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Full Length Research Paper

Effects of nacre-coated titanium surfaces on cell proliferation and osteocalcin expression in MG-63 osteoblast-like cells

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Titanium is widely used for dental implants because of its superior mechanical properties, low modulus, excellent corrosion resistance, and good biocompatibility. However, even when they are used in combination with a protective coating, such as hydroxyapatite (HA), titanium implants have been reported to have several disadvantages. These implants are unable to bond directly with the bone, and this impairs the anchorage of dental implants within the bone itself. In this study, a novel implant was obtained by implanting titanium implants into the epithelial mantle pearl sacs of a fresh water bivalve (*Hyriopsis cumingii* Lea), which has been used previously in a proof-of-concept study for the natural induction nacre coating. *In vitro* cell responses to the nacre-coated surface were evaluated by seeding MG-63 osteoblast-like cells onto the implant surfaces, and then assessing cell morphology, cell proliferation and markers of differentiation (alkaline phosphatase [ALP] activity and osteocalcin expression levels). Among all of the specimens tested, including multiple control surfaces, the nacre coatings had a stronger stimulatory on the MG-63 cells by altering cell morphology, and promoting proliferation and differentiation. These results indicate the bone-bonding capacities of nacreous coatings. However, further *in vivo* animal experiments are required to provide conclusive evidence for the bioactivity of nacreous coatings.

Key words: Dental implant, nacre, osteoblast, cell proliferation, osteogenesis.

INTRODUCTION

The insertion of dental implants to replace lost teeth is a common surgical procedure (Vanden Beucken et al., 2007). Dental implants are constructed of titanium and its alloys because of their superior mechanical properties, low modulus, excellent resistance to corrosion, and good biocompatibility. However, because of its bioinert nature, titanium cannot bond directly to the bone. After it is implanted *in vivo*, titanium implants become encapsulated by fibrous tissue that isolates it from the surrounding bone (Nishiguchi et al., 1999). In order to remain functional, titanium dental implants require a tight conjunction with the surrounding bone tissue (Vanden Beucken et al., 2007).

Several *in vitro* and *in vivo* studies have shown that modified titanium surfaces show higher levels of early osteoblast attachment than an untreated titanium surface (Zhang et al., 2004). Coatings of calcium phosphate (CaP) ceramics, such as hydroxyapatite (HA), have been reported to be beneficial for the anchorage of metal implants in bone tissue (Lacefield, 1988). Nevertheless, decades of clinical application have shown that while HA coating of implants can promote immediate bonding after implantation, brittleness (Vanden Beucken et al., 2007) and peeling (Li and Ducheyne, 1998) are the major chronic problems encountered during long-term load service in the oral environment. Furthermore, the high bioresorption rate of the implant results in loosening of

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Abbreviation: HA, Hydroxyapatite.

the implant and implant failure (Overgaard et al., 1998; Baltag et al., 2000; Darimont et al., 2003).

Various biomimetically fabricated coatings have been used in recent years to overcome these challenges. Nacre, otherwise known as mother of pearl, has been reported to be biocompatible with human bone (Lamghari et al., 1999; Mouries et al., 2002). Nacre is composed of calcium carbonate crystallized in the form of aragonite on an organic matrix scaffold. Previous studies have shown that nacre is an osteoinductive (bone inducing) material (Lopez et al., 1992; Silve et al., 1992; Atlan et al., 1997, 1999; Almeida et al., 2000), which can initiate bone formation *both in vivo* (Atlan et al., 1997, 1999) and *in vitro* (Lopez et al., 1992; Silve et al., 1992). Nacre also has outstanding mechanical properties. The resistance to fracture of nacre is 3,000 times greater than that of pure aragonite. In one study (Camprasse et al., 1990), the mechanical properties of nacre were shown to be comparable to those of titanium. Thus, both the osteoinductive and mechanical properties make nacre a promising candidate for a biological coating.

Of note, archeological studies have shown well fitted teeth made of nacre in a Mayan skull which have integrated with the bone (Westbroek and Marin, 1998). Additionally, flat sheets of pearl can be biofabricated on glass coverslips inserted between the mantle epithelium and shell of a seawater abalone (Fritz et al., 1994). In light of these findings, we previously conducted a trial experiment to insert titanium dental implants into a freshwater bivalve (*Hyriopsis cumingii* Lea), which is a mussel that is widely cultivated for pearls in China. A biologically active nacre coating was fabricated on the implants, and our results demonstrate that the nacre layer produced was sufficiently thick for solid implants to be prepared from it (Wang et al., 2005). This study was designed to evaluate the potential effects of titanium substrates coated with natural freshwater nacre on human osteosarcoma MG-63 osteoblast-like cells. Cell morphology, proliferation and differentiation were then assessed to determine the effects of nacre on these cells.

MATERIALS AND METHODS

Nacre-coated titanium substrates were obtained by inserting titanium substrates into freshwater bivalves (*H. cumingii* Lea); this technique was described in detail in our previous work (Wang et al., 2005). The HA-coated titanium substrates were obtained by the electrochemical deposition of HA onto titanium. The uncoated titanium substrates were then etched. All specimens were made of TA_2-grade titanium with the sizes of 10 × 10 × 1 mm. They were cleaned ultrasonically in deionized water at room temperature and then sterilized by γ–radiation.

Cell culture

Human osteosarcoma MG-63 osteoblast-like cells were obtained from American Type Culture Collection (ATCC, VA, USA). They were used to assay the osteoblastic cell response to the coated surface of titanium implants. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, CA, USA) and supplemented with 3% (v/v) fetal bovine serum (Invitrogen), 1% sodium pyruvate (Invitrogen), and 100 U/ml penicillin/streptomycin (Invitrogen). After reaching a confluence of 70 to 80%, the cells were subcultured using 0.1% trypsin (Invitrogen). For experiments, MG-63 osteoblast-like cells were seeded into the nacre-coated, HA-coated and uncoated substrates in 24-well plates and placed in a 5% CO_2 humidified atmosphere at 37°C.

Evaluation of cell morphology

The morphology of the cells was evaluated using scanning electronic microscopy (SEM). The cells were seeded into the sterilized substrates at a density of 1×10^5 cells. After incubation for 3 days, substrates with attached cells were washed twice with phosphate buffered saline (PBS) and fixed using 2.5% gluteraldehyde in PBS (pH 7.0) for at least 4 h. Subsequently, the substrates were washed with PBS and postfixed with 1% OsO_4 in PBS (pH 7.0) for 1 h, followed by dehydration in a graded series of ethanol (30, 50, 70, 95 and 100%, respectively). Finally, the specimen was dehydrated in a Hitachi Model HCP-2 critical point dryer with liquid CO_2 and coated with gold-palladium and observed with a Philips Model XL30 SEM.

Cell proliferation assessment by the MTT assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate cell proliferation on specimens. The MTT solution (Sigma, St Louis, MO, USA) of 2 mg/ml was prepared by dissolving MTT in PBS, after which it was filter sterilized. The cells were seeded on the sterilized coating surface at a density of 5×10^4/ml. At 1, 2, 3, 5, and 7 days post-seeding, the medium used for the experimental substrates (n = 4 for each group at each time point) was removed and 500 µl of the MTT solution was added to each sample in 24-well plates. Formazan was then formed by the action of mitochondrial dehydrogenases. After 4 h of incubation at 37°C, the MTT solution was discarded and 500 µl of the solubilization solution (dimethyl sulfoxide) was added to each well plate to dissolve the formazan crystals. Then, 100 µl of the solution was transferred to a new 96-well plate, and five data points were obtained from each sample. The optical density of the solution in each well was measured at a wavelength of 570 nm using a Microplate Reader (BioTek ELx800).

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity, which was considered as a marker for early differentiation of osteoblast-like cells, was measured with a commercial ALP Kit (JCBIO, NanJing, China) according to the manufacturer's protocol. ALP activity in the culture supernatant and the intracellular ALP activity of the cultured cells in each sample (n = 6 for each group at each time point) were both analyzed. The cells were seeded on the sterilized coating surface at a density of 5×10^4. At 3, 5, and 7 days post-seeding, a 10× lysis buffer solution (1% Triton X-100 in Tris-HCl buffer, pH 7.4) was added to each well plate for 5 min. After centrifugation at 10,000 rpm for 5 min, the supernatants were collected as cell lysates ALP activity was assessed. The optical density of the samples was measured at a wavelength of 520 nm in a spectrophotometer (Agilent 8453; Agilent, Santa Clara, CA, USA). The ALP activities in each sample were normalized to the cell protein content, as measured by the bicinchoninic acid (BCA) assay, and bovine serum albumin (BSA) was used as the standard.

Osteocalcin assay

The osteocalcin content in the cell lysates was used as a marker of late differentiation of osteoblast-like cells. Cells were seeded on the sterilized coating surface at a density of 5×10^4 cells. The amount of the osteocalcin obtained after 3, 5, and 7 days of cell culture was measured by an enzyme-linked immunoassay (ELISA, BioSource, CA, USA) according to the manufacturer's instructions. The sensitivity of this ELISA Kit was 0.4 ng/ml, the intra-assay reliability was 19.6 ± 0.4% and inter-assay reliability was 20.6 ± 1.2%. The cells were collected by detaching them from the coating surface with trypsin/EDTA (n = 6 for each group at each time point). After centrifugation, the cell pellets were washed twice with deionized water and finally resuspended by vortexing them in a 1× lysis buffer solution (1% Triton X-100 in Tris-HCl buffer, pH 7.4). After one cycle of freezing/thawing, the cells were further lysed by sonication at 4°C for 5 min. Finally, after centrifugation at 10,000 rpm for 5 min, the supernatants (cell lysates) were collected for the osteocalcin concentration assay. The osteocalcin concentration was measured in a microplate reader at 405 nm, and the osteocalcin expression level was evaluated on the basis of a standard curve.

Statistical analyses

All experiments were performed in triplicate. The results were expressed as the mean ± standard deviation (SD). Statistical significance was analyzed by a one-way analysis of variance (ANOVA), and statistical differences were considered to be significant at $P<0.05$.

RESULTS

SEM micrograph of MG-63 cells on the substrates

The morphological appearance of the cells was evaluated with SEM. The SEM micrographs of the surfaces are displayed in Figures 1a to c, and the SEM findings for MG-63 osteoblast-like cell morphology after 3 days of culture on the surfaces of titanium implants are shown in Figures 1d to f. Significant differences were observed between cell morphologies on different coatings. The cells on the nacre surface were well dispersed and had grown across the surface better than on the HA-coating or non-coated implants. The filopodia and lamellipodia were well-established and the cells covered the entire surface of the nacre coating. The cells had a predominantly flattened morphology and followed a continuous multilayered conformation, which suggested that the cells on the nacre surfaces had a good viability. In contrast, the HA surfaces and the titanium surface alone were only partially covered with MG-63 osteoblast-like cells; in these samples, the cells were confined to one region and had not extended further.

Cell proliferation increased on nacre-coated surfaces

Cell proliferation was directly monitored by the MTT assay at each culture time point (Figure 2). Within the first 2 days, cell numbers on the nacre-coated surfaces increased slightly and no significant differences were observed between the coating types ($p = 0.06$). Greater proliferation rates were observed between 2 and 5 days on the nacre coating, but this rate decreased at 7 days after cell seeding. The cell numbers on the nacre-coated surfaces were significantly higher compared to the control implants from 3 days post-seeding onwards ($p = 0.037$ at day 3, $p = 0.019$ at day 5 and $p = 0.009$ at day 7). No significant difference was observed among the HA-coated and uncoated groups throughout the entire evaluation period.

Effect on ALP-specific activity of MG-63 cells by nacre-coated surfaces

Figure 3 shows the ALP activity of MG-63 osteoblast-like cells on the different implant surfaces over the cell culture period. ALP is indicative of the early differentiation stages of the osteoblastic cell lineage. An increase in the ALP activity was observed in all experimental groups from day 3 to day 7 after cell seeding, and these results are similar to the results of the cell proliferation assay. At day 3, the level of ALP activity of MG-63 cells on nacre-coated surfaces was significantly higher ($p = 0.017$) than the control groups. By day 7, the level of ALP activity on nacre-coated surfaces was almost double the value found in both of the control groups.

Effect on expression of osteocalcin of MG-63 cells by nacre-coated surfaces

In this study, the levels of intracellular osteocalcin secreted by MG-63 osteoblast-like cells on different surfaces are illustrated in Figure 4. Until day 3 post-seeding, the levels of osteocalcin were low in each group, with no significant differences between implant types. Then, a highly significant increase ($p = 0.021$) was observed in the nacre-coated surface seeded cells compared with the other implants from day 5 onwards until the end of the experimental duration on day 7.

DISCUSSION

Bone formation induced by osteoblastic cells at the implant-tissue interface is a complex process that involves a sequence of cellular functions, including attachment, migration, proliferation, expression of markers of the osteoblast phenotype, and bone matrix mineralization (Marinucci et al., 2006). To enhance osseointegration, dental implant surfaces should possess the ability to stimulate the differentiation of osteogenic cells and matrix formation. The chemistry, morphology, and roughness of titanium implant surfaces are all important properties that influence cell-biomaterial

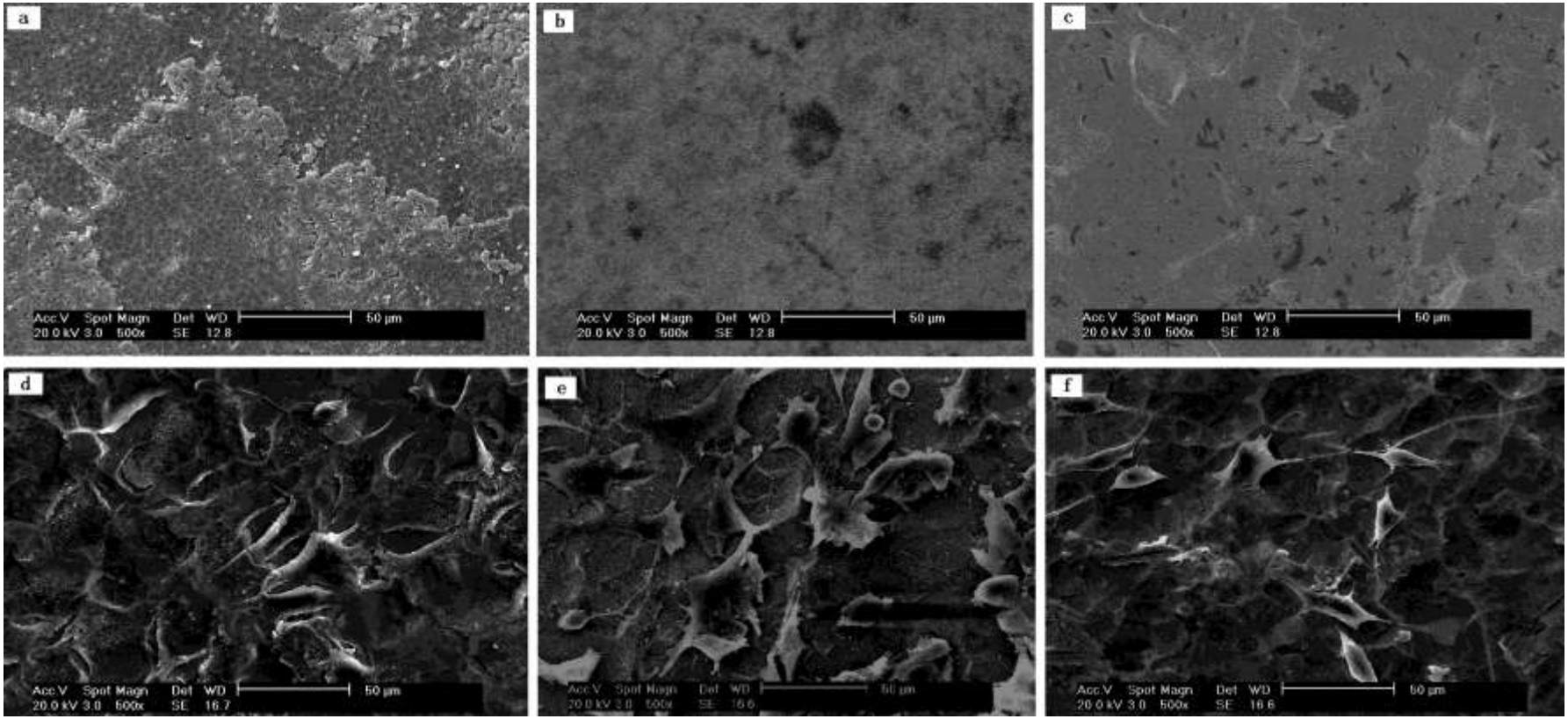

Figure 1. SEM micrograph of MG-63 osteoblast-like cells on the substrates. MG-63 osteoblast-like cells were seeded on the substrates at a density of 1 × 104. Cell morphology was observed under SEM (×500) after 3 days of culture. a: Titanium coated with nacre; b: Titanium coated with HA; c: Titanium coated with noting; d: Cell on titanium coated with nacre; e: Cell on titanium coated with HA; f: Cell on titanium coated with nothing.

interactions and bone formation, and the manipulation of implant surfaces is a promising field of research (Bagno and Di, 2004; Das et al., 2007). Nacre, commonly known as mother of pearl, has been reported to be biocompatible with bone (Lamghari et al., 1999; Mouries et al., 2002). In this study, we demonstrate that nacre could not only be induced to coat a titanium implant naturally, but also provided the implant with a good biocompatibility with a bone cell line.

Human osteosarcoma MG-63 osteoblast-like cells were chosen for this study because this cell line undergoes osteoblastic differentiation and mineralization when exposed to a variety of substrates (Wang et al., 2007; Qu et al., 2011; Lopes et al., 2011). The cells were cultured on a nacre-coated titanium surface, HA-coated titanium surface, and uncoated titanium surface, and the morphology, proliferation, ALP activity, and osteocalcin levels of the cells were investigated. The cells on the nacre-coated surface spread better and flattened more than those in the control groups, which suggests that MG-63 osteoblast-like cells have a higher ability to differentiate on nacre than the other tested substrates.

Thus, we can conclude that the topography and roughness of the nacreous layer has a particular

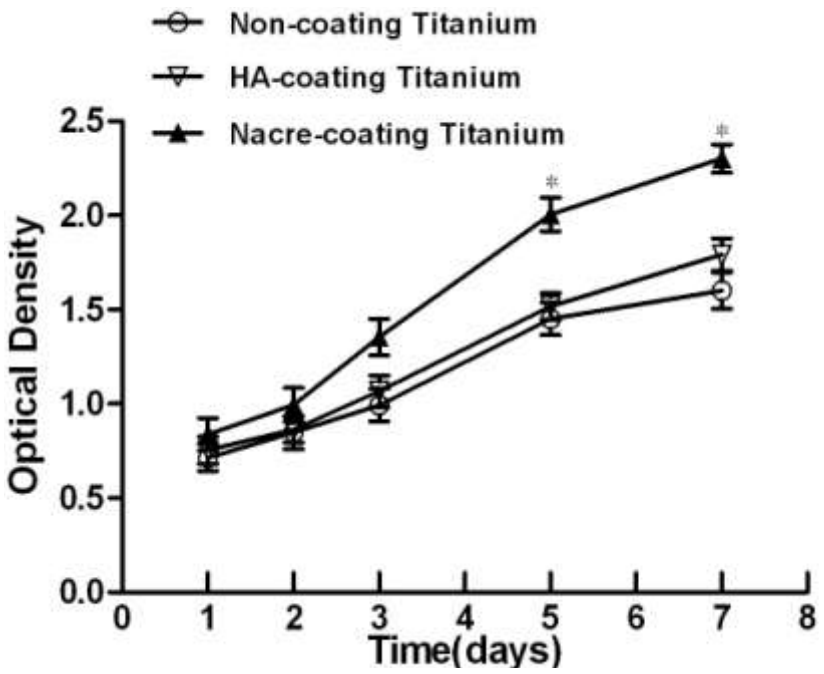

Figure 2. Assay of cell proliferation of MG-63 osteoblast-like cells on the substrates. MG-63 osteoblast-like cells were seeded on the substrates at a density of 5 × 104. Cell proliferation was evaluated using MTT assay on the 1, 2, 3, 5, and 7 day post-seeding. Nacreous group enhanced cell proliferation obviously after 3 days post-seeding (* compared with the other 2 groups, $p<0.05$).

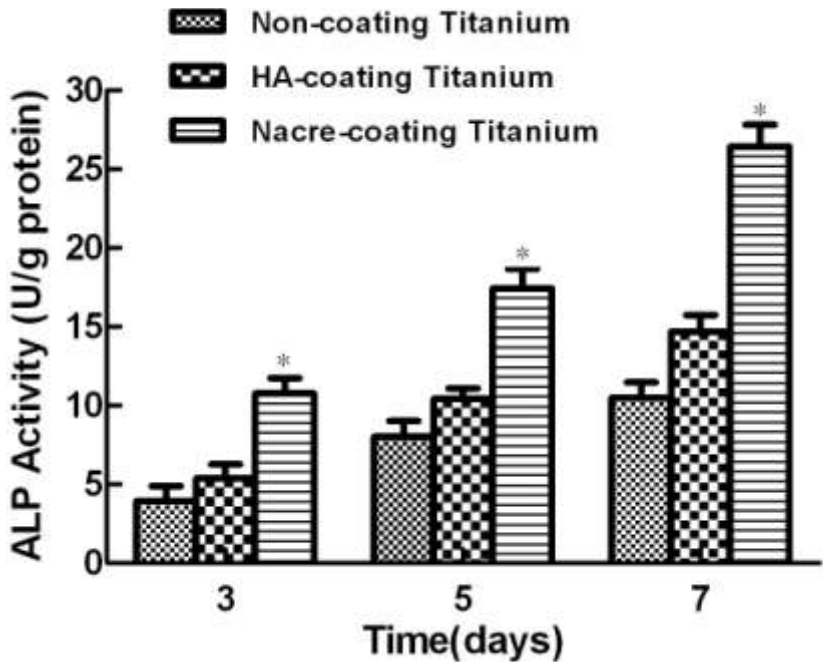

Figure 3. Assay of ALP activities of MG-63 osteoblast-like cells on the substrates. MG-63 osteoblast-like cells were seeded on the substrates at a density of 5 × 104. ALP activities were measured by a commercial ALP Kit for 3, 5, and 7 days post-seeding. Nacreous group had a significantly higher ALP activity than that of other coatings on 3, 5 and 7 days post-seeding (* compared with the other 2 groups, $p<0.05$).

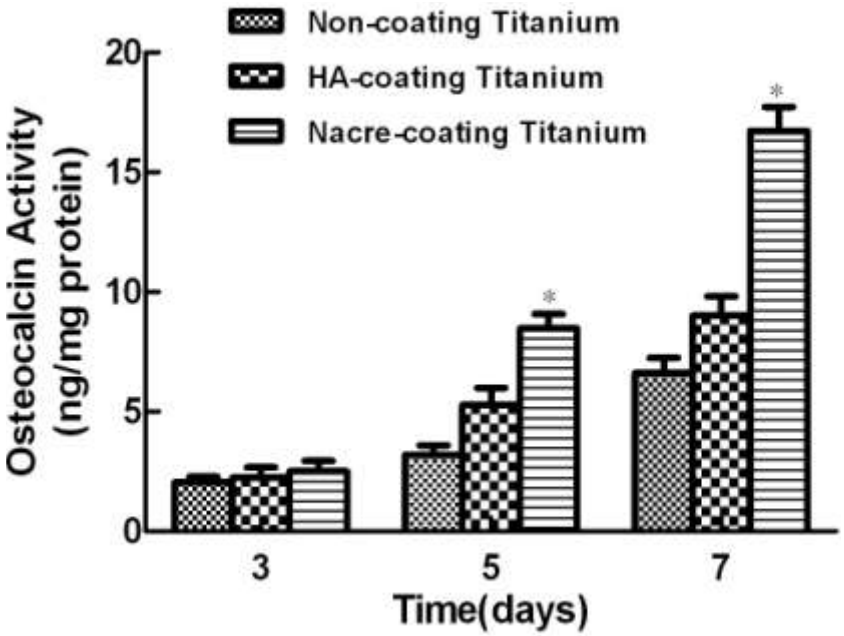

Figure 4. Evaluation of the osteocalcin level of MG-63 osteoblast-like cells on the substrates. MG-63 osteoblast-like cells were seeded on the substrates at a density of 5 × 104. The expression level of osteocalcin in the MG-63 osteoblast-like cell lysates was measured by an enzyme-linked immunoassay for 3, 5, and 7 days post-seeding. Nacreous group had a significantly higher osteocalcin level than that of other coatings on 5 and 7 day post-seeding. (* compared with other 2 groups, $p<0.05$).

influence on the osteoblastic cell morphology. The results of this study also show that nacre has a strong stimulatory effect on cell proliferation, ALP activity, and osteocalcin levels in a time-dependent manner. Cells on the nacreous surface showed greater levels of bioactivity than those seeded into the control surfaces. With increasing cell culture durations, the cell numbers and ALP activity increased significantly on the nacreous surfaces, while the osteocalcin level only increased markedly from the fifth day after seeding. This conformed to our expectation that ALP is a marker of early osteoblastic differentiation, and osteocalcin is a marker of late-stage differentiation when the cells can commence mineralization.

Throughout this study, we confirme the good bio-compatibility of nacre, but it has generally been believed that nacre and bone are not homologous (Westbroek and Marin, 1998). The osteogenic properties of nacre remain unknown. There are several explanations for this novel phenomenon, which require further investigation. Firstly, although, nacre and bone differ in terms of mineral composition and structure, they can both be regarded as biogenic composite materials that contain biological polymers and inorganic crystals. The mineral phase of the human bone is calcium phosphate in the hydroxyapatite form, whereas nacre contains calcium carbonate in the aragonite form. This difference can be attributed to the differences in the environments that surround sea shells and humans (Ni and Ratner, 2003). Previous studies (Atlan et al., 1997) have shown that the calcium carbonate crystals of nacre were surrounded by an organic matrix (mostly protein and polysaccharide), which represents approximately 1 to 5% of the nacre composition by weight. The organic matrix is commonly classified into soluble and insoluble components. Many research groups (Lopez et al., 1992; Silve et al., 1992; Atlan et al., 1997; Lamghari et al., 1999) have suggested that the nacre matrix contains chemical signals that can be released in a physiological medium, but this hypothesis could not be proved. In the study conducted by Mouries et al. (2002), four fractions designated as SE1 to SE4 were collected from the elute of the water soluble matrix (WSM) of nacre, after which size-exclusion high performance liquid chromatography (HPLC) was performed. Their molecular weights ranged from 115 to 150 kDa, 80 kDa, 65 kDa and 35 to 45 kDa, respectively, as estimated by column calibration. They also appeared to stimulate both the proliferation and ALP activity of bone marrow stem cells. This was consistent with the results in this study. Therefore, we deduce that the WSM of nacre has functions that are similar to bone morphogenetic protein (BMP) or transforming growth factor-beta (TGFβ), both of which induce bone growth. The results obtained in our study illustrate that a variety of mechanisms for osteogenesis are combined in this process (Zhu et al., 2008).

Conclusions

The present *in vitro* study showed that the nacre coating on the surface of dental implants had a beneficial effect on the proliferation and differentiation of osteoblastic cells. In comparison with HA-coated and uncoated surfaces, the nacreous surfaces induced higher levels of ALP (a specific marker involved in early osteoblast differentiation) and osteocalcin (a marker of late osteoblast differentiation). The results of this study are indicative of the osteogenic capacities of nacreous coatings. However, further studies of the molecular mechanisms of osteogenesis induced by nacre, as well as *in vivo* animal studies will be necessary to provide conclusive evidence for the bioactivity of nacreous coatings.

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