Annals of Anatomy 196 (2014) 286–295

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.de/aanat

Research article

BDNF and its TrkB receptor in human fracture healing

Olaf Kilian[a,b], Sonja Hartmann[a], Nicole Dongowski[c], Srikanth Karnati[d], Eveline Baumgart-Vogt[d], Frauke V. Härtel[e], Thomas Noll[e], Reinhard Schnettler[a,f], Katrin Susanne Lips[a,*]

[a] Laboratory of Experimental Trauma Surgery, University of Giessen, Kerkraderstr. 9, 35392 Giessen, Germany
[b] Department of Orthopedics and Trauma, Zentralklinik Bad Berka, Robert-Koch-Allee 9, 99437 Bad Berka, Germany
[c] Department of Trauma Surgery, Asklepios Klinik Lich, Goethestr. 4, 35423 Lich, Germany
[d] Institute for Anatomy and Cell Biology II, Division of Medical Cell Biology, University of Giessen, Aulweg 123, 35392 Giessen, Germany
[e] Institute of Physiology, University of Giessen, Aulweg 129, 35392 Giessen, Germany
[f] Department of Trauma Surgery, University of Giessen, Rudolf-Buchheim-Str. 7, 35385 Giessen, Germany

ARTICLE INFO

Article history:
Received 7 February 2014
Received in revised form 5 June 2014
Accepted 6 June 2014

Keywords:
Neurotrophin
Brain-derived neurotrophic factor
Tropomyosin-related kinases
Osteoblast
Bone
Blood vessel
Angiogenesis

SUMMARY

Fracture healing is a physiological process of repair which proceeds in stages, each characterized by a different predominant tissue in the fracture gap. Matrix reorganization is regulated by cytokines and growth factors. Neurotrophins and their receptors might be of importance to osteoblasts and endothelial cells during fracture healing. The aim of this study was to examine the presence of brain-derived neurotrophic factor (BDNF) and its tropomyosin-related kinase B receptor (TrkB) during human fracture healing.

BDNF and TrkB were investigated in samples from human fracture gaps and cultured cells using RT-PCR, Western blot, and immunohistochemistry.

Endothelial cells and osteoblastic cell lines demonstrated a cytoplasmic staining pattern of BDNF and TrkB in vitro. At the mRNA level, BDNF and TrkB were expressed in the initial and osteoid formation phase of human fracture healing. In the granulation tissue of fracture gap, both proteins – BDNF and TrkB – are concentrated in endothelial and osteoblastic cells at the margins of woven bone suggesting their involvement in the formation of new vessels. There was no evidence of BDNF or TrkB during fracture healing in chondrocytes of human enchondral tissue. Furthermore, BDNF is absent in mature bone. Taken together, BDNF and TrkB are involved in vessel formation and osteogenic processes during human fracture healing.

The detection of BDNF and its TrkB receptor during various stages of the bone formation process in human fracture gap tissue were shown for the first time. The current study reveals that both proteins are up-regulated in human osteoblasts and endothelial cells in fracture healing.

1. Introduction

Fracture healing is a complex physiological process that involves a sequence of coordinated activity at the cellular and molecular level. It contains discrete stages of tissue response including inflammation, recruitment and differentiation of mesenchymal stem cells, angiogenesis, callus and bone formation by recapitulation of embryonic enchondral and intramembranous ossification (Claes et al., 2012). Within this process, invasion of vessels and their stabilization by matrix is required for successful bone repair (Claes et al., 2012; Geris et al., 2008; Keramaris et al., 2008). Bone matrix is permanently reorganized through cell–cell- and cell–matrix-interactions during fracture healing. These processes are regulated by a precise orchestration of cytokines and growth factors. Changes in their expression pattern as well as an increase in reactive oxygen species (ROS) can induce delayed fracture healing (Beckmann et al., 2014). Systemic and metabolic diseases as well as inflammatory processes also have negative effects on fracture healing by disarrangement of the distinct cellular and molecular

* Corresponding author. Tel.: +49 6419939870; fax: +49 6419939879.
E-mail addresses: olaf.kilian@gmx.de (O. Kilian), Sonja.hartmann@chiru.med.uni-giessen.de (S. Hartmann), Nicole.dongowski@chiru.med.uni-giessen.de (N. Dongowski), Srikanth.karnati@anatomie.med.uni-giessen.de (S. Karnati), Eveline.baumgart-vogt@anatomie.med.uni-giessen.de (E. Baumgart-Vogt), Frauke.v.haertel@physiologie.med.uni-giesssen.de (F.V. Härtel), Thomas.noll@physiologie.med.uni-giessen.de (T. Noll), Reinhard.schnettler@chiru.med.uni-giessen.de (R. Schnettler), Katrin.s.lips@chiru.med.uni-giessen.de (K.S. Lips).

http://dx.doi.org/10.1016/j.aanat.2014.06.001
0940-9602/

cascade: Reduced levels of oestrogen and vascular endothelial growth factor (VEGF) as determined after menopause are linked to delayed bone regeneration in the systemic condition of osteoporosis (Giannoudis et al., 2007). In addition, it has been reported that, among others, brain-derived neurotrophic factor (BDNF) is increased in the systemic inflammatory profile of patients with chronic obstructive pulmonary disease (Loza et al., 2012). BDNF belongs to the family of neurotrophins that were originally identified as growth factors for neuronal cells and were detected both in central and peripheral neuronal tissue (Hallbook et al., 2006; Hohn et al., 1990; Huang and Reichardt, 2001; Kaplan and Miller, 2000; Kim et al., 2004). Additionally they were synthesized and released from non-neuronal cells such as fibroblasts, osteoblasts, endothelial cells, monocytes and mast cells (Cartwright et al., 1994; Donovan et al., 2000; Kurihara et al., 2003; Labouyrie et al., 1999). With the exception of osteoblasts these cells are involved in formation of a fracture gap haematoma, replacement of the haematoma by granulation tissue as well as angiogenesis during the inflammatory phase whereas osteoblasts are the most important cells of the repair phase of fracture healing (Claes et al., 2012). Development of osteoblasts from differentiating mesenchymal stem cells as well as building and remodelling of bone matrix are processes that are regulated by growth and osteogenic factors. One of these factors essential to normal angiogenesis and appropriate callus architecture is VEGF (Geris et al., 2008). The neurotrophin BDNF is a signalling partner of VEGF in angiogenic tube formation. Donovan et al. (2000) showed that overexpression of BDNF stimulates angiogenesis through a VEGF signalling pathway (Donovan et al., 2000). The coordinated action of these two growth factors was recently verified (Long et al., 2013).

In general, BDNF acts through binding to the highly specific tropomyosin-related kinase receptor B (TrkB) leading as well as to the p75-NTR receptor that is able to bind the other members of the neurotrophin family. When binding to the p75-NTR receptor, BDNF induces apoptosis, whereas coupling to TrkB triggers intracellular survival signalling pathways (Huang and Reichardt, 2001; Kaplan and Miller, 2000).

Recently, Usui et al. reported that BDNF leads to ROS generation via activation of TrkB (Usui et al., 2014). The phosphorylation of Akt was identified as the next step downstream which raised the assumption that BDNF induces endothelial cell migration through the PI3-kinase/Akt signalling pathway (Au et al., 2009; Kim et al., 2004). In addition to the cooperation of BDNF and VEGF, TrkB and ROS are able to stimulate VEGF receptor-2 (VEGFR-2) expression and activation (Ushio-Fukai, 2007; Usui et al., 2014). This explains the similar and sometimes even greater pro-angiogenic effect of BDNF in correlation with VEGF (Kermani et al., 2005; Long et al., 2013).

Besides acting as a survival factor and promoting angiogenesis, it is supposed that BDNF is involved in the cross talk of inflammatory cells. It has been reported that the release of BDNF is enhanced after nerve injury through mechanisms that include activation of mast cells and macrophage-like cells (Skaper et al., 2012). Additionally, BDNF plasma levels were increased in patients with osteoarthritis compared to healthy individuals (Simao et al., 2014). In both contexts it is suspected that BDNF is involved in pain perception (Simao et al., 2014; Skaper et al., 2012).

Furthermore, BDNF enhances the expression and synthesis of alkaline phosphatase, osteopontin, bone morphogenetic protein-2 in cementoblasts through the TrkB-cRaf-ERK1/2-Elk1 signalling pathway. Cementoblasts produce mineralized cementum that forms the outer covering of the tooth roots (Kajiya et al., 2008). Thus, the functions of cementoblasts are somehow similar to those of the osteoblast. Within this framework, we were interested in the role of BDNF in osteoblast function and therefore in fracture healing.

In rodent models (mouse and rat), the distribution patterns of neurotrophins and their receptors were analysed during fracture healing. BDNF as well as other neurotrophins and its receptors were detected in the fracture callus (Asaumi et al., 2000; Grills and Schuijers, 1998). To the best of our knowledge no information is available to date on neurotrophins and their receptors in the process of human fracture healing. Therefore, in this study, we analysed the expression patterns of BDNF and its receptor TrkB at different time points in bone repair of patients undergoing osteosynthesis after bone fractures.

2. Materials and methods

2.1. Tissue samples

Permission for the biopsies was obtained from the Ethics committee of the medical Faculty of the Justus-Liebig-University Giessen (138/07). Mature adult bone samples from the iliac crest were obtained from patients ($n = 6$) undergoing surgical treatment with autologous bone grafting (Table 1). Biopsies from patients with osteosynthesis after fractures at different stages of fracture healing (initial fibrin matrix, granulation tissue, woven bone) were taken from the fracture gaps ($n = 6$ per group, approximate size of 5 mm^3). Samples for RNA extraction and RT-PCR were snap frozen in liquid nitrogen and stored at −80 °C or fixed in RNA stabilizer reagent (RNAlater, Ambion Ltd., Huntingdon, UK). For immunohistochemistry, samples of fracture healing and spongeious bone were fixed in 4% PFA for 24 h. After several washes in PBS the samples were decalcified in 10% EDTA (Carl Roth GmbH, Karlsruhe, Germany), embedded in paraffin and cut into 5 μm sections with a Leica RM2155 microtome (Leica, Wetzlar, Germany).

Positive control groups for BDNF and TrkB detection were prepared from biopsies of human astrocytoma with different grading, including polycystic astrocytoma grade I, astrocytoma stage II and fibril astrocytoma grade III (Wadhwa et al., 2003; Wang et al., 1998; Yamamoto et al., 1996). Assimakopoulou et al. (2007), Chiaretti et al. (2004) showed components of neurotrophin signalling pathways are expressed in astrocytoma tissue. The neurotrophins are structurally related proteins as well as their receptors (Trk) therefore the antibodies for detection of these proteins in astroytoma tissue should be highly specific.

2.2. HUVEC

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords and cultured according to Peters et al. (2005). The harvested cells were cultured in PromoCell™ endothelial cell growth medium (PromoCell, Heidelberg, Germany) supplemented with 10 vol/vol% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and Supplement Mix™ (final concentration of the components: 0.4% endothelial growth supplement with heparin, 0.1 ng/ml human EGF, 1.0 ng/ml bFGF, 1.0 μg/ml hydrocortisone). Confluent cultures of primary endothelial cells were trypsinized in phosphate-buffered saline (PBS, composition in mM: 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4 and 8.0 Na_2HPO_4, at pH 7.4, supplemented with 0.05 wt/vol% trypsin and 0.02 wt/vol% EDTA) and seeded at a density of 2.2×10^4 cells/cm^2 on 35-mm culture dishes or chamber slides. Confluent endothelial monolayers of passage 1 were used for RNA isolation or immunocytochemistry 3–4 days after seeding. For immunocytochemistry, cells were fixed with 4 wt/vol% paraformaldehyde (PFA) in PBS for 10 min at RT followed by three washing steps with PBS.