Bone cell transfection in tissue culture using hydroxyapatite microparticles

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Abstract: Coprecipitates of calcium phosphate and DNA have been used *in vitro* for several decades for cell transfection. We evaluated the efficiency of calcium phosphate ceramics associated to plasmid DNA in the transfection of bone cells *in vitro* when they are grown in tissue culture. Newborn rat calvariae and tibia epiphyses were grown on an agar surface for a period of 48 h to 30 days. The hydroxyapatite (HA)-particles were loaded with a plasmid bearing a galactosidase reporter gene by incubation of the plasmid solution in PBS with the particles. One milligram of HA-particles was then placed in contact with the bone explants for 8 and 30 days. Histological sections were then performed and the galactosidase activity was revealed using an X-gal solution. At eight days, very few cells expressing the galactosidase activity were detected. By 30 days, however, the explants appeared uniformly stained blue. The staining of sections showed that the osteoblasts, chondroblasts, perichondroblasts, and perisoteal cells all expressed the *lacZ* gene while the number of cells stained in the control was negligible. The time dependence of the transfection suggests that transfection using ceramics is linked to the degradation of the ceramic by the cells. Furthermore, the cells are stained remote from the particles suggesting that the particles induce a coprecipitate of DNA in the explant. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 79A: 225–228, 2006

Key words: transfection; calcium phosphate ceramics; bone; tissue culture; plasmid

INTRODUCTION

Calcium phosphates have been used as transfection agents during the last decades. They are used as nanometric-sized coprecipitates of hydroxyapatite (HA) and DNA.[1,2] The precipitates are then endocytosed by the cells. The precipitate-containing vesicles fuse with lysosomes and the DNA/calcium phosphate particles are then released into the cytoplasm where the majority of the DNA is degraded.[3,4] Later, part of the DNA enters the nucleus and may be incorporated into the genetic material. The way the DNA crosses the nuclear membrane is unclear.

The coprecipitate is obtained by supersaturating the medium containing the DNA with calcium and phosphate[5]: a solution of calcium chloride is added to the medium containing the phosphates. The characteristics of the precipitate differ with the pH, temperature, and composition of the solution.

There are some drawbacks to the use of such precipitates because the yield of the transfection is variable and generally low. Furthermore, it is impossible to use this technology *in vivo*.

We tried to overcome some of these difficulties with calcium phosphate ceramics. Their surface properties can be monitored and made reproducible and have been used for orthopaedic applications in human surgery for more than two decades now.[6,7]

We used bone tissue culture to assess the ability of particles of calcium phosphate ceramic impregnated with a plasmid containing a β-galactosidase reporter gene to transfect bone cells when they are organized in a 3D pattern.

MATERIALS AND METHODS

Ceramic characteristics

The hydroxyapatite (HA) powder was obtained by precipitation. The powder was composed of more than 98% hydroxyapatite and less than 2% calcium oxide and tricalcium phosphate. The particle size was 80–125 μm, the surface area was 0.62 m^2/g. The surface potential was −35 mV. The powder was hydrophobic. SEM of the ceramic surface was performed after the stage of plasmid adsorption at the material surface.

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Plasmid adsorption on the ceramic surface

The ceramic particles (2 mg) was suspended in phosphate buffer (0.12*M*, pH 6.8) for 2 h at 60°C. The powder was then washed in demineralized water and incubated in phosphate buffer (0.12*M*, pH 6.8) containing 10 μg of the plasmid bearing a galactosidase gene.

Tissue culture

Newborn rat tibias and calvariae were aseptically dissected out. The tibias were then cut under the proximal metaphysis. The tibia extremity was deposited on the surface of an agar gel (1% in Dulbecco Modified Eagle Medium) in order to be at the interface between the air and the culture medium (DMEM supplemented with 10% fetal calf serum). Fragments of calvariae 3–4 mm in size were deposited at the agar surface. A few micrograms of powder were deposited on the explant using a Pasteur pipette and left for the whole culture period. The galactosidase activity was monitored at 48 h and 30 days of culture. The tissue samples were embedded in ethyl methacrylate and 5 μm sections were cut, they were then stained for galactosidase activity.

Galactosidase activity

The cell cultures and tissue sections were immersed in X-gal solution at 37°C for 2 h (100 m*M* sodium phosphate, pH 7.3, 1.3 m*M* $MgCl_2$, 3 m*M* $K_3Fe(CN)_6$, 3m*M* $K_4Fe(CN)_6$ and 1 mg/ml X-Gal). The cells and sections were observed under a light microscope. The cells expressing the *lacZ* gene were stained in blue.

Control

Bone explants were either grown with HA particles not impregnated with DNA or with culture medium in which plasmid, the same amount as had been in contact with the particles, was inoculated in the culture medium.

RESULTS

SEM of the ceramic surface

The surface of the ceramic was composed of an aggregation of grains. At the grain surface, mineral precipitates were visible after the incubation in the phosphate buffer containing the plasmid (Fig. 1).

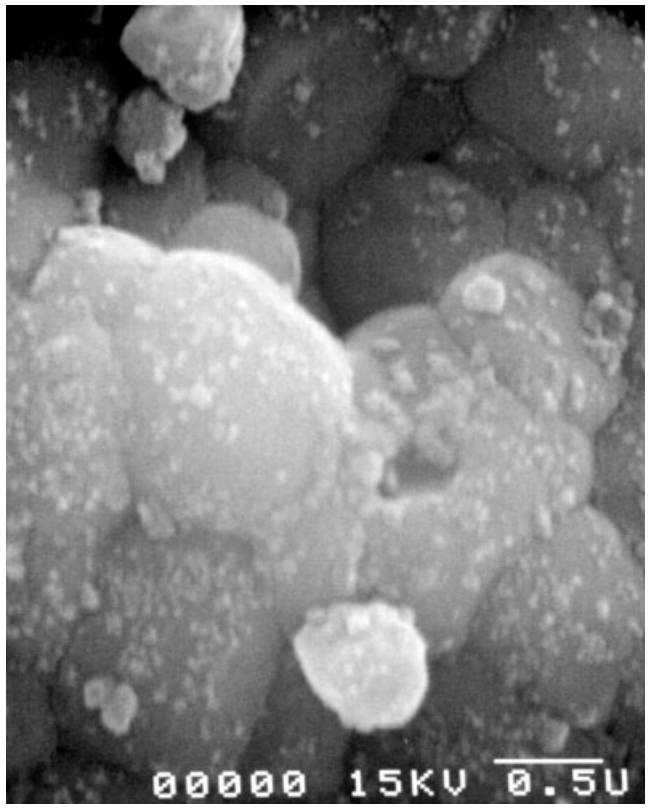

Figure 1. SEM of the particles incubated in the transfecting phosphate buffer showing calcium phosphate deposits at the grain surface. Bar 0.5 μm.

Modifications of the bone tissue structure during the culture

At 3 weeks of culture, modification of the histological structure of the different bone fragments was noted.

Calvariae

There was a proliferation of mesenchymal cells around the bone fragments that surrounded the particles. Numerous multinucleated cells were visible at the periphery of the particles. There was no sign of cell necrosis. The proliferating cells had a stacked appearance and in some places, an osteoid substance was visible.

Epiphyses

The bone marrow cells died, while the periosteal and perichondral cells had proliferated. The osteoblasts located in between the bone trabeculae lost their regular morphology and had a stellar shape while the chondroblasts looked like they usually do.

Cell transfection

Calvariae

At 2 days, there were a few cells expressing the *lacZ* gene. They were located either in contact with the particles or at some distance from them. At 30 days, all the cells exhibited a blue stain indicating that

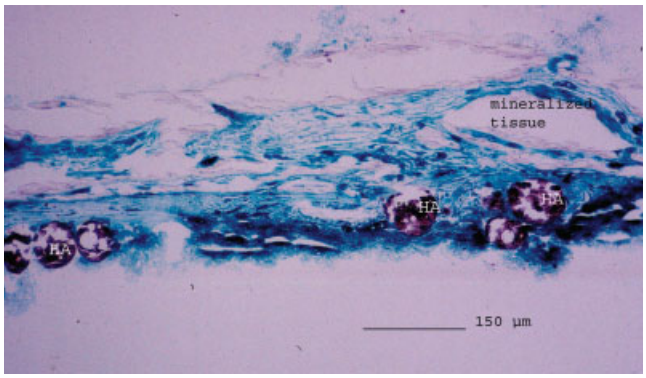

Figure 2. Histological section of rat calvaria after 30 days in contact with the particles. All the cells (multinucleated cells or cells of the osteoid tissue) in close proximity to the material are stained in blue and thus express the reporter gene. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

they were transfected. The multinucleated cells evidenced in the proximity of the particles also showed *lac-Z* expression (Fig. 2). The majority of the cells expressing the *lac-Z* gene did not show any staining of the nucleus.

Epiphyses

All the cells contained between the trabeculae identified as osteoblasts were transfected by 30 days. The chondroblasts, perichondroblasts and periosteal cells were also stained. However, the cells contained in the bone marrow cavity were not labeled (Figs. 3, 4, and 5). In both calvariae and epiphyses, most cells showed cytoplasmic staining at 30 days. Only a few cells presented nuclear staining suggesting that the DNA had found its way into the nucleus.

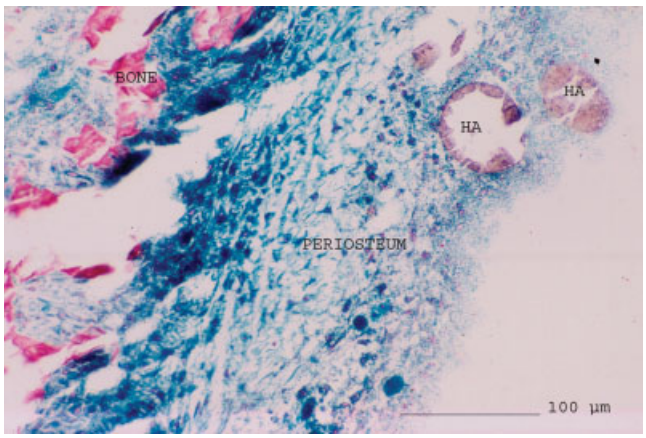

Figure 3. Histological section of a rat epiphysis after 30 days in contact with the particles (HA). The particles were in contact with the periosteum. All the cells expressed the lac-Z gene even when they were located between the bone trabeculae. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

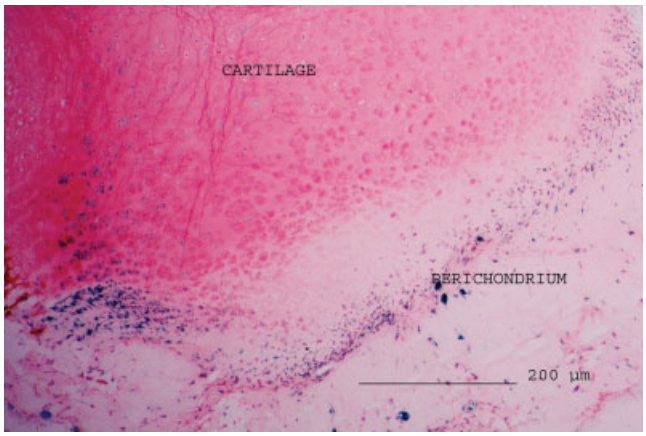

Figure 4. Light microscopy of the epiphysal bone fragments grown 30 days in contact with the particles. The cartilage anlage is surrounded by perichondrium. The perichondrium cells express the galactosidase gene and some chondroblasts are also positive. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Control

Very few cells were stained in blue (less than 0.1%) when plasmids were introduced in the culture medium.

DISCUSSION

This study shows that calcium phosphate ceramics are able to transfect bone cells of a tissue culture. The transfection is time dependent and also concerns the cells which are not in direct contact with the particles.

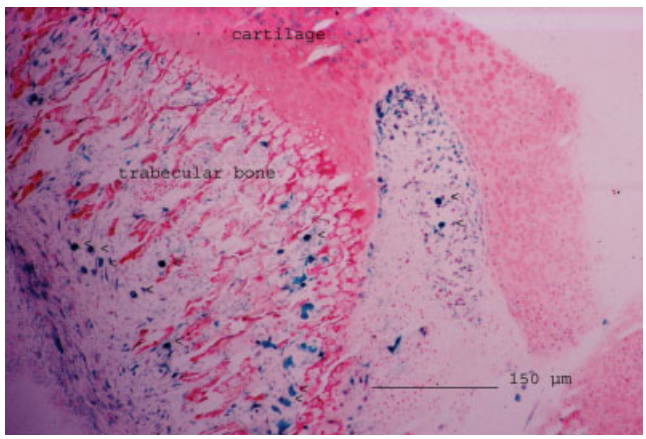

Figure 5. Histological section of the epiphysal bone fragments grown 30 days in contact with the particles. There is a zone of enchondral bone formation characterized by a high rate of cartilage resorption by chondroclasts. The chondroclasts (arrow heads) shows a strong staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The modifications of the characteristics of calcium phosphate ceramics in a saline solution or in a living organism are well known. Epitaxial growth of carbonated apatite occurs at the ceramic surface and in a period of time which varies according to the ceramic characteristics and the cells in contact with it,[8] the material is degraded at the grain boundaries and releases ceramic grains which are then phagocytosed by professional phagocytes or other cells.[9]

It is probable that the DNA immobilized at the surface of the ceramic is internalized by the cells with the ceramic grains, some of these particles are visible in the cells when the ceramics are used to transfect isolated cells.[10] It can be compared to the transfection with nanoprecipitates of calcium phosphate and DNA which are also phago or endocytosed by the cells to be transfected.

Wolff et al.[11] demonstrated that naked plasmid DNA injected into muscle of rodents could induce the expression of foreign genes. Nevertheless, in this experiment, we could not transfect a significant number of cells using the plasmid diluted in the culture medium. The advantages of this vector over naked DNA are several: time release of the DNA, partial specificity of the transfected cells, and high percentage of transfected cells.

It is very improbable that the DNA, which is a negatively charged molecule, establishes electrostatic interactions with the ceramic surface which also exhibits an overall negative charge. Regarding what we know about the formation of DNA/calcium phosphate nanoprecipitates and the dissolution/reprecipitation processes occurring at the ceramic surface,[12] it seems probable that there is a coprecipitation of DNA/calcium phosphate at the ceramic surface. The modification of the microparticle surface is visible using SEM after the particles have been soaked within a phosphate buffer. The incubation of calcium phosphate ceramics in a phosphate buffer or a simulated body fluid provokes the nucleation and growth of carbonated apatite crystals at the surface of the ceramic. The nucleation of carbonated apatite could be favored by the presence of small amounts of contaminants in the ceramic, such as CaO, which can increase the calcium concentration close to the material surface. Furthermore, plasmid DNA is known to adsorb at the surface of calcium phosphate ceramics in the pH and ionic strength conditions used in our experiments. These different events suggest that complex reactions occur at the material surface between the material and the macromolecules.

It is consistent with the time dependence of ceramic transfection as the degradation of the material seems to be necessary for the internalization of the DNA.

CONCLUSIONS

Calcium phosphate ceramics are able to transfect cells grown in tissue culture. The percentage of transfected cells is high: almost all the cells expressed the reporter gene. This newly described property of calcium phosphate ceramics could have various applications such as DNA vaccination or transient transfection for bone healing.

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