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Strategy to enhance the osseointegration process: synthetic peptides improving osteoblast adhesion on implant surface

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In order to improve the integration process between surgically placed implants and biological tissues, biomaterials of the next generation have to be designed to enhance and support osteoblast adhesion. In fact, it has been demonstrated that the quality of the early cell/material interactions is highly responsible for the long-term functional response of implants.

Polystyrene surfaces have been conditioned with different synthetic peptides and their abilities to promote osteoblast adhesion have been compared. The results obtained applying the best-performing peptides in osteoblast adhesion assays on acellular bone matrix are herewith discussed.

Key words: osseointegration, osteoblast adhesion, metallic implant, synthetic peprides

1. Introduction

Recent studies in oral implantology allowed the statement that a clinical success of implanted devices mainly relies upon osseointegration, which has to be considered as both anatomical congruency and load-bearing capacity [1]. In order to evaluate the performances of implanted materials and the development of more effective devices, we have to elucidate the mechanisms of interactions between tissues and biomaterials [2], [3]. In particular, the response of biological tissues due to the characteristics of implant surface (e.g., chemical composition and micro- and macrotopography) is under continuous investigation [4]–[7]. Moreover, exploitation of a wide range of macromolecules in the native extracellular matrix (ECM) of the tissue and on the cell membrane, or both, allows us to promote a sequence of phenomena (i.e., adhesion, migration, proliferation and differentiation of cells) [6], [8]: in fact, cell–cell and cell–surface interaction and adhesion processes are both mediated by cell membrane receptors, which are responsible for reversible, non-covalent binding to complementary ligand proteins [9].

A generation of newly conceived biomaterials is likely to be designed and used in the field of oral implantology: these materials are expected to promote advantageous functions in cells and tissues which are in contact with implant surface by specific control of their interactions at the implant interface [10].

The clinical use of biological compounds promoting osteoblast adhesion and growth, such as BMPs, fibronectin and vitronectin, is still affected by a number of relevant drawbacks. First, these proteins are instable and often insoluble macromolecules. Then, their specific biological functions require the integrity of tertiary structure. Finally, these proteins are highly expensive and seldom available in large amounts.

As an alternative, it is often possible to identify the biologically active fragment of these adhesion/growth proteins and to reproduce it by chemical synthesis [11]. These bioactive peptides allow us to overcome most of the above mentioned troubles since they are stable, soluble, cheap and available in sufficient amount with high degree of purity. Thus, the design of innovative biomaterials would be based on the so-called *peptide mimicry* approach.

It is well known from literature that osteoblast cells can adhere through different mechanisms: much research has been already concerned with their interaction with the Arg-Gly-Asp (RGD) sequence *via* cell-membrane integrin receptors [12]–[16]. An alternative, but less investigated mechanism is based on the interaction between cell-membrane heparan sulphate proteoglycans and heparin-binding sites on the ECM proteins [17].

This paper addresses the results obtained by synthesizing several peptides from different proteins and testing them through *in vitro* assays. The following sequences have been identified as bioactive peptides:

1. RGD containing peptides promoting osteoblast adhesion *in vitro* by integrin-mediated mechanism.
2. Peptides from human vitronectin (HVP) which can specifically enhance osteoblast adhesion *via* proteoglycans-mediated mechanism.

The ability of synthetic peptides to improve osteoblast adhesion has been preliminarily compared on polystyrene support chosen as standard surface; thus, the best performing peptide has been tested on acellular bone matrix which represents a biological substrate suitable for predicting the behaviour of biological systems.

2. Materials and methods

2.1. Peptide design and synthesis

Several peptides (the table) containing RGD or KRSR as the main components have been synthesized as potential osteoblast adhesion factors [11]. The design of these

peptides has been guided by the rationale for presenting active components to cells in

the most attractive manner. In particular, peptides RGD and GRGDSP are sequences of different length mapped on fibronectin, while peptides $(GRGDSP)_4K$ and MAP(RGDSP) present repeated RGD components in linear and branched molecules, respectively. Peptide $(KRSRGGRGDSGG)_2$ includes both signal sequences (for integrin-mediated and proteoglycan-mediated mechanisms) in the same molecule. KRSR sequence, already suggested as signal sequence for osteoblast adhesion via proteoglycan-mediated mechanism [17], is herewith proposed as single component of KRSR
or repeated into the branched MAP(KRSR) peptide. Other peptides have been designed to reproduce fragments of different length of the human vitronectin (339–364)
sequence.

Peptide synthesis has been performed by solid phase methods using an automated synthesizer (Applied Biosystems Model 431A) via Fmoc chemistry [11]. Crude peptides have been purified using a semipreparative Delta Pack C_{18} column (Waters) and the additional use of cation-exchange chromatography (SP-5PW, Waters) is required for the purification of (339-364)HVP and (339-351)HVP sequences. The purification of (339-351)HVP peptide gave raise to the isolation of a quantitatively relevant by-product, named (339-351)HVP/Ala, containing an additional Ala residue between Ala^{346} and Lys^{347} of the native sequence. Moreover, identity and homogeneity (over 97% for each peptide) of all synthetic products have been evaluated by means of analytical reverse-phase chromatography, capillary electrophoresis, amino acids analysis after acid hydrolysis and MALDI spectrometric analysis.

2.2. Cell cultures and adhesion assays

Osteoblasts have been obtained from newborn rat calvaria according to YOKOSE et al. [18]. Wells of 96-well tissue culture microtiter plate have been conditioned with peptides using fibronectin as reference [11]. Cells have been allowed to adhere for 5 hours under standard conditions. After incubation, cells were fixed with 10% formaldehyde in phosphate buffer overnight. Fixed cells have been stained (0.04% cresyl violet in 20% methanol for 30 minutes) and, after dye extraction, absorbance has been measured at 600 nm. The results, means of four experiments, have been expressed as percentage taking as 100% the optical density of the cultures seeded onto unconditioned wells.

2.3. Acellular bone matrix assays

Acellular bone matrices have been obtained from femurs of adult Sprague-Dawley rats, demineralised and treated as discussed in [11]. Acellular bone matrices have been conditioned with $(GRGDSP)_4K$ peptide and fibronectin. After incubation

(24 hours), cells have been fixed with 3% glutaraldehyde in cacodylate buffer and examined by SEM.

3. Results and discussion

Adhesion of osteoblast cells can take place through two alternative mechanisms: the first one is mediated by interactions between cell-membrane integrin receptors and RGD sequences, the second one requires cell-membrane heparan sulphate proteoglycans to interact with some extracellular matrix proteins. Several peptides have been designed, synthesized and characterized in order to reproduce part of the natural sequences which are present in proteins promoting osteoblasts adhesion via both of the above-mentioned mechanisms.

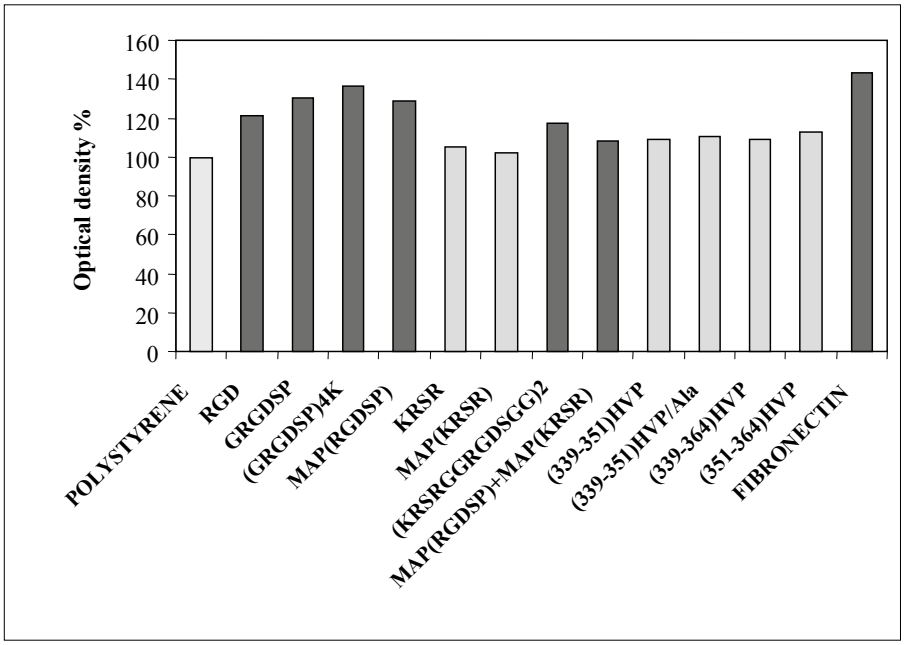

Fig. 1. Comparative results of osteoblast adhesion assays on polystyrene surface pre-treated with different peptides

The results obtained through *in vitro* assays on polystyrene surface (figure 1) suggest that:

- all RGD sequences enhance osteoblast adhesion,
- GRGDSP sequence is more effective than RGD peptide,
- $(GRGDSP)_4K$ shows the best performance; a possible reason is related to the linear structure making the adhesion sequence in a more attractive conformation than the branched peptides, or in a modification of surface density and peculiar desorption rate,

- KRSR sequence and KRSR-containing peptides do not seem to improve osteoblast adhesion,
- RGDS + KRSR-containing sequence does not increase cell adhesion in comparison with peptides containing the single component,
- (351-364)HVP, reproducing (351-364) human vitronectin sequence, is the most active molecule in promoting osteoblast adhesion *via* proteoglycan-mediated mechanism; it is worthwhile mentioning that this novel sequence is able to promote osteoblast adhesion in a highly specific manner.

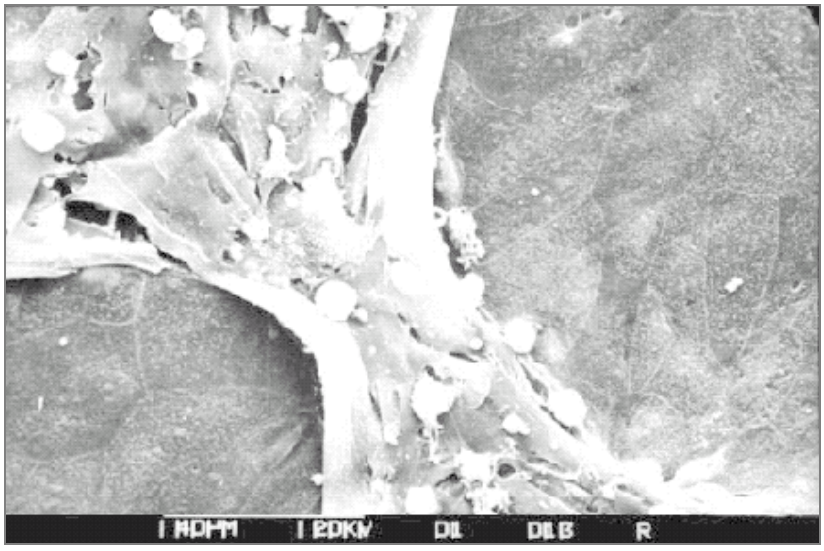

Fig. 2. Scanning electron microscopy of optimal osteoblast adhering to the acellular matrix conditioned with $(GRGDSP)_4K$ sequence

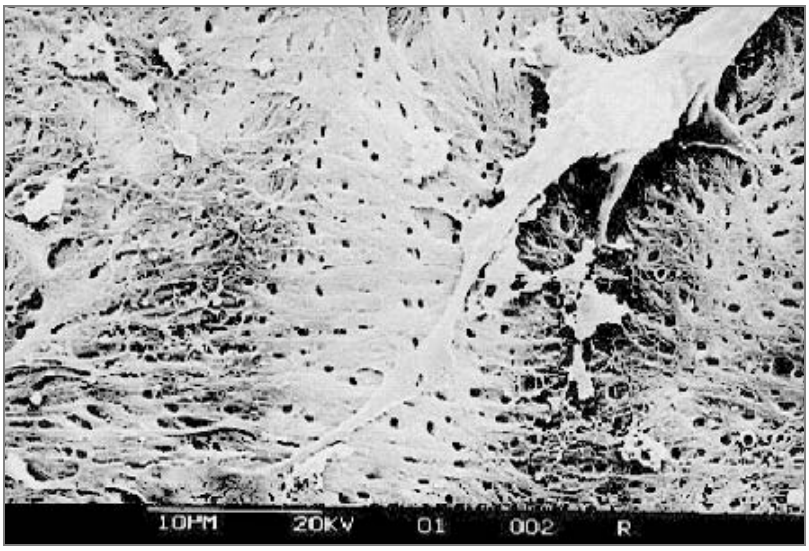

Fig. 3. Scanning electron microscopy of few osteoblasts adhering to the acellular matrix conditioned with fibronectin

Osteoblast adhesion assays have been performed on acellular matrix conditioned with the $(GRGDSP)_4K$ sequence, which exhibited the best results on polystyrene plates. Figure 2 shows that the synthetic peptide allows osteoblast to adhere forming nodular regions with polygonal shape and strict connections. Nodules are surrounded by elongated cells which tend to stratify. On the other hand, no cell adhesion has been observed on unconditioned acellular matrix (data not shown) and only few cells are present on the surface conditioned with fibronectin (figure 3).

4. Conclusions

Actually, when a metallic implant is surgically inserted, its surface comes in close contact with the exposed biological tissue: this results in a complex sequence of physicochemical and biochemical reactions which involve macromolecules from tissue and body fluids. From a general viewpoint, the overall performances of the implanted device depend on early interactions between cells and biomaterials: secure association of implant surface with biological tissue (osseointegration) is mandatory for the clinical success of implants. Thus, it is possible to state that long-term stability of the implanted prostheses is highly affected by early cell adhesion and growth on implant surface.

The results obtanied demonstrated that synthetic peptides can promote osteoblast adhesion improving the integration between surgically placed implants and biological tissues. This study represents a rational approach to the design of synthetic adhesion

peptides. Promising results have been obtained in enhancing osteoblast adhesion on acellular bone surfaces which can mimic a real biological system.

Further research will be aimed at the effect of such factors on differently treated metallic surfaces. Moreover, the development of opportune carriers for obtaining bioactive peptides at the interface between implant surface and biological tissue is in progress. The final aim of the research is to improve the overall performances of the implants and to reduce the healing phase after surgical insertion favouring the osseointegration process.

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