11

Transfection of Bone Cells *In Vivo* Using HA-Ceramic Particles - Histological Study

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1. Introduction

The non-viral introduction of genes into mammalian cells (transfection) is of growing interest for tissue engineering and used as an alternative to viral transfer of recombinant genes. The introduction of a foreign gene into cells *in vivo* is often limited to the use of viral vectors such as adeno or retroviruses (Rochliz, C.F., 2001, Kahn, A., 2000). Viral vectors may present several disadvantages or side effects, which can be disastrous. Adenoviruses produce proteins, which can trigger immune reactions. Furthermore, the expression of a gene transduced with a viral vector is transient and can be shortened when an immune reaction occurs against the viral proteins. It must also be noted that the selection of cells, which are transduced by the virus is very poor and its efficiency is dependent on the stage the cell is in.

A number of non-viral vectors have been explored and used to date i.e. lipid-based carriers, hydrogel polymers, polycationic lipids, polylysine, polyornithine, histones and other chromosomal proteins, hydrogen polymers, precipitated calcium phosphate (Maurer, N., et al., 1999; Cullis, P.R., Chonn, A., 1998; Zauner, W., 1998; Ramsay, E., et al., 2000 ; Schwartz, B, 1999; Leong, W., 1998; Perez, C., et al., 2001; Graham, F.J. et al, 1973). Most of these vectors are usable *in vitro* but are difficult to apply *in vivo*, especially when a local transfection to a specific cell line must be achieved.

Transfection using polymer matrices i.e. gel, foams or bulk material have recently been developed to overcome these difficulties (Lauffenburger, D.A., and Schaffer, D.V., 1999). They are polycationic and are able to adsorb the negatively charged DNA molecules on their surfaces (Bonadio et al.). This concept is also extended to calcium phosphate ceramics which are widely used in human surgery as bone substitutes, cell carriers, or even thin layers at the surface of metal alloys to improve their integration by bones (Frayssinet, P., et al., 1998; Frayssinet, P., et al., 1992). The use of calcium phosphate ceramics for gene delivery presents several advantages. These matrices are biocompatible and are totally degradable by the cells of the monocyte lineage (Frayssinet, P., et al., 1994). Their behaviour in the organism is well known.

This matrix was tested in jaw bones in order to transfect bone and dental ligament cells to increase bone formation during parodontal disease. We adsorbed a plasmid DNA containing an *Escherichia coli* galactosidase gene (Lac-Z) at the surface of hydroxyapatite ceramic particles which were implanted at the junction between the incisor dental ligament and bone of rabbit jaws.

2. Materials and methods

2.1 Surgical implantation

Four white New Zealand rabbits were used for each implantation period (21 and 90 days). A pouch was created at the junction between the right incisors and the bone. 0.5 mg of HA-powder (Urodelia, St Lys, France) was introduced in the pouch using a curette. The powder was aggregated in the curette using PBS and the implanted particles were covered with a mucosal flap.

Control animals: in one animal the HA-particles were implanted without any contact with plasmid and in another one, the same amount of plasmid solution as used for particle adsorption was injected at the implantation location. The histological sections were done at 21 days and 90 days.

2.2 Particle characteristics

The hydroxylapatite particle characteristics are given in table 1.

Form :	irregularly shaped micro-particles
Colour :	White
Molecular formula :	$Ca_{10}(OH)_2(PO_4)_6$
Molecular weight :	1004.6
Solubility :	Stable at neutral and basic pH, soluble in acidic pH.
Granulometry range :	45 - 80 µm
Apparent density :	1.4 ± 0.2 g/ml
Composition	HA ≥ 97%
Ca/P :	1.663 ≤ Ca/P ≤ 1.728
Surface area :	0.7 m²/g
Surface potential :	- 35 mV
Surface pH :	7,8
BSA binding capacity :	> 22 mg/g
DNA binding capacity :	> 0.1 mg/ml (pCMV plasmid – Contech)
Dry weight/volume :	2 g/ml

Table 1. Characteristics of the implanted powder

2.3 Plasmid adsorption

10 mg of powder was soaked in 0.5 ml of a 0.1 M phosphate buffer pH 7 at 60°C for 4-8 hours. The buffer was removed and the powder was washed with new phosphate buffer. The excess buffer was removed and the powder was introduced in 1 ml of a phosphate buffer solution (0.1 M phosphate buffer pH 7) containing 25 µg of plasmid DNA (Clontech, Palo Alto, California) and incubated 2 hours at 37°C. The excess solution was then removed and the powder was dried at room temperature.

2.4 Bone histology

The jaw was fixed in a mixture ethanol/acetone (50/50 V/V) at room temperature and partially decalcified in a 4% solution of diNa-EDTA for 6 days. The jaw fragments were then embedded inside hydroxyl-ethylmethacrylate. 5 µm thick sections were then performed using a microtome for calcified tissues (Reicher-Jung type K). The galactosidase activity was

evidenced using a X-gal solution at 37°C for two hours (100 mM sodium phosphate pH 7.3, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3mM $K_4Fe(CN)_6$ and 1mg/ml X-Gal). The sections were observed under a light microscope, and then counterstained by a Giemsa solution. The cells expressing the LacZ gene were stained in blue. The sections were done through the implanted particles and the same zone in the controlateral region.

3. Results

Macroscopically, the particles can be seen at the basis of the incisors at the first implantation time and they were stained in blue (fig. 1).

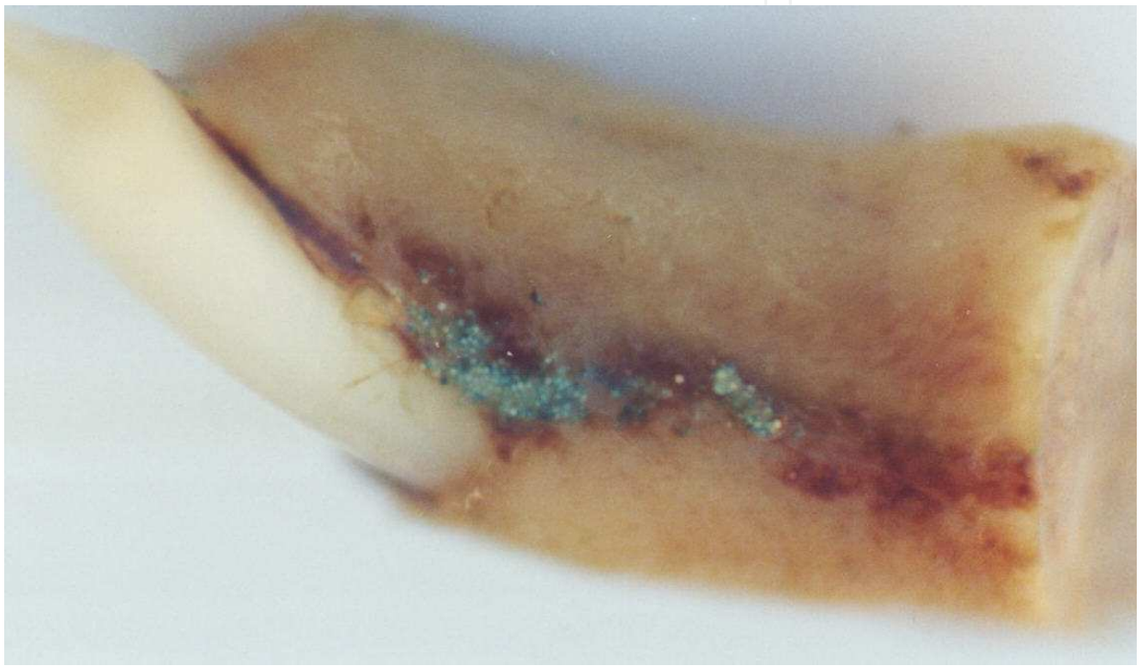

Fig. 1. Photograph of the implantation site at 21 days. The particles were visible at the junction between the incisor and the bone. The particles are stained in blue indicating that the cells having ingrown the material express the Lac-Z gene.

At 21 days, the particles were surrounded by a mild foreign body reaction constituted by mono and plurinucleated cells (fig. 2). These cells contained fragments of calcium phosphate ceramics.

The monocytes and multinucleated cells located around the particles were stained in blue (fig. 3). In the controlateral site, blue stained cells were dispersed in the stromal tissue evidenced in the bone pores. They were circulating cells such as monocytes and multinucleated cells (fig. 4). These late cells were often evidenced at the bone surface and sometimes in Howship's lacunae (fig. 4).

Some other cells expressing the galactosidase gene were found inside stromal tissue and showed a fibroblastic aspect (fig. 5). Some of these cells were identified as pericytes as they were evidenced in the immediate proximity to the capillaries.

Blue stained cells were also evidenced in the dental ligament (fig. 6). Some of them have the morphology of circulating cells as others are ligament fibroblasts.

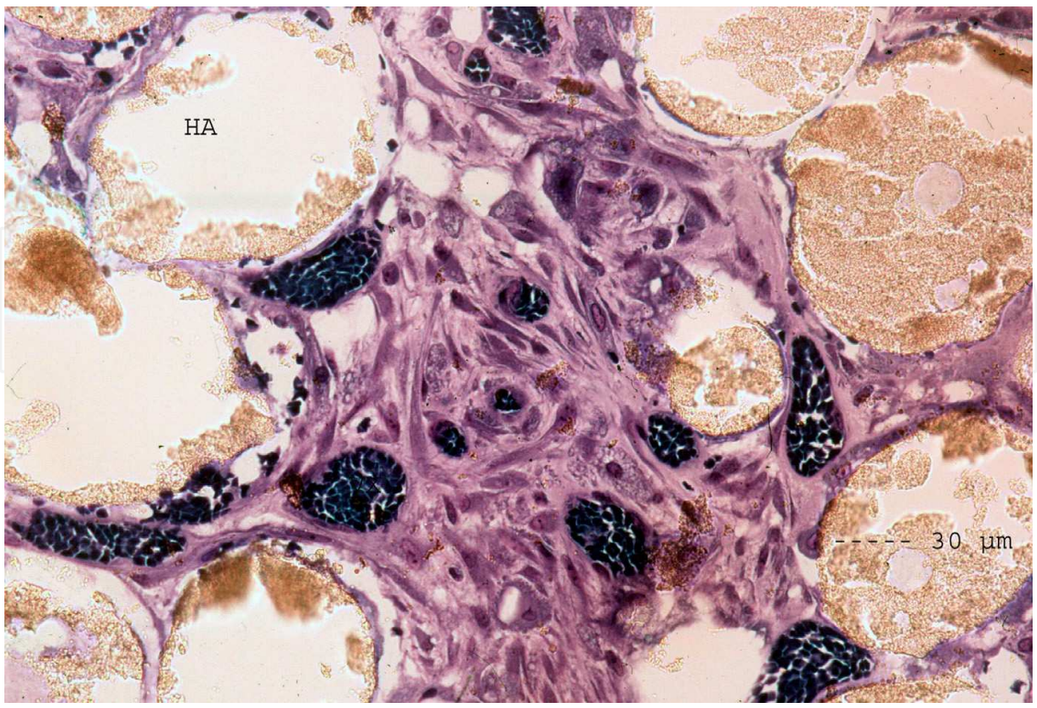

Fig. 2. Microphotograph of the implantation zone at 21 days showing that at an early implantation time, the microparticles (HA) were embedded in a mild foreign body reaction made of monocytes and multinucleated cells. Giemsa staining.

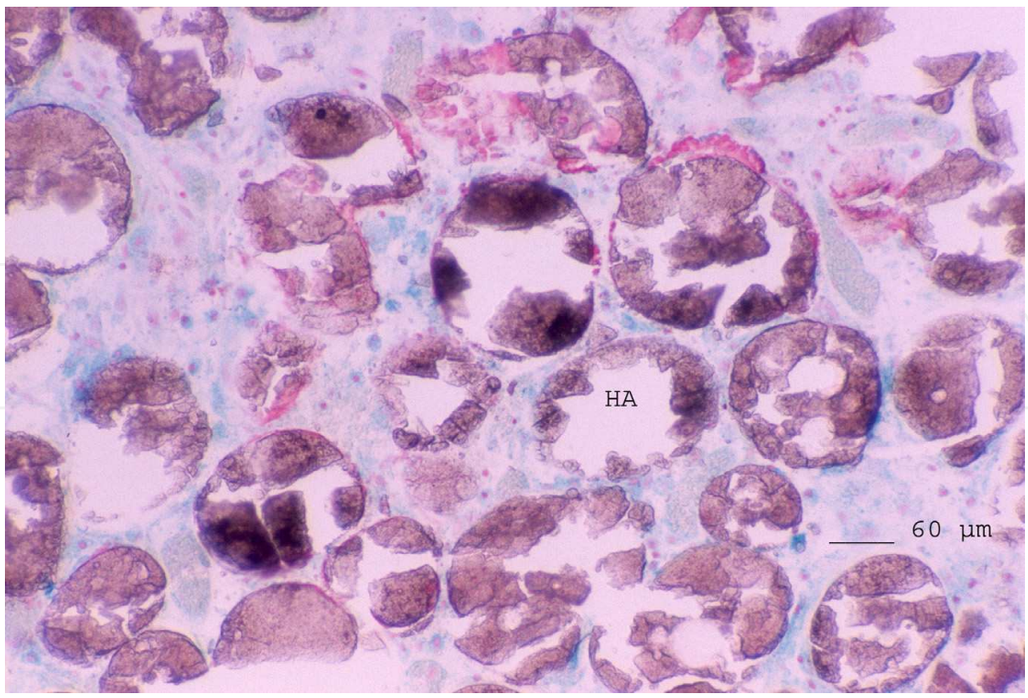

Fig. 3. Section of the implantation zone at 21 days after X-Gal staining showing that almost all the foreign body reaction cells were stained in blue. X-Gal and neutral red staining.

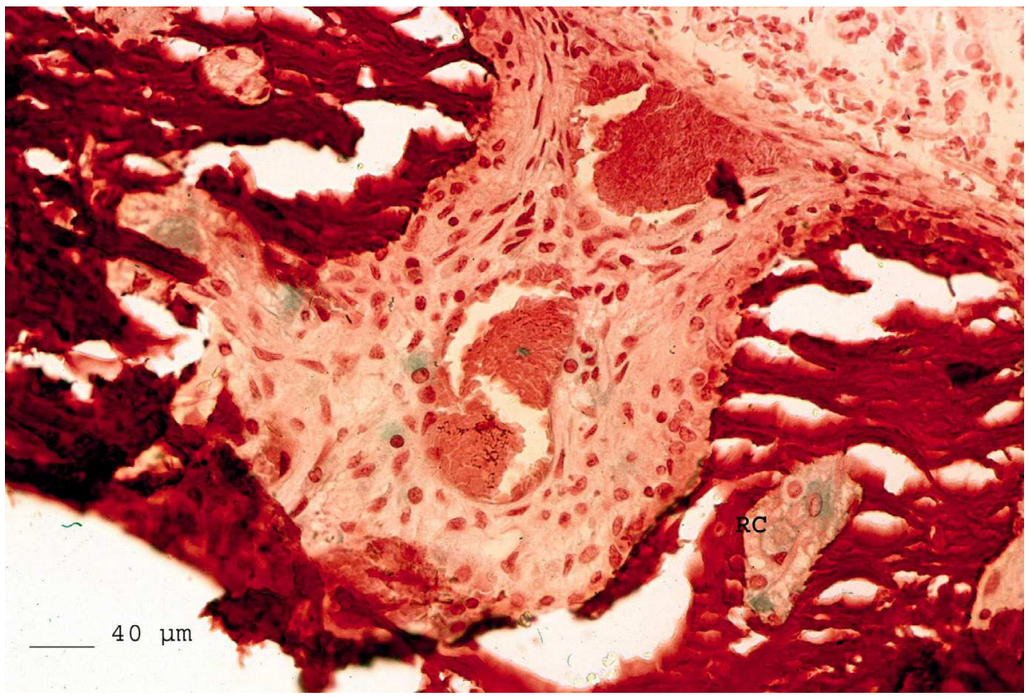

Fig. 4. Section of bone at remote distance from the implantation zone at 21 days. There were monocytes stained in blue in the pores of the bone tissue. Cells expressing the galactosidase gene were evidenced in Howship's lacunae or resorption cavities (RC). X-Gal and neutral red staining.

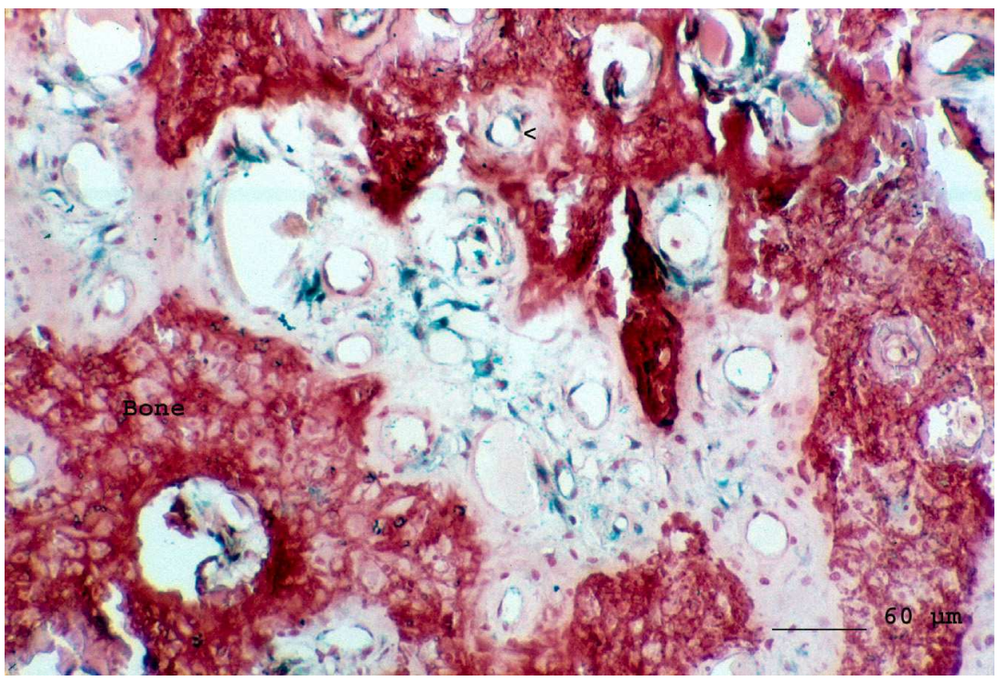

Fig. 5. At 21 days, the bone stromal tissue contained blue stained cells which were stellar shaped. Some of these cells were perivascular (<). X-Gal and neutral red staining.

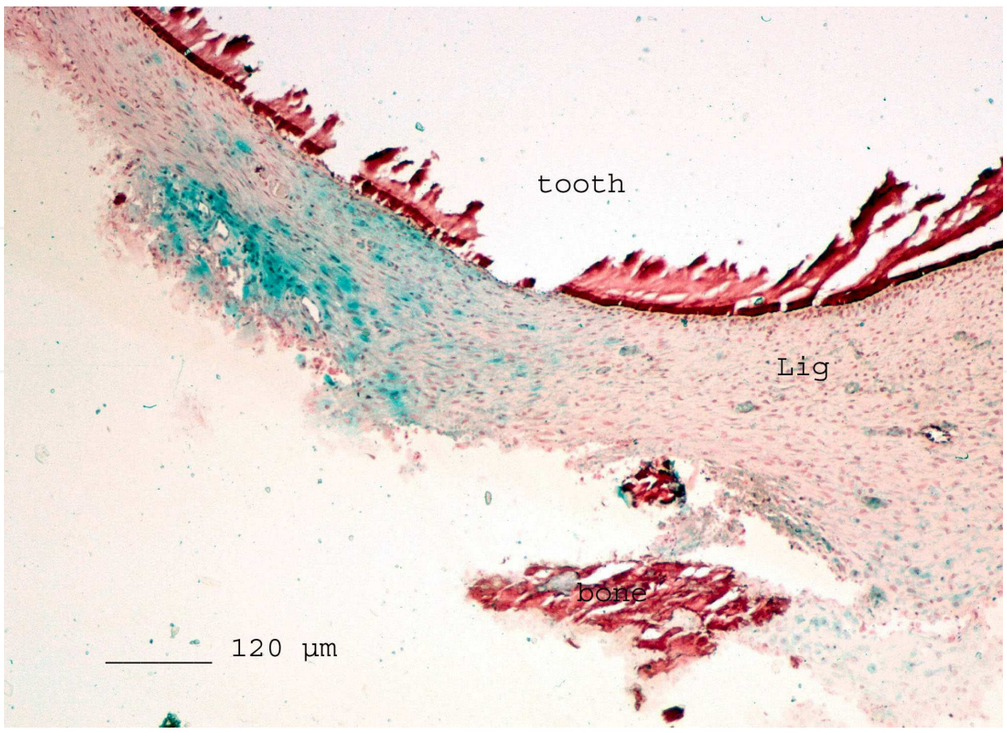

Fig. 6. At 21 days, section of the dental ligament (lig) of the implanted incisor. Some ligament cells were stained in blue. X-Gal and neutral red staining

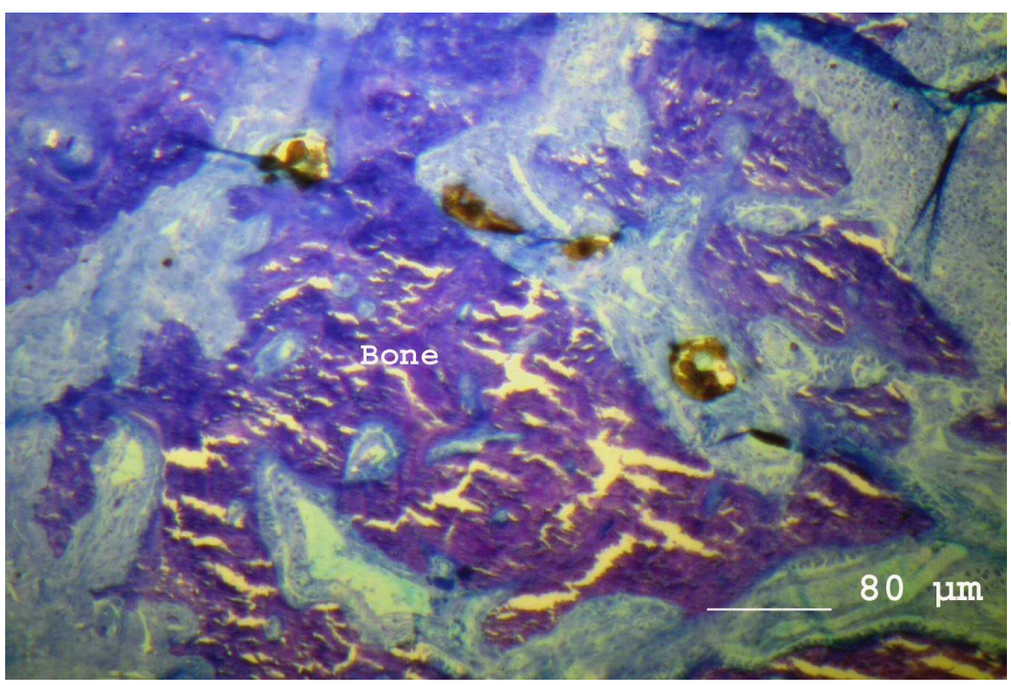

Fig. 7. At 3 months after implantation, the histological sections showed that the HA-particles (grey) were dispersed and integrated in the bone tissue without sign of foreign body reaction. Giemsa staining.

At 90 days (fig. 7), the particles were degrading and some of them were integrated inside bone trabeculae.
There were almost no circulating cells around the microparticles. The cells expressing the lac-Z gene were dispersed in the connective tissue. Some other cells showed a blue staining. Odontoblasts and fibroblasts were among these cells (fig. 8). The percentage of the labelled cells was low.
Sections of the control animals did not show any staining at any time.

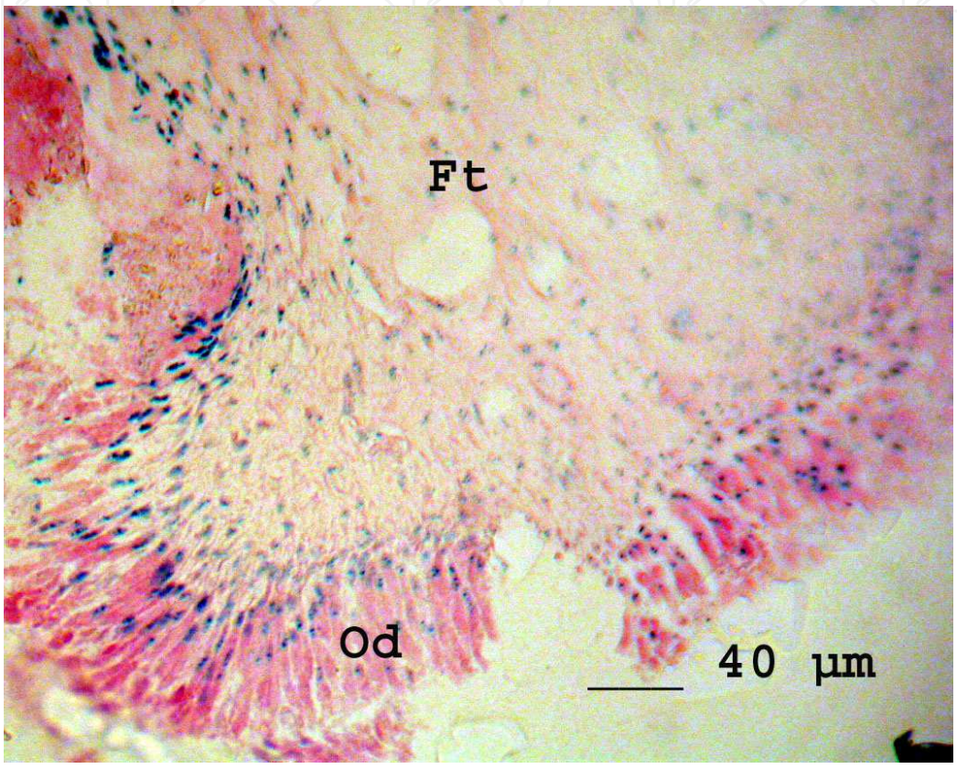

Fig. 8. Histological section of the incisor connective tissue of a three month implanted site. There were still some cells like odontoblasts showing a galactosidase gene expression. Numerous other cells mainly fibroblasts were also expressing the gene. X-Gal staining.

4. Discussion

This experiment showed that a transient transfection can be obtained using calcium phosphate ceramics with some level of specificity limited to the cells being in contact with the ceramic or located to its proximity. This "geo-specificity" allows to transfect the circulating cells i.e. monocytes, macrophages, multinucleated cells which are the first cells to come in contact with the material.
The transfection does not seem to interfere with the differentiation of the cells, as the monocytes transfected at the ceramic contact are found in Howship's lacunae and identified as osteoclasts. Furthermore, they are also found in various locations of bone indicating that the cells of the foreign body reaction are still able to circulate and differentiate from macrophages into osteoclasts. Thus, it indicates that contact with the ceramic and the phagocytosis of the ceramic particles would not impair the migration of cells toward the

lymph node and their role of antigen presenting cells. It is also indicated that the degradation of the ceramic does not release toxic particles or products responsible for a cell death or apoptosis in the site of implantation.

The cell transfection with calcium phosphate/DNA coprecipitates has been used for decades *in vitro* (Schenborn, E.T., and Goiffon, V., 2000), it is almost impossible to obtain *in vivo* in an open medium. The use of nanoparticles which are already precipitated are also difficult to use *in vivo* because it is not possible to maintain them in a particular location.

It has been reported that a direct injection of a plasmid suspension in rodent muscles could trigger a significant transfection of the muscle cells (Danko, I., et al., 1997). However, it is not known what are the other cells transfected and what is the transfection kinetic and yield. It is also difficult to ensure any specificity of the transfected cells by this way. In this study, plasmid injection did not bring a significant transfection in the injection site, probably because the injected solution did not stay in the site.

Macrophages have the reputation of being difficult to transfect. This material can thus be of interest to target these cells. During the first time, only the circulating cells are labelled, while at three months, some other cells could be evidenced such as odontoblasts or fibroblasts of the connective tissue.

The mechanism of transfection is not clear and cannot totally be dissociated from that of the co-precipitate. Furthermore, the degradation of the ceramic takes place at the grain boundaries of the ceramic particles. A release of particle grains occurs in the proximity of the implantation zone explaining the localisation of the transfected cells (Frayssinet, P., and Guilhem, A., 2004; Frayssinet, P., et al., 2006).

The material degradation leads to the release of several particle sizes and shapes depending on the degradation stage. The ceramic grains after the dissolution of the grain boundaries, can be released alone or aggregated. At this stage, they are micronic in size. Then the particles are degraded inside the low pH compartments of the cells and their size decreases and they become nanosized (Frayssinet, P., et al., 1999; Jallot, E., et al., 1999). During this time, the shape becomes round with a disappearance of the particle angles. The dissolution/precipitation process occurring at the particle surface is very complex as there is a carbonated apatite epitaxial growth at neutral pH and finally a dissolution at low pH.

The physical interaction with between the HA and the hydroxylapatite chemically stabilizes the DNA molecules increasing the denaturation temperature (Martinoson, H.G., 1978). This stabilization can partly explain the transfection mechanism as the complex DNA/calcium phosphate could impair or slow the DNA destruction in the cytoplasm (Orrantia, E., Chang, P.L., 1990).

The adsorption mechanism at the HA surface is not clear. The DNA molecule is negatively charged as is the ceramic surface. Thus the adsorption is not driven by electrostatic forces. The surface modifications occurring during the culture or implantation make it difficult for the elucidation of the adsorption mechanism.

These results have to be compared to those obtained *in vitro* with isolated cells or tissue culture. It was shown that the percentage of transfected cells was time dependent and could be very high after a few days of contact. It was also shown that, regarding bone tissue, all the cell types could be transfected by this way.

5. Conclusions

Hydroxyapatite ceramics have numerous applications relating to the field of human and animal health. Their surface properties can explain the molecule adsorption and the ability

of these materials for transient cell transfection both *in vitro* and *in vivo*. Applications for cell transfection could be numerous as the material is safe, degradable, and shows a good transfection yield. Furthermore, this material demonstrates interesting properties allowing to target antigen presenting cells. These cells can show some deficiencies in their role of antigen presentation which is essential in very different pathology such as cancers, infectiology or autoimmune diseases. It could be particularly appropriate as a DNA vaccine vector in order to bring the antigen presenting cells the properties they would need to overcome the immune evasion strategies of cancer cells.

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