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Proteomics Study of the Osteoblast Cells Proliferated on Nanostructured Hydroxyapatite Coatings

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Abstract. A big variety of bioceramics have been successfully utilized as implant materials for promoting fixation of bony tissues. Different bioceramics exhibited markedly different proliferation rates of the osteoblast cells *in vitro*. Clarification of the mechanism about the attachment and proliferation/differentiation of the cells would contribute to selecting suitable biomaterials for hard tissue replacement. Proteomics study was performed in this study employing the 2-dimensional electrophoresis assay with an aim of recognizing the changes in proteins. Nanostructured hydroxyapatite (HA) coatings have been fabricated and they have shown promising mechanical performances. Results showed that the nanostructured HA coatings promoted proliferation of the osteoblast cells. Alkaline phosphatase (ALP) assay revealed an increased ALP activity of the proliferated viable cells, and obviously the presence of the nanosized pores can enhance the anchoring and stretching of the cells. No obvious difference in the 2-D gel maps taken for the cells proliferated on the HA coating and for control can be found. This in turn suggests that the nanostructured HA coating induces minor changes in proteins of the cells.

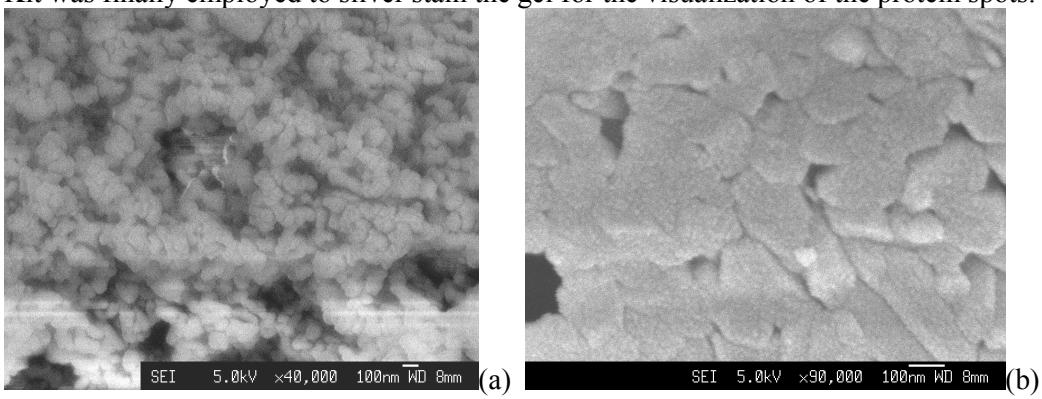
Introduction

Biomedical application of hydroxyapatite (HA) coatings has been successful for many years. The major requirement for these coatings is favorable bioactivity and sufficient mechanical properties. In recent years, nanostructured coatings attracted intense interest due to their enhanced mechanical properties [1,2]. Nanostructures within a biocompatible coating may give rise to enhanced osteoblast adhesion on it. Attachment and growth/proliferation of osteoblast cells are the essential evidences showing the biocompetitivity of the bioceramic coatings. It has been reviewed that a number of proteins played important roles in mediating the proliferation/differentiation of the osteoblasts and their content changed with the proliferation/differentiation process [3]. Therefore, the study of the proteins of the proliferated cells might be able to offer further insight into the mechanism if HA induces altered apoptosis or cell cycle. This study employed the 2-D electrophoresis assay for studying the proteins extracted from the cells proliferated on the nanostructured HA coatings. The osteoblast behaviors of the cells on the coatings were also discussed.

Materials and Methods

The nanostructured HA coatings have been fabricated using HVOF process [4]. The nanostructures of the coatings were characterized using field emission scanning electron microscopy (FESEM, JEOL JSM-6340F). For the coatings investigated, there is clear evidence of the presence of nanostructures at both their surface and cross-sections (~50-100nm). However, it was noted that one type of the nanostructured coating showed presence of rod-like grains, which resulted from the unmelted part of the starting feedstock during the spraying. Typical spherical nanostructural features and rod-shaped grains of the coatings are shown in Fig. 1. The *in vitro* cell culture work was conducted using the hFOB 1.19 human cell line. The samples were incubated in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 0.5% antibiotics. Cells were cultured in an atmosphere of 100% humidity and 5% CO_2 at 37°C. Media were changed at 48 hrs intervals. The methyl thiazole tetrazodium (MTT) assay was employed to examine the proliferation of the cells. For the MTT test, the coating samples, which have the dimension of 10mm×10mm in width and length respectively, were incubated in 24-well plate. The sterilized coating samples were

incubated in 1ml media with 5×10^4 cells. The plates were read using 490 nm wavelength on a microplate reader machine (Benchmark Plus, Bio-Rad Laboratories Inc.). Every plate was read for 3 times and each type of the coatings had 3 samples. Morphology observation of the cells attached on the coating surface was conducted using scanning electron microscopy (SEM, JSM-5600LV). Fixation of the cells was conducted using the standard glutaraldehyde/osmium tetroxidein protocol. Alkaline phosphatase (ALP) activity of the osteoblasts cultivated on the nanostructured bioceramic coatings was determined using the Sigma ALP kit (Sigma AP-F). The plate coating samples were cultured in the medium with a cell density of 5×10^4 cells/ml. A medium with no secreted ALP was prepared as the negative control to indicate the background level. A positive control was tested using the provided control enzyme (diluted) with the kit (Sigma). Fluorescence was measured immediately (excitation 360 nm, emission 440 nm). For the 2-dimentional gel experiment, the osteoblast cells (1×10^6 cells/ml) were seeded onto the nanostructured coatings in 10cm diameter culture dish with 15 ml DMEM. After 3 days incubation, the cells cultured on the nanostructured coatings were rinsed with PBS and transferred to a fresh culture dish. This was followed by trypsinization to collect the cells proliferated on them which ensured that the cells collected are entirely from the coating surfaces. Control cells were directly collected from the passaged osteoblast cells. The control and treated cells pelleted by centrifugation (2000 rpm, 5 mins, and 4^{o}C) were then washed for 4 times using FBS-free DMEM to remove potentially interfering bovine serum albumin. Total proteins extracts were prepared from the cells using lysis buffer (7M Urea, 2M Thiourea, 4% CHAPS, 10mM Tris) supplemented with proteinase inhibitor (Roche Complete Proteinase Inhibitor mix) and nuclease mix (Amersham Biosciences). Prior to Isoelectric Focusing (IEF), the protein concentration of each sample was quantified using the Amersham's 2-D Quant Kit. 80µg of total proteins were used for analytical gels. Immobilized pH gradient (IPG) gel strips pH3-10NL (Amersham Biosciences) were rehydrated with rehydration buffer (7M Urea, 2M Thiourea, 4% CHAPS, trace Bromophenol Blue) supplemented with 20mM DTT and 0.5% IPG buffer. Following rehydration, IEF was conducted by cuploading of the appropriate amount of total protein lysates supplemented with 20mM DTT and 0.5% IPG buffer. Prior to the 2nd dimension SDS-page, each IPG strip was equilibrated with equilibration buffer (50mM TrisCl pH8.8, 6M Urea, 30% Glycerol, 2% SDS, trace Bromophenol Blue) supplemented with 1% w/v Dithiothreitol (DTT) for 15 min followed by 2.5% w/v Iodoacetamide (IAA) for 15 min. The 2nd Dimension SDS-PAGE was conducted with Amersham's DaltSix System at constant 10W per gel for ~5 hr until dye front reached 0.5 cm uniformly from the base. Amersham PlusOne Silver Staining Kit was finally employed to silver stain the gel for the visualization of the protein spots.



(a) (b)

Fig. 1 Typical FESEM pictures of the nanostructured HA coatings at their surfaces showing nanosized grains of ~50-100nm, (a) spherical shaped grains, (b) rod-like grains.

Results and Discussion

Typical morphology of the cells attached/proliferated on the coating surfaces is shown in Fig. 2(a,b) (the coatings were surface-polished for getting clear cell images). It is clear that after the 2 days incubation, the cells attach and proliferate very well on both the nanostructured surfaces. There is no doubt that the well-stretched morphology refers to fast proliferation of the cells. MTT assay further gives the evidence of different proliferation rates of the cells depending on the coatings (Fig. 3a). The coating with more rod-like grains showed higher MTT value, hence the more proliferated cells (the ratio of absorbance is proportional to the cell number). The results might indicate that, compared to the spherical grains, the rod-like nanosized grains would be more capable of promoting attachment and proliferation of the cells. ALP assay suggests that the cells proliferated on the coatings are viable (Fig. 3b). It is noted that the cells proliferated on both the coatings are viable and there is no significant ALP activity difference among the coatings. In addition, the HA coatings showed clear proof of the dissolution. EDX analysis has clearly proven that the exposed

structure is nanostructured HA. Some researchers have reported that dissolution of Ca and low HA crystallinity triggered promoted absorption of certain proteins, which in turn promotes cell attachment [5,6]. And certain proteins in the media, e.g., albumin, can promote the dissolution of Ca ions at early incubation stages [7]. The dissolution of the HA coating might contribute to the enhanced proliferation of the cell. In addition, the nanostructures may provide dense surfaces, which in turn decreases the surface energy. Whilst the reduced surface energy enhances initial attachment and spreading of the cells and improve their attachment [8]. Furthermore, the present study has shown that the cells are capable of growing into the coatings through the pores inside the coatings. It nevertheless indicates that the cells recognize the micropores/cavities with the size of up to 10μm (Fig. 2c). The nanostructured coatings supply more particle/grain boundaries, which were found to be the sites where the osteoblast adhesion occurred preferentially [9]. It is clear that the proliferation of the cells is accomplished following the stretching, whilst the fibrous roots are in nanosize. Obviously, the presence of the nanosized pores can benefit the anchoring of the fibers, and in turn promotes the stretching of the cells.

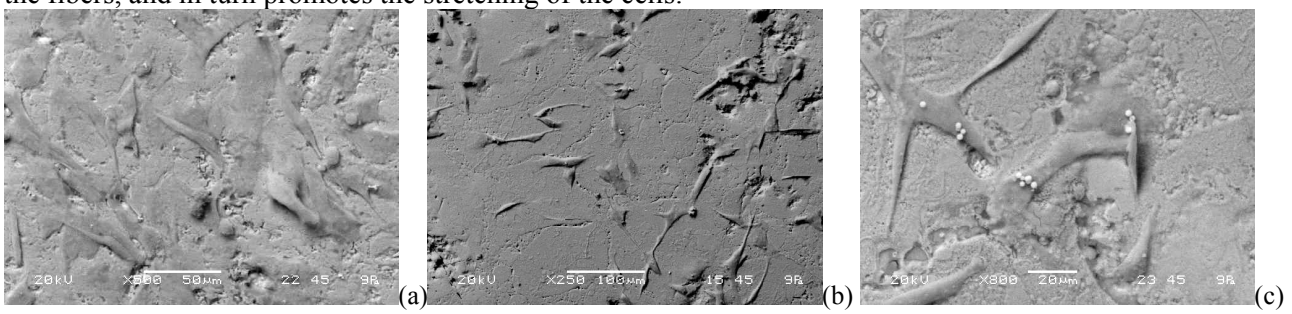


Fig. 2 Typical SEM morphology of the osteoblast cells proliferated on the nanostructured HA coatings, (a) coating with more rod-like nanosized grains, (b) coating with spherical nanosized grains, (c) anchoring and stretching of the cells by the nanosized pores.

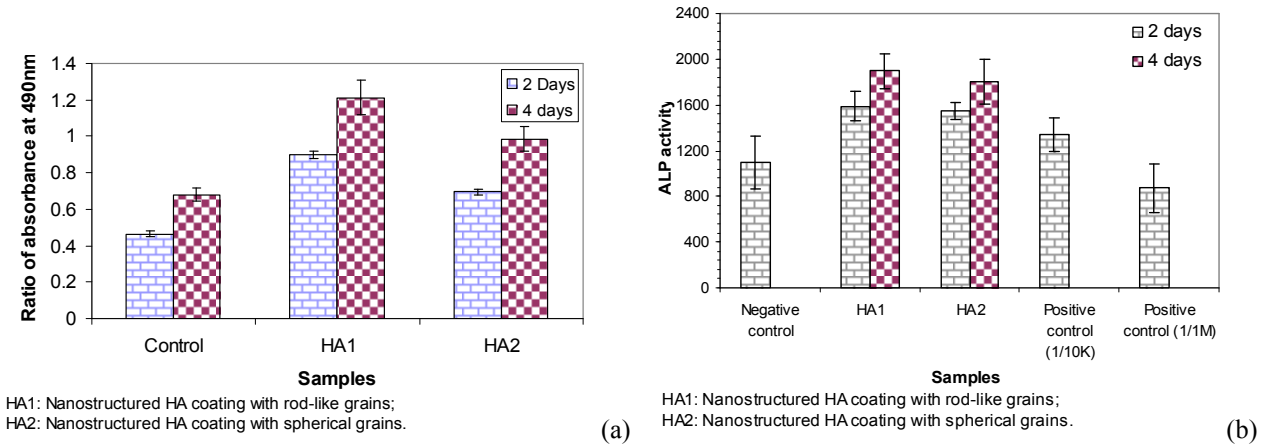


Fig. 3 (a) MTT results and (b) ALP activity of the cells proliferated on the nanostructured HA.

The proteomic study through the 2-D gel electrophoresis assay provides the information about the difference in the proteins of the cells proliferated on the nanostructured coatings (Fig. 4). It is noted that compared to the gel map for the control cells (Fig. 4a), the map for the cells proliferated on the HA coating exhibits minor differences (Fig. 4b). The cells proliferated on the HA coating only showed slight intensity differences for some spots, no remarkable changes in the protein map. As reported, the difference in the intensity of the spots suggests the quantitative changes of the proteins [10]. The enhancing effect on the proliferation of the cells brought about by the nanostructured coatings might be achieved by either their nanostructural features or their intrinsic biomimetic properties, such as their bioactive surface chemistry which was believed to be the dominant factor influencing viable cell adhesion and subsequent cytoskeletal formation [11]. The altered intensity of certain protein spots suggests enhanced/deteriorated protein expressions. While those proteins might influence apoptosis and/or cell cycle of the osteoblast cells. Nevertheless, the present study gives similar results as those reported that the nanostructured HA achieved improved cell proliferation and ALP activity [12], probably due to the nanostrcutured grains and/or nanosized pores. Furthermore, the nanostructures at the coating surface may improve the cell adhesion upon the incubation, as has been pointed out that initial contact of cells with underlying surface may influence osteoblast functions and possibly, bone regeneration and implant osteointegration *in vivo*. Early cell spreading may be an indicator of further

expression of osteoblast phenotype and may be important for application of osteogenic cells in reconstructive surgery [13]. It is very likely that the nanostructured pores are capable of enhancing cell adhesion. The effect of the specific proteins with changed intensity on the apoptosis/cell cycle is yet to be clarified. Refined 2-D gel experiment is also to be carried out for the cells to make detailed quantitative analysis possible.

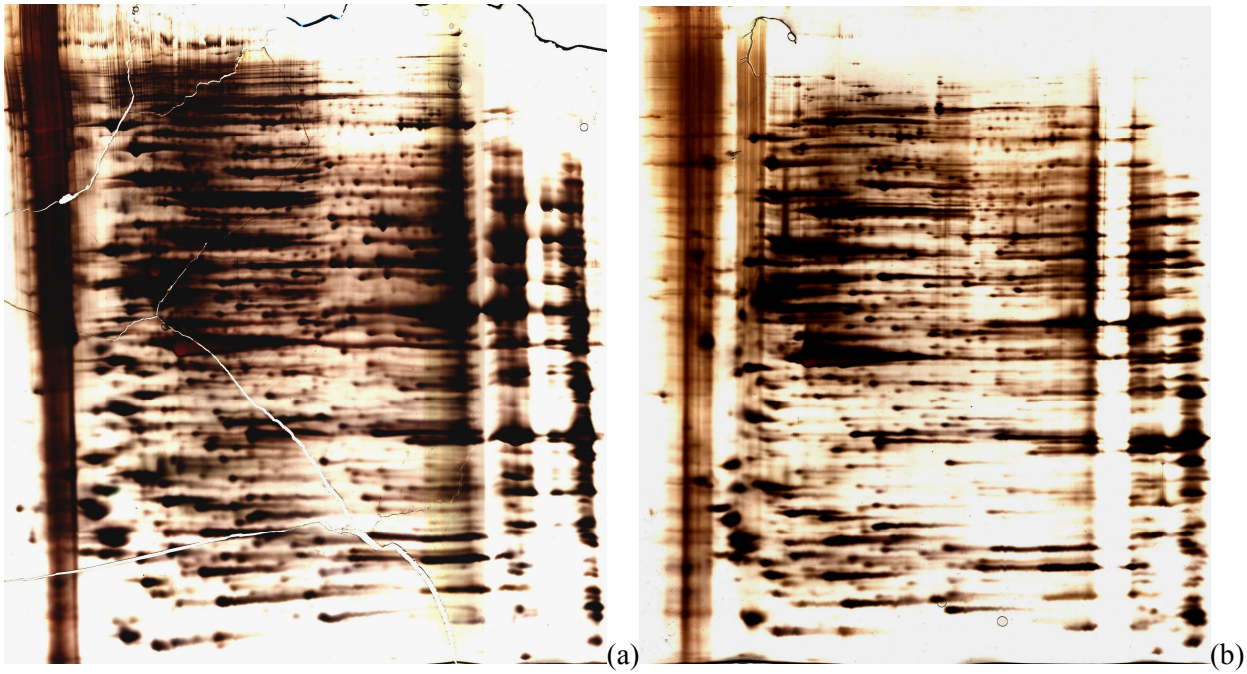

(a) (b)

Fig. 4 2-D gel protein map of the (a) control cells and (b) cells proliferated on the nanostructured HA coatings showing similar spots (proteins) pattern.

Conclusions

Nanostructured HA coatings with 50-100nm grains are capable of promoting attachment and proliferation of the viable osteoblast cells upon incubation *in vitro*. The cells proliferated on the HA coatings showed no obvious differences in extracted proteins compared to the control cells, which suggests the appropriate application of the nanostructured HA as bone mineral material. The 2-D gel electrophoresis approach was effective in identifying the proteins and useful to study the influence of the matrix materials on the proliferated cells. The nanosized pores instead of the nanosized grains might play the key roles in enhancing the attachment/proliferation of the osteoblast cells. Furthermore, it was found that the rod-like nanosized grains triggered attachment/proliferation of more cells than the spherical nanosized grains.

References

1. Y.C. Zhu, K. Yukimura, C.X. Ding, P.Y. Zhang, Thin Solid Films, 388:277-282 (2001).
2. C.C. Berndt, editor: J. Therm. Spray Technol., Vol.10(2001), p.147.
3. G.S. Stein, J.B. Lian: Endocrine Reviews Vol.14(1993), p.424.
4. H. Li, K.A. Khor, Surf. Coat. Technol., in press (2006).
5. T. Boix, J. Gómez-Morales, J. Torrent-Burgués, et l: J. Inorganic Biochem., Vol.99 (2005), p.1043.
6. Y. Yang, D. Dennison, J.L. Ong, Int. J. Oral Maxillofac: Implants, Vol.20(2005), p.187.
7. S.A. Bender, J.D. Bumgardner, M.D. Roach, K. Bessho, J.L. Ong: Biomaterials, Vol.21(2000), p.299.
8. S.A. Redey, M. Nardin, D. Bernache-Assolant, C. Rey, P. Delannoy, L. Sedel, P.J. Marie: J. Biomed. Mater. Res., Vol.50(2000), p.353.
9. T.J. Webster, J.U. Ejiofor: Biomaterials, Vol.25(2004), p.4731.
10. T. Matsumoto, M. Okazaki, M. Inoue, S. Yamaguchi, T. Kusunose, T. Toyonaga, Y. Hamada, J. Takahashi: Biomaterials, Vol.25(2004), p.3807.
11. J.M. Rice, J.A. Hunt, J.A. Gallagher, P. Hanarp, D.S. Sutherland, J. Gold: Biomaterials, Vol.24(2003), p.4799.
12. H.W. Kim, H.E. Kim, V. Salih: Biomaterials, Vol.26(2005), p.5221.
13. D. Kudelska-Mazur, M. Lewandowska-Szumiel, M. Mazur, J. Komender: Cell Tissue Bank, Vol.6(2005), p.55.