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Effect of oligonucleotide mediated immobilization of bone morphogenic proteins on titanium surfaces

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ABSTRACT

The aim of the present study was to test the hypothesis that oligonucleotides can be used for anchorage and slow release of osteogenic growth factors such as BMP to enhance the osteogenic activity of a titanium implant surface. Strands of 60-mer non-coding DNA oligonucleotides (ODN) were bound to an acid-etched sandblasted cp Ti-surface by nanomechanical fixation using anodic polarization. RhBMP2 that had been conjugated to complementary strands of DNA oligonucleotides was then bound to the anchored ODN strands by hybridization. Binding studies showed a higher binding capacity compared to non-conjugated BMP2. Long term release experiments demonstrated a continuous release from all surfaces that was lowest for the conjugated BMP2 bound to the ODN anchor strands. Proliferation of human bone marrow stroma cells (hBMSC) was significantly increased on these surfaces. Immunofluorescence showed that hBMSC grown on surfaces coated with specifically bound conjugated BMP2 developed significantly higher numbers of focal adhesion points and exhibited significantly higher levels of transcription of osteogenic markers alkaline phosphatase and osteopontin at early intervals. Biological activity (induction of alkaline phosphatase) of conjugated BMP2 released from the surface was comparable to released non-conjugated BMP2, indicating that conjugation did not negatively affect the activity of the released molecules. In conclusion the present study has shown that BMP2 conjugated to ODN strands and hybridized to complementary ODN strands anchored to a titanium surface has led to slow growth factor release and can enhance the osteogenic activity of the titanium surface.

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1. Introduction

The anchorage of endosseous titanium implants occurs through osteoconduction originating from adjacent periimplant bone. In cases of compromised bone conditions due to local or systemic diseases, bone formation can be insufficient to achieve acceptable implant stability. Modifications of implant surfaces have shown to enhance the process of osteoconductive bone formation through increased surface roughness, biomimetic coating or physicochemical modifications (for review see [1,2]), thus directing periimplant bone regeneration towards the implant surface in a more effective manner. However, in compromised sites, this approach may be insufficient and more specific activation by addition of osteoinductive signalling molecules may be required to achieve adequate periimplant bone formation.

In order to accomplish this, biologically active molecules such as attachment signals, extracellular matrix molecules and osteogenic growth factors have been tested on implant surfaces [3–15]. For biologically active molecules, the mode of binding to the implant surface is considered to be crucial for a reliable and predictable tissue reaction. The least complex way of growth factor binding is adsorption during immersion of the implant into a solution of the respective growth factor. However, titanium surfaces loaded with bone morphogenic protein (BMP) by immersion coating have lost 99 to 96% of their bioactivity during the first hours of release depending on their microstructure [16]. High dosages are therefore required to elicit a biological response that may produce untoward effects [17,18]. Alternatively, linker molecules such as phosphonate anchors [19] or Thiol anchors [10] have been employed to couple biologically active molecules to titanium surfaces. Coupling of BMP to phosphonate

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anchors has been reported to retard the release from the surface and enhance bone formation [20,21]. Finally, nanomechanical anchorage of organic molecules has been used for the partial incorporation of a cyclic RGD-peptide conjugated to phosphonate anchors [22] and fixation of collagen fibres to titanium surfaces with additional covalent binding of RGD peptides [10].

Covalent binding or direct nanomechanical anchorage of biologically active molecules have the disadvantage that sterilization of the implant prior to insertion can render the coated implant surface inactive. Therefore, the concept of nanomechanical anchorage has been modified using terminally functionalized oligonucleotides [23,24].

As the structure of oligonucleotides is likely to survive conditions of γ−radiation for sterilization under specific conditions they could be used for binding of growth factors after sterilization by hybridization with complementary oligonucleotides conjugated with a growth factor. In this way, specific binding and tailored release of different growth factors could be achieved immediately prior to the insertion.

Anchorage of biologically active molecules through terminally functionalized oligonucleotides has been successfully employed for RGD peptides [23] The aim of the present in vitro study was thus to test the hypothesis that oligonucleotides can be used for anchorage and slow release of osteogenic growth factors such as BMP from a titanium surface to enhance the osteogenic activity of the implant surface.

2. Materials and methods

2.1. Specimen fabrication

Commercially pure titanium discs of 14.7 mm diameter were prepared by sandblasting and subsequent acid-etching (KLS Martin, Tuttlingen, Germany). The samples were etched in 5.1 M hydrochloric acid and 4.6 M sulphuric acid solution for 300 s by 108 °C [25]. The 60-mer anchor strands were synthesized by Thermo Fisher Scientific GmbH (Ulm, Germany). The 31-mer Oligonucleotides (GS-ODN) to anchor strands were synthesized by Biomers GmbH (Ulm, Germany). For complementary immobilization of anchor strands (AS) an electrochemical setup was used, which has already been described by Beutner et al. [24] Briefly, the samples were mounted to the bottom of a conical cell made from polyetherimide. A conventional three-electrode setup was used with a spiral gold wire acting as auxiliary electrode and a saturated Ag/AgCl reference electrode connected to the cell via a salt bridge made from a tube filled with agarose gel (2% w/v in 2 mol l^{-1} acetate buffer, pH 4.0). Initial adsorption inside the electrochemical cell was carried out in 300 μl of 0.5 mol l^{-1} acetate buffer at pH 4.0 with additional 5 mol l^{-1} ethanol for 15 min. The electrolyte contained 800 nmol l^{-1} AS. After adsorption time 6 ml buffer (with ethanol analogous to the adsorption electrolyte) were added to the cell and the anodic polarization was started within 20 s. The potentiostat/galvanostat (Voltalab 4.0, Radiometer Analytical, Lyon, France) was operated in galvanostatic mode with 11 mA cm^{-2} to ensure a polarization time of approx. 3 s to reach a potential of 7.5 $V_{Ag/AgCl}$ Subsequently the cell was rinsed two times with 9 ml buffer, followed by dipping the samples in SDI H_2O to remove the buffer. After that treatment the samples were dried for 2 h at room temperature in the dark and finally packed in alu peel bags.

Conjugation of growth factors with complementary oligonucleotides (GS) was done by using disuccinimidyl suberate (DSS) as linker molecule. In a first step the aminomodified GS-ODN was activated with DSS in 1 mM acetate buffer pH 4.0. The excess DSS was separated using an illustra NAP-10 column (GE Healthcare, Freiburg, Germany). The DSS-activated GS-ODN was mixed with rhBMP2 (Reliatech, Braunschweig, Germany) in phosphate buffered saline (PBS) containing 2 M sodium chloride pH 7.4 and incubated at room temperature for at least 18 h.

2.2. Hybridization of anchored oligonucleotides with rhBMP2 conjugated to complementary oligonucleotides (BMP-ODN)

For each disc, 100 μl of BMP-ODN solution was prepared by mixing 10 μl of 10× PBS, 80 μl of pure water and 10 μl of conjugated growth factor solution containing 715 ng of rhBMP2.

The volumes of 100 μl were pipetted onto the discs and left for hybridization in a dark chamber at room temperature for 1 h. Care was taken to accommodate the complete volume on the disc surface. Subsequently, the specimens were washed four times in PBS. The amount of BMP2 that was washed away was measured using a custom made commercially produced sandwich ELISA (Dr. Mark Hennies, Euskirchen, Germany) in order to assess the amount of BMP bound to the surface.

2.3. Release experiments

For the release experiments, titanium discs with three different conditions were analysed in order to compare unspecific binding by adsorptive coating with specific binding through oligonucleotides:

Group R1: Sandblasted acid-etched (SAE) (Fig. 1a&b) surface with nano-anchored oligonucleotides (ODN) that were hybridized with complementary oligonucleotides conjugated to rhBMP2 as described above. Assessment of washed-out BMP2 indicated that 564.7 ng of rhBMP2 remained attached to the disc surface.
Group R2: SAE surface with nano-anchored oligonucleotides (ODN) and adsorbed non-conjugated rhBMP2 to test for the effect of non-specific binding of BMP2 to the ODN strands. Discs were loaded in an identical manner as described for Group R1. This yielded an adsorbed amount of 246.5 ng of BMP2 on the disc surface.
Group R3: Blank SAE (Fig. 1c and d) surface without nano-anchored ODN and adsorbed non-conjugated rhBMP2. Discs were loaded with rhBMP2 dripping 715 ng of rhBMP2 in 100 μl onto the disc surface. The growth factors were allowed to adsorb for 1 h in a dark chamber at room temperature after which the discs were rinsed four times in PBS, leaving 486.5 ng of rhBMP2 attached to the disc surface.

The average amount of BMP2 bound to the surface in the three groups was assessed using three specimens from each group. Measurements were performed in triplicate.

Subsequently, the discs were placed in cell culture medium (DMEM, 1 g Glc, Biochrom, Berlin, Germany) in BSA coated 24 well plates. The medium was collected and replaced after, 3 h, 24 h, 3 days and every 3–4 days thereafter until day 28. The content of released BMP2 was assessed using the custom made commercially produced sandwich ELISA mentioned above. The minimum detectable amount was 100 pg/ml BMP2. Three discs from each group were evaluated at each interval for the release experiments. All measurements were performed in triplicate.

2.4. Specific osteogenic activity of surface conditions

For this part of the experiment, the following groups were used.

Group C1 : Blank SAE surfaces uncoated (Control 1).
Group C2 : Blank SAE surface with adsorbed non-conjugated rhBMP2 (Control 2: non-specific binding by adsorption)
Group C3 : SAE surfaces with nano-anchored oligonucleotides (ODN) hybridized with complementary oligonucleotides conjugated to rhBMP2 (specific binding).

Specific osteogenic effects associated with the different surface conditions were evaluated by assessment of proliferation of primary human bone marrows stroma cells (hMSCs, P2) and the induction of osteogenic markers (Runx2, Alkaline Phosphatase (AP), Osteopontin) using real time PCR (RT-PCR). Moreover, changes in cellular morphology and structure were assessed and immunohistochemical staining of focal adhesion points/Vinculin and Actin. Also for this experiment, three discs from each group were evaluated at each interval with all measurements performed in triplicate.

2.5. Proliferation of hMSCs

Human bone marrow stroma cells (hMSCs) were expanded from bone marrow aspirations (approx. 4 ml) obtained during bone graft procurement procedures from the iliac crest with subsequent gradient centrifugation and expansion of mononuclear cells in DMEM medium (4.5 g Glucose (Gibco), www.invitrogen.com) with 15% FCS (Biochrome), 1% (5×10^{-5} M) Mercaptoethanol (Serva, Heidelberg, Germany), 1% NEAA (Gibco) and 2% Gentamicin. Procurement of bone marrow aspirates and expansion of primary hMSCs had been approved by the local ethical board.

Discs with the respective surface conditions were prepared as described above. Subsequently, they were seeded with 20,000 hMSCs and cell culture medium (3 ml of DMEM, 10% FCS and 2% Gentamicin) was added. After 24 h, 72 h, 1 and 2 weeks cells were trypsinized using 0.25% trypsin - 0.1% EDTA solution and automatically counted (CASY®, Schärfe System Reutlingen, http://www.casy-technology.com).

2.6. RT-PCR analysis for bone specific gene expression

RNA was isolated from cell lysates in RLT buffer, using RNeasy Mini Kit (Qiagen, http://www.qiagen.com) according to the manufacturer's recommendations and stored at −80 °C. Subsequently, the samples were treated with DNAse I to remove contamination by genomic DNA. RNA quality was assessed using the Agilent 2100 Bioanalyser (Agilent Technologies, http://www.agilent.com). RNA specimens were then converted into cDNA using Bio-Rad iScript cDNA Synthesis Kits (Bio-Rad Laboratories, http://www.biorad.com) and quantified on a Bio-Rad MyiQ real-time PCR Detection System with the Bio-Rad iQ SYBR Green Supermix. The gene-specific primer sets are listed in Table 1. These genes included Runx2, Alkaline

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