Eva Dohle, Andrea Sowislok, Shahram Ghanaati and Herbert P. Jennissen*

Angiogenesis by BMP-2-PDLLA-Biohybrids in Co-Culture with Osteoblasts and Endothelia

Abstract: Adsorbate biohybrids for BMP-2 delivery based on electrospun PDLLA-nanofiber fleeces loaded with $0.6 \mu\text{g}/\text{cm}^2$ rhBMP-2_{COL} (E. coli) strongly induced microvessel-like structures when incubated for 14 days in co-cultures of human outgrowth endothelial cells (OEC) together with human primary osteoblasts (pOB). Higher loaded rhBMP-2_{COL} biohybrids ($4.6 \mu\text{g}/\text{cm}^2$) were low- to non-inductive. Adsorbate biohybrids of rhBMP-2_{CHO} (CHO cells) loaded with $1.0\text{--}6.0 \mu\text{g}/\text{cm}^2$ induced only a classic cobblestone-like growth pattern without any microvessels. Concentration-response experiments (7 days) indicate a binding affinity of rhBMP-2_{COL} for microvessel induction in the picomolar range with nanomolar concentrations being non-inductive.

Keywords: BMP-2 (E.Coli), BMP-2 (CHO-cells), angiogenic sprouts, proangiogenic effects, osteogenesis

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1 Introduction

The mechanism of action of rhBMP-2 in patient therapy is still enigmatic, since the osteoinductive local dose must be 100-1000-fold higher in humans (e.g. $58 \mu\text{M}$) than in animals to be effective [1]. Therefore clinical applications of rhBMP-2 are restricted to only few indications. Fundamental BMP-2 research in the areas of osseointegration and angiogenesis is thus pertinent. A biochemical solution for reducing these unphysiologically high therapeutic doses of rhBMP-2 could be a dose reduction based on the sequential (i.e. multimodal) application of several growth factors [2]: e.g. of rhVEGF (blood vessel development) first, followed by rhBMP-2 (bone development) second. A versatile material for developing multimodal

biohybrids (see: [2]) for BMP-2 delivery in physiological concentrations is the carrier poly-(D,L)-lactide (PDLLA) in the form of electrospun nanofiber fleeces (fiber $\varnothing$ 225-295 nm). Proteins can either be immobilized inside the PDLLA nanofibers as an inclusate-biohybrid (IB) or on the surface of the nanofibers as an adsorbate-biohybrid (AB), as is the case in this report. In a co-culture system of human outgrowth endothelial cells (OEC) and human primary osteoblasts (pOB) as employed here [3,4] angiogenesis can be induced by VEGF and by sonic hedgehog. Angiogenic properties have also been attributed to rhBMP-2 at $\sim 100 \text{ ng}/\text{ml}$ (4 nM) [5,6]. Recently, rhBMP-2 released from adsorbate biohybrids appeared to be directly involved in angiogenesis in the co-culture system OEC/pOB [7]. Here we report a novel rhBMP-2 behavior.

2 Materials and Methods

Sterile lyophilized recombinant human bone morphogenetic protein 2, as rhBMP-2_{COL} ($m = 26 \text{ kDa}$), expressed in E. coli (MorphoPlant GmbH Bochum, D) and as rhBMP-2_{CHO} ($m = 36 \text{ kDa}$) expressed in CHO-cells (InductOs®, Medtronic BioPharma B.V., Heerlen, NL) were employed [8]. The dose-response derived *biological equivalent activity* was $K_{0.5} \sim 5\text{--}15 \text{ nM}$ for both types of rhBMP-2 (for details see ref. [8]). For determination of adsorbate loads on PDLLA fleeces rhBMP-2_{COL} was labeled with ^{125}I by the iodomonochloride method according to [8-10] (initial specific radioactivity: $1.1 \times 10^9 \text{ cpm}/\text{mg}$) yielding ^{125}I -rhBMP-2, which was quantified in a γ -counter (Automatic γ -Counter 1480 Wizard 3, Wallac, FIN).

For adsorption experiments, ^{125}I -rhBMP-2_{COL} was diluted with cold native rhBMP-2_{COL} or rhBMP-2_{CHO} to $1\text{--}2 \times 10^7 \text{ cpm}/\text{mg}$ [8]. PDLLA nanofiber fleeces (membranes) were prepared on a custom-made electrospinning instrument as described by Asran et al. [11]. A spinning solution (7.5 wt% PDLLA) was prepared by adding 320 mg poly-DL-lactic acid (PDLLA; Resomer® 207 S, Evonik Industries, Essen, D) to 2.96 g of hexafluoroisopropanol (HFIP; abcr, Karlsruhe, D) and 0.74 g (ml) of distilled water, thus dissolving the PDLLA under stirring for 30 min at room temperature in a solvent mixture of 80 wt% HFIP and 20 g% distilled water to a final PDLLA concentration of 112.4 mg/ml. Because of the

***Corresponding author:** Prof. Dr. H. P. Jennissen, Institute of Physiological Chemistry, University of Duisburg-Essen, University Clinics, Hufelandstr. 55, D-45122 Essen, Germany; Email: hp.jennissen@uni-due.de;

Dr. Eva Dohle, & Prof. Dr. S. Ghanaati, Klinik für Mund-, Kiefer- und Plastische Gesichtschirurgie, Universitätsklinikum Frankfurt, D-60596 Frankfurt/Main (KGU);

Dr. rer. nat. Andrea Sowislok, Universitätsklinik / Lehrstuhl für Orthopädie und Unfallchirurgie, Universitätsklinikum Essen, Hufelandstr. 55, D-45122 Essen.

possibility of fleece scrolling and shrinking in solutions the PDLA disks were framed in cell-crown 24NX inserts (Scaffdex Oy, Tampere, FIN) and inserted into 24-hole well plates. For preparation of adsorbate-biohybrids (AB) [7] fleeces were incubated for 20 hours at room temperature under gentle shaking in BMP-2 solutions (10 µg/mL and 30 µg/mL) of rhBMP-2 in citrate buffer (5 mM sodium citrate, 14.6 mM sucrose, pH 4.5). Afterwards the protein solution was removed by aspiration, washed five times under shaking with Phosphate-buffered saline (DPBS, Gibco, Thermo Fischer, Darmstadt, D) at room temperature and air dried for 20 hours.

Table 1

Synthesis of adsorbate biohybrids of two forms of rhBMP-2 with PDLA nanofiber fleeces for cell culture.*

BMP-2 concentration	Adsorbed Amount	
	rhBMP-2 _{COL}	rhBMP-2 _{CHO}
10 µg/mL	0.6 µg/cm ²	1.0 µg/cm ²
30 µg/mL	4.6 µg/cm ²	6.0 µg/cm ²

*The adsorption solution contained 10 and 30 µg/mL respectively. The amount adsorbed was determined by tracer detection with ¹²⁵I-rhBMP-2.

Primary human cells were obtained from excess tissue and their application was in accordance with the principle of informed consent and approved by the responsible Ethics Commission of the state of Hessen, Germany. Human outgrowth endothelial cells (OECs) were isolated from peripheral blood buffy coats [12] and expanded as previously described [7]. Human primary osteoblasts (pOBs) were isolated from cancellous bone fragments from healthy donors as described [13].

For co-culture experimentation, PDLA scaffolds on Cell Crowns were transferred to 24 well plates prior to cell seeding. Co-cultures [7] consisting of endothelial cells (130.000/well) and primary osteoblasts (20.000/well) were seeded together on top of the different materials and were cultivated in endothelial cell growth medium (Promocell) depending on the experiment either for 7 days or for up to 14 days at 37°C in an atmosphere of 5% CO₂ and 95% air. For immunofluorescent staining, samples were permeabilized with 0.5 % Triton X/PBS and washed three times with PBS before they were incubated with mouse anti-human CD31 (1:40, Dako, MO 08223) [14]. The cells were examined using the fluorescence microscope (Nikon Eclipse TS 100) and stained for endothelial images (CD31). Microvessel-like structures were quantified using the image processing software NIS Elements (Nikon, Düsseldorf, Germany) according to [14] either in total length (TL) of angogenic structures in [pixels] or angiogenic knots/branches (KB) measured in [nodes]. VEGF in cell culture supernatants was determined using DuoSet® ELISA Development Systems

(R&D Systems, Minneapolis, MN, USA) according to the manufacturers' protocol. Samples and standards were measured in triplicates using a microplate reader. Relative gene expression (RQ) of mRNA for the proteins osteopontin, alkaline phosphatase, and VEGF was determined using the ΔΔCt method [14] with control cultures (i.e. reference) set to 1, using the Quiagen RNeasy Micro Kit together with the Omniscript RT kit according to the manufacturer's protocol (Quiagen, Hilden, Germany). Cell viability was tested by LDH Cytotoxicity Assay CyQUANT™ according to the manufacturer protocol (Invitrogen, Carlsbad, USA).

Co-culture experiments were performed with at least 3 different donors (after informed consent). Data are presented as mean values ± SD. Statistical significance was evaluated using the paired student's t-test with MS Excel (MS Office, Microsoft) and significance was assessed by *p*-value < 0.05.

3 Results and Discussion

The non woven PDLA nanofiber fleeces are shown in **Fig. 1** at two different magnifications (**Fig. 1A,B**). A fiber diameter analysis yielded the distribution histogram

Fig. 1

SEM Images of non-Woven electrospun PDLA Fleeces (A, B) with fiber Distribution (C) and mounted on a Cell Crown (D). For further details see Methods and the text.

(**Fig. 1C**) with a frequency maximum at a diameter of 87-100 nm. Since the paper-thin fleeces (discs Ø 1.0 cm) are difficult to handle and can form rolls during incubation in well plates, they were mounted on cell crowns for rhBMP-2 adsorption experiments and cell culture incubations (**Fig. 1D**). Radiotracer labelled ¹²⁵I-rhBMP-2 was adsorbed to the PDLA nanofiber fleeces (**Table 1**) as described in Methods yielding two biohybrid adsorbates each for the two rhBMP-2 forms. On placing the cell crowns with mounted nanofiber biohybrid adsorbates of rhBMP-2 into the well plates, it was observed that the cells not only adhered and grew on the

fleece itself but also on the well bottom, indicating that the desorbed rhBMP-2 was diffusing into the well and inducing similar/identical cellular reactions not only on the fleece but also on the well bottom. Because of the well-bottom planarity and homogeneity it formed the basis for evaluation of the following experiments after 14 days in co-culture (**Fig. 2**).

Fig. 2

Action of rhBMP-2_{COL} (A,B) and of rhBMP-2_{CHO} (C,D) after 14 days in OEC/pOB co-culture system (magn. 10x). The endothelial cells were immunofluorescently stained for CD31. Scale: 300 µm; C,D: inserted blow-ups: ca. 3x.

As shown in **Fig. 2A** the fleece with the low surface concentration of 0.6 µg/cm² adsorbed rhBMP-2_{COL} leads to an induction of interconnected angiogenic tube-like structures. In contrast the fleece with a high concentration of 4.6 µg/cm² (**Fig. 2B**) only elicits a response in the range of the control without rhBMP-2 (**Fig. 2E**). Although rhBMP-2_{CHO} was biologically fully active (see Methods) and non-toxic in human use (Medtronic), no angiogenic activity, but only a classical confluent cobblestone monolayer pattern of the OEC colony in co-culture (**Fig. 2C,D**) was detected at both surface concentrations (**Table 1**). In fact this BMP-2_{CHO} also eliminated the residual spontaneous, endogenous angiogenic control activity (**Fig. 2E**). In **Fig. 2F** typical checkpoints of a NIS Elements (Nikon) analysis for determination of total length- (TL) and node number by knot/branch-analysis (KB) is shown. The quantitative results of the NIS-analysis are shown in **Fig. 3**.

Fig. 3

Length (A) and knot (B) analysis of the angiogenic tube-like structures induced by rhBMP-2_{COL} after 14 days.

*The structures of the experiments of **Fig. 2** were quantified for the two surface concentrations 0.6 and 4.6 µg/cm² according to the Software NIS-Elements. The activity of rhBMP-2_{CHO} was zero (not shown, see **Fig. 2C,D**). BMP-2_{COL} = rhBMP-2_{COL}; mean ± SD (n = 4); Control: no BMP-2_{COL}.*

At 0.6 µg/cm² a total angiogenic length of TL = 52,083 [pixels] (**Fig. 3A**) and a number of knots of KB = 52 [nodes] (**Fig. 3B**) are observed. A ca. 7-fold increase in surface concentration to 4.6 µg/cm² (**Fig. 3A**) reduces the total angiogenic length to TL = 10,012 [pixels] (20%) (p < 0.03) and the angiogenic knots to KB = 12 [nodes] (25%) (**Fig. 3B**). In the control a reduction to 10% is found in both cases, i.e. rhBMP-2_{COL} leads to a 10-fold increase in total length and in nodes of the induced angiogenic structures versus the controls. In **Table 2** a pilot concentration-response experiment of the induction of angiogenic structures as a function of defined rhBMP-2_{COL} concentrations is shown. At concentrations of 2.6 pg/ml (100 fM) and 26 pg/ml (1.0 pM) there is a strong induction of angiogenic tube-like structures (~30,000 px) decreasing to 10% at 260 pg/ml (10 pM) and disappearing at 2600 pg/ml (100 pM) with no further response at and above 2600 pg/ml. The results in **Table 2** indicate a sub-picomolar high affinity of putative receptors binding to rhBMP-2_{COL}.

Table 2

*Dependence of the appearance and total length of microvessel-like structures on the concentration of rhBMP-2_{COL} [pg/ml] in the OEC/pOB co-culture system after 7 days.**

Conc.	2.6	26	260	2600	[pg/ml]
TL	33,175	29,033	3,290	0	[pixels]

**The control of 13070 pixels [px] has been subtracted.*

For further validation of the above experiments additional parameters such as non-toxicity and cell viability by LDH and gene expression results via mRNA were obtained. These results are shown in **Table 3**. LDH-release as a measure of membrane intactness lies at 37-40% for the duration of the 7-day experiments, which is in the normal range. In parallel to the angiogenic experiments it is shown in **Table 3** (relative

expression quantification, RQ) that the osteogenic proteins alkaline phosphatase (ALP) and osteopontin (OP) are both strongly induced by the BMP-2_{COL}-biohybrids (RQ 20-34) at 4.6 µg/cm². In contrast, at a 0.6 µg/cm², which is optimal for angiogenic stimulation by rhBMP-2_{COL} (**Fig. 2A**), there is no induction of ALP and VEGF and only a very low induction of osteopontin. These data and **Table 2** provide strong evidence against the assumption that the angiogenic induction by rhBMP-2_{COL} proceeds via the pathway BMP-2/VEGF. **Table 3** provides strong evidence for a dual activity of rhBMP-2_{COL} released from the biohybrids by exhibiting a singular high angiogenic activity (**Figs. 2-3**) at low (0.6 µg/cm²) and singular potent osteogenic activity at high (4.6 µg/cm²) biohybrid surface concentrations respectively.

Table 3

Upregulated osteogenic gene expression and viability analysis in the OEC/pOB co-culture system by rhBMP-2_{COL}*

After 7 days rhBMP-2 _{COL} Biohybrid	RQ		
	Control	0.6 µg/cm ²	4.6 µg/cm ²
ALP	1.119 ± 0.0348	0.919 ± 0.10	20.38 ± 2.30
Osteopontin	0.878 ± 0.00066	4.163 ± 0.50	33.91 ± 3.99
VEGF	0.865 ± 0.0753	0.737 ± 0.00045	53.46 ± 15.03
Viability Analysis			
Rel. LDH release	100 ± 0	45 ± 4.5	37 ± 2.7

*(mean ± SD (n = 4)), RQ: relative mRNA expression quant.

4 Conclusions

The presented investigations concerning the angiogenic potential of rhBMP-2_{COL} and rhBMP-2_{CHO} immobilized on PDLLA nanofiber surfaces in the OEC/pOB co-culture system provides the following strong evidence: (i) the rhBMP-2_{COL} released from the biohybrid surfaces is biologically active and induces microvessel-like structures; (ii) biohybrid released rhBMP-2_{CHO} induces a cobblestone appearance with zero induction of angiogenic structures and even inhibition of spontaneously occurring control neo-angiogenic vessels; (iii) in a concentration-response experiment rhBMP-2_{COL} at concentrations ≤ 10 pM (**Table 2**) led to a 10-fold induction, of CD31-stainable microvessel-like structures versus the control within 14 days, but at concentrations ≥ 10 pM is non-inductive.

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E.D. and A.S. contributed equally to this work. Informed consent has been obtained from all individuals included in this study. Authors state no conflict of interest. The conducted research is not related to either human or animal use. (Supported by DFG JE 84/15-3; KI 601/9-3; MI 358/37-3).

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