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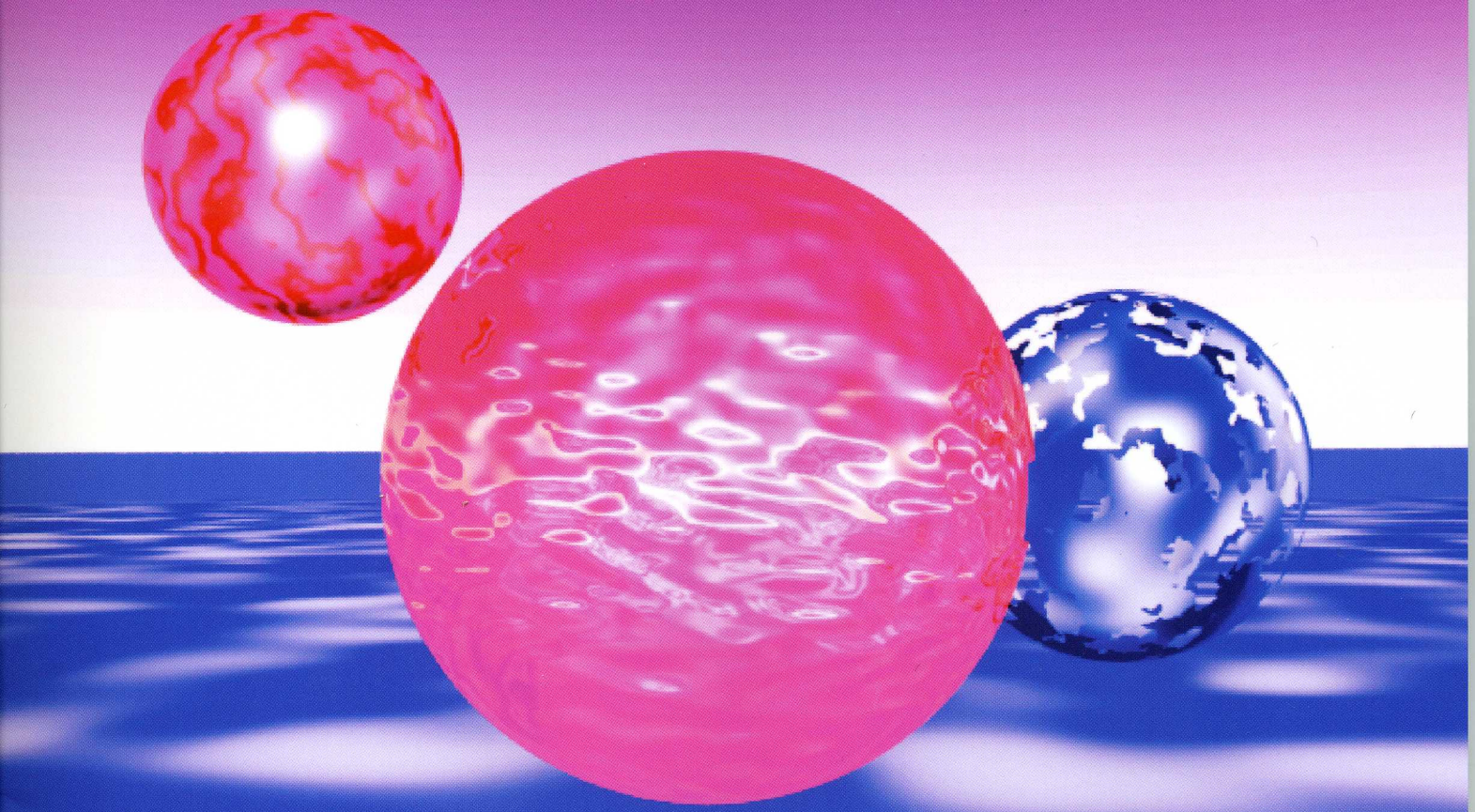

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β-TCPによるイヌ顎骨のNidogen遺伝子の発現

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要旨：β-TCPは，骨形成，創傷治癒を促進する満足のいく臨床結果をもって骨の再生医療に汎用されているが，その機序については不明な点が多い。その骨形成，創傷治癒の促進機序の解明を目途に，ビーグル犬の顎骨の骨欠損部にβ-TCPを填入し，4，7日後に骨組織から総RNAを回収した後，DNAマイクロアレイを用いて遺伝子発現レベルを解析した。β-TCPによって多数の遺伝子の発現が変動した。そのなかでnidogen (NID) 1およびNID2遺伝子の発現上昇が見いだされた。この増大は，RT-PCR，real-time PCRによって確認された。Nidogenは間葉系細胞で産生される基底膜細胞タンパク質であり，細胞外基質タンパクと相互作用して幹細胞のアセンブリーや分化に関与している。また，NID1，NID2の遺伝子ノックアウトマウスは，四肢の外胚葉系基底膜の形成の異常を生じ，ある種の動物では，成長因子と細胞外基質との相互作用に影響して骨形成に変化が起こると報告されている。これらのことから，β-TCPによるNID遺伝子発現の増大はβ-TCPの骨形成，創傷治癒促進の一機序になると示唆される。

Introduction

Beta-tricalcium phosphate (β-TCP) has been used for bone regeneration with satisfactory results in both animal models and human trials[1]. β-TCP particles in the tooth extracted sockets are clinically osteoconductive[2]. Since β-TCP is a suitable bone substitute that will biodegrade and be replaced by newly mineralizing bone tissue without fibrous tissue proliferation[3], β-TCP has received increasing attention in oral implant therapy and used as alloplastic bone graft material for maxilla sinus bone graft[4]. It was demonstrated that the early healing of an extraction socket had been grafted with β-TCP[5]. However, very little is known about the molecular basis for mechanisms enhancing the bone formation and wound healing by β-TCP. In the present study, to understand the usefulness of β-TCP in bone tissue regeneration through wound healing and bone formation, β-TCP was implanted into bone defects in beagle dog mandibles, and gene expression profiles in bone tissues were examined.

Materials and Methods

1. Implantation of β-TCP

Beagle dogs (body weight 13±2 kg ; 10 dogs) were purchased from Japan SLC (Shizuoka, Japan). The dogs were allowed access to food and water *ad libitum* at all times and were maintained on a 12 h light/dark cycle (lights on 8 : 00 to 20 : 00) at 23±1 ℃. All beagle dogs were maintained and used in accordance with the guidelines of the care and use of laboratory animals of Kanagawa Dental College (Approval # : 08.12.TTH). There were two experimental groups : the β-TCP scaffold implant group (n=3) and the no implant control (n=3). The left and right mandibles of beagle dogs were divided randomly into three time groups according to the expected time when they would be sacrificed.

All beagle dogs were injected with sodium pentobarbital (Somnopentyl®, Kyoritsu Seiyaku, Tokyo, Japan) at a dose of 35 mg/kg. The dogs then had their premolar teeth extracted. After three months' healing, the sockets of mandible defects (4.5 mm diameter, 8 mm length) were made in the mandible bone using

an implant drill with physiologic saline cooling under anaesthesia. The β-TCP was purchased (β-TCP-100, >99 % pure, Taihei Chemicals Limited, Japan) and crushed to 500-800 μm diameter particles, and was filled into left or right mandible bone defects (200 μg per one defect). After surgery, each beagle dog received an intramuscular injection of sodium ampicillin (Viccillin®, Meiji, Tokyo, Japan) at a dose of 100 mg per kg body weight. All specimens (4.5 mm diameter, 8 mm length) were taken out in a cylinder at 4 and 7 days after surgery, and immersed into RNA stabilization solution (RNA later®, Applied Biosystems, Ambion, USA).

2. RNA preparation

Implantation of β-TCP in to dog mandible and RNA preparation were described in previous report. was extracted from each bone biopsy using an optimized RNA extraction protocol based on the RNeasy® Fibrous Tissue Midi Kit Isolation System (Qiagen Ltd.) according to the manufacturer's protocol. One piece of a biopsy (a maximum of a 3 mm cube=25-35 mg of tissue) was placed into 350 ml of lysis buffer (Buffer RLT, from isolation Kit) in a Lysing Matrix A tube (FastPrep® System, MP Biomedicals, Japan) and homogenized at 6.0 m/s, 45 s, for 6 times. Samples were processed using the RNeasy Isolation System following the NanoDrop manufacturer's instructions. The RNA quality was conformed by calculating the $OD_{260/280}$ ratio by Agilent RNA 6000 Nano kits (Agilent, Santa Clara, CA, USA).

3. Gene chip analysis

The protocol for microarray processing was carried out according to the GeneChip® 3' IVT express Kit user manual (Affymetrix, Santa Clara, CA). Total RNA samples (100 ng) were subjected to two-cycle target labeling according to the Affymetrix instructions. Antisense complimentary RNA (cRNA) derived from double strand complimentary DNA (cDNA) was labeled in the presence of biotinylated deoxyribonucleotide triphosphate (dNTP) derivatives to produce cRNA probes. The probes were then fragmented and hybridized on the DNA microarray (GeneChip® Canine Genome 2.0 Array, 38,000 genes ; Affymetrix). Details of the probe set can be obtained at http://www.affymetrix.com/products_services/arrays/index.affx. After hybridization, the arrays were scanned using the GeneChip® Scanner 3000 and the scanned images were analyzed using GeneChip Operating Software (Affymetrix, Santa Clara, CA). The data was imported into GeneSpring GX software (Agilent Technologies, Inc Santa Clara, CA) for selection of induced and repressed genes in each experiment. Values below 0.01 were set to 0.01. Each time point of result was normalized against the median of the corresponding standard genes (Affymetrix manual). The presence (P) or absence (A) of signals was re-evaluated and intensity normalization was performed across the arrays. Data analysis was performed using the GeneChip Expression Analysis (Affymetrix, Santa Clara, CA) and GeneSpring (Silicone Genetics, Redwood, CA) software packages.

4. RT-PCR and Real time PCR Analysis

RT-PCR and real-time PCR reactions were carried out using a DNA thermal analyzer (RFN-Gene™ 6000 ; Corbett Life Science, Sidney, Australia). Amplification by PCR was started with an initial incubation at 95 °C for 15 s to activate the Taq DNA polymerase, and then performed at 95 °C for 5 s and 56 °C for 15 s by adequate cycles. RT-PCR products were electrophoresed on 1.5 % agarose gel, followed by staining with ethidium bromide to examine the size of PCR products. Each assay was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels.

Real-time PCR was carried out with SYBR Premix Ex Taq™ (Perfect Real-Time PCR, Takara, Japan) and a Green PCR kit (Qiagen GmbH, Dusseldorf, Germany). To calculate gene expression fold changes, the initial template concentration was derived from the cycle number at which the fluorescent signal crossed the threshold in the exponential phase of the real-time PCR reaction. The mRNA copy unit was given by the cycle threshold value from the fluorescent signal of all the samples, including the standard curve and target genes, following the method provided by Corbett Life Science Company using RFN-Gene™ 6000 software. Details were described in an operation manual, version 1.7.40, 2006.

The DNA primer sequences were 5'-tcggagta gtcgcacctttt-3' (the forward primer for NID1 gene) ; 5'-accacactactgggctggaa-3' ; (the

reverse primer for NID1 gene), (predicted size＝157 bp) ; 5'-cccaggagaagagagggttc-3' (the forward primer for NID2) ; and 5'-gatgcggtagg atcgtgttt-3' (the reverse primer for NID2 gene), (predicted size＝238 bp) ; 5'-atcaccatctt ccaggag-3' (the forward primer for GAPDH) ; and 5'-atcgactgtggtcatgag-3' (the reverse primer for GAPDH gene), (predicted size＝318 bp). Values were calculated as means±standard deviation (SD). Comparisons were made between two groups using a Student's *t*-test.

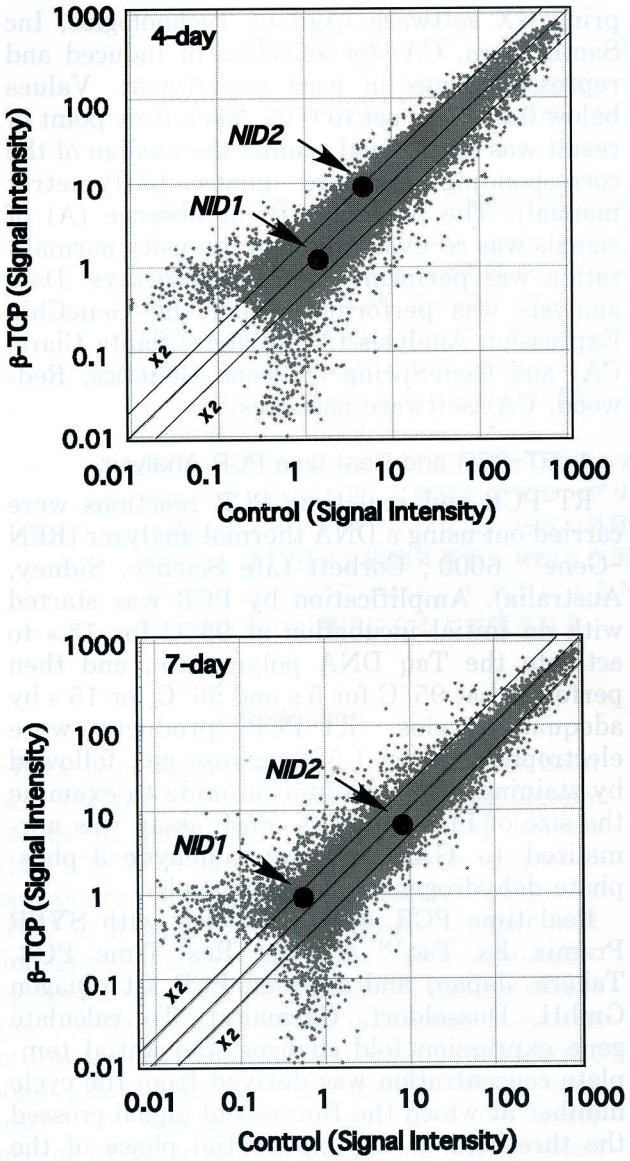

Fig. 1 A scatter plot of the mRNA levels of NID1, NID2 and GAPDH expressed in β-TCP implantation.

Results

β-TCP altered many gene expressions as shown in scatter plot analysis (Fig. 1). Among those genes we found that NID1 and NID2 mRNA levels enhanced more than 2-fold. Table 1 shows the raw mRNA signals of NID1 and NID2 in regenerated mandible bone tissues with or without β-TCP by "Present" signals.

To further investigate the elevated mRNA level of the NID1 and NID2 genes, RT-PCR was performed. As shown in Fig. 2, the mRNA level from dog mandible bone tissue implanted β-TCP was significantly higher than that of non-implantation as a control in 4-, 7-day samples. In contrast, mRNA levels of GAPDH, the housekeeping control, showed no differences between each β-TCP implanted sample and control sample.

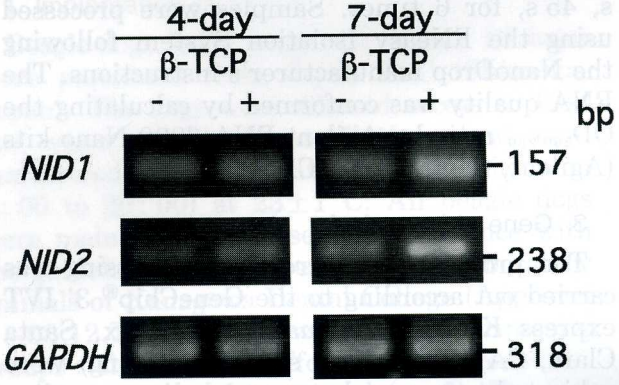

Fig. 2 RT-PCR analysis of the mRNA levels of NID1 and NID2. An ethidium bromide-staining pattern of the amplified PCR products after agarose gel electrophoresis.

Table 1 Raw intensity signals shown in GeneChip

Gene	Genebank ID	4-day β-TCP −	4-day β-TCP +	4-day Fold*	7-day β-TCP −	7-day β-TCP +	7-day Fold*
NID1	XM_546076	20.12 (A)	29.48 (P)	1.3	7.18 (A)	20.47 (P)	2.2
NID2	XM_537445	76.03 (P)	200.49 (P)	2.3	102.24 (P)	168.00 (P)	1.3

Flag (P) or (A) indicate the reliability of the data according to present (P) or absent (A) of gene expression in GeneChip. *Fold, fold change by normalization against the median of the corresponding to control.

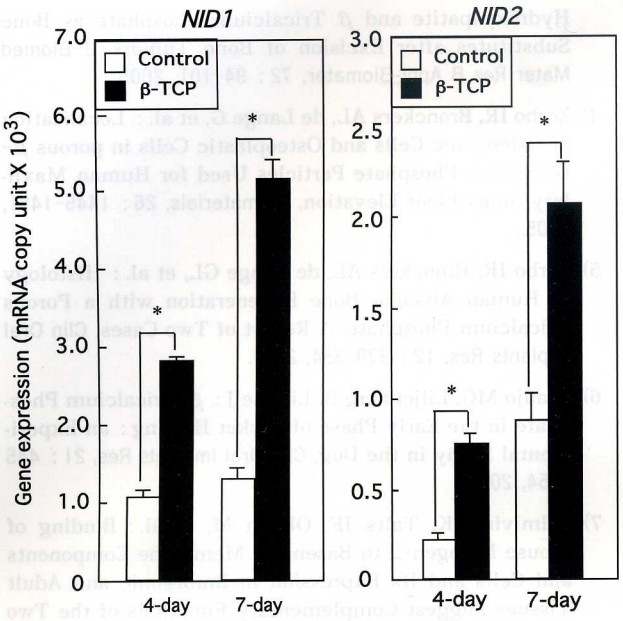

Fig. 3 Real-time analysis of NID1 and NID2 mRNA levels. Results were expressed as mRNA copy units by normalisation to a housekeeping gene GAPDH. Differences between β-TCP implantation and controls were determined using a student's *t* test. $*p<0.001$, n=3.

To determine the exact rates of enhancement of NID gene expression levels, another experiment was also performed using real-time PCR. The results were converted to mRNA copy units and the increase in NID gene expressions were shown to be significantly different between each β-TCP implanted sample and control.

The quantities of mRNA levels of NID and fold changes by β-TCP implantation in dog mandibular bone normalized by GAPDH from the GeneChip analysis and real-time RT-PCR were summarized in Table 2. Total amounts of NID1, NID2 mRNA in 4 and 7 days in β-TCP implanted sample were higher than those of control.

Discussion

β-TCP particles in the extraction sockets are clinically osteoconductive[2]. When particles of β-TCP are mixed with a blood clot and surrounded by the bony walls of the alveolar socket, osteogenic cells, including undifferentiated mesenchymal stem cells, start migrating from the existing bone surface over the surface of the particles. However, the molecular basis mechanism is not clear. In this study, we attempted to search the candidate genes which play a role of osteoinductive activity by β-TCP using dog tooth extracted socket model using a gene chip system, and found NID1 and NID2 gene expressions were enhanced by β-TCP.

The NID1 and NID2 are protein families found ubiquitously in basement membranes[7]. Human NID2 has been molecular-cloned, and been found to share 46 % sequence identity and a similar domain arrangement with the previously characterized basement membrane protein NID1. Recombinant NID2 was purified as a 200 kDa protein from transfected cell, which showed a high level of N and O-glycosylation, and could be clearly distinguished from 150 kDa of NID1[8]. Basement membranes are specialized extracellular matrices found underlying all epithelia and endothelia as well as mesenchymal cell[9]. NIDs are known ubiquitous basement membrane proteins produced by mesenchymal cells and mediated interactions with extracellular matrix proteins, and promotes human embryo stem cell assembly and differentiation[10]. Interaction of NID with laminin, collagen IV and perlecan have been

Table 2 Summary of *NID1*, *NID2* gene expression

Gene	Day	β-TCP	GeneChip (Fold)	Real-time PCR				
				mRNA copy unit	Fold	Total mRNA levels		
						Control	β-TCP	Fold
NID1	4	−	1.3	1074.00± 73.74	2.6	2390.67	7977.65	3.3
		+		2833.61± 21.26*				
	7	−	2.2	1316.67±104.62	3.9			
		+		5144.04±223.57*				
NID2	4	−	2.3	222.67± 28.15	3.4	1106.00	2829.84	2.6
		+		753.68± 48.06*				
	7	−	1.3	883.33±140.03	2.4			
		+		2076.16±223.48*				

β-TCP *vs* Control, $*p<0.01$, n=3.

considered important for basement membrane formation. The wound healing requires basement membrane reformation, and it has been studied in NID1 deficient mice. Interestingly, the impaired wound healing was found in mice lacking NID1[11]. NID1 and NID2 knockout mice showed a reduced BMPs within the developing limb. NIDs may interplay between growth factors and the extracellular matrix, and may change bone formation[12]. Taken together with our finding, the enhancement of NID gene expression by β-TCP might be one of molecular mechanisms accelerating in wound healing and bone formation.

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References

1) Artzi Z, Weinreb M, Givol N, et al. : Biomaterial Resorption Rate and Healing Site Morphology of Inorganic Bovine Bone and β-tricalcium Phosphate in the Canine : a 24-month Longitudinal Histologic Study and Morphometric Analysis, Int J Oral Maxillfac Implants, 19 : 357 -368, 2004.

2) Brkovic BM, Prasad HS, Konandreas G, et al. : Simple Preservation of a Maxillary Extraction Socket using β-tricalcium Phosphate with Type I Collagen : Preliminary Clinical and Histomorphometric observations, J Can Dent Assoc, 74 : 523-528, 2008.

3) Ogose A, Hotta T, Kawashima H, et al. : Comparison of Hydroxyapatite and β Tricalcium Phosphate as Bone Substitutes after Excision of Bone Tumors, J Biomed Mater Res B Appl Biomater, 72 : 94-101, 2005.

4) Zerbo IR, Bronckers AL, de Lange G, et al. : Localisation of Osteogenic Cells and Osteoplastic Cells in porous β-tricalcium Phosphate Particles Used for Human Maxillary Sinus Floor Elevation, Biomaterials, 26 : 1445-1451, 2005.

5) Zerbo IR, Bronckers AL, de Lange GL, et al. : Histology of Human Alveolar Bone Regeneration with a Porous Tricalcium Phosphate. A Report of Two Cases, Clin Oral Implants Res, 12 : 379-384, 2001.

6) Araújo MG, Liljenberg B, Lindhe J : β-Tricalcium Phosphate in the Early Phase of Socket Healing : an Experimental Study in the Dog, Clin Oral Implants Res, 21 : 445 -454, 2010.

7) Salmivirta K, Talts JF, Olsson M, et al. : Binding of Mouse Nidogen-2 to Basement Membrane Components and Cells and Its Expression in Embryonic and Adult Tissues Suggest Complementary Functions of the Two Nidogens, Exp Cell Res, 279 : 188-201, 2002.

8) Kohfeldt E : Nidogen-2 : A New Basement Membrane Protein with Diverse Binding Properties, J Mol Biol, 282 : 99-109, 1998.

9) Yurchenco PD, Amenta PS, Patton B : Basement Membrane Assembly, Stability and Activities Observed Through a Developmental Lens, Matrix Biol, 22 : 521-538, 2004.

10) vseenko D, Schenke-Layland K, Dravid G, et al. : Identification of the Critical Extracellular Matrix Proteins that promote Human Embryonic Stem Cell Assembly, Stem Cells Dev, 18 : 919-928, 2009.

11) Baranowsky A, Mokkapati S, Bechtel M, et al. : Impaired wound healing in mice lacking the basement membrane protein nidogen 1, Matrix Biol, 29 : 15-21, 2010.

12) Böse K, Nischt R, Page A, et al. : Loss of Nidogen-1 and -2 Results in Syndactyly and Changes in Limb Development, J Biol Chem, 281 : 39620-39629, 2006.