THE ROLE OF NIOBIUM IONS IN CALCIUM PHOSPHATE INVERT GLASSES FOR BONE REGENERATION

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Abstract: Phosphate invert glasses (PIGs) containing niobium were prepared by a conventional melt-quenching method and the structures and chemical durability of the glasses and osteoblast-like cell responses to them were examined. Raman and solid state ³¹P NMR spectra of the glasses demonstrated that two types of niobate groups, NbO₄ and NbO₆ units, were contained in the glasses and prepared to combine to pyrophosphate structure than orthophosphate one. The chemical durability in Tris-HCl solution was increased with the increase in the niobium content in the glasses, since niobium replaced to phosphorus and contributed to the formation of P-O-Nb and Nb-O-Nb bonds, which are stronger than P-O-P bond. The niobium ions released from the glasses enhanced the differentiation and mineralization of the cells, rather than the initial adhesion and proliferation. The upregulation of these cell functions by the niobium ions possessed dose-dependence; the medium containing 1 x 10⁻⁷ M of the ions exhibited the highest levels of the cell functions.

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INTRODUCTION

Since 45S5-type bioactive glass (Bioglass[®]) was developed for the use in medical and dental fields, many types of bioactive glasses have been developed. Most of them has silicate or phosphate systems. They have been applied to bone fillers, tooth fracture treatment, bone tissue engineering scaffolds and bioactive coatings for bioinert metallic materials.¹⁻⁸ Recently, several kinds of inorganic ions released from bioactive glasses, such as calcium, phosphate, silicate, magnesium, zinc *etc.*, have been reported to influence osteogenic cell functions.⁹ In particular, a trace amount of silicate ions enhance proliferation, differentiation and mineralization of osteoblasts. Xynos *et al.* found that insulin-like growth factor type II (IGF-II) of human osteoblasts was upregulated when 16.6 µg/ml of Si and 88.35 µg/ml of Ca were provided from 45S5-type bioactive glass, which enhanced the proliferation of the cells.¹⁰ Thus, to provide the ability of releasing such ions to biomaterials is one of the effective approaches for achievement of rapid bone regeneration in body. In particular, it must be useful for developing bioactive glasses with high performance, because most of glasses offer flexibility in the chemical components comprising the glasses.

Phosphate invert glasses (PIGs) are soluble and possess a neutral pH in aqueous solutions. Kasuga *et al.* developed PIGs with 60CaO-30P₂O₅-7Na₂O-3TiO₂ system (PIG-3Ti) and revealed its bioactivity of PIG-3Ti with *in vivo* and *in vitro* tests.¹¹⁻¹⁵ The PIG-3Ti coating layer on a titanium alloy was covered with a new bone tissue in 4 weeks after implantation in a Japanese rabbit. To improve the bioactivity of PIGs, we have focused on doping of niobium ions into the glass system. Niobium ions have been reported to replace phosphorus ions in phosphate glasses and form O-P-O-Nb-O type chains.^{16, 17} Additionally, the ions enhanced human osteoblast functions.¹⁸ Thus, we expected that niobium ions are able to be present homogeneously in PIGs systems, which achieves a continuous release of niobium ions in aqueous solutions in association with dissolution of the glasses and rapid bone tissue regeneration.

In this short review, we provide an overview on the role of niobium ions in PIGs and mouse osteoblast-like cell reactions to the glasses.^{19, 20} The cell tests were carried out using plates of the niobium-containing PIGs and also cell culture media containing their extracts, changing the contents of niobium in the glass systems and the culture media. Dose-dependences of upregulation of the cell

functions by the niobium in or released from the glasses were examined.

MATERIALS AND METHODS

1) Glass preparation

PIGs containing 3 – 10 mol% of Nb_2O_5 were prepared by a conventional melt-quenching method. The nominal compositions of the glasses are shown in Table 1. CaCO_3 , H_3PO_4 , Na_2CO_3 , Nb_2O_5 and TiO_2 (only for the glass as a control material) were used as the precursors. Mixed precursors were melted in an electric furnace at 1400 °C for 0.5 hr.

TABLE 1. Nominal compositions of glasses

code	CaO	P ₂ O ₅	Na ₂ O	Nb ₂ O ₅	TiO ₂
PIG-3Nb	60	30	7	3	0
PIG-5Nb	60	30	5	5	0
PIG-7Nb	60	30	3	7	0
PIG-10Nb	60	30	0	10	0
PIG-10Na	60	30	10	0	0
PIG-3Ti	60	30	7	0	3

2) Characterisation

The structures of the prepared glasses were examined with Raman spectrometer and a magic-angle-spinning nuclear magnetic resonance spectrometer (MAS-NMR), especially the changes in the structure with the changes in the Nb_2O_5 contents.

For ion release measurements, powders with <50 μm in size of each glass were prepared and soaked in Tris-HCl buffer solution with pH=7.4 and cell culture medium, minimum essential medium alpha serum containing 10 % fetal bovine serum. Then the samples were incubation at 37 °C in air for the Tris-HCl buffer solution system and in a humidified atmosphere of 95 % air and 5 % CO_2 for the cell culture medium system, respectively.

The pH value of the cell culture medium after the incubation was measured to know if the glass dissolution induces changes in the pH. Hydrophilicity of the glasses were measured using plates of each glass after polishing with sandpaper and mirror finish.

3) Cell culture tests

Cell culture tests were carried out for both the glass plates and the medium containing the extract of powders of PIG-10Nb to examine cell responses on the glass surfaces and the ions released from the glasses, respectively. The glass plates with dimensions of 4 x 4 mm were placed on 96-well plate and mouse osteoblast-like cells (MC3T3-E1 cell) were seeded on their surfaces. To prepare the medium containing the extract of PIG-10Nb, the powders of the glass were soaked in the cell culture medium, incubated for 5 days, and then removed. The resulting media were diluted in order to adjust the niobium ion concentration to 1×10^{-8} to 1×10^{-5} M (denoted by “Nb 10^{-8} ~ 10^{-5} ”), followed by being used for the cell culture using MC3T3-E1 cells.

RESULTS AND DISCUSSION

Raman spectra of the glasses demonstrated that niobium (niobate) ions are present as NbO_4 and NbO_6 groups in the glasses containing Nb_2O_5 , as shown in Fig.1. A peak around 850 cm^{-1} is assigned to the NbO_4 tetrahedra.²¹ Two peaks around 630 and 890 cm^{-1} correspond to the Nb-O vibrations of an NbO_6 octahedron corner-linked in a three-dimensional network ($\text{NbO}_6\text{-3D}$) and a short Nb-O bond in an NbO_6 octahedron ($\text{NbO}_6\text{-isolated}$), respectively.^{22, 23} A peak around 790 cm^{-1} on only spectra of the glass containing over 7 mol% is also assigned to NbO_6 ;

FIGURE 1. Raman spectra of the glasses. (a) PIG-10Na, (b) PIG-7Nb, (c) PIG-5Nb, (d) PIG-3Nb, and (e) PIG-10Nb. Reprinted with permission from reference 19.

FIGURE 2. Concentrations of (a) Nb, (b) Ca, (c) P, and (d) Na released from the glasses after soaking in Tris-HCl buffer solution. (▲) PIG-10Na, (◆) PIG-3Nb, (■) PIG-5Nb, (●) PIG-7Nb, and (▼) PIG-10Nb. Reprinted with permission from reference 19.

this indicates the NbO_6 octahedron linking into long chains (NbO_6 chain).²⁴ In addition, a peak around 980 cm^{-1} , which appeared on the spectra of only the glasses containing Nb_2O_5 , is suggested to correspond to Nb-O bonding.

Regarding the peaks of phosphate groups, two peaks around 950 and 1040 cm^{-1} of Q^0 and Q^I units in O-P-O bond were found to shift to the lower-energy side with adding Nb_2O_5 to the glasses.^{25, 26} This indicates that sodium ions interacting with PO_2 groups were substituted by niobium ions. This was followed by the results of ^{31}P MAS-NMR. The peak position corresponding to Q^0 unit shifted to higher magnetic field with increasing the Nb_2O_5 content. This is because the electronegativity is 0.9 for sodium and 1.6 for niobium. The results of deconvolution for the Raman spectra with Gaussian functions followed by estimation of peak shift for each peak demonstrated that the portion of O-P-O of Q^0 unit increased with the increase in the Nb_2O_5 content, whereas that of P-O-P decreased. The portion of O-P-O of Q^I unit kept a constant regardless of the Nb_2O_5 content.

On the other hand, for the peaks corresponding to Nb_4O and Nb_6O , the portion of the peaks of NbO_6 isolated unit to Nb-O bonding decreased with the increase in the Nb_2O_5 content, whereas that of NbO_4 tetrahedron bonding increased. The portions of the peaks of NbO_6 -3D and NbO_6 chain possessed almost no changes. Thus, the niobium ions in the glasses were supposed to form NbO_4 as a glass network former, not NbO_6 as a glass network modifier. This suggests that P-O-Nb bonds are formed in the glasses. In addition, the niobium species with different coordination numbers were found to prefer to combine to pyrophosphate structure than orthophosphate one from the results of ^{31}P MAS-NMR.

The P-O-Nb bond formation was followed by the results of the immersion test using Tris-HCl

solution. The ion amount released from the glasses decreased with the increase in the Nb_2O_5 content in the glasses (Fig. 2). This means Nb_2O_5 doping improved the chemical durability of the PIG system. This is because the P-O-Nb and Nb-O-Nb bonds are stronger than P-O-P bond.²¹

The ion release behaviour of the glasses in the cell culture media was different to that in Tris-HCl solution. Original culture medium (medium before immersing the glasses) contains several inorganic ions, including calcium, sodium and phosphate ions, proteins, amino acid, and so on. The differences in the components between the culture media and Tris-HCl buffer solution influenced the ion release behaviour of the glasses. The amounts of niobium and sodium ions released from the glasses increased with their contents in the glass system, whereas the amounts of phosphate and calcium ions eventually

FIGURE 3. ALP activities in MC3T3-E1 cells cultured on the glass plates. Error bar means SD. * $p < 0.05$. Reprinted with permission from reference 20.

reached almost similar levels after 7-d soaking. Niobium (niobate) ions released continuously from the glasses over the immersion period. This might mean that the niobium ions are present homogeneously in the glasses by replacing with phosphate ions in the glass structures.

The adhesion, proliferation, differentiation and mineralization of MC3T3-E1 cells cultured on the glass plate surfaces and in the media containing the extract of PIG-10Nb were examined. The niobium ions on the glass surfaces and in the media were found to enhance the differentiation and mineralization of the cells, rather than their adhesion or proliferation. In addition, the effects of the niobium ions on the cell functions possessed dose-dependence; only the cells cultured on the PIG-3Nb and -7Nb surfaces showed upregulated differentiation ability (Fig. 3) and the medium containing 1×10^{-7} M exhibited higher activity of differentiation and mineralization than the other samples (Fig. 4). Effects of several kinds of inorganic ions, such as silicate and zinc ions, released from biomaterials on osteogenic cell functions also possess dose-dependence.^{27, 28} Nb-containing media derived from NbCl_5 with $> 1 \times 10^{-5}$ M niobium ions were reported to upregulate mineral deposition of normal human osteoblasts, whereas no upregulation was found for the media containing 1×10^{-7} to 10^{-6} M of the ions.¹⁸ In the results of this work, the medium containing only 1×10^{-7} M exhibited enhanced activity of differentiation and mineralization. The differences in the threshold for the upregulation between the two experiments are supposed to be due

to the differences in cell types used for each test, because the effects of silicate ions on osteoblasts changed with the cell types.²⁹ At any rate, the upregulation of the niobium ions in the glasses in the experiment of this work was also found to depend on the ion concentration.

Wettability of material surfaces and pH of the culture media after immersing materials are regarded to influence abilities of cells cultured on materials. These properties in this experiments were confirmed to be almost the same regardless of the glass components. Additionally, the initial adhesion and proliferation of MC3T3-E1 cells for 7 days got no influences by the changes in the glass components. Thus, the niobium ions in the glasses are regarded to influence only the differentiation and mineralization.

SUMMARY

Niobium doped in the calcium phosphate invert glasses influenced their chemical durability and the functions of osteoblast-like cells cultured on their surfaces, especially their differentiation and mineralization. The increase in the chemical durability of the glasses was due to the substitution of niobium for phosphorus and subsequent formation of P-O-Nb and Nb-O-Nb bonds, which are stronger than P-O-P, in the glass structures. Two types of niobate groups, NbO_4 and NbO_6 units, were contained in the glasses and prepared to combine to pyrophosphate structure than orthophosphate one. All of the niobium-containing glasses released niobium ions constantly in both Tris-HCl solution and cell culture medium. The differentiation and mineralization of the cells were upregulated on the niobium-containing glasses than non-containing one, depending on the niobium content in the glass system. These upregulation was also found for the cells cultured in the media containing the extra of the glasses. This means the niobium ions released from the glasses stimulated the cells to differentiate and mineralize. Thus, the niobium-containing glasses are expected to exhibit excellent bone regeneration ability.

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REFERENCE

FIGURE 4. ALP activities in MC3T3-E1 cells cultured in the media containing niobium ions. Control means normal culture medium (containing no niobium ions). Error bar means SD. * $p < 0.05$. Reprinted with permission from reference 20.

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