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Adhesion to Extracellular Matrix Proteins can Differentiate between Human Bone Marrow Derived Mesenchymal Stem Cells and Fibroblasts

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Abstract

Mesenchymal stem or stromal cells (MSC) contribute *in vivo* to wound repair, and can be utilized for tissue regeneration. In contrast, fibroblasts may contribute to scar formation, and may even hamper functional regeneration. Depending on the clinical application, MSC are sometimes attached to a scaffold to maintain the cells in the area of regeneration. We, therefore, screened for proteins that allow a preferential binding of MSC, and avoid strong adherence of fibroblasts. The human MSC were isolated from bone marrow (bmMSC), or term placenta (pMSC). Synovial fibroblasts (SF) and dermal fibroblasts (DF) served as controls. In the first set of experiments, binding of bmMSC and SF to extracellular matrix (ECM) proteins was investigated by multiple substrate array (MSA®). From MSA® protein analyses, 57 peptides with potential MSC-binding sites were selected, and the binding of the cells to these peptides was determined. We report that MSC differ from fibroblasts in their binding to proteins of the extracellular matrix. MSC bind with higher efficiency to laminin-111, collagens-I, -III, and -IV and tenascin-C compared to fibroblasts, while both cell types bind with high efficiency to fibronectin, vitronectin and laminin-511. We conclude that overall MSC seem less selective, with respect to binding extracellular matrix components compared to fibroblasts, and fibroblasts attach to fewer proteins and peptides.

Keywords: Mesenchymal stem cells; Bone marrow; Extracellular matrix proteins; Fibroblasts

Abbreviations: bmMSC: Bone marrow derived MSC; DF: Dermal Fibroblast; ECM: Extracellular Matrix; FN: Fibronectin; LM: Laminin; MSA®: Multiple Substrate Array; MSC: Mesenchymal Stem or Stromal Cell; NC: Nitro Cellulose; PLL: Poly L-lysine; pMSC: Placenta derived MSC; SF: Synovial Fibroblast; TGF: Transforming Growth Factor

Introduction

Mesenchymal stromal cells (MSC) are multipotent progenitor cells that reside in different tissues [1-5]. Initially, MSC were isolated from bone marrow [6], and bone marrow until now remains the main source for MSC, for both diagnostic and clinical applications [3,7-10]. However, recent studies indicate that MSC from different sources, such as bone marrow, adipose tissue, umbilical cord or term placenta differ in their expression of cell surface markers [11]. Moreover, within the bulk MSC population of a given source, subsets may be identified and enriched by aid of antibodies [12-15]. These studies suggest that MSC from different sources might use distinct sets of receptors to facilitate homing or adhesion of MSC, to and in a given microenvironment. In our previous studies, we observed that bmMSC express the α1-, α2-, α3-, α6-, α7-, α9-, α11-, and β1-chains of integrins, and TGF-β1 regulated the expression of α2β1 integrin, thus facilitating the attachment of MSC to extracellular matrix proteins [16]. We hypothesized that fibroblasts might, therefore, differ from MSC in their expression of matrix-binding receptors, and investigated the binding of MSC to different proteins and peptides, in comparison to the binding of fibroblasts in more detail.

Materials and Methods

Isolation of cells

The MSC were isolated from bone marrow (bmMSC, 12 donors), or human term placenta (pMSC, 5 donors), after written consent and expanded, as described recently [16,17]. Human synovial fibroblasts (SF, 4 donors), or human dermal fibroblasts (DF, 6 donors), were isolated from surgical waste and expanded, as described recently [18,19]. All MSC preparations were characterized for adherent growth of the cells with fibroblastoid appearance, for expression of the relevant inclusion and exclusion markers (CD73, CD90, CD105, CD11b or CD14, CD34, CD45), by flow cytometry, and their tri-lineage differentiation capacity, as suggested by the International Society for Cellular Therapy (ISCT) consensus conference [20], and as described recently [16,17,21]. The study was approved by the Ethics Committee of the Medical Faculty of University of Tuebingen.

Multiple Substrate Array (MSA®)

To investigate the binding of the MSC to extracellular matrix proteins, a MSA® screening for MSC adhesion was performed [22]. In brief, 0.2 µL drops of protein solutions containing type I-, III-, IV-, V- and VI- collagens, fibronectin, laminin-111, laminin-511,

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tenascin-C or vitronectin, at optimized concentrations, were printed on nitrocellulose (NC)-coated glass slides in 8×8 arrays using a Packard BioChip Arrayer™, and air-dried (Figure 1A). The remaining surface area was sealed by covering the NC with StabilGuard® (SurModics, Eden Prairie, MN, USA) solution for 60 min at ambient temperature. A silicon frame was added on top of the arrays, to generate small cell culture chambers (Figure 1B). Then, 2×10^4 cells were added for 4 h to each array chamber and incubated on a rocking platform, placed in a humidified cell culture incubator with controlled atmosphere (37°C, 10% CO_2). During this period of time, cells were allowed to bind to the proteins (Figure 1B). Then, the floating cells were rinsed off by PBS. The adherent cells were stained by Coomassie Brilliant Blue (SIGMA-Aldrich; Figure 1C), and counted by an automated microscope-based device (Leica IRDB, with a motorized sample sled). On each array, quadruplicates spots coated with poly-L-lysine (PLL), or BSA served as positive and negative controls, respectively [22]. The results are presented as the normalized number of cells per spot (mean ± standard deviation), of three individual arrays per protein and cell type. Cells per spot counted on PLL were set as 100% (=positive control), and cells per spot counted on BSA as 0% (=negative control; Figure 1D). This kind of array investigates the avidity of the cell-matrix interaction since multiple binding sites on the ligands may interact with multiple receptors. For this type of cell-matrix interaction, we coin the term "binding" in this article. From proteins binding MSC with high avidity, candidate peptides for further analysis were delineated *in silico*.

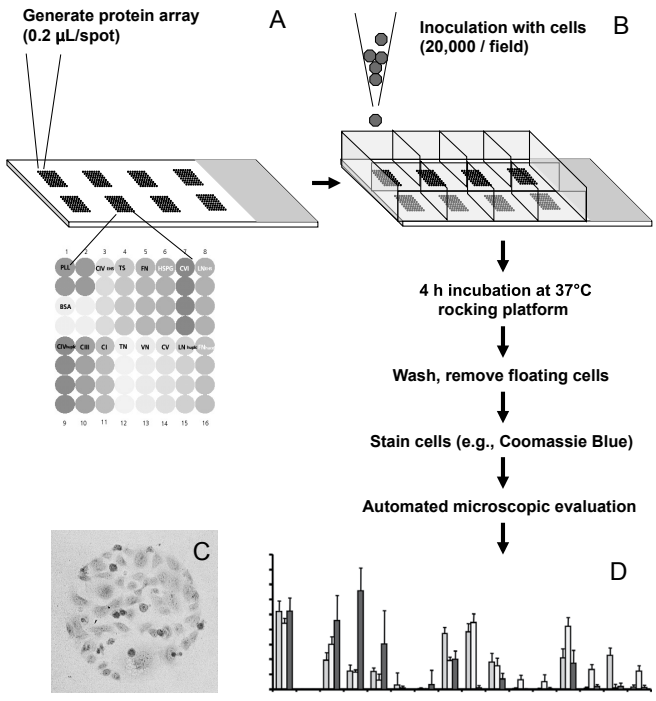

Figure 1: Investigation of cell binding to different proteins by adhesion screening on a multiple substrate array.

The basic concept of a multiple substrate array screen to investigate long-term interactions of cells on either different proteins, or on serial dilutions of a given protein, or combinations thereof, is outlined in a simplified graph. Protein arrays were generated by a micro blotter on NC-coated glass slides (A). Remaining NC was blocked by StabilGuard®, and cell culture chambers were generated by a silicon frame. Cells were added in DMEM medium (B) and incubated as indicated. After removal of floating cells, cells binding to the spots on the array were stained (C) and counted by an automated process (D). Technical details can be found in the original work published recently [22].

In a second series of investigations, binding of MSC to the selected peptides was explored in the same way, as described for proteins. However, since some small peptides may be not be accessible to cells when applied directly to the NC-coated surface, the peptides were coupled to BSA, by aid of a maleimide-facilitated reaction (Figure 2). Briefly, BSA was functionalized with maleimide groups, as described [23]. Peptides acetylated at the N-terminal amino groups (NMI TT GmbH, Reutlingen, Germany) were covalently attached to maleimide groups of the activated BSA (Cellendes GmbH, Reutlingen, Germany), *via* the thiol-function of a N-terminal cysteine residue. The peptides were used as crude trifluoro-acetic acid salts. The peptide-augmented BSA was spotted to the NC-coated glass slides, to generate the substrate array with peptides, as described [22]. The binding of the cells to the peptide-augmented BSA was enumerated, as described above. Binding of cells to PLL spots served as a positive control, while binding to unmodified BSA as a negative control.

Cell attachment assay

In a third line of experiments, the initiation of attachment of cells was investigated in more detail [24]. To this end, selected peptides were coupled to activated BSA, as described above (Figure 2), spotted to cell culture dishes (Figures 3A and 3B), and air-dried. The remaining surface was sealed with naïve (i.e. chemically not modified) BSA and 5×10^5 cells/dish were allowed to initiate attachment for only a short time (15-20 min), without rocking the samples (Figure 3C). Floating cells were then removed by aspiration of the supernatants, and by vigorously rinsing the samples with PBS. For evaluation, the spots were recorded by phase contrast microscopy, with a digital camera device (Zeiss Axiovert 200M). In these experiments, naïve BSA served as a negative control, and attachment to either laminin-111 or fibronectin served as a positive control (Figure 3D). For this type of cell-peptide interaction, the term "attachment" is used throughout this article. A cell attachment index was established to facilitate the comparison of the affinities of the peptides and cells included. The attachment index represents the number of different samples (i.e. MSC or fibroblast of a given donor), bound to a particular peptide, compared to all samples tested and expressed as % of total samples bound.

Statistics

Cell binding and cell attachment data are presented as mean values (± standard deviations) of multiple individual experiments. Statistical significance was tested by a double-sided student's T-test. P-values smaller than 0.5 were considered significant.

Results

Binding of mesenchymal stromal cells to extracellular matrix proteins

Binding of bmMSC to ECM proteins was investigated in comparison to SF. A strong binding of MSC and SF to PLL was evident, while only a few cells adhered to BSA (Figure 4). Both MSC and SF adhered well to fibronectin, either isolated from the extracellular matrix (FN_{hupls}), or from cellular sources ($_{cellular}FN$). Fibroblasts and MSC adhered to vitronectin and laminin-511. In contrast, weak or no binding was recorded for thrombospondin, heparan sulfate proteoglycan, and -with one exception in this experiment-for type V-collagen (Figure 4). Interestingly, no protein revealed a higher binding affinity to SF compared to MSC, but MSC attached more frequently, and/or more vividly to laminin-111, type IV-, III-, and I-collagens, and tenascin-C. To a lesser degree, this difference was also observed for binding of MSC

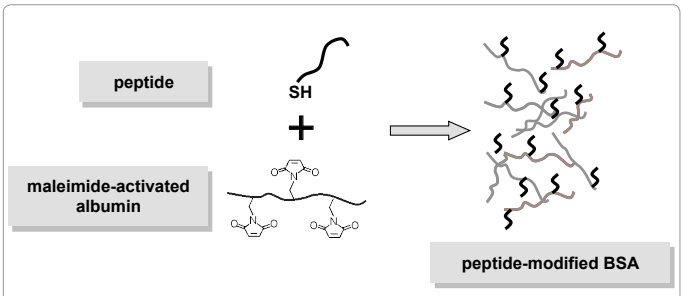

Figure 2: Covalent coupling of peptides to bovine serum albumin (BSA).

Peptides were synthesized with a short spacer and a free SH-group at the N-terminus. BSA was activated by reaction of 3-(maleimido)-propionic acid N-hydroxysuccinimde ester, attaching a maleimide group to the side chains of the lysins in BSA. The peptide was covalently coupled to BSA by nucleophilic addition of the SH-group to the maleimide double bond (Michael reaction).

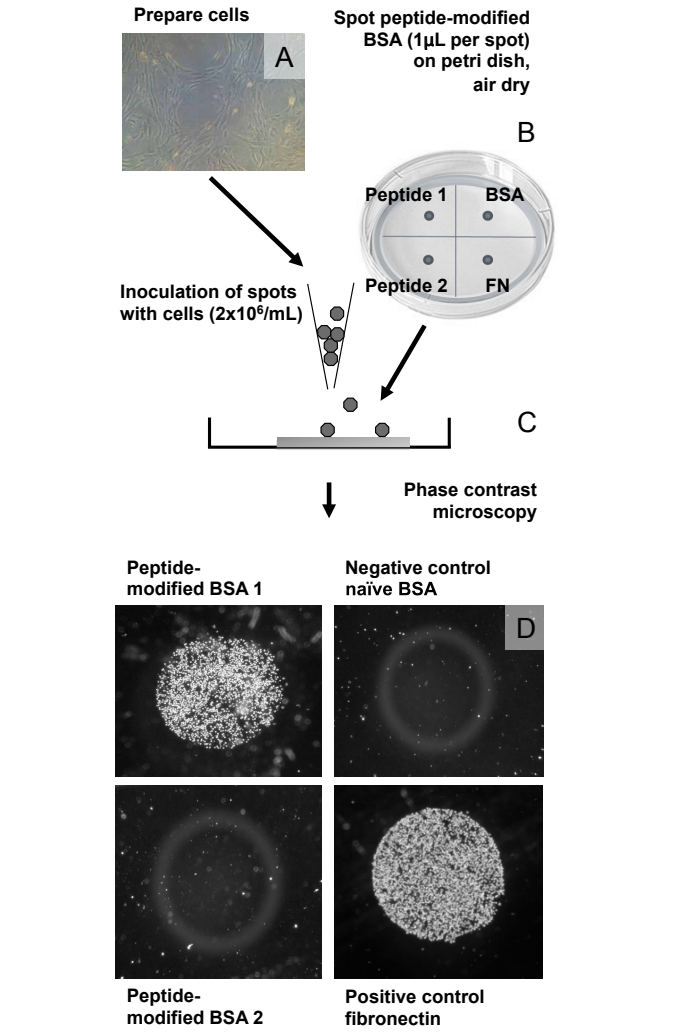

Figure 3: Short-term attachment assay.

Cells were prepared and resuspended in serum-free DMEM (2.3×10^6/mL; A). Peptide-modified BSA probes, naïve BSA or control proteins were spotted on a petri dish as indicated (B). After blocking the plastic surface with BSA, cells were added and incubated for 15-20 min in a humidified incubator (37˚C, 5% CO_2; C). Floating cells were aspirated, the samples were washed with PBS, and attached cells were visualized by phase contrast microscopy. Technical details have been described recently [24].

versus SF to type IV-collagen, when isolated from supernatants of the murine EHS cell line (Coll IV_{EHS}), or from human placenta (Coll IV_{huplc}, Figure 4).

Binding of mesenchymal stromal cells to matrix protein-derived peptides

Based on the proteins which preferentially bound MSC (Figure 4), 57 peptides (4 to 29 amino acids long) were delineated to compare binding to MSC (Figures 5A and 5B), or fibroblasts (Figures 5C and 5D) by MSA® techniques. In agreement with the total protein data, MSC bound to more individual peptides compared to fibroblasts, and the number of cells per spot was higher for several of these peptides with MSC, compared to the number of cells per spot observed with fibroblasts (Figure 5). Both cell types, MSC and fibroblasts bound to peptide P11, a control peptide containing the RGD binding motive (Figures 5A and 5C). MSC and fibroblasts bound to the laminin-derived P16, as well (Figures 5A and 5C). A somewhat stronger binding of SF was recorded with the vitronectin-derived peptide P37, compared to MSC. In contrast, stronger binding of MSC to collagen-derived P5, fibronectin-derived peptides P21 and P22 was observed. The laminin-derived peptides, P15 and P57, also showed better binding to MSC than fibroblasts. Therefore, the interaction of MSC with these peptides was investigated further in a short-term attachment assay.

Initial attachment of MSC and fibroblasts to selected peptides

Peptides P5, P15, P16 P21, P22 and P57 were further investigated for short-term cell-peptide interactions (Figure 6). Short-term attachment to laminin-111 and fibronectin served as positive controls, and attachment to BSA as a negative control. In this series of experiments, almost all MSC (87%) attached to the laminin-derived peptide P16. Interestingly, MSC failed to demonstrate rapid attachment to peptides P5, P15, P22 or P57 (Figure 6). However, less than 70% of fibroblast samples attached to peptide P16. A small number of MSC attached to the fibronectin-derived peptides P21, and a third of the fibroblast samples investigated attached to P21 (Figure 6). Attachment of fibroblasts to P5, P15, P22 or P57 was not observed. This data demonstrates that human bone marrow derived MSC and fibroblasts differ in adhesion to extracellular matrix proteins and peptides, and it provides evidence that attachment of MSC or fibroblasts (i.e. short- term assays) differs from binding (i.e. long-term assay) of the these cells.

Discussion

Binding of MSC to some of the proteins investigated in this study clearly differs from binding of fibroblasts to the same substratum. We, therefore, hypothesized that these differences in binding could possibly be applied to define peptides, and/or epitopes involved in MSC-specific cell-matrix interactions. Integrins, of course, are important for cell-matrix interactions. MSC share the expression of different integrins chains, such as α1-, α2-, α5- and β1-chains, with other mesenchymal cells, including osteoblasts, chondrocytes or fibroblasts. However, differences between MSC and osteoblasts were seen for examples for the expression of α3-integrin [25,26]. Slightly different binding patterns to extracellular matrix components were also reported for type III collagen, which binds MSC efficiently (Figure 4), but not osteoblasts [26]. The binding patterns reported in our study for MSC to fibronectin, laminin and vitronectin (Figure 4) match the previous binding patterns reported for osteoblasts [26].

Comparably, chondrocytes express the α5β1-integrin and binding to fibronectin, type-VI collagen and vitronectin, was reported [27]. In addition, chondrocytes express α1- and α3-chains at low levels,

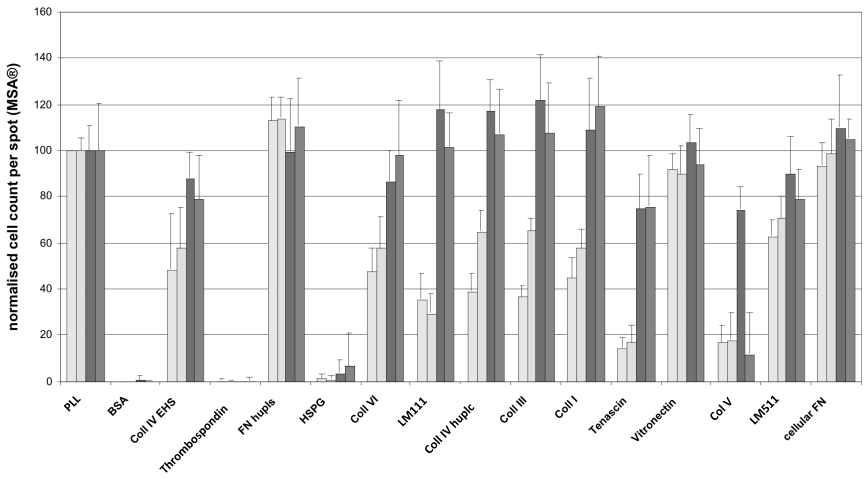

Figure 4: Comparing the long-term binding of MSC and fibroblasts to different proteins in MSA® assays.

Binding of MSCs (n=2 donors, dark grey bars) or fibroblasts (n=2 donors, light grey bars) to extracellular matrix proteins was explored as outlined in figure 1. Binding of the cells to poly-L-lysine (PLL) served as a positive control (=100%), and binding to BSA as a negative control (=0%). Binding to (from left to right) murine type IV collagen (EHS), thrombospondin, fibronectin (from human placenta), heparan sulfate proteoglycan (HSPG), human type VI collagen (from human placenta), laminin-111 (LM111), type VI collagen, type III collagen, type I collagen, tenascin-C, vitronectin, human type V collagen, laminin-511 (LM511), and cellular fibronectin was measured. MSC bound stronger to laminin-111, type-I-, type-III-, and type-IV-collagens, whereas for other proteins (e.g. fibronectin (FN), vitronectin, laminin-511), no difference was measured.

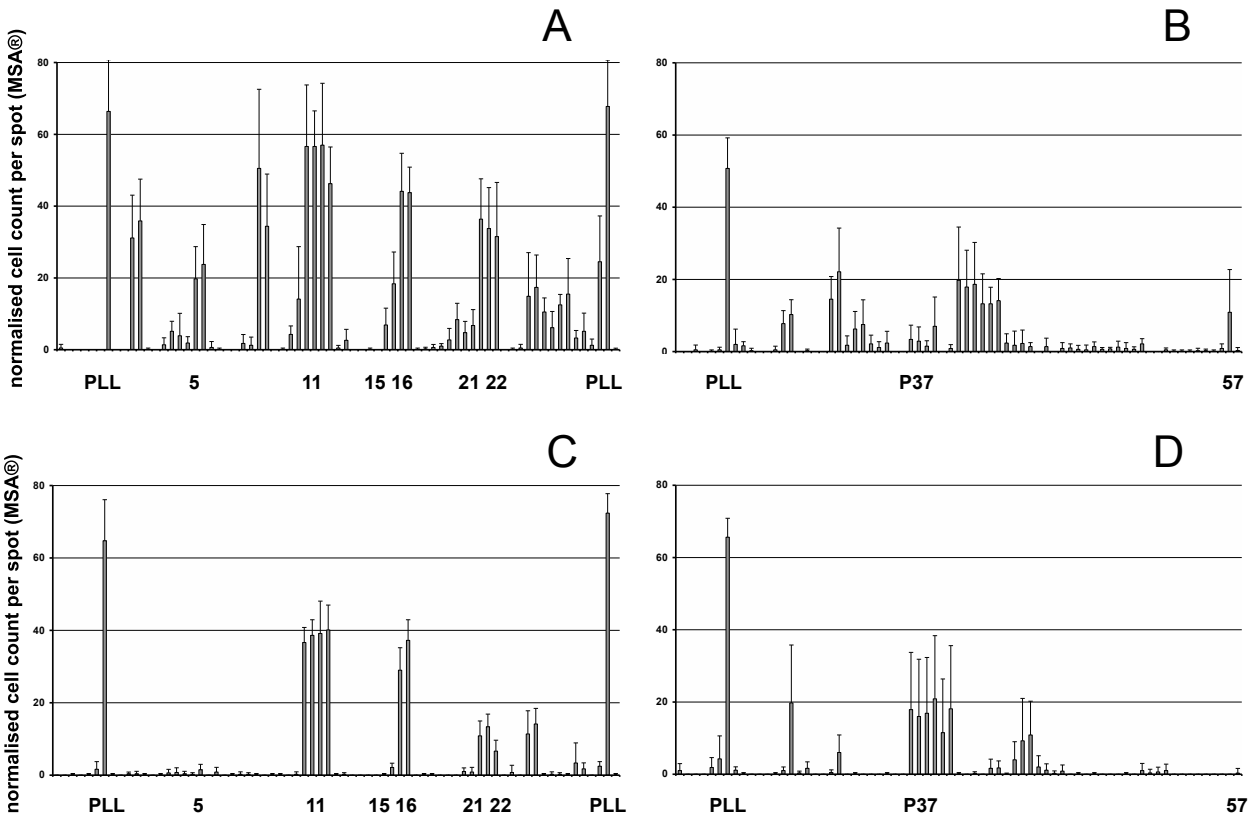

Figure 5: Comparing the long-term binding of MSC and fibroblasts to different peptides by MSA®.

Binding of MSC (panels A,B) or fibroblasts (panels C,D) to peptides attached to BSA was explored, as outlined in figure 1. Binding of the cells to poly-L-lysine (PLL) served as a positive contro,l and binding to unmodified BSA as a negative control. The binding patterns generated with relevant peptides (5,11,15,12,21, 22,37,57) are marked. The ordinate presents the normalized numbers of cells binding per spot on a scale from 0 to 80.

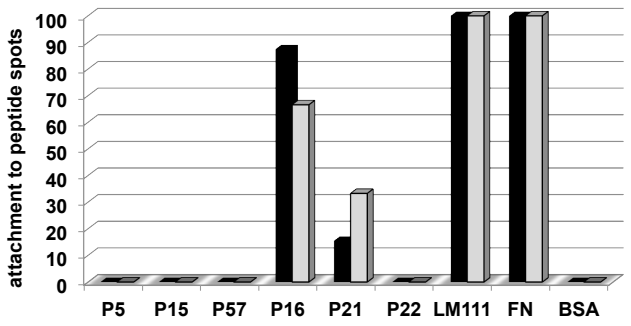

Figure 6: Exploring the short-term attachment of MSC and fibroblasts to distinct peptides.

Short-term attachment of MSC (black bars) and fibroblasts (grey bars) to selected peptides was investigated, as described in figure 3. Attachment of the cells to laminin-111 (LM111) or fibronectin (FN) served as positive controls, and attachment to BSA as a negative control. The ordinate presents the cell attachment index. This is the number of samples bound to a particular peptide compared to all samples tested, and expressed as % of total samples bound.

and not on all populations, and failed to express other α-chains, or the β3-integrin [28]. In contrast, human fibroblasts express the α1β1-integrin at high levels, but fail to express the α2-chain *in vitro,* at levels required for a measurable matrix interaction, without stimulation [29,30]. Moreover, expression of the collagen-binding integrin α11-chain was significantly higher on MSC, compared to fibroblasts [31]. Due to the differences in expression of integrins between MSC and other mesenchymal cells, it seems feasible to search for substrates that preferentially bind MSC. In addition, MSC from different sources, such as bone marrow or placenta, may differ in their expression of integrins. Our preliminary data on the transcript level would support this notion. However, this must be confirmed on the protein level. Patterns of expression of matrix proteins were not in the focus of this present study.

The proteins in the current study which yielded different binding patterns for MSC and fibroblasts were laminin-111, the type-I, -III, -IV, and -VI collagens and tenascin-C. These proteins share in terms of substrate binding to integrins [32]. For collagen binding, the α1-, α2-, α10-, and α11-integrin chains combine with the β1-integrin, while binding of laminins requires that the α1-, α2-, α3-, α6-, and α7-integrin chains combine with β1-integrin. For binding to tenascin-C, the α2β1 receptor was described [32,33]. We recently confirmed that human MSC express most of the above listed integrins (α1, α2, α3, α6, α7, α9, α11, β1) [16], required for strong binding to collagens, laminin-111 and tenascin-C. Moreover, TGF-β1 induced an elevated expression of the α2-, α6-, and β1-integrins, thus, further facilitating binding of MSC to the ECM components listed above [16].

Human fibroblasts express little α2β1-integrin *in vitro,* unless inoculated on collagen-coated cell culture dishes [29]. This indicates that expression of integrins is regulated by extracellular matrix-to-cell signaling. Moreover, as observed with MSC [16], TGF-β1 induced elevated expression of α2β1-integrin in fibroblasts, as well [30]. Therefore, cytokines regulate the expression of integrins too. But in our experiments, cells were not grown in collagen-coated dishes, nor were TGF-β1 added to the cell culture media. MSC and fibroblasts were expanded in medium containing 10% FCS. Therefore, TGF-β1, naturally occurring in bovine sera, may influence the integrin expression on MSC and other cells, unintentionally. In addition, MSC express TGF-β1 *in vitro* (our own unpublished results and [34]). We, therefore, cannot exclude that TGF-β1 may have up-regulated the expression of α2β1- or αβ1-integrins in an autocrine manner in our experiments, and may have, therefore, influenced our results. The TGF-β1-dependent expression of α2β1-, α6β1-, or α11β1-integrins on MSC might be a critical factor for compensating differences in substrate binding observed between MSC and fibroblasts.

In long-term cell binding arrays, more than 80% of MSC samples displayed strong binding to peptide P16 (Figure 5A), whereas less than 70% of the fibroblasts bound to this peptide (Figure 5C). The same pattern was observed in a short-term attachment assay (Figure 6). In contrast to P16, fibroblasts yielded a somewhat higher affinity to peptide P21 in the short-term attachment assay (incubation for 15 min, Figure 3), compared to MSC (Figure 6), whereas MSC yielded a stronger binding to peptide P21 in the long-term attachment assay (Figure 5). These differences may only reflect variables due to the technical differences applied, when studying binding versus attachment. However, it may also indicate that the initial contact of a cell to the binding epitope and short-term interactions (i.e., the "on-rate"), follows different kinetics compared to the "off-rate". In addition, cooperative effects between several binding sites of either a given integrin to a given peptide motive, or cooperative effects between different integrins sharing the affinity to that peptide, may account for the differences recorded.

At any rate, peptides 16 and P21 served as substratum for MSC and fibroblasts in the attachment assay, and in the binding array, and this was at least in part in agreement with binding of these cells the total protein. In contrast, the collagen IV- and the laminin-α-chain-derived peptides P5, 15, and P57, respectively, facilitated a measurable *binding* of MSC (Figure 5), but failed to enable a short-term *attachment* of MSC (Figure 6).

We conclude that the cell binding patterns, as determined by the MSA® method, result in considerably different substrate affinities, compared to the patterns determined by a short-term attachment assay. We show that the MSA® can address biocompatibility and inoculation characteristic of biomaterials intended for *in vitro* seeding of MSC, and for *in vitro* tissue engineering purposes. In contrast, the attachment assay can yield knowledge regarding the on- and off- rates of MSC to individual binding sites. This technique, therefore, complements the MSA®, and may enable the selection of biomaterials intended for capturing MSC *in situ* during wound healing processes. Biomaterials containing the peptide P16 motive may be a compromise serving both purposes. But based on current data, we cannot conclusively postulate that all the peptides presented in this study, which preferentially bind to expanded MSC *in vitro*, will be suitable for clinical applications *in situ,* as binding of these peptides to MSC must be confirmed with cells *ex vivo*. And a clear specificity of preferred MSC attachment or MSC binding over interactions with fibroblasts was not resolved in this first study. Again, this must be investigated in future experiments in an *ex vivo* or *in vivo* context, as well. Moreover, additional peptides have to be screened to eventually define a MSC-specific attachment, and or binding motive.

Acknowledgements

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