



Enhancement of peri-implant bone osteogenic activity induced by a peptidomimetic functionalization of titanium

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ABSTRACT

Osteoblast cell adhesion to the extracellular matrix is established through two main pathways: one is mediated by the binding between integrin and a minimal adhesion sequence (RGD) on the extracellular protein, the other is based on the interactions between transmembrane proteoglycans and heparin-binding sequences found in many matrix proteins.

The aim of this study is the evaluation in an in vivo endosseous implant model of the early osteogenic response of the peri-implant bone to a biomimetic titanium surface functionalized with the retro-inverso 2DHVP peptide, an analogue of Vitronectin heparin binding site. The experimental plan is based on a bilateral study design of Control and 2DHVP implants inserted respectively in the right and left femur distal metaphysis of adult male Wistar rats ($n = 16$) weighing about 300 grams and evaluated after 15 days.

Fluorochromic bone vital markers were given in a specific time frame, in order to monitor the dynamic of new bone deposition. The effect induced by the peptidomimetic coating on the surrounding bone were qualitatively and quantitatively evaluated by means of static and dynamic histomorphometric analyses performed within three concentric and subsequent circular Regions of Interest (ROI) of equivalent thickness ($220 \mu\text{m}$), ROI1 adjacent to the interface, ROI2, the middle, and ROI3 the farthest. The data indicated that these functionalized implants stimulated a higher bone apposition rate ($p < 0,01$) and larger and rapid osteoblast activation in terms of mineralizing surface within ROI1 compared to the control ($p < 0,01$). These higher osteoblast recruitment and activation leads to a greater bone-to-implant contact reached for DHVP samples ($p < 0,5$). This represents an initial stimulus of the osteogenic activity that might results in a faster and better osteointegration process.

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1. Introduction

Osteoblasts are anchorage-dependent cells: several functions, such as proliferation, migration, differentiation, bone matrix deposition and mineralization, strictly depend on the adhesion process. This process is mediated and activated by the extracellular proteins; in case of foreign materials, cells do not adhere directly to it, but via the protein adsorbed on the material surface (Brunette, D.M.; Tengvall, P.; Textor, Marcus; Thomsen, 2001). For this reason, extracellular matrix (ECM) composition may be useful in tailor-

ing biomaterials to stimulate tissue-specific cellular responses. In fact, each ECM protein may regulate several different behaviors of the cells including adhesion, migration, proliferation and differentiation by interacting with specific cell receptors. Re-creating the biological signals derived from the ECM by attaching bone ECM-specific protein domain onto the titanium surface could be a powerful strategy to design new peptidomimetic surfaces (Shekaran and García, 2011). Bone cells adhere to a wide variety of extracellular matrix proteins and this process is a key point in bone development, repair, and disease (Brighton and Albelda, 1992). Considered the important regulatory role that ECM molecules play on cellular responses in vivo, several full-length ECM proteins have been studied for coating bone implants in the bone defect healing approach. Attempts were made with collagen (Morra et al., 2005; Rammelt et al., 2006; Schliephake et al., 2005; Svehla et al., 2005),

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fibrin (Ben-Ari et al., 2009; Karp et al., 2004; Kim et al., 2007), hyaluronic acid (Barros et al., 2009; Paderni et al., 2009; Solchaga et al., 1999), decellularized matrix (Kurkalli et al., 2010; Suckow et al., 1999) as well as bone sialoprotein (Graf et al., 2008). The existing limitations in using full-length ECM molecules have spurred the use of peptide mimicry approach; peptides or recombinant fragments derived from their parent ECM protein by incorporating the minimal functional sequence able to maintain the bioactivity of the original protein. In fact, all of the extracellular molecules disclose in their primary structure one or more consensus sequences, – long few peptides that can be recognized by a specific cell receptor. When these receptors recognize and bind the numerous ECM components they are able to discern, they cluster together on the cell membrane and form a macromolecular structure called focal adhesion.

In particular, the majority of the interaction between bone cells and extracellular matrix proteins is mediated by integrins, a widely expressed family of transmembrane receptors, formed by a non-covalent association of an α and a β subunit (Robey, PG; John, PB; Lawrence, GR; Gideon, 2002). Osteoblasts and osteoprogenitors cells express different types of integrins, like $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha 8\beta 1$ or $\alpha v\beta 3$. The L-arginine, glycine, and L-aspartic acid (Arg-Gly-Asp or RGD) is the minimal adhesion sequence recognized by integrin receptors and it was found in many ECM proteins. For the biomimetic functionalization of biomaterials, several peptides containing the RGD sequence have been developed and studied. However, the integrin-mediated adhesion process was demonstrated to be not selective for osteoblasts, but widely used by other cell types.

Another important adhesion process in bone cells is established through interactions between transmembrane proteoglycans, like heparan sulfate proteoglycan (HSPG), and heparin-binding sequences found in many ECM proteins (Dalton et al., 1995). These types of interactions might also be considerable to control the behaviour of osteoprogenitor cells (Dee et al., 1998). A great number of evidence support the significant involvement of HSPGs in osteoblast adhesion. For example, heparan sulfate was detected immunohistochemically on the membrane of osteoblasts attached to the bone matrix (Nakamura and Ozawa, 1994). Furthermore, blocking the heparin-binding sites of fibronectin with Platelet Factor IV, inhibited approximately 45% of the subsequent osteoblast adhesion (Puleo and Bizios, 1992). Moreover, heparan sulfate added in the culture medium completely inhibited human bone-derived cell attachment to the heparin-binding region of fibronectin (Dalton et al., 1995) and the adhesion of osteoblasts to osteoactivin coated matrix was significantly inhibited by heparin, whereas the same concentration of heparin had no effect on cell attachment to fibronectin (Moussa et al., 2014). Studies focusing on the identification of bioactive peptides able to promote the osteoblast adhesion via the heparan sulfate pattern are much more recent and less studied (Robey et al., 2002). Dettin et al. found and developed the peptide reproducing the 351–359 sequence of human vitronectin, called HVP (Dettin et al., 2002; Vacatello et al., 2005) and demonstrated its specificity for bone cells and its ability in promoting *in-vitro* the osteoblast adhesion on surfaces functionalized with it (Dettin et al., 2005). Subsequent studies focused on the optimisation of the functionalisation method for creating biomimetic implantable devices. Two different methods were tested, the first was a silica gel as resorbable peptide carrier (Dettin et al., 2006), the second was the peptide covalent bond on titanium substrates (Bagno et al., 2007b). The covalent coating method resulted the best performing one, considering the physical-chemical properties and the cell *in vitro* behaviour, morphology and the gene expression analysis (Brun et al., 2013). The (351–359) HVP covalently functionalized titanium cylinders were *in-vivo* studied in rabbits to evaluate the effect of functionalisa-

tion on the osteogenic activity. The results demonstrated that the peptide enhanced the osteogenic activity accelerating the bone neodeposition in a short time frame after implantation (Cacchioli et al., 2009). Numerous experimental reads out supporting the biological activity of the (351–359) peptide were achieved, however in solution the peptide sequence resulted degraded by the trypsin (Zamuner et al., 2017). For this reason, the biomimetic peptide was fine-tuned; the new approach was based on the design of a dimeric form, in order to increase the peptide ionic interactions with cellular GAGs, and its retro-inverted analogue consisting in the reversed amino acids sequence composed of D-amino acids (Zamuner et al., 2017). *In-vitro* studies demonstrated that the dimeric form of this peptide, called 2DHVP, maintained an optimal adhesive ability and matrix mineralization induction (Giovine, M, Dettin, M, Castagliuolo, 2016). The aim of this study is the evaluation, in an *in vivo* endosseous implant model, of the early osteogenic response of the peri-implant bone to the biomimetic titanium surface functionalized with the dimeric form of the adhesive, retro-inverso 2DHVP peptide.

2. Materials and Methods

2.1. Materials

Sieber Amide resin and all Fmoc-protected amino acids were from Novabiochem (Merck KGaA Darmstadt, Darmstadt, Germany). The coupling reagents 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-Hydroxybenzotriazole (HOBt) were from Advanced Biotech (Seveso, MI, Italy). N,N-diisopropylethylamine (DIPEA) and piperidine were from Biosolve (Leenderweg, Valkenswaard, The Netherlands). Triethoxysilane (TES) was from Sigma-Aldrich (Steinheim, Germany). Solvents such as N,N-dimethylformamide (DMF), trifluoroacetic acid (TFA), N-methyl-2-pyrrolidone (NMP), dichloromethane (DCM), and acetonitrile were from Biosolve.

2.2. Synthesis of side chain-protected 2DHVP

The synthetic peptide 2DHVP (sequence: H-D-Tyr-Gly-D-Lys-D-Arg-D-Asn-D-Arg-D-His-D-Arg-D-Phe-D-Tyr-Gly-D-Lys-D-Arg-D-Asn-D-Arg-D-His-D-Arg-D-Phe-NH₂) is the retro-inverted form of HVP dimer (sequence of HVP dimer: H-Phe-Arg-His-Arg-Asn-Arg-Lys-Gly-Tyr-Phe-Arg-His-Arg-Asn-Arg-Lys-Gly-Tyr-NH₂).

The peptide 2DHVP was produced by solid-phase peptide synthesis approach (synthesizer Syro I, MultisynTech, Witten, Germany), using Fmoc chemistry. Super acid labile Sieber Amide resin (1748 mg, 0.125 mmol, substitution 0.72 mmol/g) was loaded in the reactor. The following side chain protections were used: Trt, His and Asn; Boc, Lys; But, Tyr; Pmc, Arg. After the synthesis, the peptide was cleaved from the solid support without the deprotection of side chains by 5 treatments with a 1% TFA/DCM solution for 5 min. The side-chain protected peptide (sequence: H-D-Tyr(But)-Gly-D-Lys(Boc)-D-Arg(Pmc)-D-Asn(Trt)-D-Arg(Pmc)-D-His(Trt)-D-Arg(Pmc)-D-Phe-D-Tyr(But)-Gly-D-Lys(Boc)-D-Arg(Pmc)-D-Asn(Trt)-D-Arg(Pmc)-D-His(Trt)-D-Arg(Pmc)-D-Phe-NH₂) was precipitated with cold water.

2.3. Titanium implants preparation

Commercially pure titanium (Ti grade 2) implants (diameter, 2 mm; height, 2.3 mm;) were obtained from cylindrical bars by turning. First, they were smoothed with grit paper, then sand-blasted (corundum 450 mm for 10 seconds), and finally attacked with acidic solution: hydrogen chloride (HCl) (36%v/v)/sulphuric

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