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Analysis of hydrolyzable polyethylene glycol hydrogels and deproteinized bone mineral as delivery systems for glycosylated and non-glycosylated bone morphogenetic protein-2

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ABSTRACT

Bone morphogenetic proteins (BMP), in particular BMP-2, are the growth factors primarily responsible for osteoinduction. A knowledge of interactions between bone substitute materials and growth factor variants is crucial to designing bone substitutes with an ideal release profile. Here we compare glycosylated and non-glycosylated recombinant human bone morphogenetic protein-2 (rhBMP-2) either incorporated into a hydrolyzable polyethylene glycol (PEG) hydrogel developed as a slow release system or adsorbed to a deproteinized bovine bone matrix (DBBM), a clinically well-established bone substitute material. rhBMP-2 loaded materials were immersed in cell culture medium and rhBMP-2 concentration profiles in the supernatant were determined by an enzyme-linked immunosorbent assay. The corresponding biological activities were assessed in vitro by alkaline phosphatase activity assay. We show a strong affinity of rhBMP-2 for DBBM and reduced biological activity after its release from PEG hydrogels. Glycosylated rhBMP-2 was significantly less affected by the hydrogel and interacted significantly more strongly with DBBM than non-glycosylated rhBMP-2. We therefore question the combination of PEG hydrogels with DBBM as a rhBMP-2 delivery system over DBBM alone, since rhBMP-2 released from the hydrogel will be trapped by DBBM. Moreover, our results suggest that glycosylated rhBMP-2 is favorable in combination with PEG hydrogels, since its activity is better preserved, whereas in combination with DBBM non-glycosylated rhBMP-2 is favorable, benefiting from an initially higher concentration of free rhBMP-2.

1. Introduction

Bone has a unique capacity to heal itself without leaving a scar [1]. However, if a gap derived from an injury, disease or surgical treatment reaches a critical size the body is unable to repair the void on its own. For these critical sized defects or when bone augmentation is required a bone substitute is needed. The ideal material for a bone substitute provides a three-dimensional, mechanically stable structure, serves as a carrier for growth factors that enhance bone in-growth, contains living osteoblastic cells that are accepted by the body and should eventually be replaced by newly formed bone [2–4]. Autologous bone fulfills all of these criteria. Unfortunately, its use has severe drawbacks, namely increased morbidity at the harvest site and limited supply [5]. Therefore, alternatives such as allografts, xenografts or alloplasts are used. However, none of them provide all the properties of autografts [6].

Deproteinized bovine bone matrix (DBBM) is the gold standard off the shelf bone substitute material in dentistry [7]. It consists of the mineral phase of bovine bone, containing hydroxyapatite as the main component. DBBM is devoid of any organic material, therefore, it is not as tough as natural bone and devoid of bone-inducing growth factors [8], making its combination with bone-inducing growth factors advantageous [9].

Bone morphogenetic proteins (BMP), in particular BMP-2 and BMP-7, are the growth factors mainly responsible for osteoinduction [10]. They recruit and stimulate adult mesenchymal stem cells and pre-osteoblastic cells to migrate, proliferate and mature into osteoblasts [11,12]. Currently, glycosylated Chinese hamster ovary (CHO)-expressed recombinant human BMP-2 (rhBMP-2) in combination with a collagen carrier (Infuse®, Medtronic Sofamor Danek, Memphis, TN) is commercially available and approved by the Food and Drug Administration (FDA) and the European authorities for

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lumbar spine fusion and persisting long bone defects. Glycosylated rhBMP-2 is also approved for several dental applications [13]. Non-glycosylated rhBMP-2 produced by a prokaryotic expression system using *Escherichia coli* is an alternative that could substantially reduce production costs and the price for clinical application. Despite the fact that *E. coli*-derived proteins are non-glycosylated, non-glycosylated rhBMP-2 has been reported to have bone-inducing activity similar to the glycosylated form [14–17]. In a human clinical trial we demonstrated the efficacy of non-glycosylated rhBMP-2 in a bone augmentation procedure [9].

Controlling the release profile of rhBMP-2 from carrier materials is crucial to delivering the minimal effective growth factor load needed for bone reconstruction. Analysis of BMP expression during fracture healing revealed that BMP-2 expression was up-regulated at an early stage and was elevated for up to 30 days [18]. It is therefore of advantage to release rhBMP-2 over a time period of days to weeks rather than hours [19]. Rapid diffusion away from the implantation site into the body fluids, premature degradation of the carrier material and inactivation of rhBMP-2 due to unwanted interactions with the carrier materials may limit the effectiveness of rhBMP-2 [19–24].

The combination of DBBM with hydrolysable polyethylene glycol (PEG) hydrogels is a system composed of clinically evaluated materials that provides mechanical stability and allows sustained growth factor delivery [25–30]. In such a system the release profile of the growth factor is dependent on the physical binding strength to the hydrogel, the degradation characteristics of the hydrogel and the interaction of the applied growth factor with DBBM. A tunable, hydrolysable PEG hydrogel system based on a Michael-type reaction between PEG chains functionalized with acrylate groups and thiol groups has been previously developed [31]. The PEG system has been evaluated in clinical studies [32] and it was recently demonstrated that improved bone formation can be achieved by entrapping rhBMP-2 as well as parathyroid hormone peptide in this hydrolysable synthetic matrix [25,30].

Considering the similar bioactivities of glycosylated and non-glycosylated rhBMP-2, understanding the differences in the release characteristics of these variants from the carrier materials and the effect of their interaction with the carrier materials on their bioactivity is crucial. This analysis would enable the development of a successful composite of these well-known materials that allows faster bone formation and more economical use of growth factors. We therefore investigated the differences between glycosylated and non-glycosylated rhBMP-2 in terms of their interactions with DBBM, hydrolysable PEG hydrogels and a combination of these materials, as well as the bioactivity of the released rhBMP-2 variants.

2. Materials and methods

2.1. General preparations

Non-glycosylated rhBMP-2 was expressed in *E. coli* and refolded as previously described [33]. Glycosylated rhBMP-2 was obtained from Genetics Institute Inc. (Cambridge, MA). Both rhBMP-2 variants were desalted by solvent exchange to 1 mM HCl using centrifugal filters (molecular weight cut-off 10 kDa, Millipore, Zug, Switzerland). rhBMP-2 solutions were sterilized using Millex-LG Sterilizing Filter Units (0.2 μm, Millipore, Zug, Switzerland). The concentration of rhBMP-2 was determined by Bradford assay (ThermoFisher Scientific, Lausanne, Switzerland) and samples were diluted in 1 mM HCl to 1 μg μl^{-1}.

All the following experiments were carried out under sterile conditions and all materials were sterilized by vapour sterilization (134 °C) in the central sterilization facility of the University Hospital Zurich unless specified otherwise. All experiments were carried out at room temperature (room temperature varied between 20 and 25 °C) unless specified otherwise. All samples were prepared with both glycosylated and non-glycosylated rhBMP-2.

2.2. Preparation of bone substitute components

Hydrolysable mPEG hydrogels were formed by a Michael-type reaction between acrylate and thiol groups described earlier by Elbert et al. [31] and prepared similarly to previous in vivo studies [25,30]. 15 kDa four-arm PEG functionalized with an acrylate group at the end of each arm and 3.8 kDa linear PEG dithiol dissolved in 1 mM HCl and γ-irradiated were kindly provided by the Institute Straumann AG (Basel, Switzerland). Briefly, 50 μl of PEG gel (unreacted volume) was formed in low binding microcentrifuge tubes (Eppendorf, Basel, Switzerland) in the presence or absence of 2.5 μg rhBMP-2 and/or 25 mg DBBM (Bio Oss®, small spongiosa granules 0.25–1 mm, γ-irradiated, kindly provided by Geistlich Biomaterials, Wollhusen, Switzerland) by mixing acrylate and thiol PEG in a 0.1 M triethanolamine solution. The volume of mPEG hydrogel was chosen to exactly cover the volume of the loose DBBM particles allowing a homogeneous distribution of DBBM in the hydrogel. The gelation reaction was allowed to take place for 30 min. In order to shorten the time period for this study the precursors were chosen to give a final concentration of 3% (w/v) PEG, allowing the mPEG hydrogel to be completely hydrolysed after 3 days.

In order to adsorb rhBMP-2 to DBBM from solution 25 mg DBBM was mixed with 50 μl of 1 mM HCl containing 2.5 μg rhBMP-2. The mixture was vortexed, briefly spun down (10,600g, 10 s) in a centrifuge and incubated for 60 min. Samples were used immediately without removing the liquid from the adsorption procedure. For the adsorption of rhBMP-2 to DBBM via lyophilisation 25 mg DBBM was mixed with 50 μl of 1 mM HCl containing 2.5 μg rhBMP-2. The mixture was vortexed, briefly spun down in a centrifuge (10,600g, 10 s) and frozen immediately using liquid nitrogen. Frozen samples were lyophilized overnight. Controls were prepared without the addition of rhBMP-2. The pH change of a 1 mM HCl solution 1 h after immersion of DBBM was evaluated using the same ratio between DBBM and 1 mM HCl as for the sample preparation.

2.3. Determination of rhBMP-2 concentration profiles

rhBM2-loaded samples and the respective controls were immersed in cell culture medium (Dulbecco's modified Eagle's medium (Invitrogen, Basel, Switzerland) containing 10% inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 20 mM HEPES (stock adjusted to pH 7.4, Sigma Aldrich, Buchs, Switzerland), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Penicillin/Streptomycin, Invitrogen), resulting in a final volume of 500 μl. The final sample volume was calculated including the hydrolysable mPEG hydrogel (unreacted volume) as well as the liquid remaining in DBBM samples and controls, but excluding the non-degradable (within this timespan) volume of DBBM. After pre-incubation for 1 h in cell culture medium to allow initial adsorption of serum proteins 2.5 μg rhBMP-2 was added to the controls. Positive controls of 500 μl of cell culture medium containing 2.5 μg rhBMP-2 were prepared. Thus all samples, controls and the positive controls contained 2.5 μg rhBMP-2. Empty controls were prepared without the addition of rhBMP-2. An overview of all samples and controls as well as their abbreviations is shown in Fig. 1. All samples and controls were incubated at 37 °C for 15 days. 50 μl of supernatant was taken after 1 h and 1, 2, 3, 6, 9 and 15 days incubation, which was replaced by 50 μl of fresh cell

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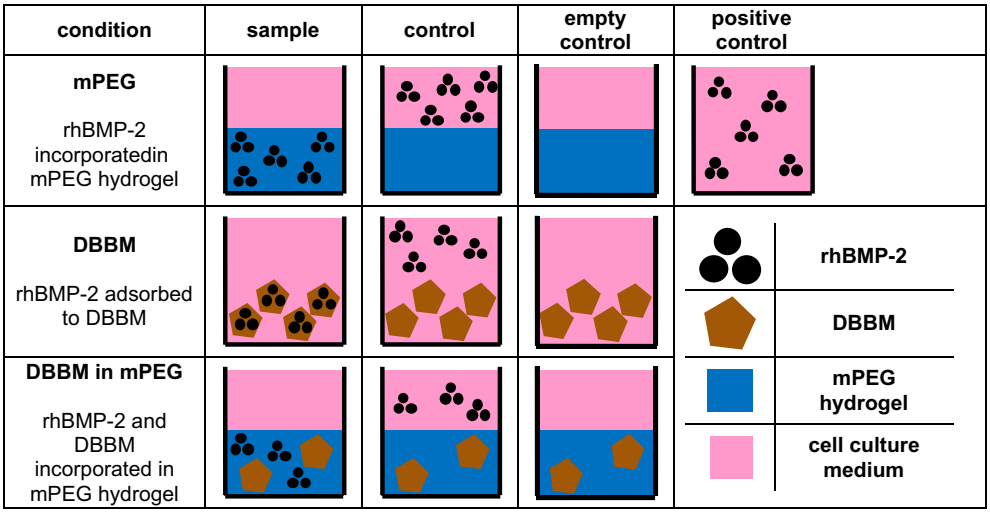

Fig. 1. Scheme of the experimental set-ups. Glycosylated and non-glycosylated rhBMP-2 was incorporated into mPEG hydrogels, adsorbed to DBBM and incorporated into a mPEG hydrogel together with DBBM. These samples were immersed in cell culture medium to investigate the concentration and activity in the supernatant over time. In order to separate effects due to the adsorption or incorporation procedures controls devoid of rhBMP-2 were prepared and immersed in cell culture medium that was subsequently supplemented with rhBMP-2. Empty controls without rhBMP-2 were prepared in order to evaluate the effect of the bone substitute material degradation products on cell viability. The low biological half-life of rhBMP-2 was taken into account by comparing all results with a positive control of rhBMP-2 in cell culture medium.

culture medium. The removed supernatant aliquots were stored at −20 °C for later analysis.

The concentration of rhBMP-2 in the supernatant was determined by an enzyme-linked immunosorbent assay (ELISA) against rhBMP-2 (Quantikine BMP-2, R&D Systems, Abingdon, UK). Standard series of glycosylated and non-glycosylated rhBMP-2 were prepared to determine the rhBMP-2 concentration in the samples. The cumulative amount of released rhBMP-2 was calculated taking into account the removal and addition of 50 µl of fresh medium at each time point. All results were standardized to the positive controls (100%).

2.4. Cell culture assays

2.4.1. Cell stimulation

Glycosylated and non-glycosylated rhBMP-2 were tested for differences in their biological activity as well as for differences in their stability in vitro at 37 °C. Standard series (100–1000 ng ml^{-1}) of both rhBMP-2 variants were prepared in cell culture medium. The stability in cell culture medium was determined by incubating rhBMP-2 at 37 °C in cell culture medium for 0, 3, 9, 15 and 30 days without cells. A pre-myoblastic cell line (C2C12, ATCC, Molsheim Cedex, France) was stimulated with the standard series and the samples prepared for the stability test and tested for alkaline phosphatase (ALP) activity as described in Section 2.4.3.

The biological activity of the rhBMP-2 variants released from bone substitute materials as described in Section 2.3, as well as the impact of bone substitute material degradation products and growth factors on cell viability, were tested by stimulating C2C12 cells. Cells were stimulated with cell culture medium supernatant after 15 days release from bone substitute materials prepared as described in Section 2.3. Stimulated cells were tested for their viability as described in Section 2.4.2 and for their ALP activity as described in Section 2.4.3.

2.4.2. Cell viability

Cell viability was evaluated after 3 and 6 days incubation at 37 °C and under 5% CO_2 in a humidified atmosphere. Viability was determined by adding the cell proliferation reagent WST-1 (Roche, Basel, Switzerland) to the cell culture medium at the concentration specified by the manufacturer. Cells were incubated in the presence of the cell proliferation reagent for 45 min at 37 °C and under 5% CO_2. The resulting colour reaction was evaluated by measuring the optical density at 450 nm using a plate reader (Synergy HT, Biotek, Luzern, Switzerland).

2.4.3. Alkaline phosphatase activity

The biological activity of rhBMP-2 was determined via an assay for ALP activity, an early marker of osteoblastic differentiation. ALP is expressed by mesenchymal progenitor cells exposed to rhBMP-2 and its activity therefore provides an appropriate functional assessment of the growth factor bioactivity [34]. In detail, C2C12 cells were stimulated for 6 days at 37 °C under 5% CO_2 and were subsequently lysed using 0.56 M 2-amino-2-methyl-1-propanol (Sigma Aldrich, Buchs, Switzerland) (pH 10, containing 0.2% Triton X-100 (Sigma Aldrich)). ALP activity in the lysis buffer was determined via the kinetics of a colour reaction based on the reduction of *p*-nitrophenyl phosphate (Sigma Aldrich) to *p*-nitrophenol. The DNA concentration of the lysate was determined using a PicoGreen assay (Invitrogen, Basel, Switzerland). The optical density of the *p*-nitrophenol reaction (410 nm) and fluorescence of PicoGreen (excitation 480 nm, emission 520 nm) was detected with a plate reader (Synergy HT, Biotek, Luzern, Switzerland). The determined ALP activity was adjusted for the total cell number by standardizing it to the DNA concentration. All results were standardized to the positive controls (100%).

2.5. Statistical analysis

All experiments were carried out with a total number of six individual and independent samples for each condition ($n = 6$), except for the ALP assay, which was carried out with 12 independent samples ($n = 12$). Each experiment was carried out twice. A Gaussian distribution of the samples was confirmed by a Shapiro–Wilk test. A two-tailed, heteroscedastic *t*-test ($P < 0.05$) was applied to determine whether or not there are statistically significant differences between the concentrations and/or activity of glycosylated and non-glycosylated rhBMP-2 after 15 days incubation. For the statistical analysis of the cell culture viability repeated measures ANOVA and subsequent pairwise Student's *t*-tests with corrected *P* values according to Bonferroni were used to detect significant differences between samples devoid of growth factors,

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loaded with non-glycosylated or loaded with glycosylated rhBMP-2.

3. Results

3.1. Interaction of rhBMP-2 with mPEG hydrogels

The cumulative concentration of both glycosylated and non-glycosylated rhBMP-2 in the cell culture medium supernatant of mPEG hydrogel samples was measured by ELISA. The concentration of both growth factor variants increased up to day 3 and subsequently remained constant up to day 15 (Fig. 2). However, only part of the initial amount of rhBMP-2 was detected after 15 days incubation. We found significant differences between the concentration of glycosylated (56 ± 4% of positive control) and non-glycosylated (26 ± 2% of positive control) rhBMP-2 on day 15. A comparison of the mPEG hydrogel controls showed reduced concentrations of both rhBMP-2 variants. There was no significant change in concentration during the incubation time and there was no significant difference between the concentration of glycosylated (58 ± 11% of positive control) and non-glycosylated (56 ± 5% of positive control) rhBMP-2 on day 15 for the mPEG controls.

3.2. Interaction of rhBMP-2 with DBBM

pH measurements showed that the pH of 1 mM HCl was neutralized by DBBM after 1 h if mixed at a concentration of 500 mg ml^{-1} DBBM (data not shown). We did not find any significant difference in the adsorption behavior of rhBMP-2 between immersion in 1 mM HCl containing rhBMP-2 and lyophilisation (data not shown). We therefore applied the adsorption procedure without lyophilization to load DBBM with rhBMP-2. After 1 h adsorption in 1 mM HCl and 1 h incubation in cell culture medium 30 ± 14% glycosylated rhBMP-2 and 32 ± 4% non-glycosylated rhBMP-2 were detected in the cell culture medium supernatant relative to the positive control. These levels dropped to 7 ± 3% glycosylated and 16 ± 7% non-glycosylated rhBMP-2 after 15 days, showing a significant difference between the two growth factor variants (Fig. 3A). Concentrations of rhBMP-2 variants in the cell culture medium supernatant of DBBM controls showed a higher initial concentration than in the samples (Fig. 3A and B). By day 3 of incubation the concentrations in the supernatants of the controls dropped to similar levels to the concentrations in the supernatant of the samples, showing significant differences in concentration between glycosylated (7 ± 2% of positive control) and non-glycosylated (19 ± 3% of positive control) rhBMP-2 after 15 days.

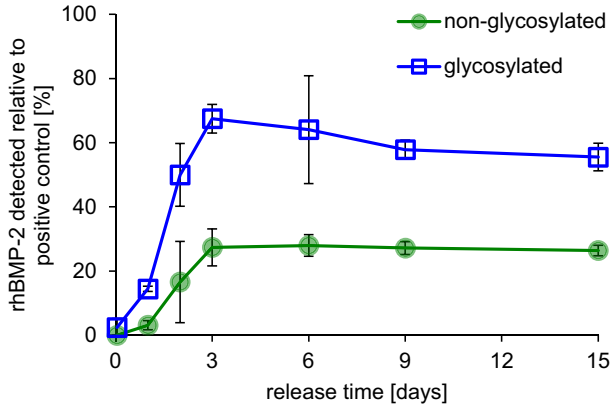

Fig. 2. Concentration of glycosylated and non-glycosylated rhBMP-2 in the cell culture medium supernatant of mPEG samples detected by ELISA.

3.3. Combination of mPEG hydrogel, DBBM and rhBMP-2

We detected strongly reduced concentrations of glycosylated and non-glycosylated rhBMP-2 in the cell culture medium supernatant of DBBM in mPEG samples compared with the positive controls (Fig. 4A). A maximum of 17 ± 3% glycosylated and 5 ± 4% non-glycosylated rhBMP-2 was detected after 6 days. After 15 days we found significant differences between the concentrations of glycosylated (11 ± 3%) and non-glycosylated (3 ± 2%) rhBMP-2. A high initial concentration of rhBMP-2 was found in the cell culture medium supernatant of the controls (Fig. 4B). After 3 days we observed a rapid drop in the concentration of both glycosylated and non-glycosylated rhBMP-2. After 15 days incubation the concentration was found to be significantly higher for the non-glycosylated (13 ± 4% of positive control) than for the glycosylated (5 ± 4% of positive control) rhBMP-2.

3.4. Cell viability – influence of degradation products and rhBMP-2

C2C12 cells stimulated with the cell culture medium supernatant from all samples were tested for their cell viability. After 3 days we found a significantly elevated cell viability compared with controls devoid of rhBMP-2 and samples with glycosylated rhBMP-2 in the positive controls and the DBBM sample group (Fig. 5A). After 6 days cell viability was significantly elevated in

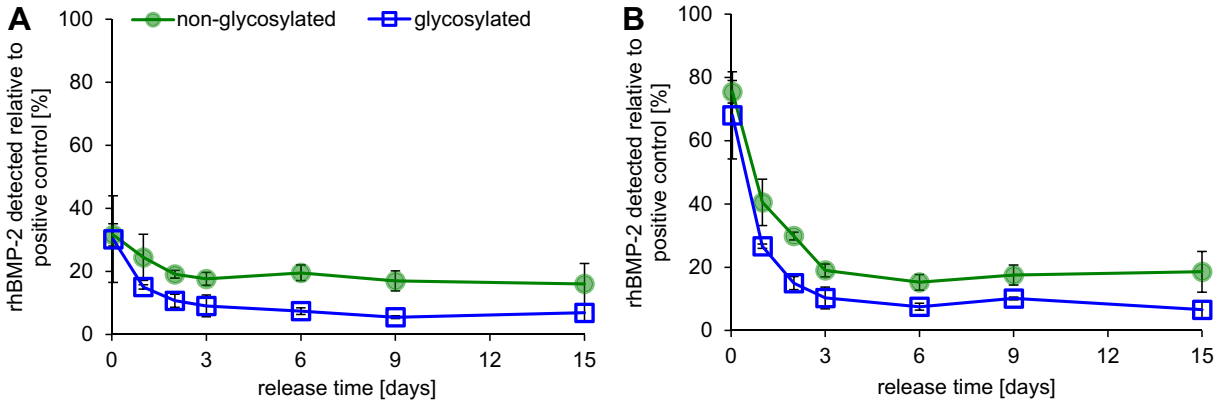

Fig. 3. Concentration of glycosylated and non-glycosylated rhBMP-2 in the cell culture medium supernatant of DBBM samples and controls detected by ELISA. (A) DBBM samples. (B) DBBM controls.

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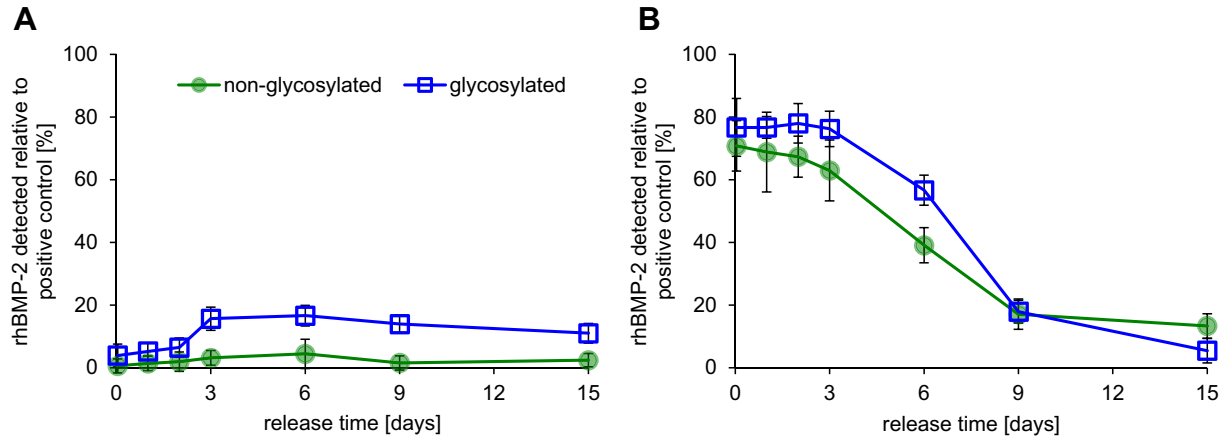

Fig. 4. Concentration of glycosylated and non-glycosylated rhBMP-2 in the cell culture medium supernatant of DBBM in mPEG samples and controls detected by ELISA. (A) DBBM in mPEG samples. (B) DBBM in mPEG controls.

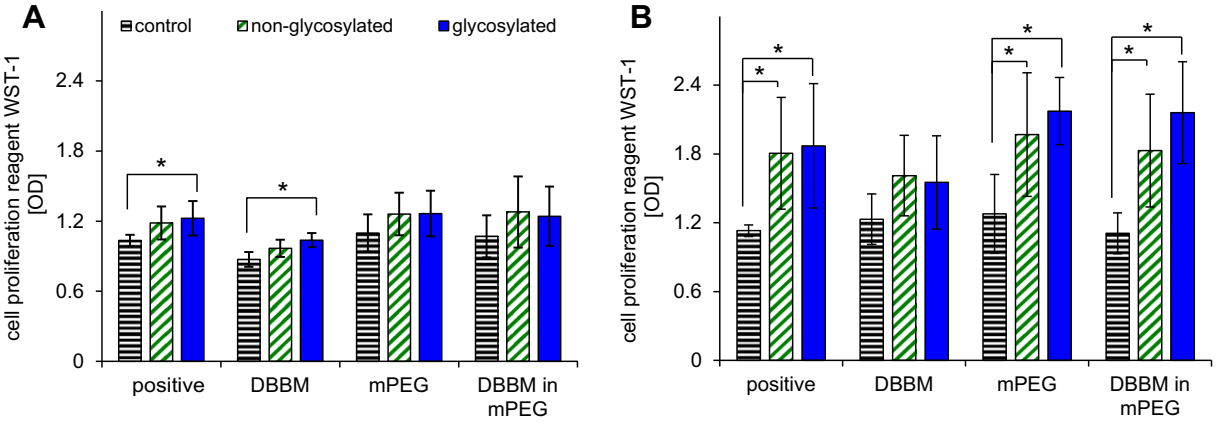

Fig. 5. Cell viability of C2C12 cells stimulated with cell culture medium supernatant from mPEG, DBBM and DBBM in mPEG measured with cell proliferation reagent WST-1. (A) After 3 days stimulation. (B) After 6 days stimulation. *Significant difference between samples ($P < 0.05$).

samples containing either glycosylated or non-glycosylated rhBMP-2 in the sample groups of positive controls, mPEG hydrogel and DBBM in the mPEG hydrogel. There were no significant differences between samples containing glycosylated and non-glycosylated rhBMP-2 (Fig. 5B). Comparing the cell viability between the bone substitute materials we did not find any significant differences.

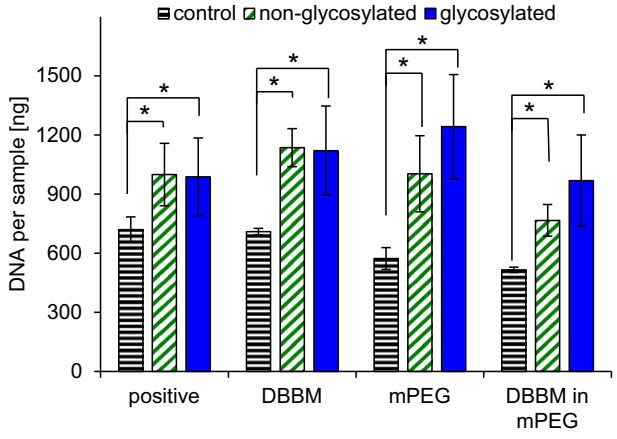

Fig. 6. DNA concentration in samples of cells stimulated with cell culture medium supernatant from mPEG, DBBM and DBBM in mPEG hydrogel samples determined using a PicoGreen assay. *Significant difference between samples ($P < 0.05$).

Along with measurement of the biological activity of rhBMP-2 (Section 2.4) we determined the total amount of DNA in the stimulated cells by PicoGreen assay. After 6 days we found a significantly higher amount of DNA in samples containing glycosylated or non-glycosylated rhBMP-2, however, we did not find any significant differences between samples containing glycosylated or non-glycosylated rhBMP-2 (Fig. 6). However, comparing the DNA content between samples stimulated with cell culture supernatant from bone substitute materials not containing rhBMP-2 we found a significant decrease for those samples containing mPEG hydrogels.

3.5. Biological activity of rhBMP-2 – impact of bone substitute materials

The biological activity of glycosylated and non-glycosylated rhBMP-2 was tested by stimulating C2C12 cells with various concentrations of both growth variants and testing for ALP activity. We did not find any significant differences between glycosylated and non-glycosylated rhBMP-2 (Fig. 7A). We found a strong decrease in ALP activity when stimulating C2C12 cells with rhBMP-2 pre-incubated for various times in cell culture medium at 37 °C

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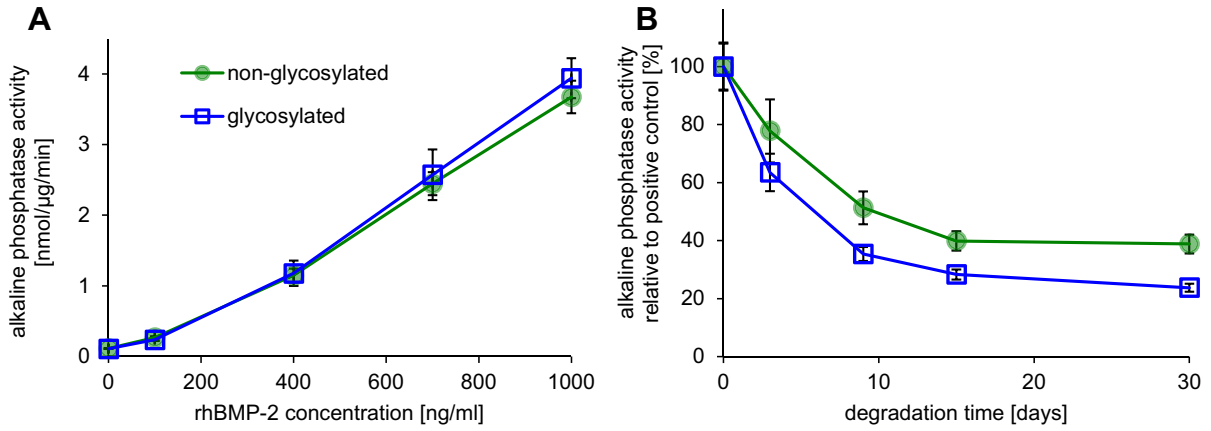

Fig. 7. Bioactivity and stability of non-glycosylated and glycosylated rhBMP-2. (A) ALP activity of cells stimulated by various concentrations of non-glycosylated and glycosylated rhBMP-2. (B) ALP activity of cells stimulated with rhBMP-2 preincubated for various times at 37 °C in cell culture medium.

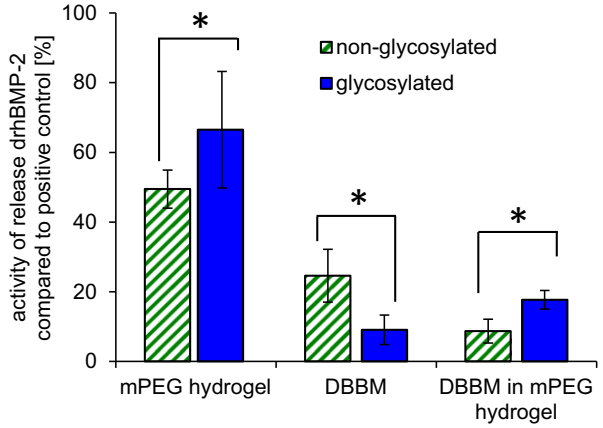

Fig. 8. Bioactivity of glycosylated and non-glycosylated rhBMP-2 in the cell culture medium supernatant of mPEG, DBBM and DBBM in mPEG samples after 15 days incubation. C2C12 cells were stimulated with cell culture medium supernatant from the samples and incubated for 6 days, followed by ALP activity assay.

under 5% CO_2 (Fig. 7B). Glycosylated (28 ± 2% initial activity) rhBMP-2 was significantly more affected than non-glycosylated (40 ± 3% initial activity) rhBMP-2 after 30 days pre-incubation.

The impact of incubating rhBMP-2 together with bone substitute materials on the bioactivity of the growth factor was investigated by stimulating C2C12 cells with the cell culture supernatant from mPEG hydrogels, DBBM and DBBM in mPEG hydrogel and testing them for ALP activity. Incorporation into mPEG hydrogels reduced the activity of rhBMP-2 in the cell culture medium supernatant. The activity of non-glycosylated rhBMP-2 (49 ± 6% of positive control) was more significantly reduced than the activity of glycosylated rhBMP-2 (66 ± 17% of positive control) (Fig. 8). The activity of rhBMP-2 in the cell culture medium supernatant of DBBM was strongly decreased. This decrease was significantly higher for glycosylated (9 ± 4% activity of positive control) than for non-glycosylated rhBMP-2 (25 ± 8% activity of positive control) (Fig. 8). The activity of rhBMP-2 released from mPEG hydrogels having incorporated DBBM and rhBMP-2 was also strongly decreased. The decrease was significantly higher for non-glycosylated (9 ± 3% activity of positive control) than for glycosylated rhBMP-2 (18 ± 3% activity of positive control) (Fig. 8).

4. Discussion

Our aim was to understand the differences between the interaction of glycosylated and non-glycosylated rhBMP-2 with clinically evaluated bone substitute materials. This analysis should ultimately allow the design of a combination of known materials and growth factors leading to improved bone reconstruction. For our investigation we chose two well-evaluated materials, mPEG hydrogels and DBBM. Both materials have previously been clinically tested for bone augmentation procedures in dentistry [7,9,25–30,32].

We evaluated the equivalence of the two variants of rhBMP-2. In agreement with the literature [9,14–17], we found the biological activity of the glycosylated and non-glycosylated rhBMP-2 to be alike in vitro (Fig. 7). The biological activity of both variants drops rapidly in cell culture medium at 37 °C. This activity decrease has to be considered for rhBMP-2 delivery in vivo, as well as for experimental design in vitro. Therefore, we compared all of our samples with a positive control of rhBMP-2 in cell culture medium. We further investigated the equivalence of cell viability stimulated by the two variants of rhBMP-2. We observed a trend for an increase in cell viability in the presence of cell culture medium supernatant from preincubated bone substitute materials and/or glycosylated or non-glycosylated rhBMP-2 after 3 days and found a significant increase after 6 days (Fig. 5A and B). This increase in viability is reflected in the significantly higher total amount of DNA per sample in those samples containing rhBMP-2 (Fig. 6). Our results agree with other works that show the stimulation of cell viability by both glycosylated and non-glycosylated rhBMP-2 [17].

In investigating the effects of an mPEG hydrogel on rhBMP-2 variants we found that incorporating rhBMP-2 into an mPEG hydrogel reduces the level of growth factor detected in the cell culture medium supernatant (Fig. 2), as well as the amount of active rhBMP-2 detected (Fig. 8) in the supernatant after 15 days incubation. We hypothesize that this reduction is mainly caused by an interaction of rhBMP-2 with the PEG starting material during gel formation. The thiol groups on the linear PEG could attack the disulfide bonds of the rhBMP-2 dimers, leading to formation of monomers, incorrectly refolded dimers, or even covalent incorporation of monomers into the mPEG hydrogel network. This hypothesis is supported by the significantly lower decrease in concentration of glycosylated rhBMP-2, since glycosylation can result in a protective effect. It is further supported by the fact that the concentration of non-glycosylated rhBMP-2 in the supernatant of the controls, where rhBMP-2 is added after mPEG hydrogel formation, is much higher than in the supernatant of the corresponding control. However, it is possible that a weaker physical interaction between rhBMP-2 and remnants of the degraded mPEG hydrogel might be responsible for the reduced detection of rhBMP-2 in the controls. Although controlled PEGylation offers the possibility of

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increasing the solubility and half-life of BMP-2 [35], or even allow enzymatically controlled growth factor release [36], it should be avoided in order to deliver functional native rhBMP-2. Therefore, if applied together with an mPEG hydrogel, glycosylated rhBMP-2 is superior.

In contrast to the samples containing mPEG hydrogel, only a small part of the initial rhBMP-2 concentration can be detected in the cell culture medium supernatant of DBBM samples after 15 days immersion. The amount of active rhBMP-2 detected in the cell culture medium supernatant is reduced by a corresponding amount. We attribute this effect to the high affinity of BMP-2 to hydroxyapatite-based materials [37], as well as to the high surface area (79.7 $m^2 g^{-1}$) of DBBM, due to its micro- and nano-structure [38]. Moreover, instead of the expected release after immersion of DBBM preloaded with rhBMP-2, adsorption of rhBMP-2 continued even in the presence of serum proteins from the cell culture medium (Fig. 3A). This result agrees with other work showing that the affinity of rhBMP-2 for hydroxyapatite is higher than the affinity of bovine serum albumin for hydroxyapatite [37]. The reduced activity of the supernatant, however, does not necessarily imply a reduced activity in vivo, as indicated by successful studies on the combination of DBBM and rhBMP-2 [9,32,39]. rhBMP-2 could be adsorbed on DBBM in an active or a denatured form, or it could be that it is reactivated upon release after slow degradation of DBBM in vivo. The significantly stronger affinity of glycosylated rhBMP-2 for DBBM compared with the non-glycosylated variant seems to recommend the use of glycosylated rhBMP-2 if applied together with DBBM. However, Brown et al. have shown in vivo that an initially high concentration of rhBMP-2 followed by slow release is favourable over slow release only [19]. This fact, together with the much lower production costs for the expression of rhBMP-2 in *E. coli*, makes non-glycosylated rhBMP-2 the preferred protein if applied together with DBBM. In the sole clinical trial where DBBM was used as carrier for non-glycosylated rhBMP-2 the results show that overall bone formation was not increased compared with DBBM alone, but maturation of bone as well as direct coverage of DBBM by bone had advanced significantly [32]. Both results suggest that non-glycosylated rhBMP-2 delivered by DBBM is still active, but the long-range effect is limited due to the high affinity of rhBMP-2 for DBBM. Whether this remaining activity is due to the low amount of rhBMP-2 dissolved in the extracellular matrix (ECM), rhBMP-2 resolubilized after the slow dissolution of DBBM in vivo or rhBMP-2 adsorbed to DBBM cannot be determined based on these results.

Combining the two previously discussed materials we Incorporated rhBMP-2 into mPEG hydrogels together with DBBM. This led to a constantly low concentration of rhBMP-2 in the cell culture medium supernatant during the 15 days of incubation, with a small increase after three days. After 15 days, in contrast to the samples with DBBM alone, a significantly higher concentration of glycosylated compared to non-glycosylated rhBMP-2 can be detected (Fig. 4A). This result is confirmed by cell culture experiments that show the same significant difference for the amount of active rhBMP-2 detectable in the cell culture medium supernatant (Fig. 8). We attribute this to the reduced detectability of non-glycosylated rhBMP-2 if present during formation of mPEG hydrogels (Fig. 2). Control samples for DBBM in mPEG hydrogels initially only show a small decrease in rhBMP-2 concentration in the cell culture medium supernatant. However, the concentration drops drastically after 3 days (Fig. 4B). This drop can be explained by the hydrolysation of the mPEG hydrogel that was covering the DBBM, leading to adsorption of rhBMP-2 on DBBM. The concentration of non-glycosylated rhBMP-2 is, in agreement with the results for DBBM alone, significantly higher than for glycosylated rhBMP-2, supporting the assumption that the only reason for the lower concentration of non-glycosylated rhBMP-2 in the samples is due to its interaction with the mPEG hydrogel components during hydrogel formation.

5. Conclusions

We have shown that mPEG hydrogels interact with rhBMP-2 and reduce its activity. Furthermore, we found a strong affinity of rhBMP-2 for DBBM. Based on these results we question the use of mPEG hydrogels as a carrier system for rhBMP-2 in conjunction with DBBM, since the use of DBBM with pre-adsorbed rhBMP-2 alone yields a higher BMP activity, which might translate to better results in vivo. Since the affinity of non-glycosylated rhBMP-2 to DBBM is lower than the affinity of the glycosylated variant, non-glycosylated rhBMP-2 may be used if an initial high concentration of rhBMP-2 is desired to chemotactically attract cells. Further research on this aspect is necessary to determine whether rhBMP-2 adsorbed to DBBM and hydroxyapatite in general is active or denatured and/or released in an active form during degradation. However, if other needs, e.g. formability or stability of the bone substitute, require the use of mPEG hydrogels, glycosylated rhBMP-2 is the preferred growth factor since its interactions with mPEG hydrogels is significantly lower compared with non-glycosylated rhBMP-2.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1–8, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.08.002.

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