JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **14** (2003) 95–102

Nerve growth factor β(NGF β) delivery via a collagen/hydroxyapatite (Col/HAp) composite and its effects on new bone ingrowth

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In craniofacial surgery, bone is needed to augment misshapen areas and to fill gaps during repair of congenital anomalies and injuries resulting into bone deficiencies. Examples of conditions requiring bone tissue include missing alveolar bone in cleft palates, bony nasal pyramid defects following removal of fistulous tracts or cysts and defects following removal of sinus and mandibular tumors. Moreover, maxillofacial neurosensory deficiencies may be caused by various surgical procedures, such as tooth extraction, osteotomies, pre-prosthetic procedures, excision of tumors or cysts, surgery of TMJ, and surgical treatment of fractures and cleft lip/palate. Therefore, a tissue engineering approach to craniofacial surgery has a crucial importance: the use of various composites with osteoconductive ceramics, polymers, bioactive factors, cells, or a combination of them, offers the possibility of rapid tissue regeneration and integration with the host tissue.

In this study, a composite consisting of two well-known biomaterials, collagen/hydroxyapatite (Col/HAp), was used as a drug delivery device for neurotrophin – nerve growth factor β (NGF β). This delivery device, enriched with neurogenic-osteogenic factor, was analyzed *in vitro* and *in vivo*. It was implanted into calvaria defects of 20 Wistar rats, weighing 200–250 g. Implants were left in place for different periods of time. Controls were as follows: (a) contralateral defect without any implant; and (b) contralateral defect implanted with composite without NGF factor. The rats were euthanized after 30 days, and the implant sites and explants were examined clinically, histologically, SEM and histomorphometrically. Our results evidenced stimulation of periosteal and endocortical woven and lamellar bone formation, with increases in bone mass and decreases in bone marrow. We found that NGF enhanced the remodeling activity in the intracortical region, and induced an increase in the intracortical cavity number and area by the end of the study. *In vitro* results were in line with *in vivo* ones. We believe that the composite proposed in this study has considerable advantages in tissue engineering and is very suitable as a biomaterial for the filling of irregular defects in maxillo-facial surgery. Two areas of clinical research will be impacted by this system. The first is pharmaceutical research on drug delivery and high-throughput screening of neurotrophic-osteogenic compounds. Transplantation research is the second area that will benefit from the system.
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1. Introduction

Development, remodeling or artificial reconstruction of the skeleton, and particularly the craniofacial skeleton, are processes that are regulated and orchestrated by a variety of factors. These factors are generated by different tissues, such as bone, nerves, skin, muscles etc. [1]. Bone metabolism is controlled by several systemic hormones, as well as by many locally-acting cytokines and growth factors [2–5]. The possibility that bone metabolism may be controlled also by neuropeptides [6–8] is suggested not only by observations in paraplegic patients and denervated rats, but also by

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immunohistochemical detection of neuropeptides containing nerve fibers in the interior of bone and periosteum [9, 10]. Clinical observations infer that nerve fibers are included in the local regulation of skeletal metabolism [11–13]. Thus, patients with neurological disorders exhibit localized osteopenia, bone fragility, increased fracture rate [14, 15], fracture healing and excessive callus formation [14]. In experimentally-induced denervation in rats, a loss of bone tissue and changes in fracture healing were observed [16]. The face, and in particular the oral and perioral regions, are among the areas with the highest density of peripheral receptors, presumably because of their remarkable importance in daily life. It is difficult to tolerate neurological disturbances in oral and maxillofacial areas [17] compared to disturbances in other parts of the body. Pain, temperature, touch, pressure, and proprioception (sense of body position) are transmitted centrally from the perioral structures via the inferior alveolar, lingual, infraorbital and mental nerves. Each of these sensations is transmitted by different types of sensory receptors and nerve fibers, each showing different susceptibility to injury and recovery. Maxillofacial neurosensory deficiencies may be caused by various surgical procedures, such as tooth extraction, osteotomies, pre-prosthetic procedures, excision of tumors or cysts, surgery of TMJ, and surgical treatment of fractures and cleft lip/palate [17].

Morphological studies showed the presence of a number of neuropeptides in the skeleton, including vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), substance P (SP) and neuropeptide Y (NPY), as well as enzymes involved in catecholamine biosynthesis [8, 12, 16, 18], and these findings were confirmed by quantitative assessments of peptides in extracts of bone tissues [12]. The distribution of VIP-immunoreactive nerve fibers was concentrated in the periosteal and epiphyseal regions of bones. The VIP positive fibers in the periosteum were localized in the areas which were the closest to bone tissue and some, but not all, were associated with blood vessels. The observation that patients with VIP-producing tumors may develop hypercalcemia is an interesting clinical finding, which further supports the assumption that neuropeptides may be involved in bone cell metabolism [12, 17]. The finding that an injection of VIP in intact rats results into decreased uptake of calcium in the skeleton is another indication that VIP may affect skeletal metabolism.

Of the numerous neurotrophic factors, we chose the nerve growth factor β (NGF β) because it is one of the known neurotrophins that was best characterized [20]. For example, kinines and neuropeptides are reported to directly or indirectly modulate the activity of bone cells in physiological and pathological conditions [21]. In medicine and dentistry, the neuro-osteological evaluation of pre-natal and post-natal pathological developmental conditions can provide substantial evidence of neuro-osteological combinations of the central nervous system, osseous development and remodeling [6, 8, 10].

Recent studies indicated that NGF β promotes recovery of several neurological deficits and stimulates wound healing in cutaneous tissues. There is also consistent with emerging evidence of the fact that NGF, either alone or synergistically with other biological endogenous mediators, plays a crucial role in cartilaginous and bone tissues [18, 22, 23]. This hypothesis is suggested by recent studies showing that the exogenous administration of NGF β improves fracture healing in laboratory animals, boosts osteogenesis and increases the rate and quality of fracture repair [22, 23]. The observations that chondrocytes express NGF and TrkA receptors and that NGF is elevated in the synovial fluid of patients with chronic arthritis, juvenile chronic arthritis, in cartilage-related diseases, is consistent with the observation that the basal NGF levels in osteoarthritis are associated with the distribution of neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP), which are involved in this pathology [18]. Based on these findings, NGF is very likely to have major therapeutic implications for bone regeneration and for skeletal reconstruction and joint replacement.

Over the past two decades, many natural or synthetic bone substitutes were developed, mostly consisting of calcium phosphate, thanks to its excellent biocompatibility. In particular, hydroxyapatite (HAp) is currently used worldwide in practical applications as a bone substitute. Dense, porous, or particulate forms of HAp are available. Particulate HAp lacks shape and cohesive strength; therefore, it tends to be dislodged and to migrate under externally applied forces during the healing period. Attempts were made to overcome this problem by combining HAp particles with a collagen fiber [24, 25], or other organic polymers [26]. The purpose was to develop new bone substitutes that resemble bone more closely than currently available materials.

Although growth factors are useful as growth-promoting substances, their application, distribution and long-term activity *in vivo* are problematic due to their sensitive organic nature. Therefore, various targeted and controlled drug delivery systems are under continuous development.

The study reported in this paper was aimed at investigating the use of a biocompatible bone-equivalent composite, such as collagen/hydroxyapatite (Col/HAp), as a delivery device for NGF β. The paper analyzes the formulation of the composite device, enriched with three doses of NGF β, and its osteogenetic potential both *in vitro* and *in vivo*.

2. Material and methods

2.1. Biomaterial design and characteristics

The composite formulation consisted of HAp and collagen. HAp was in the form of spherical porous granules (BIOHAP, Coatings Ind, France) with the following general specifications: Chemical composition: $2(Ca_5(PO_4)_3OH)$; Ca/P 1.667 ± 0.03; total heavy metals: < 50 ppm; crystallinity index $> 95 \pm 5\%$; characterization and quantification of crystalline phases: %HAp > 97; density: 1.2 ± 0.4 g/cm^3; grain size: less than 100 µm; influence of γ sterilization: properties not modified. The HAp particles had a various sizes of about 10–60 µm, with pores of about 10 ± 3 nm. Atelocollagen (Col) was purchased from Hypro Ltd. (Czech Republic). It was crystalline, native atelocollagen

type I, prepared from bovine Achilles tendon in the form of felt, with a content of non-collagenous peptides of < 0.5 wt %, and of inorganic substances < 0.5 wt %. Before use, the acidic collagen solution was dialyzed against 0.02 M PBS (phosphate buffered saline, pH 7.2) and centrifuged at 48 000 rpm (Ti 70.1, Beckman) for 3 h. The upper two thirds of the collagen solution were collected from the centrifuge tube and used for reconstitution. Nerve growth factor β (NGF β) (MW 45 000) from mouse submaxillary gland was purchased from Sigma, USA, Product No. 72183. The biological activity of recombinant rat NGF β was measured in a cell proliferation assay using the factor-dependent human erythro-leukaemic cell line, TF-1. Endotoxin tested; neurobiological tests; neuro-filament outgrowth observed at 30 ng/ml; cell culture: free of bacteria, yeasts, molds and mycoplasma; preparation: by gel filtration and ion-exchange chromatography, sterilized by 0.2 mm-filtration and lyophilized (100 mg) from 1 ml of 5 mM PBS, pH 6.8. Solubility (0.1 mg/ml H_2O) clear, colorless. NGF β was added in three different doses as follows: I 0.5 v %, i.e. 1 μg per volume/per sample; II 1 v %, i.e. 2 μg per volume/per sample; and III 2%, i.e. 4 μg per volume/per sample.

The composite material consisted of seven parts of inorganic component by weight and three parts of organic component (7 : 3, HAp vs. collagen), including a mixture of collagen (93%) and NGF in three different doses. Collagen powder was dissolved in PBS in a heparinized glass container at room temperature. The solution was mixed with HAp powders and, later, a homogenous, soft, shapeable paste was repeatedly washed and desiccated under vacuum. For *in vitro* experiments, the same samples were pressed so as to make discs of $\varnothing 1 \times 0.2$ cm to fit into a tissue culture dish 35×10 mm (Becton Dickinson Co, England). For *in vivo* experiments, a shapeable paste weighing 50–55 mg was used. All samples were sterilized by γ radiation.

2.2. *In vitro* evaluation

2.2.1. *Culturing and biochemical parameters*

The osteosarcoma cell line, MG-63, was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) and maintained in DMEM supplemented with 10% FCS. No antibiotics were added. Cells were cultured in a humidified 5% CO_2 37 °C incubator, as recommended by ATCC (USA www.atcc.org). Cells were subcultured (0.05% trypsin/ 0.02% EDTA wt/vol) and seeded in 75-cm^2 flasks. All cultures were routinely screened for mycoplasma (Boehringer Mycoplasma Detection Kit). The cells were released at the confluence with trypsin : EDTA, counted and used for experiments. Six samples of each material (formulation with dose I, II and III) were placed in 24-well culture plates, and the cell suspension (1×10^4 cell/ml in 100 μl) was directly applied on every sample. The same amount of cells was also plated in the remaining six empty wells as a control. Cells were allowed to attach for 2 h, then 900 μl of culture medium (DMEM, ascorbic acid 50 μg/ml and β-glycero-phosphate 10^{-8} M) were added. The culture was maintained under the same conditions as described above for 48 h. No contamination was found during the experiments. At the end of the experiment, osteoblasts were characterized according to well-established parameters of osteoblastic phenotypes: Alkaline phosphatase activity (ALP, Sigma, UK Kinetic method kit), calcium (Ca, Sigma, UK kit), phosphorus (P, Sigma UK kit), all tests as indicators of osteoblast activity. Mtt test – Cells from the culture were seeded on tissue culture plates (TPP, Switzerland) at a density of 5×10^3 cells/well for a 0.36-cm well. One day later, the cultures were washed and incubated for 2 h with the different samples at various dilutions in serum-free, Ham F-12 medium. The different preparations were adjusted to the composite substrate tested. Afterwards, 50 ml/well of PBS containing 1 mg/ml MTT (tetrazolium salt, Sigma S.A., Madrid) were added, and the plates were incubated for another 4 h. The intracellular formazan crystals resulting from the reduction of the tetrazolium salts, which were only present in metabolically active cells, were solubilized with DMSO (Sigma-Aldrich). The number of active cells was estimated by measuring the absorbance at 540 nm (Titertek Multiscan, performed in triplicate. ICN, Costa Mesa, CA). All samples were made in sextuplicate.

2.2.2. *Evaluation of NGF β sustaining release*

Three composite formulations of collagen/HAp/NGF β containing three different concentrations of NGF β were prepared. For the experiment, they were placed in plastic dishes and, after adding 1 ml of PBS (pH 7.4), they were incubated at 37 °C. At each time point (1, 2, 3, 5, 24 48, 72, 96, 120, 144 and 160 h), the supernatants were collected, centrifuged and replaced with fresh PBS. The supernatants developing at different times were analyzed for concentration of NGF β released by each of the composites, in triplicate, via NGF-ELISA (Boeringer Mannheim, Germany).

2.2.3. *Scanning electron microscopy (SEM)*

Representative samples of biomaterial and also cells attached to the composite were processed for SEM. Samples were rinsed three times with PBS and fixed for 60 min with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1% cacodylate buffer (pH 7.4), and fixed afterwards with osmium tetroxide, critical point-dried and sputter-coated with gold-palladium. Morphologic analysis and element analysis (KEVEX) was performed by SEM (Etec Autoscan, Etec, and Haywood, CA).

2.3. *In vivo* evaluation

2.3.1. *Rat calvarial defect model*

For implantation, healthy, skeletally mature, male Wistar rats with a weight between 200 and 250 g were used. Surgery was done according to the standard test systems following the European (OEDC) requirements for ''Good Laboratory Practice''. The experimental protocol was approved by the local Ethical Committee. All experiments were performed under general anesthesia. To reduce the peri-operative infection risk, the rats

received antibiotic prophylaxis (penicillin). The animals were anaesthetized i.p. with a combination of 75 ml/kg body weight (b.w.) ketamine (Aescoket, Boxtel, The Netherlands) and 10 mg/kg b.w. xylazine (Rompun, Bayer, Germany) in sterile water. The cranium was shaved and iodinated before fixation and surgery under aseptical conditions. A 2 cm-long incision was made along the sagittal suture to reflect skin, connective tissues and periosteum, and gain access to the parietal bones. Two full-thickness bone defects of 5 mm diameter were created bilaterally of the sagittal suture with a trephine drill (no. 227RF 050, Meissinger, Düsseldorf, Germany) in a dental electrical drill (EWL K9, KAVO, Biberach, Germany) under constant irrigation with sterile saline. The defects were filled with 53 mg of three composite formulations with three different concentrations of NGF. The periosteum was closed over the implant using 3-0 Vicryl sutures, and the skin was closed using a subcuticular Vicryl suture. One defect served as a control. A total of about 30 scaffolds of three different formulations and 10 controls (20 animals with two cranial defects) were implanted. Thirty days post-operatively, the skull bone was removed together with the defect and fixed with 10 v% of aqueous neutral formalin solution.

2.3.2. *Histomorphometrical evaluation*

The bone was dissected and left in Karnovsky's fixative for 5 days. Quantitative evaluations were carried out using a digitizing image-analyzing system (DIAS, USA). The precision of the basic area and perimeter measurements was 7%. Basic histomorphometry parameters (listed in the Tables) were measured according to the standard nomenclature for bone histomorphometry ASBMR [27].

Parameters: total cross-sectional area (T.Ar) (Se.Ar), medullary area (Me.Ar), cortical width (Ct.Wd), periosteal new bone area (Ps-NB.Ar) and osteoid perimeter (%O.Pm), were used to calculate the total bone area (T.B.Ar), cortical bone area (Ct.Ar), percent cortical area (%Ct.Ar), percent osteoid perimeter (%O.Pm/B.Pm), mineral apposition rate (MAR), and bone formation rate per unit of bone surface (BFR/B.Pm). The porosity was defined in terms of intra-cortical cavities with a diameter $> 30\,\mu$m. The intra-cortical cavity area and cortical area were used to calculate the percent porosity area. The forming osteon number (FON) was the sum of single and double-labeled surfaces. Forming osteon number, resorption cavity number, and porosity number were used to calculate the forming osteon density (%RCN) and the ratio of the forming osteon number and resorption number (FON/RCN). Results were presented as mean $\pm$ SD.

2.4. Statistics

The statistical differences within the groups were evaluated by using the one-way analysis of variance (ANOVA). $P < 0.05$ was considered as significant.

3. Results

3.1. *In vitro* evaluation

3.1.1. *SEM morphology of NGF β-loaded composite*

The morphology of the composite, comprising osteoconductive ceramic HAp and organic extracellular matrix protein collagen (10 : 1 HAp vs. collagen) with entrapped neurogenic-osteogenic factor NGF β as 2 μm per sample, show highly rough porous surface (Fig. 1). The idea to combine the biologicaly active composite Hap/Col with the further component NGF β, has originated from a requirement of multi-active scaffolds that support bone and nerve ingrowth.

3.1.2. *NGF β release profile*

The release profile of protein-NGF β in the collagen/HAp/NGF β formulation (Fig. 2) was recorded. As shown in Fig. 2, up to 30% of the NGF was released within the first 24 h, with a large initial burst for all three NGF concentrations tested. Almost 90–100% was released at 72–96 h after loading. Some delay was observed for the third dose of NGF β with respect to the other two. This fact may be due to the higher concentration of this formulation.

3.1.3. *Biochemical parameters of osteoblasts after culturing on an NGF β-enriched composite*

Biochemical results showed that during the incubation of MG63 osteoblast-like cells on the composite enriched with three different concentrations of NGF β, no signs of cytotoxicity were observed (Table I). In effect, all the values detected in the material sample cultures corresponded to the control culture values. Our results showed significant changes in the biochemical parameters of NGF II and III, i.e. for concentrations of 2 and 4 μg. ALP values proved to be higher, suggesting that the presence of a composite with NGF activity increases the osteogenetic function of osteoblasts. Calcium (Ca) and Phosphorus (P) detected in the supernatant were significantly lower than in controls (Table I). The MTT

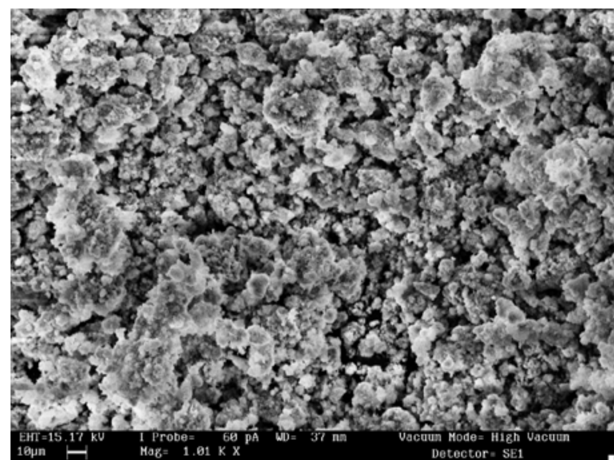

Figure 1 SEM photomicrograph of a panoramic view of the irregular face of Col/Hap/NGF β composites used as substrate for osteoblast cells cultivation (Bar 10 μm).

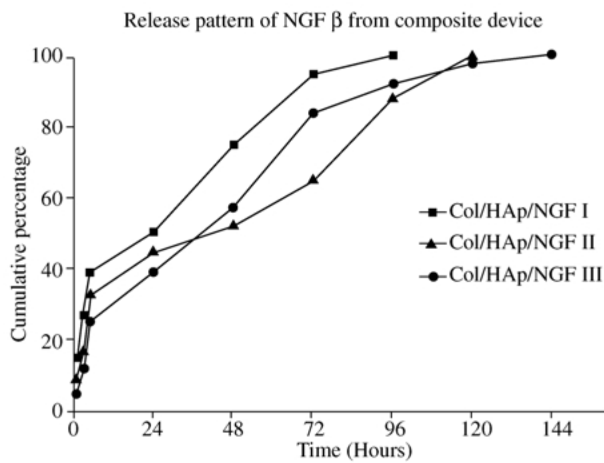

Figure 2 Release pattern of the three different doses of NGF β from a composites formulations Col/HAp/NGF I, Col/HAp/NGF II and Col/HAp/NGF III. Results are presented as cumulative percentage of sustained release of NGF β measured with ELISA, on time points: 1, 2, 3, 5, 24, 48, 72, 96, 120, 144 and 160 h.

test revealed that cell proliferation significantly improved in the presence of biomaterials with respect to control values.

SEM evaluation of osteoblast cell response to a Col/HAp/NGF device. The osteoblast-like cells cultured on HAp/Col/NGF β composite showed a wide variety of forms and appearance. On this substrate, osteoblasts were abundant, highly proliferative, closely connected and firmly attached to the substrate (Fig. 3(A) and (B)). SEM micrographs (Fig. 3(A) and (B)) indicated that the particles of HAp were anchored to the complex of biopolymer matrix, and a compact block structure had been formed. The particles were completely covered with a film of well-developed bone-forming cells. Higher magnification of the osteoblast-like cells evidenced a fine lamellar and granular structure on the osteoblast cell surfaces (Fig. 3(A)). On these cell structures, attached particles of the composite substrate were observed (Fig. 3(A)). The osteoblast-like cell outgrowth was closely related to the HAp–Col–NGF composite substrate. In the extra-cellular spaces all around the cells, there were short and thin fibrillar and granular structures, embedded in an extra-cellular matrix and also firmly bound to the nearest osteoblast-like cells and composite substrate (Fig. 3(A) and (B)).

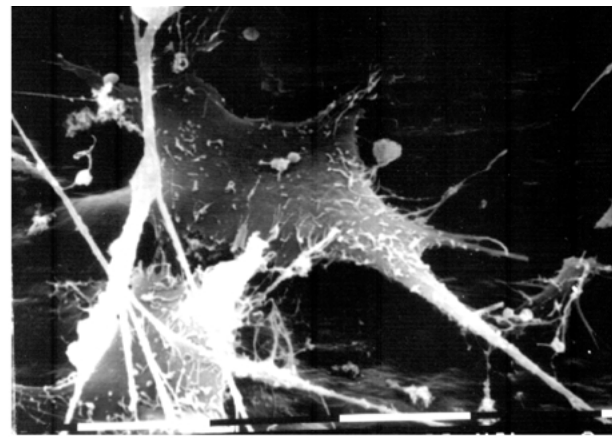

Figure 3 SEM analysis of the surface of the (A) Col-HAp-NGF β II (bar 10 mm) and (B) Col-HAp-NGF β III (bar 0.1 mm) composite formulation used for osteoblast cell cultivation. Could be seen rough structures of a composite matrix, covered with the film of well developed bone forming cells. On these cells can be observed attached HAp particles of the composite substrates.

3.2. *In vivo* evaluation

3.2.1. *Histomorphometric changes at the bone ingrowth around the Col/HAp/NGF β device*

Clinicaly, no weight loss was recorded in any group of animals during the 30 days of experiments. Histomorphometrically, three concentrations of NGF β gave gradually increasing effects on the new bone ingrowth around the implanted device (Tables II, III and IV). Significant differences were noted only for the second and third dose of NGF β. There were no changes in static histomorphometric parameters in the control groups (Tables II, III and IV). No osteoblasts or osteoprogenitor cells were seen at the endocortical surfaces in

TABLE I Biochemical parameters of the osteoblasts MG 63 cultured 48 h on the Col/HAp/NGF β composite formulation with three different concentrations of NGF β

Biochemical parameters	Controls – without NGF	Experiment – with NGF			ANOVA test
		I conc.	II conc.	III conc.	
ALP(U/l)	19.67 ± 0.70	20.5 ± 1.1	22.3 ± 1.1	23.5 ± 0.9	21.25, $*p < 0.005$
Ca(mg/dl)	7.33 ± 0.25	2.96 ± 0.8	3.07 ± 0.4	3.1 ± 1.2	113.51, $*p < 0.0005$
P (mg/dl)	3.77 ± 0.17	0.91 ± 1.0	1.14 ± 0.16	1.11 ± 0.7	85.89, $*p < 0.0005$
MTT (OD540 nm)	0.33 ± 0.053	0.43 ± 0.04	0.70 ± 0.031	0.57 ± 0.002	21.27, $*p < 0.0005$

*Experiments with all three doses of NGFβ vs. controls.

TABLE II Evaluation of effects of Col/HAp composite implantation and NGF β delivering on new bone ingrowth. Static histomorphometric changes

Groups (AN)	T.Ar. (mm^2)	Ct.Ar (%)	T.B.Ar (%)
Control 0 day (6)	3.5 ± 0.3	85.4 ± 0.4	83.7 ± 0.5
Composite without NGF (6)	3.9 ± 0.4	82.7 ± 2.1	81.9 ± 2.3
Composite with NGF I (6)	3.8 ± 0.5	85.1 ± 1.9	84.0 ± 2.2[a]
Composite with NGF II (8)	3.7 ± 0.5	85.6 ± 3.9	85.9 ± 4.6[ab]
Composite with NGF III (6)	4.3 ± 0.7	87.7 ± 2.5	92.2 ± 3.7[abc]

AN – animal number; T.Ar – total tissue area; CtAr – cortical bone area; T.B.Ar – total bone area; [a]vs. 0 day control, $p < 0.05$; [b]vs. control only composite, $p < 0.05$; [c]vs. NGF II, $p < 0.05$.

TABLE III Evaluation of effects of Col/HAp composite implantation and NGF β delivering on new bone ingrowth. Histomorphometric changes at the periosteal and endocortical surface

Groups (AN)	NBF (mm^2)	MAR (μm/day)	O.pm (%)
Control 0 day (6)	0/6	0.0 ± 0.0	2.7 ± 5.1
Composite without NGF (6)	0/6	0.0 ± 0.0	4.3 ± 3.5
Composite with NGF I (6)	0/7	0.0 ± 0.0	2.9 ± 2.3[a]
Composite with NGF II (6)	3/9	0.9 ± 0.1[ab]	64.5 ± 15
Composite with NGF III (6)	5/10	1.6 ± 0.1[abc]	56.0 ± 11[abc]

AN – animal number; NBF – new bone (woven + lamellar) frequency; MAR – mineral apposition rate; % O.Pm – osteoid perimeter; [a]vs. 0 day control, $p < 0.05$; [b]vs. control only composite, $p < 0.05$; [c]vs. composite with NGF II, $p < 0.05$.

TABLE IV Evaluation of effects of Col/HAp composite implantation and NGF β delivering on new bone ingrowth. Intracortical cavity changes

Groups (AN)	FON (%)	RCN(%)	FON/RCN
Control 0 day (6)	23.2 ± 2.1	2.3 ± 4.7	2.0 ± 0.5
Composite without NGF (6)	15.5 ± 4.5	2.9 ± 3.7	1.2 ± 1.1
Composite with NGF I (6)	16.0 ± 7.5	4.8 ± 5.3	1.1 ± 0.3
Composite with NGF II (8)	67.9 ± 8.5[ab]	5.7 ± 2.8[ab]	0.6 ± 4.7[ab]
Composite with NGF III (6)	71.2 ± 9.8[abc]	2.1 ± 1.0[abc]	33.5 ± 13.4[abc]

AN – animal number; FON – forming osteon number; FON/RCN, ration of forming osteon number to resorption cavity number; RCN – resorption cavity number; [a]vs. 0 day control, $p < 0.05$; [b]vs. control only composite, $p < 0.05$; [c]vs. NGF II, $p < 0.05$.

the control groups. Static and dynamic histomorphometric profiles, as compared with controls, were as follows: (1) total tissue area, cortical bone area, and total bone area increased gradually with NGF doses; (2) marrow space area did not differ, but the yellow marrow area decreased to 55%; (3) marrow trabecular area remained unchanged; (4) woven and lamellar bone (Table I) was attached to the periosteal surfaces as well as to the endocortical surfaces with significant increase in the osteoid surface (Table II and III); (5) newly formed bone on the endocortical surface radiated into the marrow cavity in the form of new lamellar or woven trabecular bone, together with the newly formed bone on the previously existing trabecular surface, filling a large part of the marrow cavity; (6) a single layer of osteoblasts was found adjacent to the periosteal and endocortical surfaces; (7) osteo-progenitor cells were identified on the endocortical woven trabecular surface; (8) forming osteon number (Table IV) and osteoid surface and thickness were higher in the experimental group with increasing NGF doses; (9) percentage of remodeling did not differ (Tables II, III and IV).

4. Discussion

This study has demonstrated that biodegradable bone-equivalent formulations are useful for local delivery of neurogenic-osteogenic factor NGF β and that they increase positive osteogenic effects in *in vitro* and *in vivo* applications. NGF β requires precise delivery conditions both for neurogenic and osteogenic activity. We have optimized a process to prepare an NGF β-loaded bone-equivalent composite exhibiting a high-level entrapment yield. Neurogenic NGF β factor has been added to this collagen-complex compound in order to improve bone formation and remodeling, that are influenced by the nervous system. Numerous groups examined the controlled release of neurotrophic factors [28–30]. This release can be achieved with many methods [22–30], such as delivery from polymeric microspheres or discs [29], mini-pumps, or transplanted cells engineered to produce NGF [32]. To date, a few groups have described the preparation of poly(D,L-lactide-*co*-glycolide) (PLGA) biodegradable microspheres loaded with NGF [28–30]. The design of a biodegradable device allowing a controlled and sustained release of neurotrophic factors, such as NGF β, is difficult to perform, due to the instability of these proteins, which may deteriorate during the preparation or the release phase.

Composite systems, such as the ones proposed in this study, consisting of inorganic (bio-ceramic) fibers or particles, and organic substrates may be different, depending on fine composition, bio-resorbability and/or bio-reactivity. However, the compounds that we have used retain some problems which need to be resolved,

such as: (1) rigidity of HAp, which is presently lower than that of natural cortical bone; (2) immunogenicity and degradation rate of the organic collagen component; (3) bioactivity, which is increased by adding NGF β-osteogenic molecules; and (4) long-term bioactivity of the growth factor, considering its protein nature. Undoubtedly, extracellular matrix protein collagen in combination with HAp can enhance the biological and mechanical (functional) properties of bone-equivalent implants [24, 25].

In our *in vitro* release study, we recorded a burst effect during the first 24 h, in which almost 50% of the NGF β was released. The initial burst release was followed by a slow completion of the NGF β release by the end of the week. Although this experiment did not provide data about the activity of the released NGF β, our results suggested that the same release profile would occurs *in situ*. Unfortunately, the activity of the NGF released after 24 h *in vivo* could not be ascribed with certainty to the effects of NGF β, because of possible protein denaturation *in situ*. Additionally, since the delivery increases with protein loading, we applied three different doses of NGF β, and depending on the degradation rate and inversely with the size of the device, our results confirmed an increase in bone ingrowth with increasing doses of NGF β. A major concern in the delivery of growth factors to the site of bone healing has been their short half-live in systemic circulation, as noted by Nimni [33] in his review of the various methods used for targeted delivery of growth factors to specific tissues. PDGF has a half-life of about 2 min if injected intravenously, and TGF-b in its active form is also cleared from the blood stream within a few minutes. It was demonstrated that the action of TGF-b is only a local one, with no influence on bone formation at sites that are distant from its application. Furthermore, Kasprek [34] and other investigators reported that these multiple growth factors are present at the same time at the site of bone formation and have a synergistic effect with each other. This led to the philosophy of providing higher concentrations of growth factors, as we did, at the actual site of bone formation in order to mimic the natural process of osteogenesis as closely as possible.

5. Conclusion

In conclusion, our implant system proved to have good chemical and physical characteristics and a satisfactory bone tissue tolerance when implanted. No signs of inflammation or cytotoxicity were noticed during the period of implantation. The chemical and physical properties of the Col–HAp–NGF β composites significantly affected the process of implant osseointegration, namely an increased bone ingrowth into the implant. We used the calvaria skull model, because calvariae are membranous bones of different embryonic origin; thus calvaria osteoblast precursors might have different properties [35], and be more sensitive to NGF β activity. A very positive effect on bone-producing cells, i.e. osteoblasts, was evident *in vitro*. The delivered osteogenic-neurogenic factor (NGF β) significantly affected and promoted bone ingrowth *in situ*. In the future, this implant system might undergo various changes and improvements, as well as mechanical and chemical adjustments, in order to make it suitable for better bone engineering in reconstructive craniofacial surgery. In sum, this system has obvious advantages because:

1. It may improve cosmetic and functional results in patients treated for cranio-facial tumors, malformations and traumas, contributing to the development of new surgical techniques for bone reconstruction of the splanchno- and neuro-cranium;
2. It may represent a new drug delivery device for sustaining the release of both neurogenic and/or osteogenic growth factors that are essential in tissue engineering; moreover, in combination with cell transplantation, it may lead to the development of more complex engineered tissues.

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Received 25 February
and accepted 25 July 2002