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Development of Scaffolds for Tissue Engineering Using Single-crystal Apatite Fibres and Their Biological Evaluation by Osteoblastic Cell

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Single-crystal apatite fibres were synthesized from aqueous solutions in the $Ca(NO_3)_2$-$(NH_4)_2HPO_4$-HNO_3 systems by a homogeneous precipitation method using urea. The resulting fibres with long-axis sizes of 60-100 μm were composed of carbonate-containing apatite with preferred orientation along the c-axis. We have developed porous scaffold for tissue engineering of bone using the single-crystal apatite fibres. The scaffolds have an apatite single phase, high porosities of ~90% and high absorption coefficients of ~400%. In order to clarify cellular responses of the scaffolds, the biological evaluations were performed using osteoblastic cells (MC3T3-E1). The cells cultured on the scaffolds showed excellent cellular responses, such as good cell proliferation and enhanced differentiation into osteoblasts. We conclude that such scaffolds with high porosity may be effective as a matrix of tissue engineered structures for promoting differentiation of osteoblasts.

Keywords: Hydroxyapatite, Single-crystal fibre, Homogeneous precipitation method, Tissue engineering, Scaffold, *In vitro* evaluation, Osteoblast

1. INTRODUCTION

Hydroxyapatite (HAp) has been widely applied as a biomaterial [1] and as an adsorbent for chromatography [2]. By controlling the morphology of HAp crystals, novel properties may be produced by enabling controlled orientation of the crystal planes, as HAp crystal has two crystal planes with different charges: positive on a(b)-planes and negative on c-planes [3].

We have successfully synthesized hydroxyapatite (HAp) fibre with long axis size of 60-100 μm [4,5]. It was confirmed from the results of a high-resolution transmission electron microscopy (HR-TEM) using a shadow imaging technique that the apatite fibres were of single crystals with the c-axis orientation parallel to the long axis of the fibre. Selected area electron diffractions (SAED) were performed at five points along long-axis of apatite fibre. The diffraction pattern from five points showed clear spots corresponding to an apatite structure with high crystallinity. As five diffraction patterns showed the same geometry along to long-axis of the fibre, we have concluded that the apatite fibres were not polycrystalline, but single crystal. The HR-TEM observations combined with the XRD results suggest that the single crystal apatite fibres may grow along the c-axis to develop the a(b)-plane of hexagonal HAp [6]. Thus, the present apatite fibres may have a positive charge on the surface.

Using the above fibres, we have promoted the development of i) porous HAp ceramics with well-controlled pore sizes [7] and ii) HAp/polymer hybrids possessing mechanical properties similar to those of living cortical bone by *in situ* bulk polymerisation of the monomer in the pores of the ceramic [8]. This hybrid, with mechanical properties similar to those of cortical bone, has been shown to have excellent biocompatibility both *in vitro* and *in vivo* [9,10].

Tissue engineering is an important technology that encourages regeneration of the defecting tissue utilising scaffolds, cells and growth factors. In the case of tissue engineering for bone, porous calcium-phosphate ceramics are generally used as scaffolds, together with bone marrow cells and rh-BMP-2 or TGF-β growth factors, as reported by Ogushi and co-workers in detail [11]. We have also tried development of novel scaffolds for tissue engineering of bone using the above-mentioned apatite fibres [12-14].

In the present investigation, we describe the development of novel scaffolds for 3D cell culture utilizing properties of apatite fibres: fibre-shape and a positive charge on the surface, and biological evaluations of the resulting apatite fibre scaffolds using osteoblastic cell (MC3T3-E1).

2. EXPERIMENTAL METHODS

2.1 Fabrication of apatite fibre scaffold and its characterisation

The HAp fibres were prepared from aqueous solutions in the $Ca(NO_3)_2$-$(NH_4)_2HPO_4$-$(NH_2)_2CO$-HNO_3 systems through a homogeneous precipitation method using urea, as previously reported [7,12].

The HAp fibres were suspended with pure water to prepare the HAp slurry with amounts of 1.8 mass%. The green sheets were fabricated by pouring and vacuum pumping the HAp slurry (5 cm^3) into the vinyl-chloride mould with an internal diameter of 16.5 mm. Hereafter, this green sheet is defined as "AFS". The resulting sheets (AFS) were fired at 1200°C for 1 h in an air atmosphere to develop the structure of the scaffold (1200AFS). As a control, dense HAp ceramics were also fabricated by firing at 1200°C for 1 h using crushed apatite fibres; hereafter, it is defined as "1200CAFC".

The above products were characterised as follows: i) phase identification by X-ray diffractometry (XRD) and Fourier transform infrared spectroscopy (FT-IR), ii) observation of microstructure by scanning electron microscopy (SEM), iii) examination of pore size distribution by mercury porosimetry, iv) measurement of porosity on the basis of the mass and dimension of the scaffolds, and v) absorption coefficient.

2.2 Biological evaluation of apatite fibre scaffold using osteoblastic cells

The resulting scaffolds were biologically evaluated using osteoblastic cells, MC3T3-E1 [15]. The cellular responses to three kinds of samples, AFS, 1200AFS and 1200CAFC, were examined by observing the cell proliferation, the cell morphology and differentiation of the cells. Cell differentiation was examined by determinng an alkaline phosphatase (ALP) activity normalized for DNA content of the cultured cell on the resulting scaffolds. ALP assays were measured using a kit of Wako chemicals based on the Bessey-Lowry method and DNA contents were determined using a Hoechst 33258 method. Polystyrene plate for cell culture was used as a control. Cells of 5 x 10^5 were seeded on each scaffold and cultured for the desired period of time. The medium used was α-MEM with 10% FBS (Gibco BRL); culture conditions were at 37°C in a 5% CO_2 atmosphere.

3. RESULTS AND DISCUSSION

3.1 Fabrication of the apatite fibre scaffolds and their characterization

At first, we briefly describe characterization of apatite fibres that were used to fabricate the AFS, 1200AFS and 1200CAFC. Figure 1 shows an XRD pattern of the used apatite fibres. The (100), (200) and (300) reflections of the apatite fibres were more intense than those of a typical HAp listed in JCPDS card #9-432; however, the XRD pattern of crushed fibres was more similar to that of typical HAp. The FT-IR results showed that the fibres were composed of carbonate-containing apatite (CHAp) of type AB, in which carbonate ions (CO_3^{2-}) are substituted into both the PO_4^{3-} and OH^- sites in the HAp structure. The CHAp of type A is defined that the CO_3^{2-} ions substitute for the OH^- group in the HAp. On the other hands, the CHAp of type B is defined

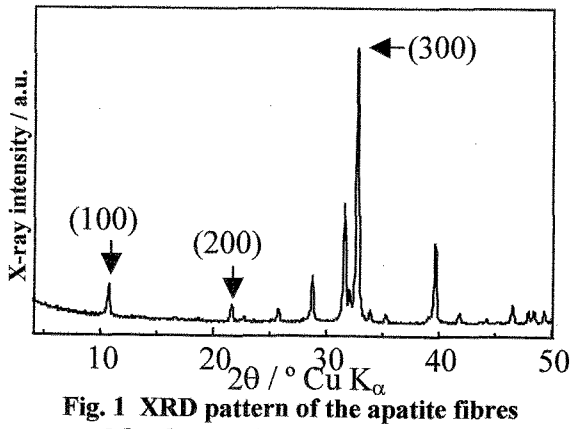

Fig. 1 XRD pattern of the apatite fibres used for fabricating the scaffolds.

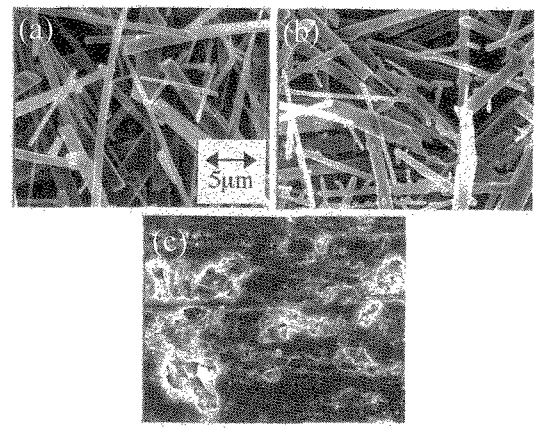

Fig.2 Microstructure of the apatite fibre scaffolds. (a) AFS, (b) 1200AFS, and (c) 1200CAFC

that the CO_3^{2-} ions substitute for the OH^- group in the HAp. The results of X-ray fluorescence spectrometry indicated that the contents of CaO and P_2O_5 were 53.91 mass% and 40.15 mass%, respectively. The Ca/P ratio of the apatite fibres was determined to be 1.69. In addition, the SEM observation showed that the apatite fibres were ~60 - ~100 μm along the long axis.

Table I gives some properties of three kinds of the scaffolds, AFS, 1200AFS and 1200CAFC, derived from apatite fibres. Single apatite phase was present in all the resulting scaffolds, although AFS was composed of carbonate-containing apatite (Type AB) because it was fabricated without firing. The apatite phase in AFS and 1200AFS had preferred orientation in the (h00) planes: (100), (200) and (300). The porosity and absorption coefficient of the AFS or 1200AFS were ~90% and ~400%, respectively. On the other hand, the 1200CAFC derived from the crushed apatite fibres showed the lower porosity (22%) and absorption coefficient (10%) than AFS or 1200AFS. Figure 2 shows the microstructures of the AFS (a), 1200AFS (b) and 1200CAFC (c). The AFS and 1200AFS derived from apatite fibres were porous structure with intertwining individual fibres, while the 1200CAFC derived from crushed apatite fibres was dense structure. According to results of pore size distribution by mercury porosimetry, the median pore size of AFS was about 5 μm.

Table I Some properties of the apatite fibre scaffolds

Sample	Phase	Porosity / %	Absorption coefficient / %
AFS	CO_3HAp type AB	90	395
1200 AFS	HAp	91	380
1200 CAFC	HAp	22	10

3.2 Biological evaluation of apatite fibre scaffolds using MC3T3-E1 cells

We next examined a cellular response to the AFS, 1200AFS and 1200CAFC, together with cell culture plate (polystyrene) as a control. Figure 3 shows the

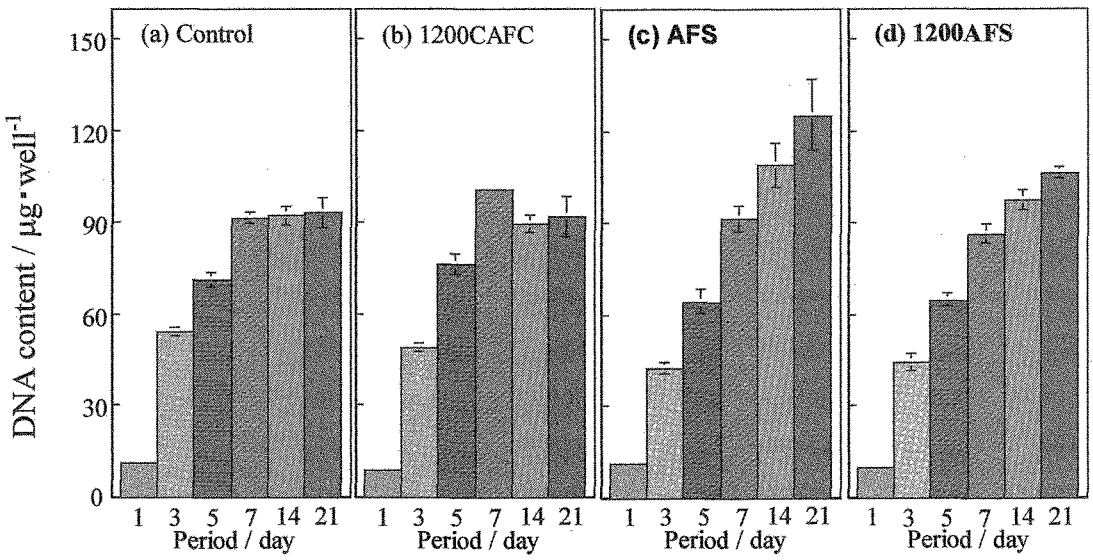

Fig. 3 Proliferation of cells cultured on the apatite fibre scaffolds: (a) Control, (b) 1200CAFC, (c) AFS and (d) 1200AFS.

results for proliferation of the cells seeded on four kinds of samples. Cell proliferation was defined by determining the DNA contents produced from cells via a Hoechst 33258 method. About 2 x 10^4 of cells correspond to 1 μg of DNA. The cells on AFS and 1200AFS proliferated in a similar manner to those on the 1200CAFC and the control during the incubating periods from 1 day up to 7 days. However, the cells on the AFS and 1200AFS proliferated more than those on the Control and 1200CAFC after the longer incubating periods of 7 days up to 21 days. The error bars in Fig.3 are based on a standard deviation of 10 samples. The present good cell proliferation may be due to the 3D structure of the AFS and 1200AFS.

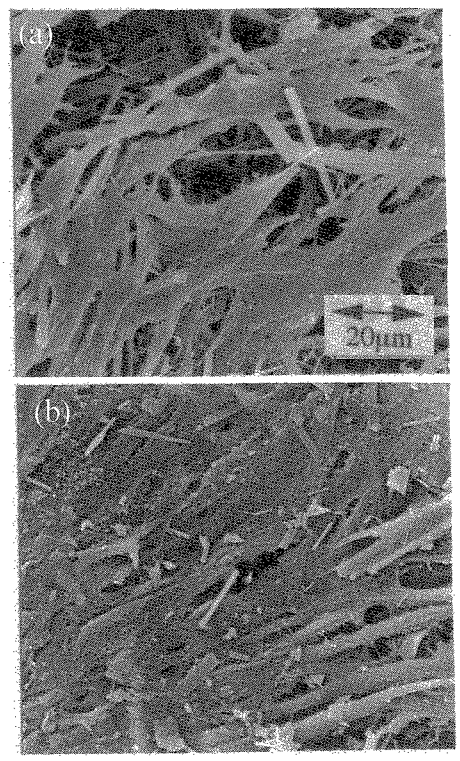

Fig. 4 Morphologies of cells cultured on the AFS for (a) 7 and (b) 14 days.

Figure 4 shows the SEM observation of the cells cultured on the AFS for 7 (a) and 14 days (b). The cells in 7 days attached on the AFS to spread with forming pseudopodium and then the cells further proliferated to be confluent after 14 days.

Figure 5 shows the ALP activity normalized for the DNA content of the cells cultured on the four samples for 7, 14 and 21 days. The ALP activity normalized for DNA content is an indication of differentiation of MC3T3-E1 cells into osteoblast. The error bars in Fig. 5 are based on a standard deviation of 10 samples. In the four samples, the ALP activity increased with incubating periods. For 7, 14 and 21 days, the ALP activities of the AFS and 1200AFS showed higher values than those of the 1200CAFC and the control. Although the difference in the results for 7 days was not regarded as significant ($P>0.05$) by ANOVA, those of 14 and 21 days could be regarded as significant ($P<0.01$). The ALP activity of cells cultured on the 3D-structured matrix, i.e., AFS and 1200AFS, is significantly higher than that on the 2D-structured one, i.e., 1200CAFC and the control. Especially, the ALP activity of the AFS is higher than that of 1200AFS. These results show that the AFS and 1200AFS have an excellent differentiation-inducing ability into osteoblast.

Based on the above findings for cell proliferation and differentiation, we can conclude that the present apatite fibre scaffold has excellent cellular responses and biocompatibility. This may be due to the following reasons: (i) presence of a(b)-plane developed by c-axis orientation of apatite fibres, (ii) carbonate-containing apatite, and (iii) 3D-structured apatite fibre scaffold. We have a plan to perform a further investigation..

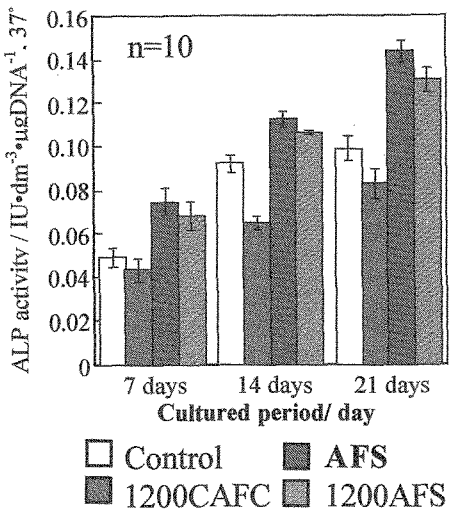

Fig.5 ALP activity normalized for DNA content of cells cultured on the apatite fibre scaffolds.

4. CONCLUSIONS

Porous apatite fibre scaffolds for tissue engineering of bone were developed using the single-crystal apatite fibres. The scaffolds have an apatite single phase, high porosities of ~90% and high absorption coefficients of ~400%. The present apatite fibre scaffolds have an excellent cellular response, such as good cell proliferation and enhanced differentiation into osteoblasts. We conclude that such scaffolds with high porosity may be effective as a matrix of tissue engineered structures for promoting differentiation of osteoblasts.

REFERENCES

[1] L. L. Hench, *J. Am Ceram. Soc.*, **81**, 1705-28 (1998).
[2] K. Ota, H. Monma and T. Kawasaki, *Inorg. Mater.*, **6**, 224-30 (1999).
[3] T. Kawasaki, *J. Chromatogr.*, **544**, 147-84 (1991).
[4] M. Kinoshita, A. Kishioka, H. Hayashi and K. Itatani, *Gypsum & Lime*, No. 219, 23-29 (1989).
[5] M. Aizawa, T. Terado, F. S. Howell and K. Itatani, *Mater. Res. Bull.*, **34**, 1215-25 (1999).
[6] M. Aizawa, A. E. Porter, S. M. Best and W. Bonfield, *Key Engineering Materials (Bioceramics 15)*, **240-242**, 509-512 (2003).
[7] M. Aizawa, F. S. Howell, K. Itatani, Y. Yokogawa, K. Nishizawa, M. Toriyama and T. Kameyama, *J. Ceram. Soc. Jpn.*, **108**, 249-253 (2000).
[8] M. Aizawa, Y. Tsuchiya, K. Itatani, H. Suemasu, A. Nozue and I. Okada, *Bioceramics*, **12**, 453-456 (1999).
[9] M. Aizawa, M. Ito, K. Itatani, H. Suemasu, A. Nozue, I. Okada, M. Matsumoto, M. Ishikawa, H. Matsumoto and Y. Toyama, *Key Engineering Materials (Bioceramics 14)*, **218-220**, 465-468 (2002).
[10] M. Aizawa, M. Ito, K. Itatani, I. Okada and M. Matsumoto, *Phosphorus letters*, (2003), in press.
[11] H. Ogushi, *J. Jpn. Soc. Biomater.*, **20**, 296-304 (2002).
[12] M. Aizawa, H. Ueno, K. Itatani, *Material Integration*, **12**, 75-77 (1999).
[13] M. Aizawa, (2001) *Japan patent*: tokugann 2001-300791.
[14] M. Aizawa, H. Hiroki, H. Uchida, K. Itatani, I. Okada, M. Matsumoto, H. Morisue, H. Matsumoto, Y. Toyama, *Key Engineering Materials (Bioceramics 15)*, **240-242**, 647-650 (2003).
[15] H. Sudo, H. Kodama, Y. Amagai, S. Yamamoto and S. Kasai, *J. Cell Biol.*, **96**, 191 (1983).

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