The International Journal of Biochemistry & Cell Biology 45 (2013) 1410–1418

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Identification of fibronectin as a major factor in human serum to recruit subchondral mesenchymal progenitor cells

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ARTICLE INFO

Article history:
Received 21 January 2013
Received in revised form 12 April 2013
Accepted 14 April 2013
Available online 21 April 2013

Keywords:
Cell migration
Cell recruitment
Subchondral mesenchymal progenitor cells
Fibronectin
Serum fractionation

ABSTRACT

Human serum has the potential for mesenchymal progenitor cell recruitment in repair of articular cartilage lesions. It is unclear which factor(s) in serum mediate this migratory effect. Our goal was to identify cell recruiting factors in human serum fractions obtained by ion exchange chromatography. The recruiting activity of serum fractions on human subchondral mesenchymal progenitor cells was analyzed using 96-well chemotaxis assays. Protein composition of recruiting serum fractions were analyzed by mass spectrometry and showed 58 potential candidates. Fibronectin, gelsolin, lumican, thrombospondin-1 and WNT-9a were identified as key candidates for progenitor cell recruitment. Only human plasma derived and recombinant fibronectin showed significant recruiting activity on progenitors reaching 50–90% of the recruiting activity of normal human serum. Presence of fibronectin in all human serum fractions with recruiting activity was verified by Western blot analysis. This study shows that fibronectin is a key factor in human serum to recruit mesenchymal progenitor cells and might be involved in subchondral mesenchymal progenitor cell migration into cartilage defects after microfracture.

1. Introduction

Cell migration plays an important role in numerous physiological and pathophysiological processes, for example in embryogenesis, wound healing, angiogenesis or stem cell growth (Balkwill, 1998; Luster, 1998; Vorotnikov, 2011). Basically, cell motility is the locomotion of a cell to another position, either by chemotaxis or by chemokinesis. Chemotaxis is defined as the directed migration of a cell towards (positive chemotaxis) or against (negative chemotaxis) a gradient of a stimulus (Balkwill, 1998), whereas chemokinesis is the undirected motion of a cell at random or due to a change in its environment (Lauffenburger and Horwitz, 1996).

The ability of cells to migrate can be utilized in regenerative medicine. Regenerative medicine is a science field with the aim to restore human tissue, e.g. by the use of stem and progenitor cells (Sng and Lufkin, 2012; Mimeault et al., 2007) and already offered several new clinical approaches like treating cartilage defects (Brittberg et al., 1994), myocardial infarction (Zhang et al., 2010) or non healing wounds (Brower et al., 2011). Besides all improvements these therapies include, there are also critical issues to consider such as the risk of an immune response against transplanted cells, developing tumors or a possibly incorrect biodistribution of injected cells (Jones et al., 1989; Kruse and Gratz, 2009; Herberts et al., 2011).

Therefore, strategies have been developed, to recruit stem and progenitor cells in vivo to a defect (Bueno and Glowacki, 2009). Such a strategy is already applied in combination with microfracture when treating articular cartilage defects. For this therapy, first, the subchondral bone beneath the defected cartilage is microfractured to form an access to the spongious bone (2–3 mm deep) (Steadman et al., 2003). Then, a resorbable implant, soaked with autologous serum is fixed into the cartilage defect. Mesenchymal progenitor cells located in the subchondral spongious bone migrate into the implant recruited by factors in the serum and form cartilaginous repair tissue (Erggelet et al., 2009; Patrascu et al., 2010; Siclari et al., 2012).

Although human serum is successfully used to recruit the cells, it is unclear, which factors in serum are responsible for cell migration and recruitment. Concerning identification of a single protein it has to be considered that the human serum proteome is composed of roughly estimated 1 million different proteins (Anderson, 2005). There are proteins secreted by solid tissue (e.g. liver) and receptor ligands (e.g. hormones and cytokines). Furthermore, the serum

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http://dx.doi.org/10.1016/j.biocel.2013.04.016

contains proteins that are usually found within the cell but are released to the plasma as a result of cell damage or death. Moreover, foreign proteins are brought into serum by parasites or infectious organisms. In addition, human serum contains around 10 million different immunoglobulins. Another difficulty for serum proteome analyses is the fact that the concentration of these proteins covers a range of 10 orders of magnitude. Only 21 proteins account for ~99% of all serum protein content, whereas 55% of the whole serum protein content is constituted by a single protein: albumin (Anderson and Anderson, 2002; Tirumalai et al., 2003; Anderson, 2005; Luque-Garcia and Neubert, 2007).

Due to the fact, that there is a great diversity in human serum proteins among different individuals, the results achieved with cartilage repair treatment with a cell-free graft vary from patient to patient and make the clinical outcome difficult to predict (Corzett et al., 2010; Nedelkov et al., 2005). Therefore, it is required to determine which protein or which protein combination is responsible for the recruitment of subchondral mesenchymal progenitor cells. In previous studies it was shown that there are many proteins which are responsible for a significant increase of migration of mesenchymal stem and progenitor cells. These proteins are for example several growth factors such as the platelet-derived growth factor and the bone morphogenetic protein-2, -4 and -7 (Fiedler et al., 2002, 2004) the vascular endothelial growth factor-A and the placenta growth factor-1 (Fiedler et al., 2005), the insulin-like growth factors I and II (Fiedler et al., 2006) and the myristoylated alanine-rich C-kinase substrate (Miller et al., 2010) as well as several chemokines like CCL25 (thymus-expressed chemokine), CXCL7 (platelet basic protein), XCL1 (Lymphotactin) (Kalwitz et al., 2009; Endres et al., 2010), CXCL8 (Interleukin-8) (Mishima and Lotz, 2008), CXCL10 (interferon gamma-induced protein), CXCL11 (interferon gamma-inducible protein 9) (Kalwitz et al., 2010) and CXCL12 (stromal cell-derived factor-1) (Landsberg et al., 2011).

The variety of substances found to induce cell migration in mesenchymal stem and progenitor cells leads to the hypothesis that further substances or substance classes are involved in cell recruitment. Furthermore, the cell numbers which were stimulated to migrate is relatively low compared to the originally applied cells in the used assays (Kalwitz et al., 2009; Endres et al., 2010).

2. Materials and methods

2.1. Isolation and characterisation of subchondral mesenchymal progenitor cells

Human subchondral mesenchymal progenitor cells were isolated from subchondral cortico-spongious bone derived postmortem from femoral condyle or tibia head from 8 individual donors (3 females, 5 males, age 29–71 years), as described previously (Neumann et al., 2008). Cell identity was characterized using flow cytometry analysis and multilineage differentiation capability of subchondral mesenchymal progenitor cells was analyzed using common methods as reported previously (Kruger et al., 2012). The study was approved on 08/15/2007 by the ethical committee of the Charité-Universitätsmedizin Berlin.

2.2. Chromatography of human serum on Q Sepharose FF

The column XK 26/40 (GE Healthcare, Chalfont St Giles, Great Britain) for anion exchange chromatography (IEC) was packed with Q Sepharose FF (GE Healthcare) and equilibrated with 50 mM potassium phosphate buffer (Merck, Darmstadt, Germany), pH 7.5 with a linear flow rate of 56.6 cm/h.

Human serum (German Red Cross, Berlin, Germany) from three individual donors, obtained after centrifugation using no anticoagulant or preservatives, was pooled and diluted 1:2 with distilled water. Subsequently, 37 ml of the diluted serum was applied onto the column with a linear flow rate of 56.6 cm/h. Non-bound proteins were removed from the column by washing with 50 mM potassium phosphate buffer, pH 7.5. Bound proteins were eluted with 50 mM potassium phosphate buffer, pH 7.5 containing increasing amounts of NaCl (Roth, Karlsruhe, Germany). In the first step the buffer contained 150 mM NaCl, followed by a NaCl concentration of 200 mM and finally the elution was performed with a linear salt gradient from 200 to 500 mM NaCl. All washing and elution steps were performed with a linear flow rate of 56.6 cm/h. The eluted proteins were collected in fractions of 50 ml volumes. 2 ml of each fraction were used for cell migration analysis. To prevent protein denaturation, protease inhibitor cOmplete ULTRA tablets, EDTA free (Roche Applied Science, Basel, Switzerland) was added to the remaining part of each fraction which was used for mass spectrometry. All fractions were stored at −80 °C. Protein concentration of the collected serum fractions was determined using the bicinchoninic acid assay (Sigma–Aldrich, St. Louis, MO, USA) according to the manufactures protocol using bovine serum albumin as a standard.

2.3. Cell migration assay

The recruiting activity of non-fractionized serum, serum fractions, human plasma derived fibronectin (Fn, Biochrom, Berlin, Germany), recombinant human (rh) Fn (R&D, Minneapolis, MN, USA) rh WNT9a (Abnova, Taipei City, Taiwan), human plasma derived gelsolin (Sigma), rh lumican (R&D) and rh thrombospondin-1 (R&D) were tested using a 96-well cell migration assay with polycarbonate membranes (pore size 8 μm; Neuroprobe, Gaithersburg, MD, USA). The assays were performed in triplicates using three independent cell preparations each ($n = 9$ assays per sample and dose). Briefly, 3×10^4 subchondral mesenchymal progenitor cells (passage three) were resuspended in migration medium (DME-medium containing, 100 U/ml penicillin, 100 mg/ml streptomycin, 100 μg/ml gentamycin, 100 ng/ml amphotericin B (all Biochrom) and 0.1% human serum) and seeded in the upper wells.

All samples to be tested were given to the lower wells. IEC fractions were tested in a four times dilution. As a control, non-fractionized human serum was tested at a concentration of 10%. Human plasma derived Fn and gelsolin were tested at 15 different concentrations between 500×10^3 ng/ml and 0 ng/ml. Rh Fn, rh lumican and rh thrombospondin-1 were tested at 10 different concentrations between 20×10^3 ng/ml and 0 ng/ml. Rh WNT9a was tested at 10 different concentrations between 5×10^3 ng/ml and 0 ng/ml. All substances were solved in 50% PBS/50% migration medium. As negative control (0 ng/ml sample), 50% PBS/50% migration medium was used. Chemokinesis was determined by testing cell migration upon adding the recruiting factors to the upper and lower wells.

The assay chambers were incubated for 20 h at 37 °C and 5% CO_2. After removal of non-responding cells on top of the filter, subchondral mesenchymal progenitor cells that migrated through the membrane were fixed in methanol/acetone (Avantor, Center Valley, PA, USA), stained with Hemacolor (Merck) and enumerated microscopically by counting the number of stained cells in four representative fields. Data were normalized by calculating the migration index (MI). This index was determined as the average number of migrated cells in stimulated wells divided by average number of migrated cells in the negative control. Moreover, statistical analysis was performed.