Parathyroid hormone-related protein (107-111) improves the bone regeneration potential of gelatin–glutaraldehyde biopolymer-coated hydroxyapatite

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Abstract

Biopolymer-coated nanocrystalline hydroxyapatite (HA) made as macroporous foams which are degradable and flexible are promising candidates as orthopaedic implants. The C-terminal (107-111) epitope of parathyroid hormone-related protein (PTHrP) exhibits osteogenic properties. The main aim of this study was to evaluate whether PTHrP (107-111) loading into gelatin–glutaraldehyde biopolymer-coated HA (HAGlu) scaffolds would produce an optimal biomaterial for tissue engineering applications. HAGlu scaffolds with and without PTHrP (107-111) were implanted into a cavitary defect performed in both distal tibial metaphysis of adult rats. Animals were sacrificed after 4 weeks for histological, microcomputed tomography and gene expression analysis of the callus. At this time, bone healing occurred only in the presence of PTHrP (107-111)-containing HA\textsubscript{Glu} implant, related to an increase in bone volume/tissue volume and trabecular thickness, cortical thickness and gene expression of osteocalcin and vascular cell adhesion molecule 1, but a decreased gene expression of Wnt inhibitors, SOST and dickkopf homolog 1. The autonomous osteogenic effect of the PTHrP (107-111)-loaded HA\textsubscript{Glu} scaffolds was confirmed in mouse and human osteoelastic cell cultures. Our findings demonstrate the advantage of loading PTHrP (107-111) into degradable HA\textsubscript{Glu} scaffolds for achieving an optimal biomaterial that is promising for low load bearing clinical applications.

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1. Introduction

The number of traumatic and non-traumatic fractures, particularly if producing secondary bone defects, has increased enormously in the past few decades, associated with the increase in lifespan in our societies [1]. In this context, the development of optimal strategies to accelerate bone repair after fracture is likely to have a great socioeconomic impact.

Bone healing involves a variety of cellular and molecular events that lead to new bone formation [2,3]. This process recapitulates most of the features of normal bone development during embryogenesis, but it is highly influenced by factors such as mechanical loading and the relative abundance of osteoprogenitors (e.g. low in older patients). In addition, injured bone tissue revascularization, providing oxygen, nutrients and cell precursors, is critical for bone healing [4]. However, the resolution of fractures often requires the use of synthetic materials as implants to replace bone tissue damage.

Different types of ceramics have been widely used in this respect, because of their similarity with the mineral component of natural bone [5,6]. Current interest is focused on bioactive and biodegradable bioceramics as scaffolds exhibiting suitable osteointegration and osteoconductive features in bone tissue engineering applica-
radiation. Supplementary details of the characterization of these scaffolds for PTHrP (107-111) loading were reported elsewhere [30]. Scanning electron microscopy (SEM) in a JEOL 6400 microscope (Tokyo, Japan) was used to characterize the macroporous 3-D architecture of the gelatin–glutaraldehyde biopolymer-coated HA scaffold. The radioactivity released into the incubation medium was sequentially monitored by counting in a γ-spectrometer. By using this method, the mean uptake of PTHrP (107-111) by these scaffolds after 24 h of loading was 60%, equivalent to 0.7 ng of peptide per mg of scaffold. Meanwhile, 80% of this amount was released to the surrounding medium within 1 h, and virtually 100% at 48 h. It was previously shown that minor amounts of this peptide (in the sub-nanomolar range) still sustained biological activity [18,23,25,26].

2. In vivo rat model of bone healing

Our protocol, using a limited number of male Wistar rats (6 months of age; n = 5 per experimental group) was approved by the Institutional Animal Care and Use Committee at the IIS-Fundación Jiménez Díaz, according to the European Union guidelines for decreasing the pain and suffering of the animals. The rats were placed in cages under standard conditions (room temperature 20 ± 0.5 °C, relative humidity 55 ± 5% and illumination with a 12 h/12 h light/dark photoperiod), given food and water ad libitum and allowed to move without restriction. Surgical interventions were performed under aseptic conditions and general anesthesia was induced by injection of xylacine (10 mg kg⁻¹) and ketamine (25 mg kg⁻¹). Both knees were shaved, and a transcortical defect was generated by drilling a hole (2 mm in diameter and 3 mm in depth) through the cortex of both distal tibial metaphyses, using continuous irrigation with physiological saline to prevent bone necrosis. The healing response in this simple defect recapitulates that of a stabilized fracture, minimizing animal morbidity, trauma and infection [31,32]. The unloaded HA scaffold was implanted into the left tibial defect, whereas the right tibial defect received the PTHrP-derived pentapeptide-coated scaffold. Rats were sacrificed after 4 weeks for histological, microcomputerized tomography (μCT) and gene expression analysis of the callus.

2.3. μCT analysis

Both tibiae were scanned using GE eXplore Locus μCT scanner (GE Healthcare, London, Canada). The X-ray tube settings were 80 kV of energy and 450 μA of current. The μCT image acquisition consisted of 400 projections collected in one full rotation of the gantry. The resulting raw data were reconstructed using a filtered back-projection algorithm to a final image with a resolution of 93 μm in all three spatial dimensions. The reconstructed images were viewed and analysed using MicroView software, version 2.2 with Advanced Bone Analysis plus (GE Healthcare). Bone volume/tissue volume (BV/TV) as well as trabecular and cortical thickness (Tb.Th and Ct.Th, respectively) were calculated.

2.4. Histological evaluation

Tibiae were removed and fixed in 10% neutral formaldehyde, followed by decalcification with OsteoSoft (Merck, Madrid Spain) for 4 weeks. Bone specimens were dehydrated before paraffin embedding using a Leica TP 1020 tissue processor. All histological and immunohistochemical determinations were carried out onto sagittal 4 μm sections of each bone sample in a Zeiss Axioskop optical microscope (Carl Zeiss, Oberkochen, Germany). For histological analysis, haematoxylin & eosin and Masson’s trichromic staining were used. Osteoblasts (cubic cells adjacent to the bone surface) and osteoclasts (polynucleated cells with a rough border close to bone surfaces) were also quantified in the same trabecular area (5 mm²) around the scaffold, loaded or not with PTHrP (107-111), in which μCT evaluation was carried out as described above.

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Evaluations were performed by at least two independent observers in a blinded fashion for each rat.

2.5. Cell culture studies

Cell culture experiments were performed using the well-characterized mouse osteoblastic cell line MC3T3-E1 (subclone 4, ATCC, Mannassas, VA), which responds to C-terminal PTHrP peptides [25,26,33,34]. The tested scaffolds were placed into each well of 6- or 24-well plates before cell seeding. MC3T3-E1 cells were then plated at a density of 10,000 cells cm$^{-2}$ in 2 ml of osteogenic medium consisting of α-minimum essential medium containing 10% heat-inactivated foetal bovine serum (FBS), 50 μg ml$^{-1}$ ascorbic acid, 10 mM β-glycerol-2-phosphate and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere of 5% CO2, and incubated for different times. Human osteoblast-like (hOB) cells, isolated from trabecular bone explants obtained from knee samples discarded at the time of surgery on two osteoarthritic subjects (aged 69 and 80 years) [34], were cultured in Dulbecco’s modified Eagle’s medium containing 15% FBS and the same aforementioned supplements for 12 days. Some wells contained no scaffolds as controls. Medium was replaced every other day.

Cell proliferation was assessed after MC3T3-E1 cell incubation with the different materials for 4 days. At this time, 10 vol.% Alamar blue solution (ABD Serotec, Oxford, UK) was added to the cell culture. Four hours later, 1 ml samples of the cell-conditioned medium were added to a 24-well plate and the fluorescence intensity was measured using excitation and emission wavelengths of 540 and 620 nm, respectively. Following incubation with the tested materials for 4 days, the cells were washed with PBS and the alkaline phosphatase (ALP) activity was measured in cell extracts obtained with 0.1% Triton X-100 using p-nitrophenolphosphate as the substrate, as described previously [26,34]. The ALP activity was normalized to cell protein content, determined by bicinchoninic acid (Thermo Scientific, Rockford, IL, USA) method with bovine serum albumin as standard. Matrix mineralization in MC3T3-E1 or hOB cells exposed to the tested materials for 12 days was determined using Alizarin S red staining, with the absorbance at 620 nm being measured as described previously [26,34].

2.6. Real-time PCR

Total RNA was isolated from osteoblastic MC3T3-E1 cells and the rat tibia callus by a standard procedure (Trizol, Invitrogen, Groningen, The Netherlands), and gene expression was analysed by real-time PCR using an ABI PRISM 7500 system (Applied Biosystems, Foster City, CA), as reported [26,34]. Real-time PCR was done using Sybr premix ex Taq (Takara, Otsu, Japan) and the following rat-specific primers: 5’-GCTGATAGCCACGTAT-3’ and 5’-AGGCGATCATATCGTATT-3’ (dickkopf homolog 1, Dkk-1); or 5’-GAGTACCCAGAGCCTCCTCA-3’ and 5’-GCTGCATGAGGCACGCTAT-3’ (dickkopf homolog 1, DKK-1); or 5’-GAGTACCCAGAGCCTCCTCA-3’ and 5’-GCTGCATGAGGCACGCTAT-3’ (Sost). Osteocalcin (OC), osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL), vascular endothelial growth factor (VEGF) and vascular cell adhesion molecule 1 were amplified in parallel with tested genes. The number of amplification steps required to reach an arbitrary intensity (Ct) was computed. The relative gene expression was represented by 2$^{-}\Delta{Ct}$, where $\Delta{Ct} = \Delta{Ct}_{target\ gene} - \Delta{Ct}_{18S}$. The fold change for the treatment was defined as the relative expression compared with control, calculated as $2^{-\Delta{Ct}_{treatment} - \Delta{Ct}_{control}}$.

2.7. Statistical analysis

Results are expressed as mean ± standard error of the mean (SE). Statistical evaluation was carried out with non-parametric Kruskal–Wallis test and the post hoc Dunn’s test or Mann–Whitney U-test, when appropriate. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Characterization of HAGlu scaffolds

The preparation technique employed on HAGlu foams enables one not only to synthesize and fabricate macroporous scaffolds in a one-step process, but also to create a hierarchical interconnected structure from the macroporous to the mesoporous range. HAGlu foams take up Trypan blue stain by diffusion through the pores, indicating good interconnected porosity (Fig. 1A). SEM micrographs of HAGlu foams show interconnected macroporosity with a porosity range of 1–400 μm (Fig. 1B). The XRD pattern corresponds to pure nanocrystalline HA (ICDD PDF 9-432) (Fig. 1C). The average crystallite size calculated was 20 nm, based on all the reflections by Rietveld refinement [35]. As previously reported, transmission electron microscopy indicated a mesoporous network with a pore size of 10–15 nm (Supplementary Fig. S1). The total porosity measured by Hg intrusion of three representative HAGlu specimens is approx. 70% (Supplementary Fig. S2). Thermogravimetric analyses of these samples confirmed their content of 80% wt. HA and 20% wt. gelatin (Supplementary Fig. S3). These scaffolds behave as a hydrogel, i.e. the network is able to absorb fluid maintaining its overall structure. The swelling ratio (W) was calculated as (%): 100 × (Wt – Wd)/Wd, where Wd is the weight of dried foam and Wt is the weight of hydrated foam. HAGlu foams have a W of 400% wt. when immersed in aqueous solution due to the hydrophilic nature of glutaraldehyde-crosslinked gelatin (Supplementary Fig. S4).

3.2. PTHrP (107-111) loading onto HAGlu scaffolds improves bone healing of a cavitary defect in the rat tibial metaphysis.

3.2.1. μCT analysis

The bone tissue response to the implanted scaffolds tested was examined by μCT at the tissue/biomaterial interface and the peripheral area of the implant. We found that the PTHrP (107-111)-loaded scaffold completely healed the bone defect at 4 weeks after implantation. This contrasts with incomplete bone union observed by implanting the unloaded HAGlu scaffold instead (Figs. 2A and 3A). Osteoinduction related to the presence of the PTHrP-derived pentapeptide on this scaffold was confirmed by quantitating the bone volume per total volume (BV/TV) at the cortical and trabecular compartments, as well as trabecular (Tb.Th) and cortical thickness (Ct.Th) (Figs. 2B and 3B), corresponding to at each skeletal site in the regenerating tibia, respectively.

3.2.2. Histological findings

At 4 weeks after implantation, no signs of inflammation were observed in the vicinity of HAGlu materials. Complete repair of the cavitary bone defect was not observed in the unloaded HAGlu control group at this time (Fig. 4A), though rats implanted with PTHrP (107-111)-loaded scaffolds showed good healing of the cavity (Fig. 4B). Consistent with the μCT results, the histological studies showed that, compared to the unloaded material, the implantation of PTHrP (107-111)-loaded HAGlu scaffolds promoted the appearance of a lot more new trabeculae around the bone inter-
scaffolds display a clear advantage over peptide-un- scaffolds would provide a more appro- 

scaffolds in bone regeneration in

scaffolds. Cell 

foamy scaffolds indicate that they fulfil 

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111) in the implanted scaffolds

was also examined in the rat tibial defect at 4 weeks post-implan-

Using this approach, we demonstrate that PTHrP (107-111)-con-

armed PTHrP (107-111) from SBA15-based ceramics as implanted

載 scaffolds into a rabbit bone defect[27,29]. This type of Si-enriched

ceramic has a narrow mesopore size distribution (in the nanometre

range) but a large surface area that governs the interaction with

material to affect osteoblastic function. It was found that the pres- 

ence of PTHrP (107-111) in the scaffold increased the expression of

various genes related to osteoblast differentiation, namely OC and

OPG, whereas it decreased that of RANKL – a key factor for induc-

ing osteoclastogenesis – thereby increasing the OPG/RANKL mRNA

ratio, at 4 days of culture (Fig. 7B). In addition, this type of scaffold

containing this pentapeptide stimulated ALP activity at this time

point in MC3T3-E1 cells (Fig. 8A), and promoted matrix mineraliza-

tion in these cells and also in hOBs cells at day 12 of culture (Fig. 8B

and inset). VEGF gene expression was also up-regulated in this sce-

nario (Fig. 7B), which is consistent with the action of PTHrP (107-

111) and the native PTHrP (107-139) fragment in various osteo-

blastic cell preparations[25,26,28,33,34]. The unloaded HA_{CNU} scaf-

folds failed to affect either cell growth or matrix mineralization in

MC3T3-E1 cells within the time of the study (4–12 days), empha-

sizing the notion that PTHrP (107-111) gives bioactivity to these

scaffolds (Figs. 7 and 8).

4. Discussion

Recently, we showed the osteoinductive actions of locally deliv- 
ered PTHrP (107-111) from SBA15-based ceramics as implanted carriers into a rabbit bone defect [27,29]. This type of Si-enriched ceramic has a narrow mesopore size distribution (in the nanometre range) but a large surface area that governs the interaction with the host bone tissue. However, these materials were non-degrad- able and induced the formation of a thick fibrous cup around the implant [3,27,29]. This prompted us to evaluate whether loading PTHrP (107-111) into HA_{CNU} scaffolds would provide a more approp- riate biomaterial as an implant for improving new bone forma-

tion. These scaffolds were therefore implanted into a cortical defect in the rat tibial metaphysis, in which bone regeneration is known to proceed through intramembranous ossification [12]. Using this approach, we demonstrate that PTHrP (107-111)-containing HA_{CNU} scaffolds display a clear advantage over peptide-un- 
loaded scaffolds in promoting bone healing, as assessed by bone structure and histology, as well as molecular criteria. The cell autonomy of the osteogenic effects of this biomaterial was further confirmed using in vitro osteoblastic cell cultures.

An idoneous bone filler should provide structural support and a three-dimensional matrix to favour bone in- and on-growth, and gradually degrade to non-cytotoxic products [37,38]. Previous characterization of HA_{CNU} foamy scaffolds indicate that they fulfill these criteria [11,12]. In fact, the nanocrystalline structure of HA_{CNU} scaffolds, which is similar to that of native HA in bone, was found...
Fig. 2. (A) Representative frontal plane images by μCT of the area surrounding the HA_{Glu} implants, with or without loaded PTHrP (107-111), showing newly formed bone at 4 weeks after implantation into a cavitary defect in the rat tibia. (B) Trabecular bone volume/total volume (BV/TV) and trabecular thickness (Tb.Th) corresponding to the evaluated bone area around the implant. The circle denotes the bone defect, whereas the square shows the area close to the defect where the trabecular parameters were measured by μCT. Results are mean ± SE (n = 5). *p < 0.05 vs. the corresponding unloaded HA_{Glu} scaffold.

Fig. 3. (A) Representative transverse plane images by μCT of the area surrounding the HA_{Glu} implants, in the presence or absence of PTHrP (107-111), containing new bone at 4 weeks after implantation into a cavitary defect in the rat tibia. (B) Cortical bone volume/total volume (BV/TV) and cortical thickness (Ct.Th) corresponding to the evaluated bone area around the implant as remarked by the circle. Results are mean ± SE (n = 5). *p < 0.05 vs. the corresponding unloaded HA_{Glu} scaffold.

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to promote bone formation, avoiding the generation of fibrous tissue around the implant [12]. The biological performance of HAGlu scaffolds allows their definition as third-generation materials for orthopaedic use [39]. It was recently found that these scaffolds display excellent osteointegration properties when implanted into a cavitary bone defect in the rabbit epiphysis; thus, complete bone healing was observed at 4 months after implantation [12]. In the present study, we examined the performance of both types of implants tested – with and without PTHrP (107-111) – in a rat cortical bone defect at 4 weeks, which is an insufficient time period for complete bone healing [40]. This approach allowed us to more easily disclose the osteoinductive advantage represented by the PTHrP (107-111)-loaded foams.

Dramatic differences were observed in the pattern of bone repair of this non-critical bone defect in the rat tibia between both types of implanted HAGlu scaffold evaluated. The occurrence of bone regeneration leading to a completely sealed cortical defect was strictly associated with the presence of PTHrP (107-111) in the scaffold after 4 weeks. Consistent with previous data using other types of non-degradable ceramics as carriers of this pentapeptide [26], we observed here that, in vitro, PTHrP (107-111) was released very rapidly (within 2 days) from HAGlu scaffolds into the surrounding medium. Using the former materials, even small amounts of this peptide (in the nanomolar range or lower concentrations) remaining in the non-degradable ceramic seemed to elicit osteogenic actions [26,27,29]. The HA Glu scaffolds have been shown to be stable for about 2 weeks, but progressive degradation occurs thereafter [11]. Thus, and consistent with previous observations in a rabbit model [12], we found here, using μCT and histological analysis, scarce HA Glu material (although still detectable) in the bone defect area in our rat model at 4 weeks after implantation. Most proposed release strategies (i.e. using BMP2) provide a burst immediately after the local (surgical) application [41]. However, it is presently debatable whether maintaining the peptide bioactivity and its release burst are equally important factors in this respect. Assuming similar PTHrP (107-111) kinetics in our present in vivo setting, it seems that, besides the initial burst, even the remaining material containing a small amount of this peptide in the 2 days to 4 weeks time frame might contribute to improving bone healing in this model.

Our present data show that PTHrP (107-111)-loaded HA Glu scaffolds promote trabecular formation, with abundant osteoblastic cells adhering to the trabecular surface in the vicinity of the degrading biomaterial. This was related to an increased gene expression of OC, a late osteoblast differentiation marker, and accompanied by gene overexpression of VCAM 1, a vascular endothelial marker [42], in the regenerated callus. In this regard, previous studies have shown that local or systemic administration of...
Fig. 5. Relative abundance of osteoblasts and osteoclasts observed on trabecular surfaces in the bone healing area around each type of implanted HA_Glu scaffold into a cavitary defect in the rat tibia (as described in the legend to Fig. 2), 4 weeks after implantation. The arrow and the star denote the presence of multinucleated osteoclast and cuboidal osteoblasts, respectively. Results are mean ± SE (n = 5). *p < 0.05 vs. the corresponding unloaded HA_Glu scaffold.

Fig. 6. Gene expression (by real-time PCR) of bone-related factors and the angiogenic factor VCAM 1 in the callus generated at 4 weeks after implantation of PTHrP (107-111)-loaded or unloaded HA_Glu scaffolds into a cavitary defect in the rat tibia. Results are mean ± SE (n = 5). *p < 0.05 vs. the corresponding unloaded HA_Glu scaffold.
PTHrP (107-111) or the native PTHrP (107-139) fragment, respectively, increased angiogenesis in other in vivo models of bone regeneration in mice and rabbits [19,20,27,29]. The present in vitro data further support the idea that PTHrP (107-111) loading confers osteogenic and angiogenic potential to HA<sub>Glu</sub> foams.

PTHrP (107-111)-loaded HA<sub>Glu</sub> scaffolds induced the opposite effect (i.e. a decrease) on two well-known inhibitors of the canonical Wnt pathway, DKK1 and Sost, in the healing bone defect. Of note, the putative PTHrP (107-139) fragment has been reported to decrease the expression of both genes in bone after its systemic administration for 4 weeks to ovariectomized mice, and also in rat osteoblastic UMR-106 cells [21]. Moreover, Sost downregulation has been shown to be an important event in the early phase of fracture healing in humans [43]. Furthermore, a previous study demonstrates that systemic or local injection of a DKK1 adenovirus hampered cortical defect healing in the mouse tibia of mice [36]. It has also been shown that an anti-DKK1 antibody injection was efficient to stimulate bone healing after trauma caused by a stainless steel screw inserted into the rat tibia metaphysis [44]. Hence, our present findings add credence to the notion that PTHrP (107-111) loaded onto HA<sub>Glu</sub> scaffolds may promote bone healing through targeting the Wnt pathway.

Our results also indicate that PTHrP (107-111) loading onto HA<sub>Glu</sub> scaffolds decreased the abundance of osteoclasts resorbing new bone around the implant. This was not surprising, considering the ability of this peptide to reduce the number of trabecular osteoclasts when administered subcutaneously, as recently reported [45], and the observed increase in OPG/RANKL mRNA ratio induced by PTHrP (107-111)-loaded HA<sub>Glu</sub> scaffolds in osteoblast cultures in this work. In fact, PTHrP (107-139) has consistently been shown to display anti-resorptive features in rodents [17,20-22], apparently by interacting with osteoclasts directly or indirectly through targeting osteoblasts [19,23,26,28]. Also in this regard, the local presence of PTHrP (107-111) was shown to inhibit the transient inflammatory response as well as the appearance of osteoclasts in a cavitary bone defect in the rabbit femur [27]. Together these data strongly suggest that PTHrP (107-111) may inhibit osteoclastogenesis during bone regeneration.

5. Conclusions

The present findings demonstrate the suitability of our experimental combined strategy, adding credence to the notion that loading these degradable HA<sub>Glu</sub> scaffolds with PTHrP (107-111)
produces an optimal cavity filling biomaterial that is promising in low load bearing clinical applications.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.actbio.2014.03.025](http://dx.doi.org/10.1016/j.actbio.2014.03.025).

**Appendix B. Figures with essential colour discrimination**

Certain figures in this article, particularly Figs. 1, 4 and 5 are difficult to interpret in black and white. The full colour images can be found in the on-line version, at [http://dx.doi.org/10.1016/j.actbio.2014.03.025](http://dx.doi.org/10.1016/j.actbio.2014.03.025).

**References**