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Morphological study of bone regeneration in the presence of 6-oxychitin

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Abstract

Surgical lesions in rat condylus were treated with *N,N*-dicarboxymethyl chitosan and 6-oxychitin sodium salt. Morphological data indicate that the best osteoarchitectural reconstruction was promoted by 6-oxychitin, even though healing was slower compared to *N,N*-dicarboxymethyl chitosan. Complete healing was obtained with *N,N*-dicarboxymethyl chitosan within three weeks. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Carbohydrate polymers exert a variety of biological actions in modulating the intra- and extracellular microenvironment. Substituted dextrans bind growth factors and protect them from enzymatic degradation. Heparin-like dextrans enhance the healing of bone in an environment where bone would otherwise not regenerate (Albo et al., 1996). The binding of heparin like polysaccharides to fibroblast growth factor (FGF) induces a conformational change in FGF, resulting in the formation of FGF dimers or oligomers, and this biologically active form is 'presented' to the FGF receptor for signal transduction (Venkataraman et al., 1996). Osteoinduction of the BMP-chitin complex was accompanied by excellent biocompatibility (Miyazawa, 1995).

Hyaluronan shows morphogenetic activities suitable for a correct bone architecture. Thanks to the high concentration of hyaluronan, the wounds in the foetus heal with the correct tissue reconstitution (West, Shaw, Lorenz, Adzick, & Longaneker, 1997), where the scar imprint typical of the adult tissue is absent.

Recent evidence points to the presence of DG42 protein (a chitooligomer synthase) during embryogenesis, that produces chitooligomers acting as primers in the synthesis of hyaluronan (Bakkers, Semino, Stroband, Kijne, Robbins, & Spain, 1997). Most preparations of hyaluronan have chitooligomers at their reducing end core region, that act as templates for hyaluronan synthesis (Varki, 1996).

The interactions between cell and extracellular matrix (ECM) provide cells with information essential for controlling morphogenesis, migration, repairs and death (Werb, 1997). In the reparative processes, chitosans may act as bridges between carboxylated and sulphated polysaccharides in the non-fibrous ECM and cells. Chitosans are believed to release chitooligomers capable of macrophage activation, favourable influence on collagen deposition, and incorporation into ECM components. About the bone tissue, it should be observed that the increase of extracellular Ca^{2+} is perceived by osteoblasts via specific receptors that lead to mutagenic and chemiotactic action. Our previous work showed that chelating modified chitosans carrying calcium phosphate accelerate bone wound healing (Muzzarelli et al., 1998). In contact with bone, chitosan promotes direct endochondral ossification.

Bone defects surgically produced in sheep and rabbit models have been treated with freeze-dried modified chitosans. Moreover the pattern of bone regeneration has been studied in an osteoporotic experimental model with bone morphogenetic protein (BMP) linked to chitosan (Muzzarelli et al., 1993; 1997).

The scope of the present work was to test the bone healing capacity of 6-oxychitin, a modified chitin obtained via regiospecific oxidation of chitin. This novel modified chitin,

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Table 1
Study design

No of animals	Right femur	Left femurs
1	Empty	DCMC
1	Empty	6-Oxychitin
1	DCMC	6-Oxychitin
6	DCMC + osteoblasts	6-Oxychitin + osteoblasts

constituted by relatively short $\beta(1-4)$ chains of 2-acetamido-2-deoxy glucuronic acid, sodium salt, is functionally similar to hyaluronan and is endowed of water-solubility, anionic character and chelating ability (Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999).

2. Experimental

2.1. Polysaccharides

6-Oxychitin and *N*,*N*-dicarboxymethyl chitosan (DCMC) were fully characterised from the chemical and enzymatic standpoints (Muzzarelli, Ilari, & Petrarulo, 1994a; Muzzarelli et al., 1994b; 1999). The products were sterilised by γ-ray irradiation at 25 KGy; their aspect was that of soft spongy and hydrophilic materials. They were water-soluble.

For the preparation of 6-oxychitin, the stable nitroxyl radical 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo™, Aldrich, Milano) was used as a catalyst, together with NaBr, to regiospecifically oxidize chitin with either a 4 or a 13% NaOCl solution (Muzzarelli et al., 1999). The average molecular weight of 6-oxychitin was close to 10 000 Da, and the degree of substitution was 1.0.

2.2. Cell culture

Osteoblasts were isolated from newborn mouse calvariae by sequential digestion for 20, 40 and 90 min at 37°C in 1 mg/ml collagenase and 0.25% trypsin. The cells of the first two digests were discarded; those released in the third one were plated in complete DMEM supplemented with 10% foetal calf serum and penicillin/streptomycin (all from Sigma) (Oliva, Marrone, & Della Ragione, 1992). Osteoblasts were characterised with the aid of alkaline phosphatase (ALP) cytochemical stain and by biochemical evaluation of the ALP increment subsequent to vitamin D3 administration. The ALP assay (Sigma Diagnostic Kit) depends upon the hydrolysis of *p*-nitrophenylphosphate by the enzyme, yielding *p*-nitrophenol and phosphate. When made alkaline, *p*-nitrophenol is converted to a yellow complex readily measured at 400 nm where colour intensity is proportional to phosphate activity (Bissey, Lowrey, & Brock, 1946). For the ALP stain, cultured cells on 35 mm culture dishes were stained with Naphthol AS-MX phosphate and fast violet B salt using the Sigma kit.

2.3. Study design

Nine Sprague Dawley female rats aged 16 months and weighing 400 ± 70 g were used. General anaesthesia was obtained by means of an intramuscular injection of Ketamine 87 mg/kg and xylazine 13 mg/kg. In aseptic conditions the condyles were exposed via longitudinal lateral skin incisions. For each condyle a bone defect having a diameter of 2 mm and a depth of 4 mm was drilled and filled with 1 cm^2 of spongy material either as such or coated with osteoblasts (1.5×10^3 cells/cm^2), as given in Table 1. The surgical wounds were sutured in two layers.

The animals were stabled in single cages, fed a standard pellet diet and water ad libitum, and under standard environmental conditions (T: 20.5 ± 0.5; HR: 55 ± 10%). As antibiotic therapy, flumequine (3 mg/100 g) was administered subcutaneously during the three days post-operatively. Three weeks after surgery, the animals were pharmacologically euthanised. Immediately after sacrifice, femurs were fixed for 24 h in 4% buffered paraformaldehyde, dehydrated in graded series of alcohol and embedded in methylmethacrylate resin. After polymerisation a series of 80 μm thick sections were obtained longitudinally to the shaft with a Leica 1600 diamond saw microtome. They were then stained with Fast Green and observed with a Zeiss Axioscop light microscope. Histomorphometry was performed by means of a computerised image analysis system Kontron KS 300. The following parameters were considered:

- Residual bone defect, if present, measured as the area observed in the section where no sign of bone repair was evident.
- Trabecular bone volume (Bv/TV), measured as the percentage of trabecular bone in the defect area.
- Trabecular thickness (Tb.Th), obtained by direct measurement on the trabeculae.
- Trabecular number (TbN), number of trabeculae per mm. According to Parfitt, Mathews, Villanueva, Kleerekoper, Frame and Rao (1983) and Parfitt et al. (1987), TbN = (BV/TV) × 10/TbTh.

2.4. Microanalysis

Methylmethacrylate embedded slices 200 μm in thickness were mounted onto stubs with colloidal graphite, coated with carbon for vacuum evaporation and observed with a Philips XL 20 SEM equipped for X-ray microanalysis (EDS-PV 9800). The operative condition for the analysis were: voltage 25 kV, magnification × 400, counts per second (cps) not less than 2000, tilt angle 15°C, count time 250 s. Only the K_α values of each element were considered, and the semi-quantitative percent concentration were calculated using ZAF (Z = atomic number, A = adsorption, F = fluorescence) correction. Five random detections for lesion were performed.

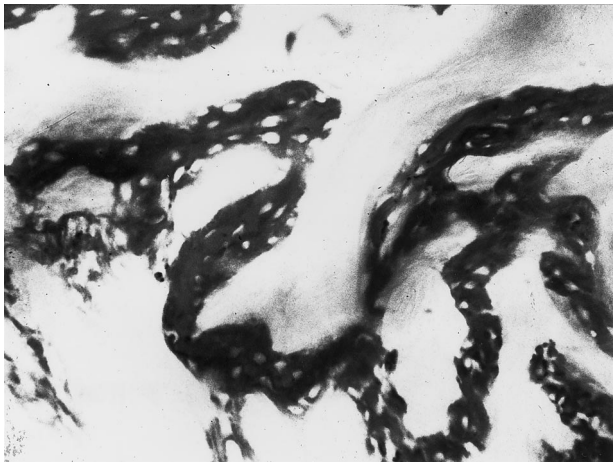

Fig. 1. Good trabecular reconstitution in a surgical lesion treated with 6-oxychitin sodium salt (× 250).

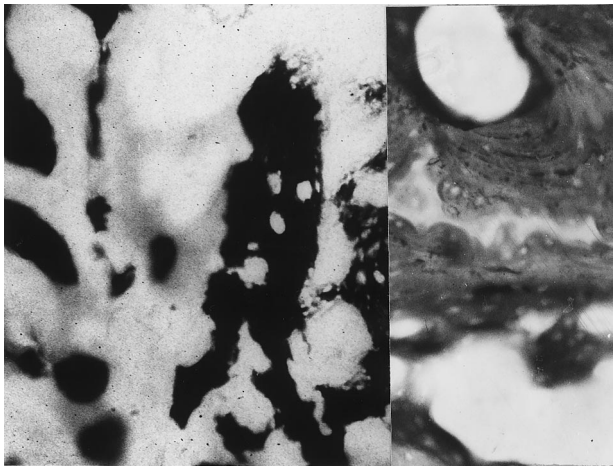

Fig. 2. Control lesion showing poor histoarchitecture (× 250). Inset: high magnification of bone trabecular structure in the presence of DCMC + osteoblasts. (× 400).

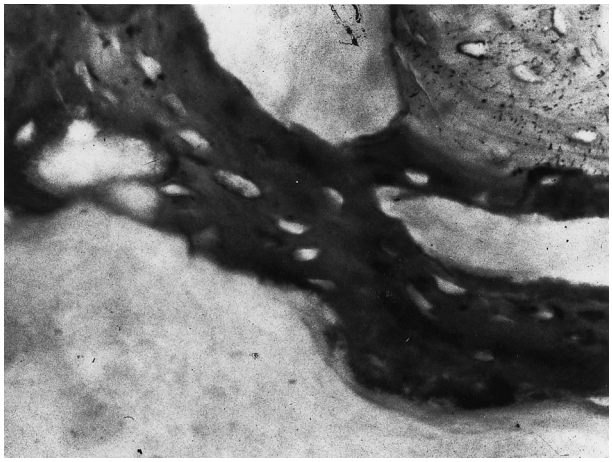

Fig. 3. Osseous trabeculae in a lesion treated with 6-oxychitin + osteoblasts.

3. Results and discussion

3.1. Morphological analysis

Both DCMC and 6-oxychitin, applied to femoral surgical defects for three weeks produced a good histoarchitectural order in the newly formed bone tissue. There were no really evident differences between the two polysaccharides, but it was remarked that 6-oxychitin gave a more ordered bone structure (Fig. 1). When osteoblasts were associated to the polysaccharides these preparations showed enhanced tissue mimicking capacity. The spongious trabecular architecture was restored in the defect site (Figs. 2 and 3). The association of the chitin derivatives with the osteoblast cells seems to be the best biomaterial in terms of bone tissue recovery. Fig. 4 shows different morphologies of the trabeculae (marked with asterisks) generated in the presence of the two different polysaccharides.

3.2. Histomorphometry

Control lesions (empty) showed the larger residual defect (3.1 mm^2), the lowest trabecular bone volume and

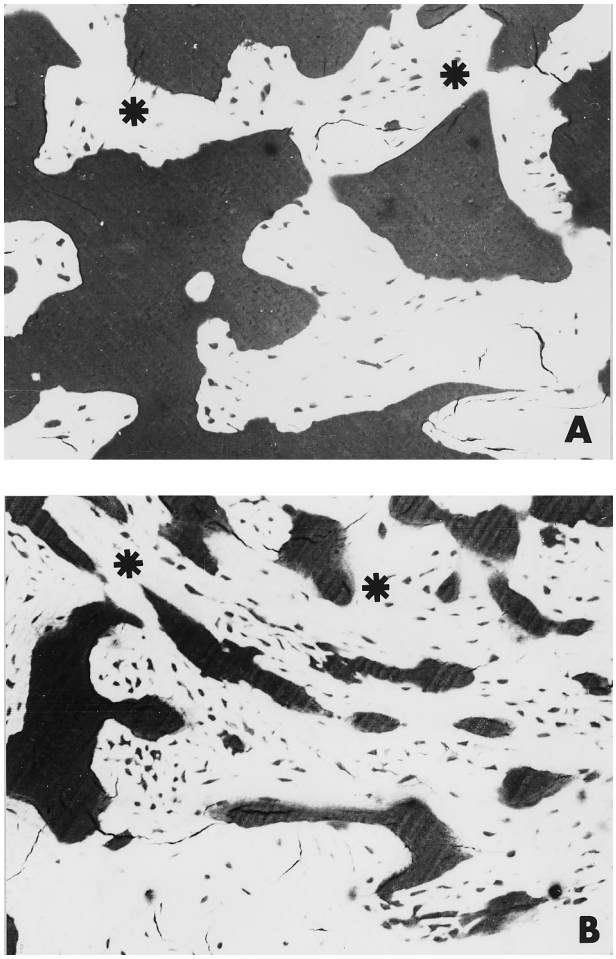

Fig. 4. Back scattered electron image of bone regeneration in the presence of (a) 6-Oxychitin + osteoblasts; (b) DCMC + osteoblasts.

Table 2
Results of morphometric analysis (mean values are reported, standard deviation = ± 2%)

	Residual bone defect (mm^2)	Trabecular bone Volume (%)	Thickness (μm)	Number (mm^{-1})
Control lesions (empty)	3.1	43.6	53.0	8.2
DCMC-treated lesions	–	44.7	61.8	7.2
DCMC + osteoblasts-treated lesions	–	47.0	69.6	6.7
6-oxychitin-treated lesions	2.2	45.3	54.7	8.2
6-oxychitin + osteoblasts-treated lesions	1.8	45.2	61.8.	7.4

Table 3
Microanalytical evaluations (values are expressed as weight %, standard deviation = ± 2%)

	P	S	K	Ca
Control lesions (empty)	31.69	0.29	1.75	66.0
DCMC-treated lesions	28.64	0.27	0.96	70.13
DCMC + osteoblasts-treated lesions	31.23	0.27	1.58	66.93
6-Oxychitin-treated lesions	32.62	0.26	1.87	65.26
6-Oxychitin + osteoblasts-treated lesions	30.92	0.23	1.45	67.42
Normal bone	30.30	0.11	0.61	68.99

thickness, with a trabecular number higher than other ones (Table 2).

The DCMC treated lesions were all healed. In the DCMC + osteoblasts (Fig. 4) treated defects the trabecular bone volume and thickness values were the highest recorded (47.0 and 69.6, respectively) (Table 2).

Even though none of the treated lesions with 6-oxychitin healed completely within the three weeks observation period, the average residual defect was much smaller than in controls. The addition of osteoblasts improved the performance of 6-oxychitin (Table 2).

3.3. Microanalysis

The sulphated component (non-mineralised ECM) was more evident, compared to regular bone under all experimental conditions. The presence of osteoblasts favoured the formation of crystals with both polysaccharides (Table 3).

4. Conclusion

The present study shows that 6-oxychitin sodium salt gives better osteoarchitectural reconstruction than DCMC, the same holds in the presence of added osteoblasts. Healing seems to be slower, but the spongious trabecular architecture is superior. 6-Oxychitin therefore represent an advance in the experimental study of the osteoinduction process and preludes to novel applications intended to reconstruct the correct morphology of bone tissues, even in the presence of important mechanical stress. It also seems reasonable to expect that 6-oxychitin would be helpful in the healing of the cartilagineous tissue.

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