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Technical note

Simultaneous detection of ERK-, p38-, and JNK-MAPK phosphorylation in human adipose-derived stem cells using the Cytometric Bead Array technology

Ralf Schubert [b], Helmut Geiger [a], Stefan Zielen [b], Patrick C. Baer [a,*]

[a] Division of Nephrology, Department of Internal Medicine III, Goethe University, Theodor Stern Kai 7, 60590 Frankfurt/M., Germany
[b] Department of Pediatrics, Goethe University, Theodor Stern Kai 7, 60590 Frankfurt/M., Germany

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ABSTRACT

Despite expanded research in stem cell biology, little is known about the mechanisms underlying migration, growth, and differentiation of adipose-derived adult mesenchymal stem cells (ASC). The simultaneous measurement of intracellular pathways opens new avenues to gain further insights in these processes. We used the Cytometric Bead Array (CBA) Flex Set technology to simultaneously analyze protein phosphorylation after stimulation of ASC and compared the results with data generated by corresponding Western blots.

Signal transduction of ASC was stimulated by epidermal growth factor (EGF) and analyzed by determining phosphorylation of mitogen-activated protein kinases (MAPKs) ERK, p38, and JNK by Western blotting and CBA. After incubation with EGF, all MAPKs were significantly but differentially phosphorylated depending on time and dose. Furthermore, the ERK-response was abolished by EGF-R antagonist AG 1478 and kinase inhibitor PD98059, whereas p38 and JNK were only inhibited by AG1478. The stimulation and inhibition profiles between the two assays were highly comparable and the data were significantly correlated.

In the present study we demonstrated that the CBA technology offers a reliable and convenient method for multiplexing of phospho-proteins in the evaluation of signal transduction pathways of adipose-derived mesenchymal stem cells.

1. Introduction

The analysis of intracellular signaling cascades using antibodies directed against phospho-specific proteins provides an excellent tool for evaluation of signal transduction pathways. Available techniques to measure protein phosphorylation can differ heavily in their convenience and their outcome. Western blots (WB) or enzyme-immunoassays had been typically used for the estimation of protein phosporylation. Both techniques allow the evaluation of only one phospho-protein per approach, and need large amounts of sample. Furthermore, Western blotting is very time-consuming and the quantification by densitometry is not undisputable. We here used the Cytometric Bead Array (CBA) Flex Set technology to analyze protein phosphorylation after stimulation of cultured, adherent-growing mesenchymal stem cells. The CBA technique is based on different bead populations with distinct fluorescence intensities that are coated with capturing antibodies specific for different cytokines, chemokines, or phospho-proteins (Varro et al., 2007; Wagner

Abbreviations: ASC, adipose-derived stem cells; CBA, Cytometric Bead Array; EGF, Epidermal growth factor; E100/10/1, EGF 100/10/1 ng/ml; ERK, extracellular signal related kinase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; WB, Western blot.

* Corresponding author. Goethe University, Department of Internal Medicine III, Division of Nephrology, Theodor Stern Kai 7, 60590 Frankfurt/M., Germany. Tel.: +49 69 6301 5554; fax: +49 69 6301 4037.

E-mail addresses: ralf.schubert@kgu.de (R. Schubert), h.geiger@em.uni-frankfurt.de (H. Geiger), stefan.zielen@kgu.de (S. Zielen), P.Baer@em.uni-frankfurt.de (P.C. Baer).

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et al., 2007). These beads are mixed with phycoerythrin-conjugated detection antibodies to form sandwich complexes, and simultaneously quantificated due to their different bead populations by flow cytometry.

Recently, little is known about the mechanisms underlying the stimulation of adipose-derived mesenchymal stem cells (ASC) by cytokines, chemokines, and growth factors. The epidermal growth factor (EGF) is involved in physiological and pathophysiological processes of various tissues and organs, like morphogenesis and repair, and in migration, proliferation, and differentiation of stem cells (Tamama et al., 2006; Fan et al., 2007). EGF's multiple cellular actions are mediated by binding to its receptor (EGF-R), followed by receptor dimerization, autophosphorylation, and recruitment of kinase substrates. These events result in phosphorylation and activation of the Ras / Raf / mitogen-activated protein kinase (MAPK) pathway, leading to phosphorylation of regulatory proteins, and transcription factors and culminating in cell migration, proliferation, or differentiation. Using CBA technology and Western blot technique, we estimated phosphorylation of ERK1/2- (extracellular signal related kinase, p44/42), JNK1/2- (Jun N-terminal kinase), and p38-MAPK, molecules known to be activated by EGF-R ligands.

In this study, we found a good correlation between the results from CBA and the corresponding WB data. Therefore, the CBA technology offers a reliable and convenient method for multiplexing of phospho-proteins in the evaluation of signal transduction pathways of adipose-derived mesenchymal stem cells.

2. Materials and methods

2.1. Human adipose-derived mesenchymal stem cells

ASC were isolated from lipoaspirates from patients undergoing cosmetic liposuction, as described by Zuk et al. (2002). In accordance with a consensus reached by the investigators, we will refer to this adherent cell population as adipose-derived mesenchymal stem cells (ASC). Our study was approved by the ethics committee of the clinics of Goethe University, Frankfurt.

Briefly, the tissue was digested for 45 min with 0.075% collagenase I (Worthington). The stromal-vascular fraction was separated from the remaining fibrous material and the floating adipocytes by centrifugation at 300*g*. The sedimented cells were filtered through a 100 µm pore filter. Erythrocyte contamination was reduced by density gradient centrifugation with Bicoll (Biochrom). High contamination with erythrocytes was found to markedly decrease cell adherence and proliferation. A preceding density gradient separation provided a better yield of adherent cells than treatment with an erythrocyte lysing buffer. For initial cell culture and expansion of the cells, Dulbecco's modified Eagle's medium (DMEM, Sigma) with a physiologic glucose concentration (100 mg/dl) supplemented with 10% fetal calf serum (FCS; PAA) was used. Primary cell isolates and cultured cells were characterised as further described (Brzoska et al., 2005). Cultured ASC were $CD29^+$, $CD44^+$, $CD49a^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD166^+$, and $CD14^-$, $CD31^-$, $CD45^-$. In vitro differentiation potential of ASC was proven by specific media and described earlier (Baer et al., 2009a,b). For our experiments, we used the 2nd–5th passage of ASC isolated from five different donors.

2.2. Stimulations with EGF

For CBA and corresponding WB experiments, we used ASC from the same isolation and in the same passage which are identically treated. Cells were grown to subconfluence (70%), washed and kept in serum-starved for 2 h. Thereafter, cells were incubated in pure medium containing EGF (100 ng/ml, 10 ng/ml, or 1 ng/ml; Immunotools, Friesoythe, Germany) or PBS as a control at 37 °C for the indicated time points and processed as described below. Furthermore, we used PBS with 10% FCS as a stimulation control. Selected experiment cells were pretreated with the EGF receptor inhibitor AG1478 (50 nM, Calbiochem) or the MEK-inhibitor PD 98059 (10 µM, Sigma) for 30 min prior to stimulation with EGF. In these experiments the inhibitor was additionally coincubated with EGF during stimulation.

2.3. Western blot analysis

In brief, cells were lysed using 10 mM Tris pH 7.4, 0.1% SDS, 0.1% Tween20, 0.5% TritonX100, 150 mM NaCl, 10 mM EDTA, 1 M urea, 10 mM NEM, 4 mM benzamidine, and 1 mM PMSF and collected by scraping. After centrifugation, the pellet was suspended in Laemmli's buffer and heated at 95 °C for 5 min prior to electrophoresis on a 10% SDS polyacrylamide gel. Protein content was determined by a standard assay and equal volume of protein was loaded on each lane. The separated proteins were electrophoretically transferred to Immobilon transfer membrane (Millipore). Membranes were blocked for 2 h. Immunoblotting was performed by incubating with an antibody against phospho-JNK (pJNK), phospho-p38 (pp38), phospho-ERK1/2 (pERK) (anti-ACTIVE, Promega, Germany, Cat.-No V7931, V1211, and V8031), or total ERK (SantaCruz, Germany, Cat. No. sc-154), and by a secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG; Amersham Pharmacia). Protein bands were made visible using an enhanced chemiluminescence system (Amersham Pharmacia) and recorded on radiographic film followed by densitometric evaluation using ImageJ 1.36 (NIH, www.nih.gov). Data were calculated as ratio phospho-mAb versus whole ERK and expressed as x-fold versus PBS as control.

2.4. Cytometric Bead Array flex set

Preparation of samples was done according to the manufacturer's protocol for adherent cells (Becton Dickinson, Germany). Briefly, activation of cells was halted by adding denaturation buffer 1/5 (v/v) and cells were collected by scraping to dislodge from plate. Afterwards, samples were placed immediately in a boiling water bath for 5 min. Cell lysates were centrifuged at 14,000 rpm for 5 min and supernatants were stored at −70 °C until measurement. pJNK1/2 (T183/Y185), pp38 (T180/Y182), and pERK1/2 (T202/Y204) were quantitatively determined using antibodies from the multiplex Flex Set Cytometric Bead Array (Becton Dickinson, Cat. No. 560213, 560010, and 560012). Serial dilutions (1/2 v/v) of the standards were prepared, cell lysates were (1/4 v/v) diluted using assay diluent and transferred to a 96-well plate. Then 50 µl of mixed capture beads were transferred to each well. After a 3 h incubation period at RT, 50 µl PE detection