demonstrate that adhesive phenotype of rat BM-MSCs is very dynamic in the first week after seeding but with further incubation stem cell adhesion to FN, VN and Coll I decreased significantly suggesting that aging processes affect the interaction of stem cells with ECM proteins.

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