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Characterization of absorbable collagen sponges as recombinant human bone morphogenetic protein-2 carriers

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Abstract

For clinical use recombinant human bone morphogenetic protein-2 (rhBMP-2) is soaked onto an absorbable collagen sponge (ACS) for bone regeneration. Therefore, loss of rhBMP-2 upon mechanical handling during implantation and a potential effect of the carrier on in vivo retention is of interest. The interactions between drug and carrier were looked at from the application mode and the amount of protein which can be mechanically expressed from the combination was investigated. The results indicated that rhBMP-2 binds to the collagen system. The most hydrophilic double extended homodimer showed the least binding affinity to ACS. By extending the waiting time between soaking and implantation, protein incorporation could be increased. In addition, the amount of rhBMP-2 which could be expressed was reduced by heavier ACS material and allowed for a shorter waiting period, especially at lower rhBMP-2 concentration. Crosslinking of ACS with formaldehyde led to reduced binding of rhBMP-2 to collagen either by direct hindrance of binding or reduction in swelling and number of binding sites available. Higher product pH or anion concentration enabled to increase rhBMP-2 incorporation but was limited by the potential precipitation of rhBMP-2. Despite a variety of chemical changes of ACS by ethylene oxide sterilization incorporation was not changed significantly. The in vivo release kinetics of ^{125}I-rhBMP-2 from the collagen sponge were studied using a rat ectopic implant model. The ACS/rhBMP-2 systems tested demonstrated small but significant differences in the in vivo retention of rhBMP-2. Consequently, it is important to have as little variability in pH, anion concentration, crosslinking, and ACS mass as possible to achieve consistent or maximum binding and to avoid rhBMP-2 precipitation. Furthermore, these characteristics can be important for other in vivo applications.

Keywords: Collagen; Bone morphogenetic protein; Sterilization; Crosslinking

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1. Introduction

The use of collagen as a biomaterial in gel form or as porous sponges currently experiences a renaissance in the tissue engineering field. The biotechnological applications focus on the aspects of cellular growth ex vivo or delivery of growth factors to stimulate cellular response in vivo (Slavin et al., 1992; Royce et al., 1995; Toolan et al., 1996). Collagen is of interest since it is well biocompatible, biodegradable and enhances cellular penetration to form extracellular matrix (Hubbell, 1995; Friess, 1998). Using porous collagen systems in combination with proteins or glycoproteins, success or failure can depend on the appropriate application based on understanding of the processing technology and basic knowledge about the chemical and physicochemical properties of the carriers. In addition, understanding of interactions between a protein drug and a biomaterial scaffold as well as the delivery aspect of the combinations helps to design optimized systems for different applications and to expand the spectrum of use. Furthermore, these applications face a broad variability in the biological cellular response and it has to be evaluated whether variability based on the carrier can be neglected or triggers inconsistency in the performance. Interest lies in the use of collagen sponges for bone regeneration using recombinant human bone morphogenetic protein-2 (rhBMP-2). In the first part of the studies the chemical and physicochemical properties of the sponge were evaluated, such as number of free amino groups, denaturation temperature, or collagenase sensitivity as a function of collagen crosslinking, sterilization, and mass (Friess et al., 1999). For implantation, the sponge is soaked with aqueous protein formulation and pH and salt concentration was investigated in the expressed liquid and its influence on rhBMP-2 precipitation. In this paper, the focus was on the interaction of collagen with rhBMP-2 and the pharmacokinetics of rhBMP-2 retained at the implant site to understand the effect of these parameters and interactions on the in vivo properties.

2. Materials and methods

rhBMP-2 was produced at Genetics Institute Inc. using a Chinese hamster ovary cell expression system (Israel et al., 1996). Purified protein was formulated at 4 mg/ml in a 5 mM glutamic acid buffer pH 4.5 (glu I; 2.5% glycine, 0.5% sucrose, 0.01% polysorbate 80, 5 mM NaCl, 5 mM glutamic acid). Dilution was performed using a 30 mM glutamic acid buffer pH 4.5 (glu II; 2.5% glycine, 0.5% sucrose, 0.01% polysorbate 80, 30 mM glutamic acid).

2.1. Absorbable collagen sponge (ACS) manufacturing

Briefly, purified, fibrillar, bovine collagen dispersion (pH approximately 6.3) was lyophilized to achieve ACS of 7.5×10 cm area and height corresponding to 2.6, 4.4, or 6.2 mg/cm^2 weight (Friess et al., 1999). Sponges were treated with formaldehyde (CH_2O) vapour for 30, 90, or 240 min and sterilized with ethylene oxide (EtO).

2.2. Incorporation analysis

ACS pieces of 6.5 cm^2 were soaked with 650 μl of 1.5 mg/ml rhBMP-2 in glu I/II buffer pH 4.5. At the designated time points the sponge pieces were squeezed using two tweezers. The rhBMP-2 concentration was determined via UV absorbance at 280 and 320 nm or reversed phase-HPLC (Vydac C_4 column, 4.6 mm × 50 mm, 300 Å, 5 μm particle size; injection volume: 100 μl; mobile phase A: 0.1% TFA, mobile phase B: 90% acetonitrile, 0.1% TFA; ramp from 0 to 100% mobile phase B within 10 min at 2 ml/min; detection at 214 nm).

Incorporation was calculated using the following equation:

$$\% \text{ incorporation} = 100 \times [1 - ([\text{rhBMP-2}] \times \text{expressate volume/mg rhBMP-2 applied})]$$

In addition, a series of expressates was analyzed for isoform distribution using a cation exchange HPLC method (Pharmacia Mono S column 5.0 mm × 50 mm, injection volume: 100 μl, mobile

phase A: 20 mM sodium acetate, 10% acetonitrile, pH 5.0, mobile phase B: 20 mM sodium acetate, 10% acetonitrile, pH 5.0; ramp from 15 to 70% mobile phase B within 19 min at 1 ml/min; detection at 280 nm) (Rathore et al., 1995).

To study the effect of mass and time on incorporation, ACS pieces were trimmed to specific weights cut from sterilized sponges of 4.4 mg/cm^2 average weight with 90 min CH_2O treatment.

2.3. Anion and pH dependence of rhBMP-2 incorporation

ACS pieces (7.5 × 10 cm) were rinsed with water (2 l exchanged four times over 48 h) and re-lyophilized. First, 325 μl of 3.0 mg/ml rhBMP-2 solution in glu I/glu II was added to 6.5 cm^2 pieces followed by 325 μl of pH and salt adjusted glu II buffer. After 30 min the expressate was gathered and analyzed as described above.

2.4. Equilibrium binding studies

ACS pieces (3–5 mg) were placed in 625 μl rhBMP-2 at various concentrations and pH in glu I containing 25 mM glutamic acid. After 24 or 48 h, samples were analyzed for rhBMP-2 concentration via HPLC and the amount of rhBMP-2 bound to ACS calculated.

2.5. rhBMP-2/ACS implant pharmacokinetics

A rat ectopic assay (Sampath and Reddi, 1983) was used to determine the residence time of implanted rhBMP-2. A volume of 200 μl rhBMP-2 (0.4 mg/ml) solution was added onto 14 × 14 mm sponges (4.4 mg/cm^2), allowed to soak for 30 min and implanted subcutaneously in Long–Evans rats (2 implants/rat). The protein solution contained a trace amount of radioactive ^{125}I-rhBMP-2. At indicated time points, two rats were sacrificed, the implants removed and the implant associated radioactivity was determined. Then the explants were homogenized with 4 M guanidine and the homogenate precipitated by 10% TCA solution which ensured that >95% of the radioactivity were protein bound. The pharmacokinetic was analyzed by WinNonLin software (Scientific Consulting Inc., Apex, NC, USA) non-compartmentally and compartmentally using a bi-exponential model. A difference greater than ±20% between two pharmacokinetic parameters was considered significant.

3. Results

3.1. Time and ACS mass dependence of incorporation

The incorporation assay represents an approach to study rhBMP-2 interactions with ACS based on the wet sponge as used in the clinical application. Fig. 1 shows incorporation of rhBMP-2 as a function of soak time and ACS weight and the results demonstrated binding of rhBMP-2 to ACS material. The sterilized ACS material in this experiment was treated with CH_2O for 90 min. The amount of rhBMP-2 incorporated by sterilized collagen sponges increased significantly with ACS mass. Comparison of the two rhBMP-2 concentrations tested indicated higher incorporation rates for 0.75 mg/ml. At heavy ACS weights, 90% were reached already after 5 min and the lightest material resulted in more than 70% after 30 min. The difference between lightest and heaviest ACS group tested was wider for 1.5 mg/ml. Approximately 90% of the rhBMP-2 load could not be removed by squeezing from sponges of the 5.7 and 6.3 mg/cm^2 groups after 30 min, whereas the light ACS material resulted in incorporation as low as 44.1 ± 5.8%. Incorporation increased significantly over time depending on rhBMP-2 concentration and ACS weight.

3.2. pH and anion concentration dependence of incorporation

To evaluate the influence of pH and anion concentration, the ACS was rinsed with excess of water and readjusted to chloride and sulfate concentrations and pH values found in ACS expressates (Friess et al., 1999). The sterilized ACS material in this experiment was treated with CH_2O for 90 min. With rising pH, incorporation

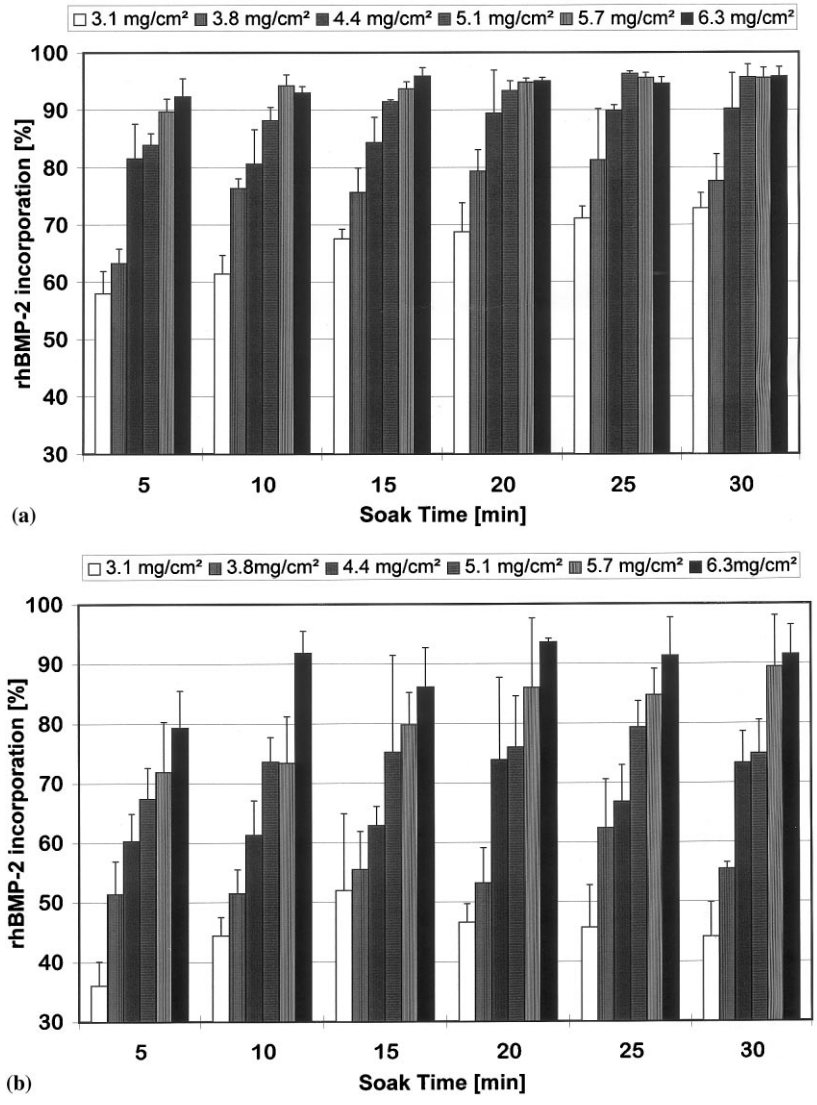

Fig. 1. Recombinant bone morphogenetic protein-2 (rhBMP-2) incorporation as a function of ACS mass and waiting time at (a) 0.75 mg/ml rhBMP-2; (b) 1.5 mg/ml rhBMP-2.

increased from approximately 50% for pH 5.1 or 5.2 to 90% without extra anions added (Fig. 2a–c). The same pH effect resulted by addition of sodium chloride, sodium sulfate, or a combination of both anions. Above 20 mM chloride, precipitation of rhBMP-2 occurred at pH 6.45 and 6.7 and precipitation was also found at pH 5.75 and 50 mM chloride (Fig. 2a; full symbols). As shown in Fig. 2b, rhBMP-2 is more sensitive to the presence of sulfate than to chloride and both incorporation was increased and precipitation occurred at much lower concentrations as compared to chloride.

Incubation of ACS pieces in excess rhBMP-2 liquid formulation demonstrated that equilibrium binding was less than 0.01 mg/mg ACS at pH 3 and 4 (Table 1). At pH 4.5 0.02 mg rhBMP-2 were bound per mg ACS. With further increase in pH, the amount of rhBMP-2 bound was 0.1 and 0.18 mg/mg at pH 5.2 and 6.5, respectively.

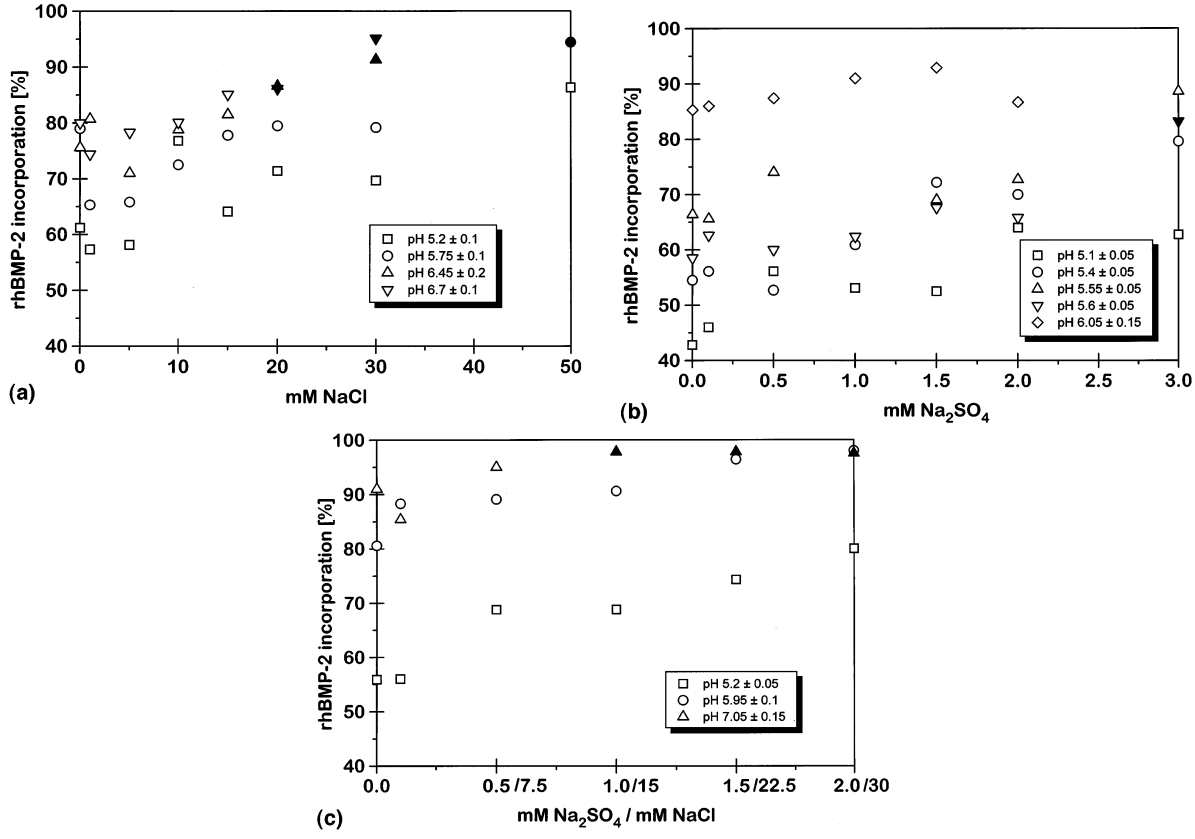

Fig. 2. Recombinant human bone morphogenetic protein-2 (rhBMP-2) incorporation as a function of pH and (a) NaCl concentration; (b) Na_2SO_4 concentration; (c) $NaCl + Na_2SO_4$ concentration (full symbols denote precipitation).

3.3. Effect of crosslinking and sterilization on incorporation

Fig. 3 summarizes the effect of crosslinking and sterilization on rhBMP-2 incorporation. With longer exposure time to CH_2O the percentage of rhBMP-2 incorporated decreased. The effect was ACS mass dependent. Incorporation of rhBMP-2 strongly increased with weight of sponges treated for 90 or 240 min. ACS material of 6.4 mg/cm^2 weight and 90 min CH_2O treatment gave more than 90% incorporation both sterilized and non-sterilized. In contrast, incorporation was reduced to 35–40% for the lightest ACS material tested. Sterilization was not connected with a clear trend in incorporation and resulted in only minor changes except for a 41% increase for 2.6 mg/cm^2, 30 min CH_2O treated ACS and a 16% decrease for 4.4 mg/cm^2, 240 min CH_2O treated ACS.

3.4. Incorporation of rhBMP-2 isoforms

The six different isoforms of rhBMP-2 showed different affinity to ACS material (Fig. 4). The

Table 1
pH dependence of recombinant bone morphogenetic protein-2 (rhBMP-2) equilibrium binding to absorbable collagen sponge (ACS)

pH	rhBMP-2 binding (mg/mg)
3.0	<0.01
4.0	<0.01
4.5	0.02
5.2	0.10
6.5	0.18

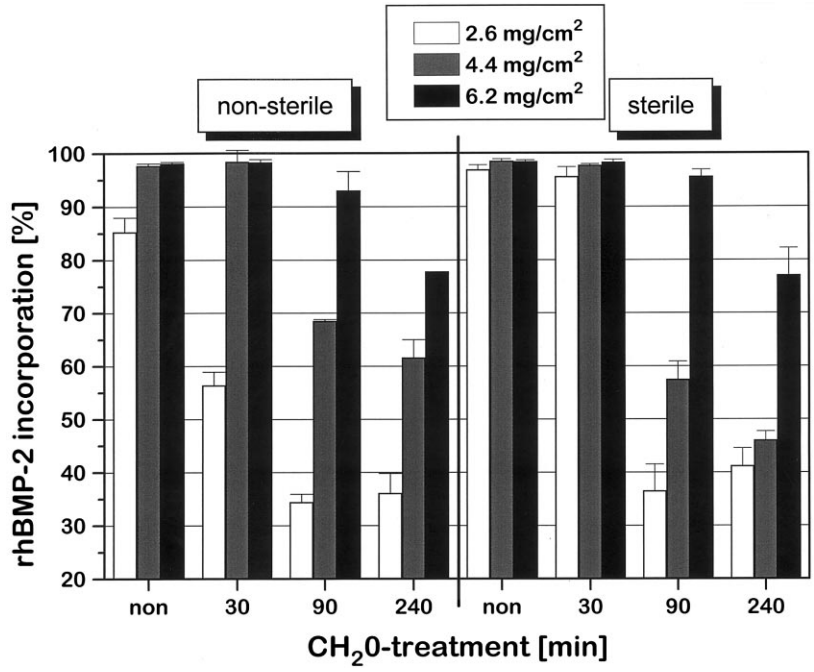

Fig. 3. Recombinant bone morphogenetic protein-2 (rhBMP-2) incorporation at 1.5 mg/ml in ACS as a function of sterilization, sponge mass and CH_2O treatment time.

most hydrophilic double extended homodimer (T/T) resulted in the least percentage (37%) of incorporation. Retention in the collagen sponge was approximately 55% for the less hydrophilic mature forms with insignificant differences between glutamate and pyroglutamate forms as well as between dimers with only one or both chains in the mature form. The total amount of rhBMP-2 incorporated into the ACS was 53%.

3.5. rhBMP-2 pharmacokinetics after rat ectopic implantation

The in vivo release kinetics of ^{125}I-rhBMP-2 from the collagen sponge were studied using a rat ectopic implant model. Analysis of the data suggested that the pharmacokinetics of rhBMP-2 implanted with fully processed ACS (crosslinked/sterilized) could be described by a bi-exponential model with $t_{1/2a}$ and $t_{1/2b}$ of approximately 10 min and 89 h, respectively and a partial AUC (between $t=0$ and $t=10$ days) of 269.7 h (Fig. 5). Non-crosslinked/non-sterilized ACS showed the highest initial retention (87.1%) and partial AUC (345.5 h) but higher loss rate (secondary $t_{1/2}$ approximately 51 h). Crosslinking with CH_2O caused a decrease in initial retention in vivo (47.8%), and partial AUC (195.6 h) whereas secondary $t_{1/2}$ increased (74.4 h). Additional sterilization led to higher initial retention of 71.6% and a slight increase in $t_{1/26}$ to 89 h, significantly higher than the $t_{1/26}$ of non-crosslinked material. The analysis resulted in a mean residence time of 60 h, 72 h and 81 h for non-crosslinked/non-sterile, crosslinked/non-sterile and crosslinked/sterile sponges.

4. Discussion

The effects of ACS modifications on the interaction with rhBMP-2 and on rhBMP-2 being removed from the system during handling upon implantation were studied by testing incorporation of rhBMP-2. Incorporation is a measure of rhBMP-2 which cannot be expressed from the

soaked carrier. It represents the combination of rhBMP-2 absorption onto ACS plus the amount of rhBMP-2 dissolved in the liquid which is not removed by the squeezing procedure. Approximately 20% of the soak liquid cannot be expressed from the product and represents a minimum level of incorporation assuming no changes in the state of rhBMP-2 solution. The results indicated that rhBMP-2 did bind to the collagen sponge at various degrees.

4.1. Soaking time

With prolonged soaking time, an increase in incorporation was observed. For clinical application the product is kept open in a sterile surgical field during this period. Consequently, an increase in incorporation could be caused by both time-dependent binding and evaporation of liquid. Evaporation led to a decrease in expressed volume over time at a rate of 1.8 μl/min in this study. Calculations correcting for the reduced expressate volume showed that there was only a minor increase in rhBMP-2 binding to ACS after 5 min. This may be a time dependent effect or a consequence of changes in the incubation medium itself over time. With liquid evaporation, higher ion concentrations were found and pH increased by approximately 0.2 U. Both effects led to an increase in rhBMP-2 binding to ACS. These findings were reproducible for several ACS lots.

4.2. ACS mass

Higher ACS mass resulted in both less expressate volume and lower rhBMP-2 concentrations in the expressed liquid. On the one hand, more rhBMP-2 binding sites were available, and on the other side the simultaneous increase in expressate pH and anion concentration led to a higher incorporation capacity of the ACS material. With increasing sponge weight, the pH rose from 4.5 of the original rhBMP-2 formulation to 4.7 for 2.6 mg/cm^2 weight and 5.15 for 6.2 mg/cm^2 for non-sterilized material. The increase in pH was independent of formaldehyde treatment (Friess et al., 1999). This pH change influenced the interactions between rhBMP-2 and collagen. Equilibrium binding studies, in which ACS was soaked with excess rhBMP-2 solution, showed that binding at pH 3 and 4 was negligible (Table 1). At pH 4.5 significant amounts of rhBMP-2 were bound which further increased at pH 5.2 and 6.5. The effect could be explained by the isoelectrical point of the two proteins. The collagen material used is treated intensively with alkali which leads to cleavage of asparagine and glutamine residues on the collagen molecule and brings the isoelectrical point down to 5.1. The ligand rhBMP-2, has an

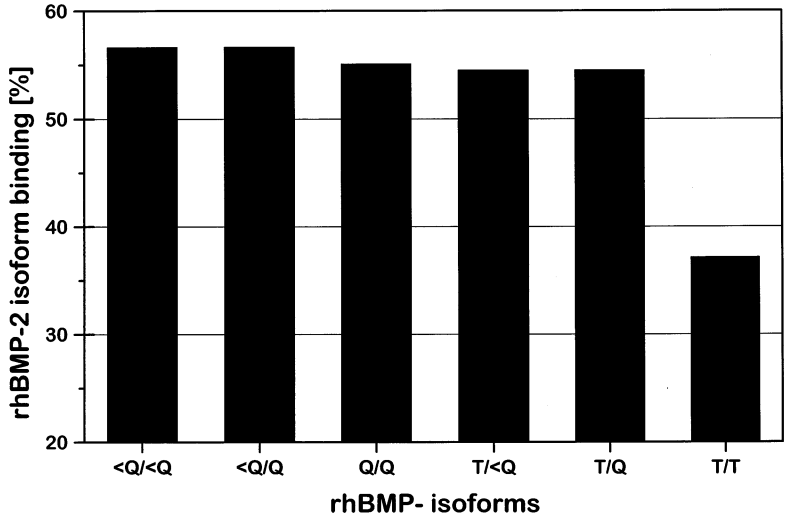

Fig. 4. rhBMP-2 Isoform binding (dimers of extended form (T), mature form (Q), and pyroglutamate mature form (< Q)).

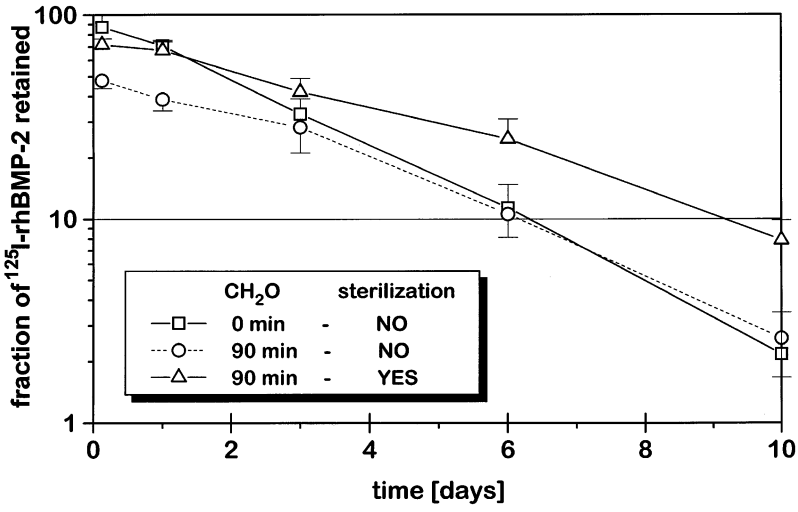

Fig. 5. Recombinant human bone morphogenetic protein-2 (rhBMP-2) retention at implant site in rat ectopic test.

isoelectrical point of approximately 8.5 and positive net charge at the pH of interest. It has a primary sequence characterized by a high number of hydrophobic residues which cause precipitation of the protein with pH increase towards its isoelectric point. Collagen at the same time shows a high number of negatively charged residues which could explain the protein/protein interactions.

Consequently, sponge mass played an important role for the properties of the product in vitro. The waiting time necessary to maximize incorporation in order to reduce protein loss during mechanical manipulation upon implantation could be reduced by increasing the ACS mass. In addition, the waiting period could be kept shorter at lower rhBMP-2 concentrations.

4.3. Crosslinking

Non-crosslinked material incorporated almost 100% of the rhBMP-2 load independent of ACS weight. Treatment with CH_2O led to chemical modifications of the ACS material as well as changes in the physico-chemical characteristics (Friess et al., 1999). Especially, crosslinking resulted in reduced swelling and stronger interaction between the collagen structures. As a consequence less surface area was made available made available upon soaking with aqueous liquid. This reduction in binding sites by crosslinking could be compensated by an increase in ACS mass.

4.4. Sterilization

Sterilization with EtO caused various chemical modifications of the ACS. Approximately 50% of the aminogroups are modified by EtO (Reich and Burgess, 1992; Friess et al., 1999). In addition, inorganic chloride reacts with EtO resulting in ethylene chlorohydrine. As a reduction in denaturation temperature indicated, the interactions between the collagen structure were reduced and the ACS sensitivity to collagenase increased (Friess et al., 1999). The most pronounced effect was the increase in the expressate pH to 4.9 for 2.6 mg/cm^2 and to 5.7 for 6.2 mg/cm^2. Despite this pH increase, which leads to stronger interactions between collagen and rhBMP-2, these manifold changes seemed to compensate and incorporation was not changed significantly in the set-up used.

4.5. Anions

Based on the studies investigating the rhBMP-2 precipitation in solution (Friess et al., 1999) and testing rhBMP-2 incorporation into ACS, the effect of anion concentration and increased pH on

both precipitation and incorporation is synergistic. Thus, incorporation could be enhanced by an increase in formulation pH or ion concentrations but the effect is limited by the potential precipitation of rhBMP-2.

4.6. Isoforms

Distribution of rhBMP-2 dimers in the expressed liquid indicated that the double extended, most hydrophilic form showed the lowest degree of binding to collagen. This isoform is the most sensitive to anions with respect to precipitation (Abbatiello and Porter, 1997). Since the single and double mature forms bound equally, there was only one lipophilic chain necessary to bind. The same conclusion was reached from binding of rhBMP-2 to porous hydroxyapatite/tricalciumphosphate carrier systems (Friess and Sargeant, 1997).

4.7. In vivo pharmacokinetics

Different studies have demonstrated a positive correlation between the retention of rhBMP-2 upon implantation and the osteoinductive activity, i.e. systems with a higher rhBMP-2 retention resulted in significantly higher bone scores (Uludag et al., 1998; Winn et al., 1998). The highest initial 3 h retention with non-crosslinked/non-sterile ACS corresponded to the highest incorporation found in vitro (Fig. 3). The loss rate appeared to be higher for these systems which were more susceptible to collagenase in vitro and degraded faster. Crosslinking with CH_2O led to an increase in the in vivo persistence and prolonged mean residence time and secondary $t_{1/2}$. At the same time, incorporation was decreased as reflected in reduced initial retention in vivo (Fig. 5). Sterilization did not affect the loss rate, but led to slightly higher initial retention in vivo, which was not reflected by a difference in incorporation. In summary, only minor, but partly significant, differences were found in the rhBMP-2 pharmacokinetics.

The results indicated that rhBMP-2 binds to ACS. It is possible to increase rhBMP-2 incorporation by adjustment of pH and anion concentration or by controlling the weight of ACS. The characteristics of the product have to be controlled because the spectrum is limited by insufficient binding on the one end and by rhBMP-2 precipitation at the other end. Consequently, it is important to have as little variability in pH, anion concentration, crosslinking, and ACS mass as possible.

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