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Controlled release of fibrin matrix-conjugated platelet derived growth factor improves ischemic tissue regeneration by functional angiogenesis

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ABSTRACT

Sustained, local, low dose growth factor stimulus of target tissues/cells is believed to be of imminent importance in tissue regeneration and engineering. Recently, a technology was developed to bind growth factors to a fibrin matrix using the transglutaminase (TG) activity of factor XIIIa, thus allowing prolonged release through enzymatic cleavage. In this study we aimed to determine whether TG-PDGF.AB in fibrin could improve tissue regeneration in a standard ischemic flap model. *In vitro* determination of binding and release kinetics of TG-PDGF.AB allowed proof of concept of the developed binding technology. A single spray application of TG-PDGF.AB in fibrin matrix at a concentration of 10 and 100 ng/ml significantly reduced ischemia-induced flap tissue necrosis *in vivo* on day 7 after ischemic impact compared to controls. TG-PDGF.AB at a concentration of 100 ng/ml fibrin induced distinct angiogenesis as reflected by significantly improved tissue perfusion assessed by laser Doppler imaging as well as enhanced von Willebrand factor (vWF) protein expression determined by immunohistochemical means. In addition, significantly more mature microvessels were observed with 100 ng/ml TG-PDGF.AB in fibrin compared to control and vehicle groups as evidenced by an improved smooth muscle actin (sma)/vWF protein ratio. In conclusion, PDGF.AB in a conjugated fibrin matrix effectively reduced ischemia-induced tissue necrosis, increased tissue perfusion and induced the growth of a mature and functional neovasculature. The sealing properties of the fibrin matrix in conjunction with the prolonged growth factor stimulus enabled by the TG-hook binding technology may present an innovative and suitable tool in tissue regeneration.

Statement of significance

In our experimental study we elucidated recombinant platelet derived growth factor (PDGF) as a potential candidate in inducing angiogenesis. To avoid preterm growth factor degradation *in vivo* PDGF.AB was covalently linked to a fibrin scaffold using a bi-domain functionalized peptide (FXIII substrate site and plasmin cleavage site). This allowed PDGF binding to fibrin during spray application to the donor site and subsequent prolonged release via endogenous plasmin. This resulted in a mature vascular network thus enhancing tissue perfusion and consequently improved clinical outcome. With our present work we could certainly provide researchers and clinicians with an innovative versatile and reproducible technology not only to induce functional vascularity but also to improve attempts in tissue engineering in general by e.g. using different growth factors. Hence, we believe that this approach studied in the present work may provide a valuable input in an effort to drive the aim forward bringing experimental work in tissue engineering to clinic by using a clinically well characterized and used fibrin scaffold in combination with a human recombinant growth factor (fibrin scaffold linked with the specific binding technology).

1. Introduction

Current strategies to regenerate tissues of various origin focus on the exogenous delivery of growth factors [1]. Angiogenic

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growth factors seem to be of particular interest due to the necessity of adequate neovascularization during each regeneration process. The formation and expansion of a vascular network is critical to ensure sufficient perfusion and to preserve tissue viability within regenerating areas. Essentially, the induced vascular network has to be mature and functional. To effectively induce therapeutic angiogenesis, a bimodal approach is of critical interest. First, the local support of angiogenic growth factors is required and second that this delivery should be persistent over a certain time period until the de-novo formed vessels are mature. Several potential growth factor and scaffold candidates are available and have already been tested in various experimental models [2–6].

Platelet-derived growth factor as an indirect stimulator of angiogenesis is of particular interest. PDGF is an important growth factor expressed by many different cells early in wound healing in response to e.g. low oxygen tension [7], thrombin [8,9], and various other cytokines [10]. Cationic homo- and heterodimers of disulfide bonded A- and B-polypeptide chains (PDGF-AA, PDGF-AB, PDGF-BB) are independently expressed and regulated at the transcriptional as well as posttranscriptional levels [11]. Typical effector cells are fibroblasts and smooth muscle cells, and the cellular effects mediated by PDGF include cell growth, chemotaxis, actin reorganization, and prevention of apoptosis [12]. Furthermore, PDGF indirectly stimulates angiogenesis by inducing an angiogenic response [13] and is responsible for recruitment and proliferation of smooth muscle cells and pericytes which are known to be essential for a functional vascular network [14].

The sustained delivery of a growth factor is a pivotal prerequisite to effectively realize tissue regeneration. Biomatrices seem to be especially suitable scaffolds due to their inherent biocompatibility and biodegradability by cell-based reorganization and the lack of toxic degradation products. Amongst the many different matrices applied in tissue regeneration, fibrin exhibits unique properties and was shown to be effective in both *in vitro* and *in vivo* wound healing studies [15–19]. The use of fibrin sealants (FS) to affix tissues is appealing due to their inherent hemostatic and adhesive properties [19–21]. In addition, FS provides a physiological, biodegradable, and three- dimensional, open, porous matrix for exogenous as well as endogenous factors such as growth factors which are then subsequently released locally to support wound healing [5,22–25]. We have previously evaluated fibrin sealant as a biodegradable matrix for the local release of vascular endothelial growth factor (VEGF), which is bound to the matrix due to natural binding sites on fibrin(ogen) [5].

Since fibrinogen lacks a defined binding site for PDGF, a specific binding technology was developed to avoid an initial burst release of the growth factor and to ensure sustained release from the fibrin matrix [26–29]. TG-PDGF.AB is a recombinant protein consisting of human PDGF (A and B chain) with a FXIII substrate site and a plasmin cleavage site located on the N-terminal end of each chain (TG or TG-hook). During the process of the fibrin clot formation, TG-PDGF.AB is covalently linked to the fibrin matrix via activated FXIII (FXIIIa) [26]. In this construct, release of growth factors takes place simultaneously, with fibrin matrix degradation by enzymatic cleavage of the TG-hook [27] enabling prolonged local growth factor stimulus. Transglutaminase is one of the few natural crosslinking agents which is found in plasma, thus also present in plasma derived fibrin sealants. Although the activity is somewhat lower than in unprocessed plasma due to the viral inactivation procedure, macromolecule crosslinking to fibrin can be achieved successfully [26]. A key feature is that no foreign substances need to be introduced in the fibrin sealant.

The objective of the present study was to test the clinical efficacy of the prolonged delivery of TG-PDGF.AB released locally from a sprayed fibrin matrix in an ischemia model. The influence of this approach on tissue necrosis and the potential to induce functional angiogenesis was determined.

2. Materials and methods

2.1. TG-PDGF.AB delivery system/fibrin matrix

TG-PDGF.AB is a recombinant variant of PDGF-AB specifically engineered for covalent incorporation into fibrin delivery matrices. Both the A and B chains of PDGF-AB are prolonged by an additional 21 amino acids (TG-hook) at the N-terminus. The TG-hook contains two physiological substrate sites. The transglutaminase crosslinking substrate site enables the spontaneous enzymatic linkage of PDGF-AB to fibrin via FXIIIa during the clotting process upon simple mixing. The plasmin cleavage site enables the release of PDGF-AB from fibrin via plasmin during fibrin matrix degradation.

Tisseel™ VH S/D Duo 4, a physiological, coarse-type two-component fibrin sealant (Baxter Healthcare Inc., CA) was used in the FS group and as a fibrin matrix in the TG-PDGF.AB delivery system. This FS contains 4 IU/ml thrombin resulting in slow solidification of the fibrin clot and was recently approved by the Food and Drug Administration (FDA) as Artiss®.

2.1.1. Binding assay

In 2 ml reaction tubes, fibrinogen solution (75 μl) containing 300 μg/ml TG-PDGF.AB and 75 μl of thrombin solution (4 IU/ml) were mixed. Solutions were allowed to clot at 37 °C and, after different incubation times (15 and 30 s, 1, 2, 3 5, 10, 20, 30, 45, 60, and 120 min), the clots were overlaid with 1 ml denaturation buffer and incubated for 5 min at 80 °C. Samples were stored frozen prior to further analysis by Western blot.

2.1.2. Release assay

A volume of 400 μl fibrin containing bound TG-PDGF.AB (mixture of 200 μl fibrinogen, 200 μl thrombin and TG-PDGF.AB at 600 μg/ml solution) was generated in 24-well plates and spread homogeneously through shaking (20 s). Emerging clots were incubated for one hour at 37 °C before overlaying with 2.0 ml of diluted urokinase solution (1 IU/ml) and then again incubated in a humid chamber at 37 °C. The entire volume of the overlay was collected prior to adding 2.0 ml of a fresh urokinase solution (1 IU/ml). This procedure was repeated twice a day at defined time points. After aspiration, the supernatants were stored frozen at −20 °C until further analysis. The change of the supernatant was repeated until all clots were fully dissolved. The amount of released PDGF.AB in the supernatants was measured with a commercially available ELISA (Quantikine® Human PDGF-AB Kit; R&D Systems, Minneapolis, MN), and with Western blot analysis (see below).

2.1.3. Western blot analysis

After separating proteins on a 10% SDS PAGE, bands were transferred to a nitrocellulose membrane. Running conditions for transfer were 50 V for 1 h. After transfer, membranes were blocked with TBS/0.1% Tween/1% milk powder for one hour. During the detection process, membranes were incubated for one hour with primary antibody (Rabbit anti-human PDGF.AB BB, US Biological, Cat.; 1:1000). Thereafter membranes were washed four times for 10 min to remove unbound antibody and were further incubated for 1 h with the secondary antibody (Goat pAB to Rabbit IgG, Abcam, 1:1000). After further washing steps (4 × 10 min) the target protein was visualized by an ECL reaction using SuperSignal WestPico Chemiluminescent Substrate (incubation of membrane

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