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Biological effects of aluminium diffusion from plasma-sprayed alumina coatings

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Plasma-sprayed alumina (Al_2O_3) coatings on metal stems of hip prostheses are used to favour bone apposition on the stem without fibrous interposition. We tested both *in vitro* and *in vivo* in rabbits, alumina coatings in order to evaluate the biological effect of this material on bone. Mice fibroblasts were grown on Al_2O_3-coated discs and time course of aluminium concentration was recorded in two phosphate and citrate buffers (pH 4 and 7) bathing the alumina coated discs. Alumina-coated cylinders were implanted into femur condyles of ten rabbits for periods of time from 1–6 months. Then, they were histologically analysed using light and scanning electron microscopy, and X-ray microanalysis. Cell proliferation was not affected on alumina coatings compared to controls. In pH 4 buffer, aluminium was released from the coatings. From a period of implantation of 4–6 months an increasing demineralization process took place in the bone at the coating contact. Aurine staining showed the presence of aluminium at the interface between the non-mineralized and the mineralized bone. These results suggest that aluminium is released from alumina coatings and leads to bone demineralization at the coating contact.

1. Introduction

Ceramic coatings of metal hip prosthes and acetabular components are widely used for the purpose of material osseointegration. Hydroxyapatite (HA) and alumina coatings are generally used [1, 2]. The stability of these two compounds used under a bulk material form is admitted once implanted. When plasma-sprayed coatings are performed using HA or alumina powder, a change of phase may occur in the material. Furthermore, the porosity of the coating may be high. These two factors are in favour of a coating degradation after implantation.

The degradation of HA-coatings has been established after implantation into animals or humans [3]. Amorphous phase is first dissolved, then HA-grains are phagocytosed by macrophages and osteoclasts and are dissolved into low pH cell compartments.

Degradation of alumina coatings has been evoked by Stea *et al.* [4] from histological study made on human implanted hips. Osteomalacia lesions were observed at the coating contact suggesting an Al^{+++} release from the coating. In order to measure the leakage of Al^{+++} from alumina coating and the effect of this release on bone formation at their contact, we have implanted some stainless steel cylinders coated with alumina into rabbits condyles. Alumina coatings were tested for their *in vitro* effects on the proliferation of isolated cells. The concentration of Al^{+++} into buffers bathing the coating was measured during several months.

2. Materials and methods

2.1. Specimen preparation

Pure α-alumina powder (99.8%) (Plasma-tecnik, Switzerland, PT1001 having a granulometry of 25–45 μm) was plasma sprayed on stainless steel cylinders having a diameter of 5 mm and a length of 7 mm or a diameter of 25 mm and a thickness of 5 mm. Alumina coatings had a thickness of 110 μm ± 50 μm. The roughness parameters were: $R_a = 5$ μm, $R_t = 33$ μm, $R_p = 13$ μm. The porosity was 10–12%. Thick alumina coatings (1 mm ± 50 μm) for aluminium release and cytotoxicity studies were plasma sprayed on stainless steel alloy discs bearing hydroxyapatite coatings. Once the thick alumina coating had been plasma sprayed on the HA, the calcium phosphate was dissolved using an acid solution in order to obtain an alumina coating independent of the support. The crystallographic structure of the coatings has been checked on powdered coating using quantitative X-ray diffraction. X-ray diffraction patterns were recorded on a Phillips goniometer TW 1050. X-ray emission is obtained by a cobalt anode ($\lambda = 0.178\,892$ nm). Roughness parameters were determined using a microrugosimeter (Mitutoyo 407 microrugosimeter).

2.2. Implantation and histological procedure

Implants having a 5 mm diameter were implanted in the external condyle of 10 rabbits. *Animals were operated on under general anesthesia with ketamine and the*

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implants were handled aseptically. For insertion, the external condyle of each femur was exposed through a lateral approach. A hole 5.5 mm in diameter was drilled in the condyle and the implant was inserted inside. No micromovement was allowed. After periods of time of 1, 2, 4 and 6 months, animals were sacrificed with a nembutal injection and implanted cotyles were retrieved and fixed in a 5% formaldehyde solution in PBS. They were dehydrated in increasing alcohol concentrations and embedded in polymethylmetacrylate (PMMA). Sections were made using a low speed cooled diamond saw. They were polished with a polisher using silicon carbide discs until a thickness of 50–100 μm. Staining was made on non-deplasticized sections. Mineralized extracellular matrix of the sections was stained using Von Kossa method. Aluminium in bone tissue was detected using ammonium salt of the aurine tricarboxylic acid.

For realizing such an histochemical staining, aluminon buffer solution was used, prepared by mixing 60 ml of 5 M ammonium chloride, 60 ml of 5 M ammonium acetate and 10 ml of 6 N HCL. A 2% solution of the aluminon was made by first dissolving 2 g of the reagent in 10 ml of the buffer, then adding more buffer to make 100 ml and heating at 75 °C. The solution was filtered and used while still hot. The differentiating solution was prepared by mixing 50 ml of the buffer solution and 22 ml of 1.6 M ammonium carbonate. The staining was carried out with two 15 min dips in warm acetone. Stained sections were then briefly rinsed in distilled water and differentiated in the ammonium carbonate solution for 5–10 s.

Some 6-month implanted sections were stained using silver methenamine and were examined using a field emission scanning electron microscope (Hitachi S4000) in a back-scattered mode under a tension of 5 or 15 kV. Chemical analysis using energy dispersive spectrometer (EDS) was made using a detector for light elements. X-ray dot maps were performed.

2.3. Cell culture assay

The 25 mm diameter discs were inserted into polystyrene wells of the same diameter. 40 discs were tested. A near confluent monolayer of established mouse fibroblasts line (L929) was suspended using a trypsin EDTA solution (Gibo, Cergy Pontoise, France) into DMEM medium (Gibco, Cergy Pontoise, France) supplemented with 10% foetal calf serum (Gibco, Cergy Pontoise, France) and L-glutamine. One millilitre of the suspended cells at a density of 10^6 cells ml^{-1} was inoculated into each well at the surface of the alumina coating. The cells were incubated at 37 °C into a 5% CO2 and 98% humidity atmosphere for periods from 24 h up to 400 h. At the end of the incubation period, the cells were resuspended and were counted in a Malassez's cell.

2.4. Aluminium release

Independent plasma-sprayed alumina coatings weighing approximately 5.4 g were immersed in 100 ml of phosphate buffer and citric buffer (buffer pH 7.00 and buffer pH 400 Riedel de Haen, Germany) having different pH (7 and 4). Each month during a period of 6 months, 1 ml of the buffer was sampled and diluted into 10 ml of distilled water. The content of the solution in Al^{+++} was measured using inductive coupled plasma process (Perkin Elmer, Plasma 40 Emission Spectrometer).

2.5. Statistical analysis

The unpaired Student's *t*-test was used to compare the different cell growth values at different culture periods.

3. Results

An α-Al_2O_3 alumina powder was used for the plasma spray to obtain a γ-Al_2O_3 coating (Fig. 1).

The implanted cylinders were in contact with bone trabeculae during the first 4 months. No fibrous tissue was visible during this period between bone trabeculae and the coating. Von Kossa staining showed that the bone in contact with the coating was mineralized (Fig. 2). Six months after implantation, unstained sections showed that bone trabeculae were in contact with the coatings. Von Kossa staining showed at this implantation time, a thin layer of unmineralized bone matrix between the mineralized bone and the coating (Fig. 3).

Aurine staining did not show Al^{+++} deposit into the bone until 4 months after implantation. At 4

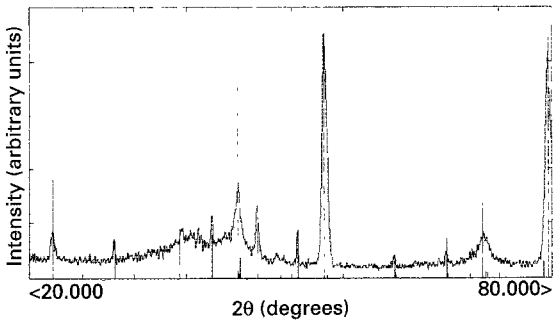

Figure 1 X-ray diffraction pattern of powdered alumina coatings used in the experiment.

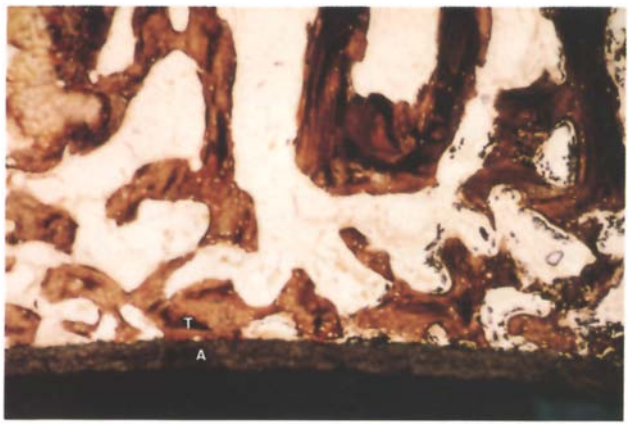

Figure 2 Von Kossa stained section of a 2-month implanted cylinder. Bone trabeculae (T) are at the contact of the alumina coating (A). Von Kossa × 50.

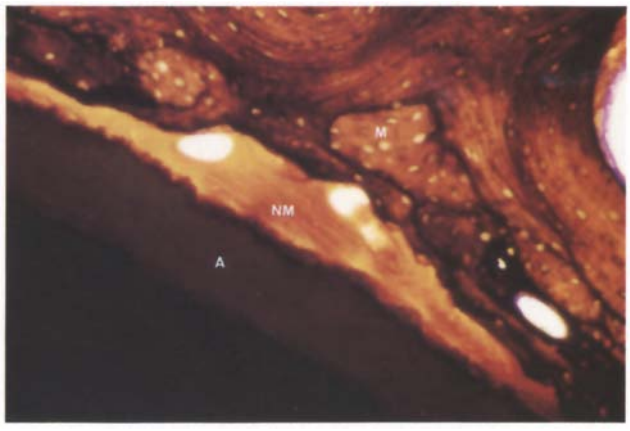

Figure 3 Section of a 6-month implanted cylinder Non-mineralized bone matrix (NM) still having a haversian structure is located between the regular bone (M) and the alumina coating (A). Von Kossa × 350.

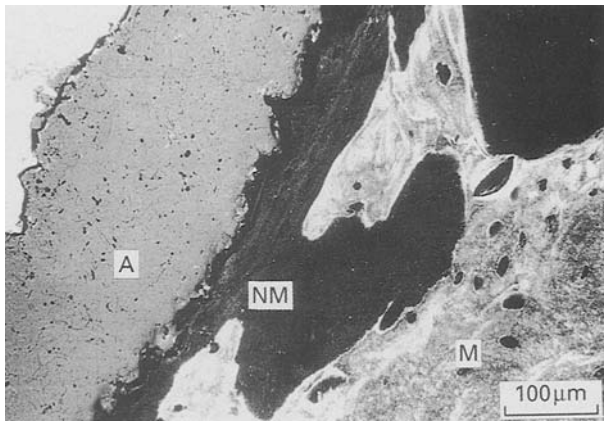

Figure 5 Back-scatter SEM of a silver methenamine stained section of an alumina-coated cylinder implanted for 6 months: there is non-mineralized matrix zone (NM) between the bone (M) and the coating.

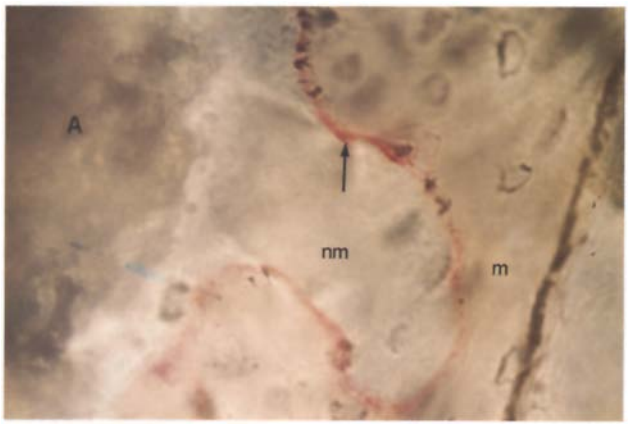

Figure 4 Aurine staining of a section of a 6-month implanted cylinder. A pink line showing a high aluminium concentration (↑) is located with the non-mineralized zone (nm) and the mineralized bone (m). A = alumina-coating. X 800

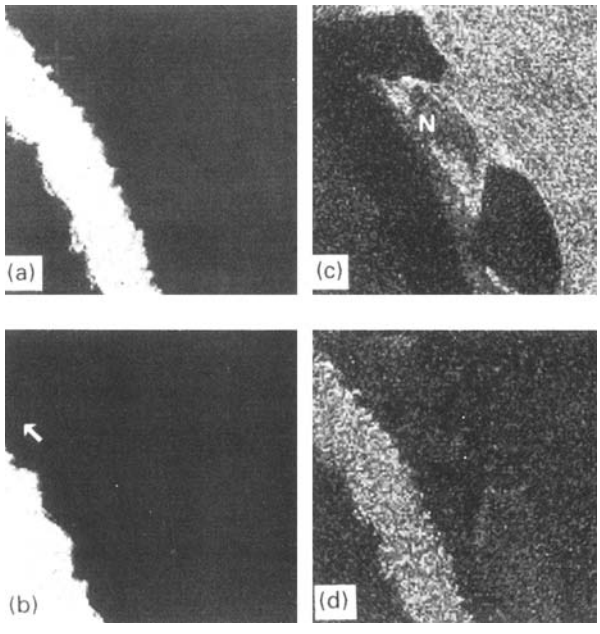

Figure 6 X-ray dot map of the same region for aluminium (a), iron (b), silver (c) and oxygen (d). No aluminium is clearly visible in the bone region. Silver (methenamine) is located in the mineralized region. A non-mineralized bone matrix exits (N) between the silver-stained region and the coating.

months after implantation, a pink line was observed on the coating and a demineralized zone was either not apparent or very thin (< 100 μm). At 6 months, a pink line was located between the mineralized and the unmineralized matrix (Fig. 4) and the unmineralized zone was up to 1000 μm thick.

Back-scatter electron microscopy of the sections from the 6-month implanted specimens showed a bone matrix in contact with the coating different from the bone located far away from the alumina coating (Fig. 5). Microanalysis of the region at the junction between the two types of bone did not detect Al^{+++} (Fig. 6).

Cell proliferation on alumina coatings was lower than on the negative control material for the period 124–184 h (Fig. 7), however, the difference was not significant ($p > 0.05$).

Aluminium content of the pH 7.00 buffer solution was constant during the experiment. The content increased in the pH 4.00 buffer solution (Fig. 8).

4. Discussion

This experiment shows that, although cell proliferation is not affected at the initial contact of Al_2O_3 coatings, bone formation can be altered in the long term. The newly formed bone extracellular matrix is

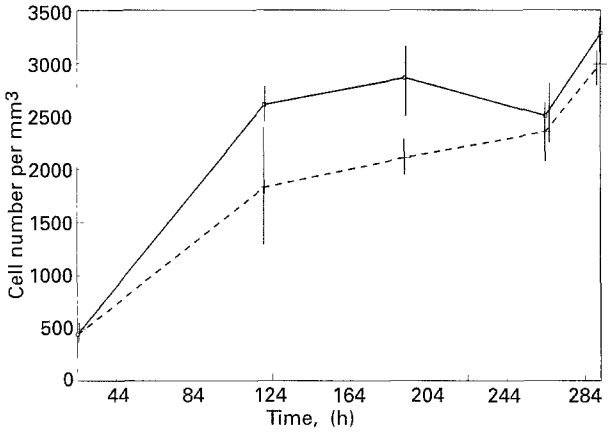

Figure 7 Cell growth curve of a L929 cell line on alumina coating (——) and polystyrene wells as negative control (– – –). Data are indicated as mean ± SD.

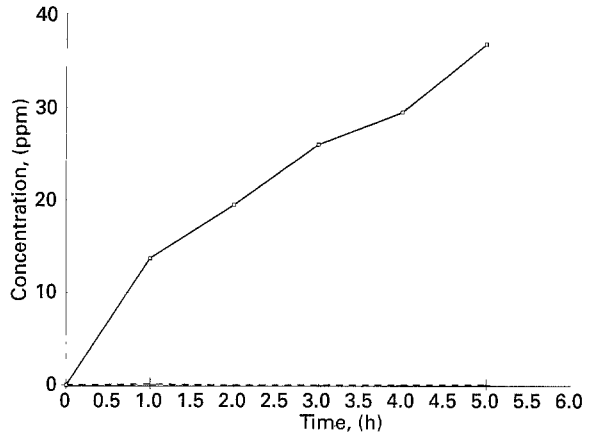

Figure 8 Aluminium content of pH 7.00 (——) and pH 4.00 (– – –) buffers in contact with alumina coatings as measured using an inductive coupled plasma process.

firstly mineralized, then submitted to a demineralization process which does not disturb the structure of the bone tissue at the light microscope level. This anomaly in bone tissue mineralization in close contact with the coating is concomitant with an *in vitro* Al^{+++} release from the coating at a pH which can be found in some cellular or extracellular compartments. This variation of pH in some microdomains of the medullary cavities is supposed to be responsible for the degradation of HA-coatings, which has been observed over long periods of time [3]. Moreover, aurine staining shows an Al^{+++} deposition at the interface between bone and unmineralized matrix. The amount of Al^{+++} is low: probably < 500 ppm which is the concentration below which the detector used for X-ray microanalysis could not detect an element.

The presence of aluminium in bone has already been related to defects in the mineralization of bone ECM. Smith and McClure [5] have localized aluminium by histochemical (aurine staining) and electron probe X-ray microanalytical techniques at the interface between the unmineralized and mineralized ECM in the region of renal osteodystrophy lesion. Ott *et al.* [6] showed that aluminium deposition is associated with impaired bone formation or mineralization and with a poor response to calcitriol therapy. Boyce *et al.* [7] related the hypercalcaemic osteomalacia resistant to vitamin-D therapy found in patients with chronic renal failures due to the high content in aluminium of their bone and plasma. Goodman [8] demonstrated that aluminium did not affect the previously formed bone. Data from culture with chick bone [9] suggested that aluminium interferes with osteoid calcification by preventing calcium phosphate crystal growth. The localization of aluminium at the interface with the mineralization front suggested that aluminium could act as a physico-chemical inhibitor of calcification. Severson *et al.* [10] studying the effect of aluminium on the osteogenic properties of demineralized bone matrix in rats showed that it appeared to alter the differentiation and calcification of developing cartilage and bone.

The results obtained in this experiment show that aluminium does not affect only the mineralization of forming bone but is also able to affect the mineralization of previously formed bone. Mineralized bone formed in contact with Al_2O_3 during the first weeks of implantation demineralized when Al^{+++} was released from the coating. This suggests also that aluminium does not only interfere with calcium phosphate crystal growth: Goodman *et al.* [11] demonstrated that aluminium affected the proliferation and differentiation of osteoblasts. The aluminium effect on bone cell biology could play a role in bone tissue demineralization.

Furthermore, it seems that the excellent osseointegration which has been demonstrated at the contact point of alumina coatings the first time of implantation evolves during the implantation. During the period of aluminium release from the coating a demineralized ECM is interposed between the mineralized trabeculae and the coating without fibrous tissue. Due to the limited duration of our experiment, we could not determine how long the aluminium release lasted and how long the demineralized matrix between the prosthesis coating and the regular bone persisted. The amount and the time of release depends mostly on the porosity and the roughness of the coating. However, the amount of aluminium available for diffusion from the alumina coating is very low and cannot give any sign of aluminium toxicity exept from a very narrow strip of bone at the coating contact. This demineralization process could modify the biomechanics of the implant by introducing an interposition zone having intermediary mechanical properties between the bone and the material. These results are consistent with the results found by Stea *et al.* [4] at the contact of hip prostheses coated by alumina and implanted into humans.

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