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Epidermal growth factor as a mechanosensitizer in human bone marrow stromal cells

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ABSTRACT

Epidermal growth factors (EGFs) e.g. EGF, heparin-binding EGF and transforming growth factor alpha and their receptors e.g. EGFR and ErbB2 control proinflammatory signaling and modulate proliferation in bone marrow stromal cells (BMSC). Interleukin-6 and interleukin-8 are EGF targets and participate in the inflammatory phase of bone regeneration via non-canonical wnt signaling. BMSC differentiation is also influenced by mechanical strain-related activation of ERK1/2 and AP-1, but the role of EGFR signaling in mechanotransduction is unclear. We investigated the effects of EGFR signaling in telomerase-immortalized BMSC, transfected with a luciferase reporter, comprising a mechanoresponsive AP1 element, using ligands, neutralizing antibodies and EGFR inhibitors on mechanotransduction and we found that EGF via EGFR increased the response to mechanical strain. Results were confirmed by qPCR analysis of mechanoresponsive genes. EGF-responsive interleukin-6 and interleukin-8 were synergistically enhanced by EGF stimulation and mechanical strain. We show here in immortalized and primary BMSC that EGFR signaling enhances mechanotransduction, indicating that the EGF system is a mechanosensitizer in BMSC. Alterations in mechanosensitivity and -adaptation are contributors to age-related diseases like osteoporosis and the identification of a suitable mechanosensitizer could be beneficial. The role of the synergism of these signaling cascades in physiology and disease remains to be unraveled.

1. Introduction

The family of epidermal growth factors (EGFs) describes a group of proteins with high structural and functional similarities. Family members besides EGF are heparin-binding epidermal growth factor (HbEGF), transforming growth factor alpha (TGFα), betacellulin, amphiregulin, epiregulin, epithelial mitogen and the neuregulins 1–4 (Abdallah et al., 2005). The proteins are ligands for members of the EGF receptor family of protein tyrosin kinases, which are ubiquitously expressed. The EGF receptor family consists of four members: EGFR (ErbB1, Her1), ErbB2 (Her2), ErbB3 (Her3) and ErbB4 (Her4), which differ in their ligand binding and phosphorylation capacities as ErbB2 is lacking the ligand-binding and ErbB3 the phosphorylation domain. After ligand binding receptor homo- or heterodimerization is induced, followed by subsequent phosphorylation and activation of signaling cascades (Ahmed et al., 2003). EGFR family signaling induces activation of RAS/MAPK, PLCγ/PKC, PI3K/AKT, and STAT signaling pathways, but also nuclear translocation and signaling have been reported (Alexander, 2001; Azizi et al., 2012). EGFR systems control proinflammatory signaling cascades and proliferation and are targeted for anticancer treatment, ErbB2 and EGFR targeting in breast (Brand et al., 2011) and colon cancer being prominent examples (Chandra et al., 2013; Cohen et al., 2015).

In recent years a role for EGF family members has been identified in bone biology: EGF enhances proliferation and migration in bone marrow stromal cells (BMSC) (Cruz-Lopez et al., 2011; De Luca et al., 2011), which can give rise to e.g. osteoblasts, chondrocytes and adipocytes and thus are the source of mesenchymal tissue regeneration. Stimulation of BMSC with EGFR ligands increases the production of growth and differentiation factors and cytokines like vascular endothelial growth factor (VEGF), angiopoetin-2, platelet-derived growth factor BB (PDGF-BB), granulocyte-colony stimulating factor (GCSF), hepatocyte growth factor (HGF), and interleukin-6 and -8 (IL-6, IL-8) in BMSCs and osteoblast precursors (Ebert et al., 2015; Eccles, 2011). Similar to other target tissues EGFR family stimulation in BMSCs and

Abbreviations: AP-1, activator protein 1; BMSC, bone marrow stromal cell; EEF1A1, eukaryotic translation elongation factor 1 alpha 1; EGF, epidermal growth factor; EGFR, EGF receptor,; ELISA, enzyme linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GCSF, granulocyte-colony stimulating factor; HbEGF, heparin-binding EGF-like growth factor; HGF, hepatocyte growth factor; IL6, interleukin 6; IL8, interleukin 8; hMSC-TERT, telomerase-immortalized human mesenchymal stem cells; PDGF-BB, platelet-derived growth factor BB; PI3K/Akt, Phosphatidylinositol 3-kinase/AKT Serine/Threonine kinase; PTGS2, prostaglandin-endoperoxide synthase 2; PTH, parathyroid hormone; PU, polyurethane; TGFα, tumor growth factor; VEGF, vascular endothelial growth factor; wnt, wingless-type MMTV integration site family.

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osteoblasts activates the above mentioned signaling cascades. Furthermore, extensive crosstalk to osteogenic signaling pathways such as wnt signaling, estrogen receptor(s), prostaglandins and PTH receptor type 1 signaling has been reported (Fedorchak et al., 2014). Proinflammatory genes like IL-6 and IL-8 are direct EGF targets and participate in the inflammatory phase of fracture healing/bone regeneration where they enhance osteogenic differentiation and mineralization via non-canonical wnt signaling via Wnt-5a and ROR2 (Hahn and Schwartz, 2009; Hedhli et al., 2014; Hess et al., 2004). EGFR knockout animals and dominant negative variants for EGFR indicated that EGFR plays an anabolic role in the skeleton, but recent work has also shown that EGFR signaling keeps osteoblasts in an undifferentiated stage, inhibits the expression of key osteogenic differentiation markers and is dispensable for the anabolic effect of intermittent PTH treatment (Huang et al., 2013; Humphrey et al., 2014; Iskratsch et al., 2014; Kerpedjieva et al., 2012).

Mesenchymal fate decision, lineage commitment and cell differentiation processes in mesodermal and mesenchymal precursors are also influenced by mechanotransduction following e.g. cyclic stretching or fluid flow, but the role of EGFR signaling in this context is unclear. Mechanical forces in the microenvironment are translated into biochemical cues by e.g. integrins and calcium channels that are associated to the cell membrane and the basis of the primary cilium (Kim et al., 2003; Klein-Nulend et al., 2012). Mechanoresponsive transcription is mediated via strain-related activation of e.g. ERK1/2 and nuclear translocation of transcription factors that bind to so called strain sensitive response elements (Kolar et al., 2010). AP1-response, SP1-response and other shear stress DNA-response elements have been described to mediate and modulate mechanoresponse (Lemmon, 2009; Liedert et al., 2006; Liedert et al., 2009; Liedert et al., 2010).

Alterations in mechanically orchestrated remodeling and adaptation are contributors to disease mechanisms and age-related tissue degeneration like in osteoporosis, a syndrome of dysadaptation caused by polygenetic susceptibility and lifestyle changes (Marie et al., 2014; Marmor et al., 2004; Mehta et al., 2010). The identification of a suitable "mechanosensitizer" could be of great benefit to these patients.

We have previously described a reporter gene system for mechanotransduction in BMSC using stably transfected telomerase-immortalized human bone marrow stromal cells (hMSC-TERT) cultured in polyurethane dishes where cyclic stretching can be applied with a homogenous stretching profile (Seefried et al., 2010). Here we show in hMSC-TERT cells that EGF is a mechanosensitizer that enables and amplifies the mechanoresponse of intrinsic AP-1 reporter gene system and downstream targets of mechanical stretch like Prostaglandin-Endoperoxide Synthase 2 (PTGS2) and Fos Proto-Oncogene, AP-1 Transcription Factor Subunit (FOS) (Liedert et al., 2009; Noriega et al., 2010). While strain alone does not significantly activate EGF-responsive genes like IL-6 and IL-8 on mRNA level, cyclic stretching is able to enhance EGF-responsive gene regulation in the presence of EGFR activating ligands. Since AP-1 transcription complexes are well described pro-osteogenic systems, the mechanotransduction via EGFR should drive osteogenic differentiation and regeneration and explain anabolic effects of EGFR on bone (Ozcivici et al., 2010). We identified EGFR and its ligands as powerful modulators of mechanosensitivity in early stages of mesenchymal commitment and differentiation.

2. Materials and methods

2.1. Cell culture

Media for cell culture were obtained from Thermo Fisher Scientific (Darmstadt, Germany), FCS was obtained from Biochrom GmbH (Berlin, Germany). Primary human bone marrow stromal cells (BMSC) were isolated from bone marrow from seven different donors (three male, four female, mean age 65.6 ± 10.0 SD) and cultivated up to four weeks by a standardized protocol (Hess et al., 2004). Bone marrow was obtained with informed consent from the femoral head of patients undergoing elective hip arthroplasty. The procedure was approved by the local Ethics Committee of the University of Würzburg. Briefly, bone marrow preparations were washed with Dulbecco's modified Eagle's medium, (DMEM/F12) supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μg/ml ascorbate (Sigma-Aldrich GmbH, Munich, Germany), and centrifuged at 1200 rpm for 5 min. The pellet was reconstituted in medium and washed four times, and the supernatants of the washing steps containing the released cells were collected. Cells were centrifuged and cultivated at a density of 1×10^9 cells per 175 cm^2 culture flask. Adherent cells were washed after 2 days and cultivated until confluence. Telomerase immortalized human bone marrow stromal cells (hMSC-TERT), established from a 33 year old male donor by the group of M. Kassem, and the stable hMSC-TERT-AP-1 cells generated therefrom, comprising a luciferase-based AP-1 reporter, were used as a tool to perform mechanistic studies. HMSC-TERT cells display a high proliferation capacity, while maintaining their mesenchymal differentiation capacity in vitro and in vivo (Liedert et al., 2010; Peake and El Haj, 2003; Pelaez et al., 2012). hMSC-TERT cells were cultivated in Earle's MEM containing 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin. All cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

2.2. Biochemical activation, cyclic stretching of hMSC-TERT cells and luciferase assay

3×10^4 cells per well were seeded on 24-well polyurethane (PU) plates where they adhere tightly after a few hours as shown before (Liedert et al., 2010) and stimulated on the following day with different concentrations of recombinant human EGF (PeproTech GmbH, Hamburg, Germany), Gefitinib (Biozol Diagnostics GmbH, Eching, Germany), Mubritinib (Sigma Aldrich GmbH), human EGF antibody (monoclonal mouse IgG clone #10825, R&D Systems, Wiesbaden, Germany) or human HbEGF antibody (monoclonal mouse IgG clone #406316, R&D Systems) as indicated. For cyclic stretching the same cell number was used, seeded on 24-well PU plates and cultivated for 24 h. After 24 h preincubation with different factors and inhibitors, PU dishes were placed in a bioreactor as previously described (Liedert et al., 2010) and cyclic stretching was performed twice for 30 min at 1 Hz and 1% extension with a 60 min pause in between. This stimulation regime resulted in a 2.5-fold higher induction of luciferase activity compared to the application of cyclic strain for 120 min without pausing in between (data not shown). Cells were harvested after another 24 h and lysed in 150 μl Reporter Lysis Buffer (Promega GmbH, Mannheim, Germany). 20 μl of each extract was analyzed for luciferase activity using the reporter gene assay provided by Promega GmbH in an Orion II Luminometer (Berthold Detection Systems, Pforzheim, Germany) in 96-well plates. Relative light units were normalized to protein content determined by using RotiQuant Protein Assay (Carl Roth GmbH, Karlsruhe, Germany) and the data from four wells were used to calculate the mean value.

2.3. Cyclic stretching of hMSC-TERT cells, RT-PCR and quantitative PCR

For cyclic stretching, 5×10^5 cells per well were seeded on 4-well PU plates and cultivated for 48 h overall. Stimulants were added 24 h hours before stretching or as indicated. PU dishes were placed in a bioreactor and cyclic strain was applied as previously described (Liedert et al., 2010). Immediately after stretching, cells were harvested and total RNA was isolated by using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed with MMLV reverse transcriptase (Promega GmbH) in a volume of 25 μl. For RT-PCR 1 μl of cDNA was used as a template in a volume of 50 μl. Taq DNA polymerase was obtained from Promega GmbH and primers were obtained