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IV. 研究成果の刊行物・別刷

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Conversion of functions by nanosizing—from osteoconductivity to bone substitutional properties in apatite

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Abstract. Synthetic hydroxyapatite, in the usual case, of a macroscopic size, exhibits excellent osteoconductivity. However, it is not substituted with natural bone and remains permanently in the body; therefore it is suitable for using as an implant. It is well known that natural bone is composed of collagen and nanocrystallites of apatite with the size of approximately 50 nm. When the composite with collagen and nanoapatite synthesized in the biomimetic aspects is implanted, phagocytosis and inflammation are induced. Osteoclasts and osteoblasts are then differentiated and activated. The bone resemblant material and its phagocytizable nanometer size provide the conditions that composite is biologically degradable through phagocytosis by osteoclasts, and new bone formation by osteoblasts is simultaneously activated and proceeded. As a result, nanocomposite leads to the bone substitutional properties. Thus the conversion of functions is attained for apatite by nanosizing—from osteoconductivity in macroscopic size to bone substitutional properties in nano/micro scale. This tendency is more enhanced for carbonated hydroxyapatite. The mineralization surrounding collagen fibrils determines the crystallization of apatite for their size and orientations. Nanoparticles cause the reaction of cells/tissue and stimulate the occurrence of inflammation, which works as a stimulus in most cases or pronounces the conversion of functions leading to the bioactive properties for some cases, depending on the situation. Nano structure is essential for these stages to be processed.

Key words. nanosizing, apatite, tissue regeneration, inflammation, nanotoxicology

Introduction: non-resorbable and resorbable apatite

Synthetic hydroxyapatite in the usual case, that is, in a macroscopic size, exhibits excellent osteoconductivity. However, it is not substituted for natural bone and remains permanently in the body; therefore it is suitable for using as an implant.

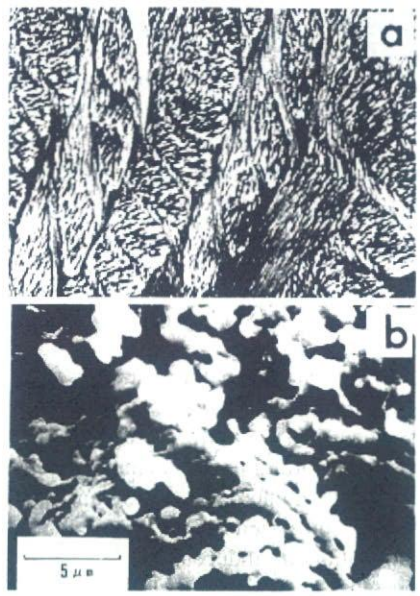

Fig. 1. Difference of morphology of hydroxyapatite. **a** Enamel of molar of rat, **b** sintered synthetic apatite

It is well known that natural bone is composed of collagen and nanocrystallites of apatite with the size of approximately 50 nm. In Fig. 1 the SEM photographs compare the difference in morphology of hydroxyapatite for natural hard tissue, in this case, enamel of molar of rat (a) and sintered synthetic apatite (b). In synthetic apatite the size of particles is a few microns, and they agglomerate in random, while in enamel, enamel prism of about 5 μm is composed of a bunch of apatite crystallites of about 50 nm. It is known that apatite crystallites are grown in their *c*-axis along collagen fibrils. Thus natural hard tissue is regarded as a kind of composite with the preferably oriented structure of nanocrystallites.

There is the difference in behavior between synthetic apatite and bone. Bone is continuously remodeled by resorption and new bone formation. Thus there exist apatites with different behaviors, non-resorbable and resorbable apatites. The problem arises: what is their difference and its cause? We will first see the nanosizing effect in general, and then the case of apatite and its mechanism.

Materials and methods

Both biochemical cell functional tests and animal implantation tests were done to investigate the reaction to fine particles of 99.9% pure Ti, Fe, Ni, and TiO_2 for the various sizes from 300 nm to 150 μm [1]. Human neutrophils were used as probe

cells for various cell toxicity tests, after mixed with particles in Hank's balanced salt solution (HBSS) at 37°C. Histological investigations were done after implanting in the subcutaneous connective tissue of rats.

Hydroxyapatite-collagen composites were synthesized biomimetically on mineralized collagen type I. They have the three-dimensional scaffold structures with the interconnecting pores. They were implanted into the subcutaneous tissue, and bone defects made in the femur of rats for 1–12 weeks and observed histopathologically [2].

Results

Micro/nanosizing effect onto cell/tissue reaction

Figure 2 shows the dependence of TNF-α release from human neutrophils on the size of Ti particles. TNF-α was increased with the decrease in particle size. The increase was pronounced for 0.5 and 3 μm. The release of LDH, superoxide and cytokine Il-1β showed the similar behavior as TNF-α, while cell survival rate showed the inverse decreasing tendency. Under these conditions ICP elemental analysis indicated that the dissolution from Ti particles was negligible below detection limit [1].

Figure 3 shows the SEM image of human neutrophils of control (a) and the one exposed to 0.5 μm Ti particles (b) where a neutrophil extends its pseudopod to phagocytize Ti particles for the size less than 10 μm [3]. For the particles larger

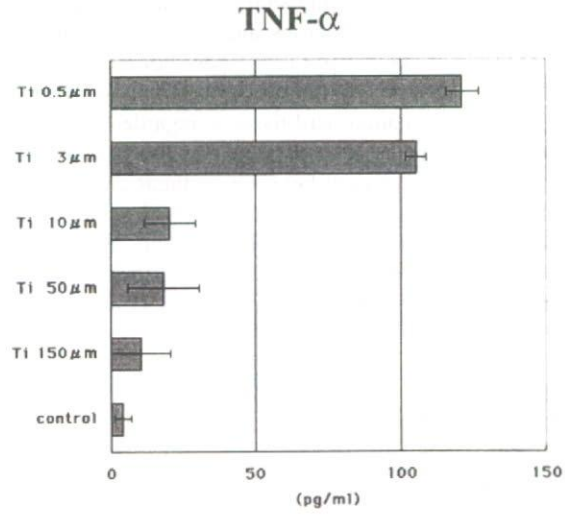

Fig. 2. Dependence of TNF-α release from human neutrophils on Ti particle size [1]

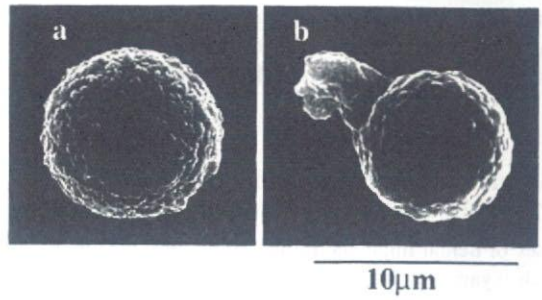

Fig. 3. SEM images of human neutrophils. **a** Control, **b** exposed to particles of Ti (500 nm) [3]

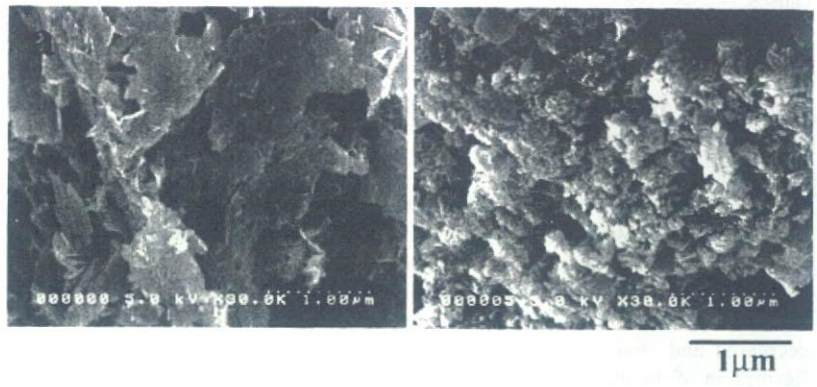

Fig. 4. Hydroxyapatite synthesized without (**a**) and with (**b**) collagen

than about 10 μm, phagocytosis was not observed. The pronounced phenomena of biochemical cell reaction for below 10 μm in Fig. 2 are closely related to the phagocytosis shown in Fig. 3.

The histological image of tissue reaction of rat to the different sizes of Ti particles showed a similar size dependence to those in vitro shown in Figs. 2 and 3.

These phenomena occur commonly in any bioactive and bioinert materials other than Ti, such as Fe and TiO_2 where particles induce non-specifically phagocytosis to cells and inflammation to tissue for the size below 10 μm, about the cell size. It is different from the usually observed toxicity due to the ionic dissolution effect in the macroscopic size [4].

Apatite formation with and without collagen

Figure 4 shows the comparison of morphology of hydroxyapatite synthesized without (a) and with (b) collagen by SEM observation. The particle size of

apatite is mostly a few microns for without-collagen, while under the coexistence of collagen the product becomes the agglomerate of apatite crystallites of less than 100 nm with the lower crystallinity, revealed from X-ray diffraction analysis.

Failure of dental implants by bone desorption

In clinical cases of dental implants, failure sometimes occurs. Figure 5 shows the example of hydroxyapatite-coated titanium implant: before (a) and after (b) implantation. Failure occurs through inflammation and the resorption of apatite and the surrounding alveolar bone. Inflammation is induced by various reasons. The breakage of apatite-coating film or release of fine dust of apatite powders is one of the causes.

Resorption of nanoapatite and simultaneous osteogenesis in bone circumstances

When the biomimetic nanocomposites of apatite and collagen fibrils were implanted in the subcutaneous tissue, they were covered with fibrous connective tissue and then resorbed mostly at 8 weeks by phagocytosis.

Figure 6 shows the histopathological image when they were implanted in the bone marrow of rat for 8 weeks [2]. The area of nanocomposites (asterisks) was decreased and covered with new bone (white asterisks) of lamellar structures. Resorption of the nanocomposites and replacement by new bone proceeded. This tendency was progressed with time by 12 weeks. As shown in Fig. 6, phagocytosis

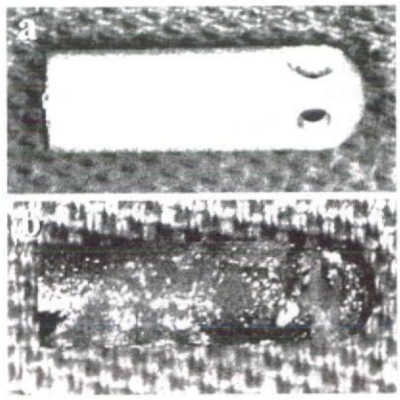

Fig. 5. Example of failure of dental implant of apatite-coated titanium: before (**a**) and after (**b**) implantation

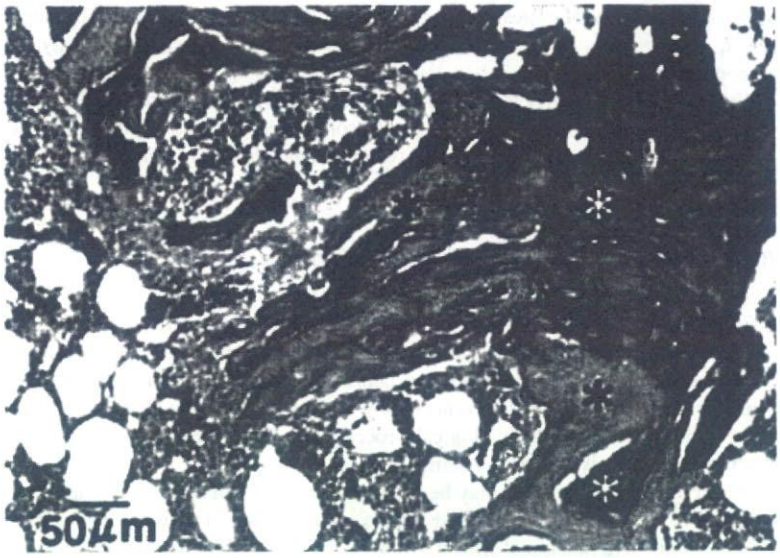

Fig. 6. Histology at 8 weeks after implantation in the bone marrow of rat. Materials (*asterisks*) were decreased and covered with new bone (*white asterisks*) with lamellar structures. AZ stain [2]

of nanoapatite by osteoclasts and osteogenesis by osteoblasts occurred adjacently to each other. Resorption and remodeling were similar to the case of autologous bone graft. As a result nanoapatite composites work as bone substitute materials for hard-tissue reconstruction.

Discussion

Nanosizing effect (general)

Nanosizing effect of materials onto living organism is usually interpreted as the aspects of the increase in specific surface area, which pronounces the chemical reactivity with the decrease in particle size. Effects related to the ionic dissolution correspond to this category, such as the acceleration of toxicity observed in Ni where tumor was generated in the long-term implantation for 0.5 μm particles [4], compared with necrosis that occurred in short term for macroscopic size [5]. There are, however, other kinds of effects [4]. Biocompatible titanium causes inflammation in abraded fine particles, when produced in the sliding parts of artificial joints, and asbestos [6], a kind of clay mineral, induces mesothelioma after a long

term, large quantity of exposure. They can be understood as the physical particle effect, apart from the chemical material properties of either toxicity or biocompatibility.

Figure 2 showed clearly the cytotoxicity due to fine particles and its size dependence. Cytotoxicity and inflammation were pronounced when the particle size was smaller than 10 μm, about the cell size, where phagocytosis was induced.

Bioactive properties induced by nanosizing

Specific surface area effect is based solely on the material properties, and material-dependent, whereas the physical particle size effect has the origin in the relative size relationship between particles and cell/tissue and independent of materials. Stimulus arises as non-specific events to any bioinert, bioactive materials of metals [7], ceramics, polymers by biological process, which induces the occurrence of functionality of body defense system.

The term "biocompatible" may be classified into two categories: "bioinert" and "bioactive". "Bioinert" may be used for the materials which give neither harmful effects nor positive functional effects. Alumina, carbon, and Ti may also be included in this category. "Bioactive" is used for the materials which induce the intrinsic functional effects of the living organism, usually in a positive sense, for example, apatite inducing osteoconductivity.

The judgment of positive or negative is based on the evaluation system in the application for human beings whether they work usefully or obstructively, and indifferent from their generation mechanism. If we enlarge the definition of "bioactive" as the potential properties to induce the intrinsic functional effects of the living organism, including both the positive and negative sense, nanosizing effect can be classified as bioactive whether it generates inflammation or osteogenesis.

Nanosizing induces the non-specific phagocytosis of particles, which gives rise to the superoxide production, cytokine emission and differentiation/activation of cells which lead to inflammation in tissue.

Nanosizing effect in apatite

In the case of apatite, nanosizing effect induces phagocytosis and leads to the apparent inflammation, which causes bone resorption in some cases like Fig. 5 and bone formation in other cases like Fig. 6, depending on the bone circumstances of these events. The former causes the failure of dental implants where the hydroxyapatite-coating film on titanium implant and the surrounding newly formed bone were resorbed. The breakage of apatite-coating film and release of fine dust of nanoapatite powders could be one reason to activate osteoclasts and other phagocytizing cells. The similar phenomena are also well known for other materials. For

example, abrasion particles produced from the sliding parts of artificial joints cause inflammation, whether material is polymer (polyethylene, etc.), metal (Ti, Co–Cr) or ceramics (alumina), and lead to osteolysis in the surrounding bone tissue, which determines the lifetime of using artificial joints.

When nanoapatite-collagen composites [2] or their derivatives reinforced with PLA or PLGA [8] were implanted into the bone defects of hard tissue, it leads apparently to the inflammation where cytokine emission and the differentiation or activation of osteoclasts and osteoblasts occur. Then the phagocytosis of nanoapatites by osteoclasts and osteogenesis by osteoblasts occur adjacently to each other, and the resorption of nanoapatite composites and new bone formation proceed simultaneously with time as shown in Fig. 6. As a result nanoapatite composites are substituted with new bone. Thus nanoapatite induces bioactive functions and works as bone substitutional. This tendency is more enhanced for carbonated hydroxyapatite. These phenomena are very similar to the bone remodeling process which occurs in natural bone.

Conversion of functions by nanosizing

Apatite in macroscopic size works as osteo-conductive but non-bone substitutional, while nano-size apatite works as bone substitutional.

Here, there is a conversion of functions of materials by nanosizing—from osteo-conductivity to bone substitutional properties in apatite.

Stimulus and bioactive properties of nanomaterials induced by biological process

Nanosizing causes the reaction of cells/tissue and stimulates to the occurrence of inflammation, which works as the stimulus in most cases. This toxicity is very weak compared with endotoxin [4]. Inflammation generates the conversion of functions leading to the bioactive functions for some cases, depending on the situation. These stimuli are different from those by specific surface effect where origin is solely from materials.

Conclusions

Synthesized hydroxyapatite, usually in macroscopic size, is osteoconductive but non-bone substitutional. Nanosizing of apatite induces bioactive reactivity to tissue where bone resorption or bone substitutional functions arise through the expression of inflammation, depending on the circumstances. Adjacent occurrence of resorp-

tion of nanoapatite composite by osteoclasts and simultaneous new bone formation by osteoblasts is very similar to the remodeling process of natural bone. Nanosizing works as a bioactive and causes inflammation, which leads to the conversion of functions through biological process such as from biocompatible to stimulative or from osteoconductive but non-bone substitute to bone substitute. Thus nanosizing of apatite is essential for hard tissue reconstruction and bone remodeling in the living organism.

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Biomaterials based on mineralised collagen—an artificial extracellular bone matrix

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Abstract. Extracellular matrix (ECM) of bone tissue consists of a highly organised nanocomposite made of fibrillar collagen type I and calcium phosphate mineral phase hydroxyapatite. We have developed a process to synthesise a material, mimicking bone ECM, and produced several biomaterials out of this mineralised collagen, suitable for use in oral medicine and maxillofacial as well as in general surgery. Synthesis of the nanocomposite, development of the different types of scaffolds, some of their properties, and possible applications are discussed.

Key words. scaffold, collagen, hydroxyapatite, biomimetic, nanocomposite

1 Introduction

Bone biomineralisation is a complex and multi-step process, starting with collagen type I biosynthesis, followed by precipitation of hydroxyapatite nanocrystals. Calcium phosphate crystallisation is induced by expression of the enzyme alkaline phosphatase (ALP) and controlled by several non-collagenous proteins such as osteocalcin and osteopontin, secreted by osteoblasts. The resulting mineralised extracellular bone matrix can be interpreted as a highly organised nanocomposite material with not only unique mechanical, but also biological properties [1]. In our lab we have been working on synthetically mineralised collagen as biomimetic implant material, scaffold for bone tissue engineering, and in vitro model for bone biomineralisation and remodelling for several years. In this study, a review of the biomaterials based on mineralised collagen and those suitable for use in oral medicine and maxillofacial as well as in general surgery is given. This includes membrane-like materials for covering of bone defects as well as non-mineralised collagen membranes for tissue engineering of oral mucosa, porous three-dimensional scaffolds, and biphasic implant materials for the therapy of osteochondral defects and also resorbable calcium phosphate bone cements, functionalised with mineralised collagen as fibre reinforcement.

2 Materials and methods

Preparation of mineralised collagen

Preparation of mineralised collagen fibrils was described in detail elsewhere [2]. Briefly, a collagen type I solution in hydrochloric acid is combined with calcium and sodium chloride, TRIS- and phosphate buffer (final pH = 7.0). Warming up the mixture to 37°C starts the fibril reassembly process which occurs coevally with precipitation of nanocrystalline hydroxyapatite (HAP). Here, the collagen acts as a template for mineral deposition. Finally, a homogenous nanocomposite consisting of about 30% collagen and 70% mineral is formed which can be isolated by centrifugation as a soft, wax-like material.

Biomaterial development

Using the mineralised collagen as a basic material, several types of scaffolds have been developed. Applying a vacuum filtration process, followed by chemical crosslinking with *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC), a flat, membrane-like material ("tape") was achieved [3, 4]. Treatment of these mineralised tapes with acidic TRIS buffer led to dissolution of the HAP nanocrystals and therefore to the formation of a pure collagen membrane, suitable for tissue engineering of oral mucosa. By freeze–drying mineralised collagen suspensions, followed by EDC crosslinking, porous 3D scaffolds were obtained [5, 6]. By combining these with a layer of a non-mineralised collagen hyaluronic acid composite, joint freeze–drying and crosslinking, biphasic, but monolithic scaffolds for therapy of osteochondral defects were developed [7]. The modification of a resorbable calcium phosphate bone cement with mineralised collagen fibrils was described in detail in previous studies [8, 9].

Cell culture and animal experiments

Cell seeding and analysis of the cell matrix constructs were carried out under the same conditions as described elsewhere [4]. Briefly, bone marrow derived human mesenchymal stromal cells (hBMSC) were seeded on membranes of mineralised as well as demineralised collagen. For this experiment, collagen, isolated from calf skin (Collaplex 1.0, GfN, Waldmichelbach, Germany) was used. Adhesion of cells was investigated by fluorescence microscopy. In addition, proliferation and osteogenic differentiation of hBMSC, growing on mineralised collagen membranes (collagen isolated from bovine tendon, kindly provided by Syntacoll, Saal/Donau, Germany), was studied. Cells were cultivated in DMEM with 10% foetal calf serum (FCS) without (–OS) and with osteogenic supplements (dexamethasone, β-glycerophosphate and ascorbic acid 2-phosphate; +OS). Cell number was determined by measurement of lactate dehydrogenase (LDH) activity, and osteogenic

differentiation was monitored by ALP activity quantification. Porous 3D scaffolds made of mineralised collagen were tested in an animal model. The material was implanted in a defect made in rat femur. Details of the procedure were published by Yokoyama et al. [6].

3 Results

Synchronous collagen fibril reassembly and mineralisation with nanocrystalline HAP led to a homogenous composite material which mimics extracellular matrix (ECM) of healthy bone tissue [2]. Using these mineralised collagen fibrils, several different types of scaffolds have been developed.

Membranes made of mineralised or demineralised collagen

Densification of resuspended mineralised collagen by means of vacuum filtration leads to a flat, membrane-like material ("tape") [3]. After stabilisation by chemical crosslinking of the collagen with carbodiimide derivative EDC, the tapes can be used as a model substrate for osteogenic differentiation of hBMSC [4] and for in vitro studies on bone matrix remodelling by co-culturing osteoblasts and osteoclasts [10]. A scanning electron microscopy (SEM) image of the microstructure of the tape is given in Fig. 1a, showing the close interaction between the reconstituted collagen fibrils and the nanocrystalline HAP phase and the microporosity of the material.

Proliferation of hBMSC seeded on tapes was investigated by measurement of LDH activity after cell lysis at different time points of culture. An increase in the number of living cells could be observed in the presence (+OS) as well as in the absence (–OS) of osteogenic supplements (Fig. 2a). However, "–OS" cells showed higher proliferation rates in comparison with "+OS" cells. Thus, a more than sevenfold increase was detected for non-induced cells over a period of 21 days compared to a fourfold increase of osteogenically induced cells. To analyse the osteogenic differentiation of hBMSC, ALP activity in the same cell lysates were determined and related to the cell number. Specific ALP activity of the "+OS" cells

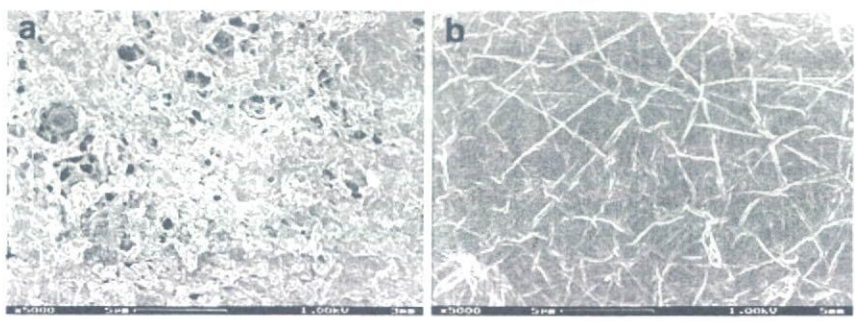

Fig. 1. SEM micrographs of the surface of **a** mineralised collagen membrane ("tape") and **b** demineralised collagen membrane. ×5,000

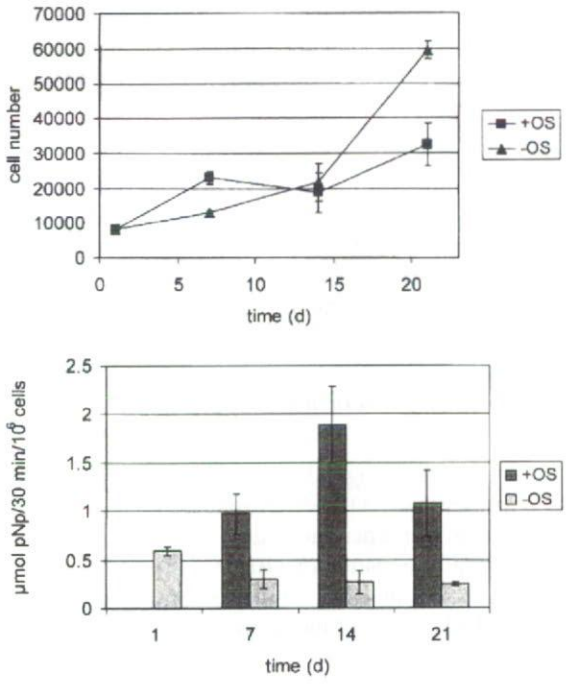

Fig. 2. Comparison between cell culture without ("–OS") and with osteogenic supplements ("+OS"). **a** Proliferation of hBMSC, and **b** specific activity of alkaline phosphatase (ALP) of hBMSC, growing on membranes of mineralised collagen

raised over the cultivation period of 21 days with a maximum on day 14 (Fig. 2b). In contrast, ALP activity of non-induced cells was not increased.

To utilise the tapes for non-mineralised tissues like oral mucosa too, they were demineralised by storage in acidic 0.1 M TRIS-buffer (pH 2) for 2 days. After dissolution of the mineral phase the surface of the tape is smooth and the micropores had disappeared (Fig. 1b). Both materials support hBMSC attachment: there were no obvious differences between mineralised and demineralised collagen. At early stages of culture many cells had already attached to both types of membrane and showed initial spreading. After 24 h most of the cells were attached and exhibited the characteristic fibroblast-like morphology known for mesenchymal stromal cells (Fig. 3). These findings were confirmed by biochemical analysis (data not shown).

Porous three-dimensional scaffolds

Applying freeze–drying and chemical crosslinking, porous 3D scaffolds can be prepared out of mineralised collagen which exhibit interconnecting pores with diameters of about 200 µm [5]. Due to their elastic properties in the wet state the material is suitable to act as a scaffold for cell culturing under cyclic mechanical stimulation. The material already has been tested successfully in an animal model [6]. Figure 4 shows a histological image taken 2 weeks after implantation of the material in a cavity made in the rat femur. The pores of the scaffolds are heavily invaded by osteoblasts which produce new mineralised matrix, deposited directly on the inner