Acta Biomaterialia 6 (2010) 4405–4421

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Peri-implant reactivity and osteoinductive potential of immobilized rhBMP-2 on titanium carriers

M. Chatzinikolaidou [a,1], T.K. Lichtinger [b], R.T. Müller [b], H.P. Jennissen [a,*]

[a] Institut für Physiologische Chemie, Abtlg. Biochemische Endokrinologie, Universität Duisburg-Essen, Hufelandstrasse 55, D-45122 Essen, Germany
[b] Orthopädische Universitätsklinik, Universitätsklinikum Essen, Universität Duisburg-Essen, Hufelandstrasse 55, D-45122 Essen, Germany

ARTICLE INFO

Article history:
Received 14 December 2009
Received in revised form 4 June 2010
Accepted 8 June 2010
Available online 15 June 2010

Keywords:
Peri-implant bone induction
Functionalized nanostructured titanium
Immobilized BMP-2 rate constants
Immobilized BMP-2 binding constants
Osseointegration in ovine model

ABSTRACT

Recombinant human BMP-2 (rhBMP-2) was immobilized non-covalently and covalently as a monolayer on plasma vapour deposited (PVD) porous commercially pure titanium surfaces in amounts of 5–8 μg cm^{-2}, providing a ca. 10-fold increase vs. previously reported values [37]. Dissociation of the immobilized [^{125}I]rhBMP-2 from the surface occurred in a two-phase exponential decay: a first rapid phase (ca. 15% of immobilized BMP-2) with a half-life of 1–2 days and a second slow sustained release phase (ca. 85% of immobilized BMP-2) with a half-life of 40–60 days. Dissociation rate constants of sustained release of $k_{-1} = 1.3–1.9 \times 10^{-7}$ s^{-1} were determined, allowing an estimation of the binding constants (K_A) for the adsorbed rhBMP-2 monolayer, to be around 10^{12} M^{-1}. The rhBMP-2-coated surfaces showed a high level of biological activity, as demonstrated by *in vitro* epifluorescence tests for alkaline phosphatase with MC3T3-E1 cells and *in vivo* experiments. *In vivo* osteoinductivity of rhBMP-2-coated implants was investigated in a gap-healing model in the trabecular bone of the distal femur condylus of sheep. Healing occurred without inflammation or capsule formation. The calculated concentration of released rhBMP-2 in the 1 mm gap ranged from 20 to 98 nM – well above the half-maximal response concentration ($K_{0.5}$) for inducing alkaline phosphatase in MC3T3-E1 cells. After 4, 9 and 12 weeks the bone density (BD) and bone-to-implant contact (BIC) of the explanted implants were assessed histomorphometrically. Implants with immobilized rhBMP-2 displayed a significant (2- to 4-fold) increase in BD and BIC values vs. negative controls after 4–9 weeks. Integration of implants by trabecular bone was achieved after 4 weeks, indicating a mean "gap-filling rate" of ~250 μm $week^{-1}$. Integration of implants by cortical bone was observed after 9 weeks. Control implants without rhBMP-2 were not osseointegrated. This study demonstrates the feasibility of enhancing peri-implant osseointegration and gap bridging by immobilized rhBMP-2 on implant surfaces which may serve as a model for future clinical applications.

1. Introduction

Macroimplants such as artificial hips or knees [1,2], mini-implants in the form of dental or oral devices [3] and microimplants applied as ossicular constructs [4] or stents [5,6], all made of biocompatible metals, have revolutionized implantology in the last 30 years. Worldwide, millions of such implants are placed each year. The success rates (see Ref. [7] for definitions thereof) still vary widely – between 70% and 98% – depending on methodology, implant type and implant residence times in patients [1–3,8,9]. Thus widespread efforts are being made to improve implants. In general, the failure of such implants is due not so much to structural as to surface incompatibilities. One of the methods of the last decade to improve the surface compatibility is based on the affinity technology concept [10] of coating implants with biomolecular materials such as growth factors [11].

Bone morphogenetic proteins (BMPs) belong to the group of transforming growth factor (TGF-β) proteins with three key effects in osteogenesis [12]: chemotaxis, mitosis and differentiation. BMP-2, BMP-7, TGF-β and TGF-β-related proteins have been classified as anthelix proteins [13–15]. The surface of BMP-2 consists of a mosaic of hydrophilic and hydrophobic patches [16]. In 1979 Urist et al. [17] first isolated BMP from bovine bone matrix, and in 1988 Wozney and co-workers [18] isolated cDNA clones encoding human BMPs. This allowed the recombinant production of human BMP-2 as a glycoprotein (rhBMP-2_{CHO}) in eukaryotic cells (e.g. Chinese hamster ovary cells [19,20] or insect cells [21]). In *Escherichia coli* (rhBMP-2_{COLI}) BMP-2 is synthesized in the non-glycosylated form [11,22], which displays neither toxicity nor genotoxic effects in cell culture [23]. No differences in biological activity between

* Corresponding author. Tel.: +49 (0) 201 723 4125; fax: +49 (0) 201 723 5944.
E-mail address: hp.jennissen@uni-due.de (H.P. Jennissen).
[1] Present address: Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas (FO.R.T.H) and Department of Biology, University of Crete, Heraklio, Greece.

1742-7061/$ - see front matter © 2010 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.actbio.2010.06.009

these two forms of BMP-2 have been detected in vitro [24]. However, this might be different in vivo [25].

Therapeutically, rhBMP-2 is recommended for non-implant use, such as fracture stabilization and spine fusion, being applied in large amounts of 12 mg (rhBMP-2_{CHO}, InductOs, Wyeth), together with the surfactant Tween 80 and a carrier such as bovine collagen [26–28]. Release rates of rhBMP-2, locally administered in rats, have been reported with half-lives between 2 and 4 days [29]. This is in agreement with release rates from collagen in vitro with half-lives of 2–3 days [30]. Although the number of side effects is low, the application of rhBMP-2 in large amounts (e.g. 12 mg) has been reported to induce antibodies [28] and to inhibit bone ingrowth into porous implants [31]. A recent report indicates the risk of heterotopic ossifications in humans [32].

The use of rhBMP-2 in implantology for enhancing peri-implant bone growth has not yet found its way into clinical practice. In 1999 we reported the chemical immobilization of rhBMP-2_{COLI} on small titanium plates for developing chemotactic and juxtacrine osteoinductive surfaces [11]. Immobilization models were based on covalent [11] and non-covalent mechanisms [33–35], the latter being subdivided into hydrophobic [16] and hydrophilic interactions [36]. In 2000 and 2001 we first reported the in vivo activity of Ti-immobilized rhBMP-2 (0.15–0.20 $\mu g\,cm^{-2}$) in ovine pilot experiments [33], and in an ectopic healing model in rabbits [34] there was a statistically significant increase of induced de novo bone volume on titanium. In subsequent pilot experiments in dogs with immobilized rhBMP-2 (0.60–0.80 $\mu g\,cm^{-2}$) on dental implants a significant increase in peri-implant bone density was also indicated in the tiba but not in the mandible [37]. Work by other groups on peri-implant bone formation *in vivo* by rhBMP-2 applied together with diverse carriers has achieved varied degrees of success or has failed [38–41]. These unsatisfactory results indicate that the biochemical and physicochemical mechanisms underlying bioactive BMP-2 coatings are still only poorly understood.

Based on our aim to produce an implant which positively influences its own integration, it will be shown that rhBMP-2 immobilized in amounts of 5–8 $\mu g\,cm^{-2}$ on titanium implants can induce integrating amounts of peri-implant trabecular and cortical bone. These results are supported and facilitated by extensive fundamental and quantitative data on the binding, release and in vitro biological activity of the immobilized rhBMP-2.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

3-Aminopropyltriethoxy silane (APS), 1,1′-carbonyldiimidazole (CDI) and toluene were obtained from Sigma Aldrich Chemie GmbH (Munich). Concentrated chromosulfuric acid (CSA, 92% H_2SO_4, 1.3% CrO_3, density 1.84 g cm^{-3}), dry acetone ($\leqslant$0.01% H_2O) and methanol were obtained from Merck (Darmstadt, Germany). The endogenous phosphatase detection kit ELF-97 (2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone) was obtained from Molecular Probes, Inc. (Oregon, USA). Dulbecco's phosphate-bufferred saline without calcium and manganese (DPBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.4) was obtained from PAA (Linz, Austria). Levamisol hydrochloride (1-2,3,5,6-tetrahydro-6-phenyl-imidazole thiazole) was obtained from Synopharm GmbH (Barsbüttel, Germany). All chemicals were of the highest available or analytical grade. Highly pure water was prepared by distillation of deinonized water followed by its passage through a MilliQ System (Millipore, D-65428 Schwalbach, Germany).

2.1.2. Cells and cell culture media

The embryonic mouse calvaria osteogenic cell line MC3T3-E1 [42] was purchased from DMSZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany); DPBS, α-modified Eagle's medium (α-MEM) and fetal calf serum (FCS) were from Gibco (Invitrogen GmbH, Karlsruhe, Germany).

2.1.3. Metals

For in vitro experiments, Ti-miniplates (15 × 10 × 1 mm), either electropolished (EP) or anodically oxidized (AO), were stamped from commercially pure (cp) Ti grade 2 sheets (ASTM B265, ASTM F67; gb Implantate GmbH, Essen) [11,16,43]. Ti-EP miniplates were provided with a SLA-Ti-type surface (sand-blasted with large-grit and acid-etched; Promote® 2004) by Altatec GmbH (Wimsheim, Germany; see Ref. [43]) for controls. Miniplates with a plasma vapour deposited (PVD) Ti (Ti-PVD) surface were employed in two sizes: 15 × 10 × 1.6 mm (type I), for contact angle measurements, functionalization and [^{125}I] rhBMP-2 immobilization studies; and 10 × 5 × 1.6 mm (type II), for cell culture experiments in microtiter plates. The Ti-PVD implants and miniplates consisted of the base material Ti–6Al–4V (ISO 5832-3; Fa. gb Implantat-Technologie GmbH, Essen, Germany), which was custom coated (375–450 μm) with porous titanium by PVD (cp-titanium powder ISO 5832-2; DOT GmbH, Rostock, Germany) on the top and bottom sides, leading to the same surface coating as employed for the dumbbell implants described below. The PVD coating (thickness 1.6 mm) has a porosity of 20–40%, with a pore diameter of 40–300 μm and an adhesiveness withstanding >45 MPa (DOT GmbH).

For the *in vivo* experiments non-functional, 10 mm long cylindrical dumbbell-shaped implants (see Fig. 1A), consisting of a short bar or cylinder (length 7.5 mm, ∅ = 4.2 mm) with a disk for fixation (∅ = 7 mm) at each end, were prepared from Ti–6Al–4V titanium alloy. Implants of this type are based on the design described by Soballe in 1993 [44] for dogs. This technology, in which a ~1 mm circumferential gap is created between the bone and the implant, i.e. a critical-size defect, was adapted to sheep [45]. The detailed structure of the dumbbell-type implants are described in Fig. 1. The special form of the implants allows study of the bone's gap-bridging ability in the 1 mm cleft between the implant and the bone. In agreement with forms of cementless total hip prosthesis technology, the bar of the implant was circumferentially PVD coated (thickness 375–450 μm), leading to a total bar diameter of ~5 mm (=Ti-PVD surface implants). Some implants were additionally coated with brushite ($CaHPO_4{\cdot}2H_2O$, thickness 20 μm; Bonit® coating, DOT GmbH, Rostock, Germany) on the PVD coating (=Ti-brushite surface implants) (see Refs. [46,47]). The fixation disks of the dumbbell implants were not coated by the above PVD or brushite procedures (see Fig. 1B).

2.2. Methods

2.2.1. In vitro methods

2.2.1.1. Bmp-2. Recombinant human bone morphogenetic protein 2 (rhBMP-2_{COLI}) was expressed in *E. coli* strain BL21-DE3 containing the expression vector pET24c and refolded from inclusion bodies as described elsewhere [11]. Crude refolded rhBMP-2_{COLI} was concentrated by pressure filtration to 1–2 mg ml^{-1} followed by gel filtration on Sepharose 6 pg (Amersham Biosciences/General Electric, Freiburg Germany) on a column with specifications 5 cm i.d., 15 cm bed height, flow rate 50–60 ml h^{-1}, sample 15 ml refolded rhBMP-$2_{COLI,}$ 1–2 mg ml^{-1}, equilibrated and run with 25 mM Tris, 0.5 M NaCl, 2.5 mM EDTA, 1 mM reduced glutathione, 0.5 mM oxidized glutathione, 33 mM CHAPS, pH 8.0 (buffer A) at 5 °C [23], leading to highly enriched (>90% purity) rhBMP-2_{COLI}. Fractions containing rhBMP-2_{COLI} were identified by 17% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and concentrated

ID	Title	Pages
1723	Peri-implant reactivity and osteoinductive potential of immobilized rhBMP-2 on titanium carriers	17