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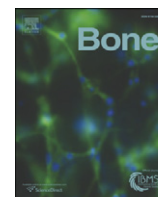

Original Full Length Article

Role of platelet-released growth factors in detoxification of reactive oxygen species in osteoblasts

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ABSTRACT

Introduction: Oxidative stress can impair fracture healing. To protect against oxidative damage, a system of detoxifying and antioxidative enzymes works to reduce the cellular stress. The transcription of these enzymes is regulated by antioxidant response element (ARE). The nuclear factor (erythroid-derived 2)-like2 (Nrf2) plays a major role in transcriptional activation of ARE-driven genes. Recently it has been shown that vascular endothelial growth factor (VEGF) prevents oxidative damage via activation of the Nrf2 pathway in vitro. Platelet-released growth factor (PRGF) is a mixture of autologous proteins and growth factors, prepared from a determined volume of platelet-rich plasma (PRP). It has already used to enhance fracture healing in vitro. The aim of the present study was to elucidate if platelets can lead to upregulation of VEGF and if platelets can regulate the activity of Nrf2–ARE system in primary human osteoblast (hOB) and in osteoblast-like cell line (SAOS-2).
Methods: Platelets and PRGF were obtained from healthy human donors.
HOB and SAOS-2 osteosarcoma cell line were used. The ARE activity was analysed using a dual luciferase reporter assay system. We used Western blot to detect the nuclear accumulation of Nrf2 and the amount of cytosolic antioxidant Thioredoxin Reductase-1 (TXNRD-1), Heme Oxygenase-1 (HO-1) and NAD(P)H quinine oxidoreductase-1 (NQO1). Gene expression analysis was performed by real-time RT PCR. ELISA was used for the quantification of growth factors.
Results: The activity of ARE was increased in the presence of PRGF up to 50%. Western blotting demonstrated enhanced nuclear accumulation of Nrf2. This was followed by an increase in the protein expression of the aforementioned downstream targets of Nrf2. Real-time RT PCR data showed an upregulation in the gene expression of the VEGF after PRGF treatment. This was confirmed by ELISA, where the treatment with PRGF induced the protein level of VEGF in both cells.
Conclusions: These results provide a new insight into PRGF's mode of action in osteoblasts. PRGF not only leads to increase the endogenous VEGF, but also it may be involved in preventing oxidative damage through the Nrf2–ARE signalling. Nrf2 activation via PRGF may have great potential as an effective therapeutic drug target in fracture healing.

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Abbreviations: ARE, Antioxidant response element; HO-1, Heme Oxygenase-1; hOB, primary human osteoblast; NQO1, NAD(P)H quinine oxidoreductase-1; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; PPP, Platelet-poor plasm; PRGF, Platelet-released growth factor; PRP, Platelet-rich plasma; ROS, reactive oxygen species; TXNRD-1, Thioredoxin Reductase-1; VEGF, vascular endothelial growth factor.

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Introduction

Large bone defects still challenge the orthopaedic surgeon. Although multiple approaches to overcoming the lack of osteogenic cells at bone healing sites have been tried, difficultly persists. Practicing surgeons have introduced various kinds of treatment regimens for defects too large to heal by themselves, including bone strut grafting, segmental transport and allogenic bone augmentation. The emerging techniques in molecular biology have given rise to a hunt for bone growth accelerating substances like bone-morphogenic proteins and growth factors. In addition the use of autologous cell and thrombocyte preparations has gained popularity. Today mesenchymal stem cell preparation kits are readily available by several companies as well as kits for the preparation of platelet-rich plasma (PRP) and similar substances [1,2]. Of course PRP has given rise to hope for the ultimate healing agent in many conditions, but it has not been yet applied by the majority of orthopaedists and trauma surgeons. Nevertheless in maxillofacial surgery PRP has become a standard adjunct for osteointegration and defect healing. The safety of its use and application makes it an easy to use substance at low cost. PRP has been proposed to be the curing agent in many medical fields — with no clinical trials and pre-clinical investigations that prove a beneficial effect on any of the conditions that it was proposed for on high level of evidence. Nevertheless the majority of investigations seem to support positive effects of PRP for wound healing [3,4], bone healing [5], tendon healing [6], in treating inflammatory conditions [7,8] and infections [9, 10] and in many more uses (for a recent review see [11]). Only recently Sanchez et al have administered PRP intra-articularly in osteoarthritic patients, with beneficial effects [12]. PRP can provide a high local concentration of extrinsic factors during the initial phase of any injury [13,14]. Platelets adhere immediately, form a fibrin mesh and subsequently release growth factors and cytokines. By the iatrogenic increase of such extrinsic factors the intrinsic osteogenic capacity can be influenced. Studies on the influence of different PRP concentrations on osteoblasts show a positive effect on osteoblast growth [15]. Nevertheless to our knowledge, there is no investigation showing an induction of osteogenic markers in vitro when PRP is used as a media supplement. Classic indicators of osteogenic potential, alkaline phosphatase (ALP) activity and calcium deposition and osteoprotegerin (OPG) were even reduced when bone marrow stromal cells and oral osteoblasts were stimulated by different PRP concentrations [16,17]. PRGF is a pool of intrinsic growth factors released by activated platelets which are concentrated in a plasma-free solution without fibrinogen. Because of the lack of plasma protein in PRGF solution, we can assume that, the effect of PRGF is due to the pure platelets released factors. PRGF have been clinically used in the same way as PRP [6,18].

Aside from “classic” osteogenic indicators there are an increasing number of alternative pathways that can influence bone healing. Our group has recently described the importance of the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and subsequent activation of the Antioxidant response element (ARE), within callus formation after femur fractures in mice. Nrf2-deficient mice show signs of impaired bone healing (data submitted for publication).

The Nrf2–ARE system represents an important mechanism for tissue regeneration by detoxifying reactive oxygen species (ROS) and various other toxic substances [19]. The promoter region of so called “phase II” enzymes and other detoxifying enzymes such as Thioredoxin Reductase-1 (TXNRD-1), Heme Oxygenase-1 (HO-1) and NAD(P)H:quinine oxidoreductase-1 (NQO1) carry ARE, a cis-acting enhancer element, in their promoter region for these enzymes. ARE binds Nrf2, a member of the Cap“n”Collar family of transcription factors [20], and upregulate the transcription of the detoxifying [21, 22]. In fractures tissue concentrations of ROS were shown to be increased in a rodent model [23] and markers of increased ROS were similarly induced [24]. With regard to osteogenic differentiation upregulation of Nrf2 was demonstrated in multipotent MSC after adrenaline exposure [25]. Thereby the vulnerability of chondrogenic and osteogenic precursor cells to ROS could be reduced. Certainly one of the best-known factors that ROS activates is VEGF. VEGF is particularly important in bone regeneration for compensatory angiogenesis if oxidative stress occurs after trauma [19,26,27]. Recently our team has established a link between Nrf2 and VEGF. In this study VEGF prevents in vitro the oxidative damage via activation of Nrf2 pathway [28].

Here we have deliberately not investigated in vitro effects of PRGF on classic osteogenic marker molecules. Instead the aim of this study was to elucidate possible alternative mechanisms by which PRP could positively influence bone healing. To test this hypothesis we investigated the effect of PRGF on Nrf2–ARE system in osteoblast cultures, thereby in cellular protection against ROS-mediated damage.

Materials and methods

Preparation of PRGF

PRGF was produced from liquid-preserved platelet concentrates obtained by platelet apheresis that were not more than one day old, in accordance with the current German ethics laws (EK116/10 local ethical board RWTH Aachen University). The platelet concentrate of 9×10^9 per ml containing less than 5×10^4 leukocytes was centrifuged at 2000 *g* for 5 min and the pellet was resuspended in a total of 1.1 ml culture medium to prevent gelifying and achieve 5–10 times blood concentration. The concentrate was stored at −20 °C until thawed and thereby activated at least 30 min prior to use. Activated platelets were centrifuged at 18,000 *g* for 1 min. The collected supernatant is PRGF. PRGF were added at a concentration of 5% and 10% to the medium.

Isolation of hOB

HOBs were isolated from the femoral head of three male patients (33, 47 and 74 years old) undergoing hip replacement surgery. All patients had written informed consent as approved by the Medical Ethical Committee of Aachen University (EK135/09 local ethical board RWTH Aachen University). Trabecular bone fragments were cut into pieces, rinsed several times in phosphate buffered saline (PBS). Then pieces were transferred into Dulbecco's modified eagle's medium (DMEM) with 10% FCS, glutamine (58.5 μg/ml), 100 units/ml penicillin and 100 μg/ml streptomycine (Gibco, Paisley, UK). Medium were changed thrice weekly. Cells were grown on the culture dish after two till three weeks.

Cell culture

Human SAOS-2 osteosarcoma cells were cultivated in McCoys (Gibco, Paisley, UK) plus 15% FCS, 100 units/ml penicillin and 100 μg/ml streptomycine (Gibco, Paisley, UK). Both cell types were incubated at 37 °C in a humidified 95% air and 5% CO_2 atmosphere. For subculture, cells were detached by 1% trypsin (5×, Gibco, Paisley, UK) treatment. For Life cell imaging and phase contrast microscopy a biostation was used (Nikon, Düsseldorf, Germany). A scratch assay was performed using a pipette tip. Osteoblasts were incubated for 0, 2, 4 or 6 h with 10% PRGF or with additional PBS (control). The capturing of the videos took 6 h.

Cell viability assay

For 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST) assay, media were supplemented with 10 μl/well WST 2 h before spectrophotometric evaluation. Conversion of WST to formazan was measured at 450 nm by microplate spectrophotometry (Infinite M200, TECAN). This reaction reflects the reductive capacity of the cells, which represented the viability of the cells, and is

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