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Gene Expression of Four Adhesive Proteins in the Early Healing of Bone Defect and Bone-implant Interface

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[Abstract] The objective of this study was to evaluate the gene expression of four bone-related adhesive proteins during the early healing of bone defect and bone-implant interface in animal experiments. T-shaped hollow pure titanium implants with dual acid-etched surfaces were placed into femurs of 17 Sprague-Dawley rats, and bone defects with the same size were made in the same site in 15 rats. Newly formed bone was harvested at 5 days, 8 days and 16 days respectively. The gene expression of fibronectin (FN), collagen I (COL I), bone sialoprotein II (BSP II) and osteopontin (OPN) in non-implant and bone-implant defects were examined using semi-quantity reverse transcription-polymerase chain reaction. The gene expression of OPN in the non-implant defect was slightly higher than that in the bone-implant interface. At 8 days postoperation, FN, COL I and BSP II expression were significantly up-regulated in the bone-implant group. All four proteins peaked at 8 days. The results indicate that the gene expression of the four adhesive proteins is different between bone defect and bone-implant interface. Intracellular synthesis of FN, COL I and BSP II was accelerated in the early healing stages of the bone-implant interface.

INTRODUCTION

Osseointegration, which means a direct structural and functional connection between living bone and the surface of a load carrying implant, is the foundation of long-term clinical success of endosseous dental implants. The peri-implant osteogenesis can proceed from the host bone to the implant surface (distant osteogenesis) and from the implant surface to the host bone (contact osteogenesis)[1]. In the healing of a non-implant bone defect, only distant osteogenesis occurs, which new bone growing from the host bone. It is obvious that the healing process between the bone-implant interface and non-implant bone defect is not exactly the same. Cell adhesion to extracellular matrix is essential to the development, maintenance, and remodeling of osseous tissues[2]. Contact osteogenesis, the adhesion of osteoblasts to the implant surface, which is mediated through proteins that have adsorbed to the substrate, is a prerequisite for the secretion of new bone matrix on the implant surface[3]. Therefore, the adhesion of osteoblasts to the implant surface is the vital factor in bone-implant integration that different from bone defect healing.

Until now on, most researchers focus on the effect of peptides or adhesive protein coatings on osteoblasts adhesion in vitro[4], or biomimetic surfaces in corporating bio-adhesive motifs from extracellular matrices compared with unmodified control in vivo[7]. Fibronectin (FN), collagen I (COL I), osteopontin (OPN) and bone sialoprotein II (BSP II) are common adhesive proteins in bone extracellular matrices. Establishment a profile of mRNA expression of these proteins in the bone-implant interface and bone defect healing processes are essential to characterize their possible function in the two kinds of osteogenesis. In vivo changes in mRNA expression can yield valuable clues in the regulation of osteogenesis at the molecular level.

MATERIALS AND METHODS

Implant design and surface analyses

Experimental T-shaped implants of 3.0×3.0×2.0mm were fabricated from commercially pure titanium (Baoji Special Iron and Steel Co.LTD.,China) and had a hollow inner chamber (2.5×3.0×1.0mm). The implant surface was polished and treated by dual acid-etching (DAE), specimens were immersed in 98%(w/w) H_2SO_4 and 37%(w/w) HCL (1:1 in volume), 60°C for 30min. After the DAE treatment, the implants were gently washed with distilled water and dried.

The surface of the implants was examined through SEM (Quanta 200, Fei, Netherlands). The implants were cleaned with acetone, 75% ethanal, distilled water separately and sterilized before placement surgery.

Animal surgery

37 male Sprague-Dawley rats of 8-week-old (SPF, Animal Experimental Center, Wuhan University) were randomly separated into three groups: implant group, non-implant group and control group. The experimental rats were anesthetized with 846 anesthetic (The Academy of Military Medical Sciences, China). After the leg was shaved and decontaminated with 10% povidone-iodine solution, the distal part of the femur was carefully exposed, and periosteum flaps were created using a small periosteal elevator. 17 rats were engaged in implant group. The

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implant site was prepared at 4mm from the distal edge of the femur to make sure that implant was in trabecular bone. Irrigation with sterilized isotonic saline solution was used for cooling and cleaning. The implant was placed into the site until the hollow chamber was totally embeded in the bone, and the implant roof structure reached the femur exterior. Mechanical fixation of the implant was confirmed to achieve primary stability. Muscles and skins openings were closed separately. The non-implant defect with the same size was prepared at the corresponding site of other 15 rats as non-implant group. The other 5 rats as control group received no surgical treatment. Each rat of the three groups received 30, 000 unit penicillin postoperative 3 days.

Histological observation

Two rats of implantation group were sacrificed at 8, 16 days postoperation, respectively. The distal part of the femur with implant were fixed in 4% paraformaldehyde, embedded in Spurr's resin, and sectioned into wafers about 100μm in thickness, using the Leica large scale heavy-duty sectioning system (SP1600, Leica, Germany) with a diamond- wafering blade. The undecalcified ground sections were stained by toluidine blue (Applied by Wuhan Bell Chemical Reagent Limited Company, China). Sections were observed under a Leica inverted microscope and photographed.

Semi-Quantity RT-PCR

The animals in the implant group and non-implant group were divided randomly into 3 groups of 5 each and sacrificed at 5 days, 8 days and 16 days. Tissues that had grown inside the inner chamber were harvested, and total RNA was isolated using TRIZOL™ reagent (Invitrogen Life Tech). Tissues were also scratched from the 4 walls of non-implant defect using a knife edge at day 5 days, 8 days and 16 days, in addition, normal bone of the untreated rats, total RNA was isolated respectively in the same way.

Reverse transcription-polymerase chain reaction (RT-PCR) assay was used to analyze the expression of ECM-related genes. To avoid amplification of genomic DNA, a DNase I digest was performed prior to RT-PCR reaction. 1μg of total RNA was reverse-transcribed into a cDNA template using AMV reverse-transcriptase (TaKaRa Biotechnology Co., Ltd, China) and oligo(dT) primer at 50 ^{o}C for 30min, followed by 95^{o}C for 5min. The cDNA products were amplified by PCR using Tag DNA (TaKaRa Biotechnology Co., Ltd, China) for the following ECM related genes: fibronectin, collagen I, bone sialoprotein II and osteopontin. Each pair of primers of the four proteins was showed in Table 1.

Preliminary PCR trials were performed to determine the annealing temperature and the optimal number of cycles that yields the linear range of PCR amplification for each primer set. PCR reaction from three separate experiments was performed. The PCR products were electrophoresed on 1%(wt/vol) agarose gel with 0.1μg/ml ethidium bromide. The intensity of the bands was quantified under ultraviolet light and normalized with respect to those for β-actin(housekeeping gene) mRNA.

Tab. 1 Primers and PCR conditions for SQ-PCR experiments

Primer	Annealing temperature	No.of cycle	Size of PCR products(bp)
FN			
5'-CCTTAAGCCTTCTGCTCTGG-3'	60	24	300
5'-CGGCAAAAGAAAGCAGAACT-3			
COL I			
5'-GGCGAAGGCAACAGTCGAT-3'	54	22	173
5'-TCCATTCCGAATTCCTGGTCT-3'			
BSP II			
5'-CAGGAGGCGGAGGCAGAG-3'	56.5	27	487
5'-CATACTCAACCGTGCTGC-3'			
OPN			
5'-TCCAAGGAGTATAAGCAGAGGGCCA-3'	60	22	200
5'-CTCTTAGGGTCTAGGACTAGCTTGT-3'			
β-actin			
5′-GAGACCTTCAACACCCCAGCC-3′	54	23	400
5′-TCGGGGCATCGGAACCGCTCA-3′			

Statistical methods

Data for the molecular experiment was analyzed using one-way ANOVA. Significant differences among the three experimental groups were set at the level of $P < 0.05$.

RESULTS

Surface topography of implants

Surface morphologies of the DAE modified implant were observed through SEM (Fig. 1). The surface of DAE treated implant exhibited irregular microtextures with consistent roughness.

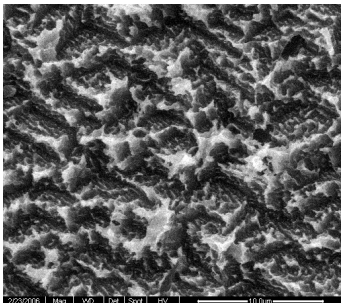

Fig. 1 SEM image of the DAE implant

General Observation

Health conditions were found to be normal in all of the experimental rats. No infection was observed in the surrounding tissue and all the implants were immobile.

Histological Analysis

Fig. 2 showed that there were immature woven bone tissues or bonelike tissues filled the inner chamber of the implant at 8 days. In some areas, bone contacted with the implant surface directly without fibrous connective tissue interposing (Fig.2b). After 16 days of wound healing, new

bone formation appeared to be more intense in the inner chamber of implants and was seen to occupy most of surface regions of the devices.

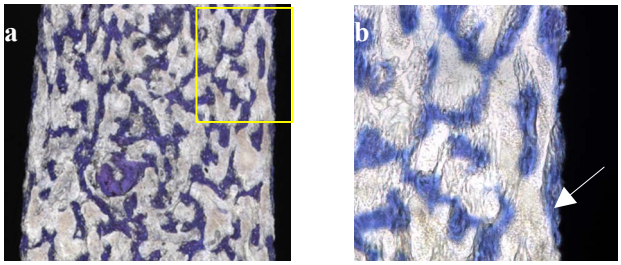

Fig. 2 (a) Histological sections of the DAE implant at 8 days. (b) Bone contacted with the implant surface in some areas as indicated by the arrow. Magnification×10 (Toluidine blue stain)

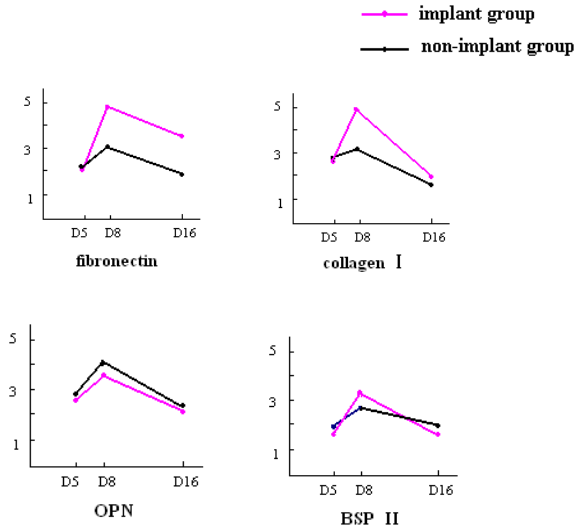

Fig. 3 Gene expression of four adhesive proteins in the implant group and non-implant group.

Bone matrix gene expression

Compared with the control group (gene expression of FN, OPN, BSP II, COL I in untreated normal bone), the gene expression in both non-implant defect group and implant group were accelerated at the three experimental times ($P<0.05$). The gene expression of OPN of the non-implant group was slightly higher than that in the implant group at 5, 8, 16 days ($P<0.05$). At 8 days postoperation, FN, COL I and BSP II expression were significantly up-regulated in the bone-implant group. ($P<0.05$). The accelerated mRNA expressions of COL I and BSP II were not obvious in implant group compared to the non-implant group at 5 days and 16 days ($P>0.05$). All four proteins in the two experimental group peaked at 8 days. (Fig.3)

DISCUSSION

T-shaped hollow implants were used to allow ingrowth of the implant-induced de novo tissue for molecular analyses in this experiment. The results of histological observation confirmed osseointegration of the implant if primary stability and regular operation procedures of implantation have been achieved. New bone can be observed on the implant outer and inner surface in some areas. It can be concluded that the osteogenesis on the host bone surface and the implant surface occurred simultaneously.

The significantly higher gene expression of the surgery groups compared with that of the control group suggests that the cell activity of synthesizing bone extracellular matrices is accelerated in surgery site.

Titanium possesses an ability to alter cellular phenotypes and tissue micro- and ultrastructure that result in enhanced intrinsic biomechanical properties of mineralized tissue. In vitro, it has been proved that osteoblasts accelerate gene expression of collagen I and generate intensive collagen deposition on titanium than on polystyrene[5]. Titanium implants also induce increased expression of matrix metalloproteinases in bone during osseointegration[6]. It is supposed that bone-bonding material, titanium, influences gene expression of extracellular matrix in order to facilitate osseointegration.

Osteopontin is a highly phosphorylated sialoprotein containing a conserved sequence of RGD motif that mediates cell attachment and signaling. This protein is expressed at high levels in mineralized tissues and may inhibit the in vitro formation of hydroxyapatite (HA) in physiological fluids of high supersaturation[7, 8]. The expression of OPN is affected by a large number of hormones, cytokines, and growth factor. The gene expression of OPN in the non-implant defect was slightly higher than that in the bone-implant interface at 5, 8, 16 days in this experiment may due to the versatility of OPN, such as wound healing, immunological responses, bone resorption, and vascular calcification[9, 10]. BSP II is a bone-specific highly glycosylated and sulphated phosphoprotein that is found almost exclusively in mineralized connective tissues. Newly formed osteoblasts induce expression of the BSP II gene, which is up-regulated by hormones and cytokines that promote bone formation and down-regulated by factors that suppress bone formation. BSP II has been considered as a potential nucleator of hydroxyapatite[11, 12] and as a specific marker of osteoblast differentiation. Furthermore, BSP II can regulate cell attachment and signaling through RGD motif as OPN[13]. As the results showed here, an increased expression of BSP II for the bone-implant interface compared with that of the bone defect at the early healing stage of 8 days, maybe suggested that BSP II played an important role during the early healing stage of bone-implant integration. The results of gene expression of OPN and BSP II in this study were generally consistent

with previous relevant experiment[14], which supported our findings in turn.

It has been demonstrated that integrin interactions, in particular α5β1, with fibronectin are essential for osteoblast survival, proliferation, osteoblast-specific gene expression, and matrix mineraliztion[15]. FN can also irregular cell skeletal arrangement and induce cell migration[16]. In this study, the obviously up-regulated gene expression of FN and COL I in implant group showed the critical effect of FN and COL I in osteoblast adhesion, migration during the process of early bone-implant integration. As we know, COL I forms the scaffold for the rest of the extracellular matrix including the mineral phase. It is integral to tissue remodeling, wound healing and hemostasis. Moreover, it has been reported that the presence of collagen is necessary to facilitate all stages of osteogenesis including cell migration, proliferation and differentiation. The results were consistent with previous findings about α2β1 integrin receptors of cell membrane interactions with COL I play critical roles in osteoblast survival, proliferation, differentiation and matrix mineralization, as well as in bone formation[17-19], and coating of titanium alloy and polystyrene with FN and COL I increased the adhesion, spreading, and proliferation of osteoblasts[20, 21]. All of them suggest that gene expression of FN, COL I and BSP II may play an important role in the early healing stages of bone-implant integration. These results cast a light on the effect of bone-related adhesive proteins in osseointegration and may contribute to biological modification of the implant surface. However, further researches on proteins expression in bone defect healing and bone-implant interface are needed.

CONCLUSIONS

An accelerated mRNA expression of BSP II was evoked in the bone-implant interface compared with bone defect healing at 8 days. While the gene expression of FN and collagen I in the implant group were significantly higher than that in the bone defect group at the same time. The results indicate that the gene expression of the four adhesive proteins is different between bone defect and bone-implant integration, intracellular synthesis of FN, COL I and BSP II was accelerated in the early healing stages of bone-implant interface.

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REFERENCES

[1] Davies, J.E., Understanding peri-implant endosseous healing. J Dent Educ, 2003. 67(8): p. 932-949.

[2] Damsky, C.H., Extracellular matrix-integrin interactions in osteoblast function and tissue remodeling. Bone, 1999. 25(1): p. 95-96.

[3] Garcia, A.J. and C.D. Reyes, Bio-adhesive surfaces to promote osteoblast differentiation and bone formation. J Dent Res, 2005. 84(5): p. 407-413.

[4] Ferris, D.M., et al., RGD-coated titanium implants stimulate increased bone formation in vivo. Biomaterials, 1999. 20(23-24): p. 2323-2331.

[5] Saruwatari, L., Aita, H. et al Osteoblasts generate harder, stiffer, and more delamination-resistant mineralized tissue on titanium than on polystyrene, associated with distinct tissue micro- and ultrastructure. . J Bone Miner Res., 2005. 20(11): p. 2002-16.

[6] Shubayev, V.I., et al., Titanium implants induce expression of matrix metalloproteinases in bone during osseointegration. J Rehabil Res Dev, 2004. 41(6): p. 757-66.

[7] Hunter, G.K., C.L. Kyle, and H.A. Goldberg, Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. Biochem J, 1994. 300 (Pt 3): p. 723-728.

[8] Hunter, G.K., et al., Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. Biochem J, 1996. 317 (Pt 1): p. 59-64.

[9] Kubota, T., et al., Influence of an intermittent compressive force on matrix protein expression by ROS 17/2.8 cells, with selective stimulation of osteopontin. Arch Oral Biol, 1993. 38(1): p. 23-30.

[10] Denhardt, D.T. and X. Guo, Osteopontin: a protein with diverse functions. Faseb J, 1993. 7(15): p. 1475-1482.

[11] Hunter, G.K. and H.A. Goldberg, Nucleation of hydroxyapatite by bone sialoprotein. Proc Natl Acad Sci U S A, 1993. 90(18): p. 8562-8565.

[12] Hunter, G.K. and H.A. Goldberg, Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. Biochem J, 1994. 302 (Pt 1): p. 175-179.

[13] Ross, Interactions between the bone matrix proteins osteopontin and bone siloprotein and the osteoclast integrin alpha v beta 3 protentiate bone resorption.
J Biol Chem, 1993(268): p. 9901-9907.

[14] Ogawa, T. and I. Nishimura, Different bone integration profiles of turned and acid-etched implants associated with modulated expression of extracellular matrix genes. Int J Oral Maxillofac Implants, 2003. 18(2): p. 200-210.

[15] Moursi, A.M., R.K. Globus, and C.H. Damsky, interactions between integrin receptors and fibronectin are required for calcarial osteoblast differentiation in vitro. J Cell Sci, 1997. 110: p. 2187-2196

[16] Fava, R.A. and D.B. McClure, Fibronectin-associated transforming growth factor. J Cell Physiol, 1987. 131(2): p. 184-189.

[17] Mizuno, M., R. Fujisawa, and Y. Kuboki, Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-alpha2beta1 integrin interaction. J Cell Physiol, 2000. 184(2): p. 207-213.

[18] Xiao, G., et al., Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. J Biol Chem, 1998. 273(49): p. 32988-32994.

[19] Jikko, A., et al., Collagen integrin receptors regulate early osteoblast differentiation induced by BMP-2. J Bone Miner Res, 1999. 14(7): p. 1075-1083.

[20] Geissler, U., et al., Collagen type I-coating of Ti6Al4V promotes adhesion of osteoblasts. J Biomed Mater Res, 2000. 51(4): p. 752-760.

[21] Cowles, E.A., L.L. Brailey, and G.A. Gronowicz, Integrin-mediated signaling regulates AP-1 transcription factors and proliferation in osteoblasts. J Biomed Mater Res, 2000. 52(4): p. 725-737.