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Effect of Bioactive Glass Templates on Osteoblast Proliferation and In Vitro Synthesis of Bone-Like Tissue

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Abstract Using in vitro synthesized bone tissue with cells aspirated from the patient's marrow is an appealing idea to avoid the profound limitations of biological and synthetic grafts. Procedures to synthesize bone tissue in vitro primarily relied on seeding various substrates with cells that have osteogenic capacity in culture. It should be noted that in an in vitro system, osteoprogenitor cells, as well as bone cells themselves can rapidly change their phenotype, hence the substrate needs to promote the expression of the bone cell phenotype. Furthermore, it needs to provide a template for bone deposition while gradually resorbing once bone tissue has been laid down. This paper presents initial evidence that bioactive glass, a synthetic material with documented extensive bone bioactivity properties, represents a material that optimally combines the requirements of the ideal template for in vitro synthesis of bone tissue. When made in porous form, and conditioned to develop a bone-like surface prior to being seeded with pluripotential cells capable of expressing the osteoblastic phenotype, these templates lead to expeditious and abundant in vitro synthesis of extracellular matrix with most important characteristics of bone tissue.

Key words: bioactive glass, in vitro synthesis of bone tissue, osteoblast, bone tissue

Materials were first used to provide structural support during healing of bones, or to replace damaged or diseased bone tissue. Historically, the most important material selection criterion was inertness. It was believed that the implant material should only provoke the slightest of reactions in the body. It is important to realize, though, that no matter how chemically inert a material may be, it always provokes a reaction upon implantation. The intensity of reaction does not only depend on the surface and bulk properties of the implant material, but also on the trauma at the time of surgery, the site of implantation, and the relative motion at the tissue-implant interface [Albrektsson et al., 1983]. These observations prompted the use of bioactive materials [Hench et al., 1972; Jarcho et al., 1976; Aoki et al., 1979; DeGroot, 1980; Ducheyne et al., 1980; Yamamuro et al., 1984] instead of so called inert materials. The implication is that a bioactive material must provoke a beneficial tissue response; specifically it must elicit the formation of the normal tissue at its surface and create an interface that promotes long functional life. The field of calcified tissue reconstruction has achieved this goal of synthesizing materials that enhance bone tissue formation and bond to bone in vivo. We suggest that this achievement is the stepping stone for an even more ambitious goal: create materials that are capable of serving as a template for in vitro bone tissue formation. This is part of the true future of biomaterials: create materials that help to regenerate the tissues, rather than replace them. In what follows we review some of the studies that bear significantly on the issue of achieving extensive extracellular matrix formation by osteogenic cells.

The synthesis of bone-like tissue in vitro can be pursued by culturing cells capable of expressing the osteoblastic phenotype. Maniatopoulos et al. [1988] documented that bone marrow cells extracted from the femora of young adult male Wistar rats were capable of expressing the osteoblastic phenotype when cultured in a plastic culture flask for 20 days. Subsequent studies determined the variation in biological effect arising from the use of different substrate materials. Using neonatal rat calvaria osteoblast cells,

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Puleo et al. [1991] reported on variations in osteoblast attachment, osteoblast proliferation, and collagen synthesis by using 316 L stainless steel, Ti-6A1-4V, Co-Cr-Mo, PMMA, hydroxyapatite, borosilicate glass, and tissue culture polystyrene substrates: the data, however, did not allow to discern crisp substrate materials dependent effects. As indicated, one of the materials of the study was hydroxyapatite, a so called bioactive calcium phosphate ceramic of which one would have expected a beneficial effect. The data of Puleo et al. [1991] are not surprising, though, in the context of all the studies that used calcium phosphate ceramics in cell culture experiments: both beneficial as well as adverse phenomena were reported. Cheung et al. [1989] studied the effect of porous calcium phosphate ceramic on growth and hormonal response of periosteal fibroblasts, osteoblasts, and chondrocytes. The number of these cells increased 29-, 23-, and 17-fold, respectively, during a 10 week time period. Osteoblasts retained their phenotypic expression by producing only type I collagen. Before, Cheung [1985] had shown that the phenotypic expression of canine chondrocytes had been retained up to 13 months, when cultured on porous hydroxyapatite ceramic granules. The elaboration of extracellular matrix started to appear at week 1 and increased throughout the 13 month period. Hyakuna et al. [1989] studied the attachment and subsequent growth of Chinese hamster fibroblasts in contact with various calcium phosphate ceramics and found that cell growth was markedly inhibited by hydroxyapatite, and slightly inhibited by tricalcium phosphate and glass ceramics. Under conditions supporting phagocytosis of small bioactive ceramic powders, Gregoire et al. [1990] found that RNA transcription and protein synthesis of osteoblast populations were stimulated. This phenomenon was also observed for phagocytosing fibroblasts. Orly et al. [1989], from the same lab, suggested that the increase of ^{3}H-thymidine incorporation into DNA and the decrease of alkaline phosphatase activity probably resulted from secondary calcium messenger pathways. Elgendy et al. [1993] used the osteogenic cell line (MC3T3-E1) to study cell attachment and cell growth on the surfaces of 50:50 poly (lactide-co-glycolide), hydroxyapatite, 50:50 hydroxyapatite/poly(lactide-co-glycolide), and the poly(anhydride), poly(bis p-carboxyphenoxy) propane. Cell adhesion and growth of MC3T-E1 cells on the polymer and ceramic systems were higher on (lactide-co-glycolide) and hydroxyapatite/poly(lactide-co-glycolide) than hydroxyapatite.

Few studies have attempted to modify the material properties to maximize desirable biological effects in vitro. If the eventual goal is to implant in vitro synthesized bone tissue in sites of major tissue deficiency, bone tissue must be synthesized abundantly and expeditiously. Thus, in this paper we document alterations in the characteristics of the biomaterial substrate that markedly affect the synthesis of extracellular matrix by cells expressing the osteoblastic phenotype.

Over the last 3 years we have demonstrated that neonatal rat calvaria osteoblasts elaborate substantial amounts of extracellular material when inoculated on porous bioactive glass disks [El-Ghannam et al., in press]. Disks of porous 45S5 bioactive glass were prepared by the foaming method. The nominal glass composition was, in weight percent, 45% SiO_2, 24.5% CaO, 24.5% Na_2O, and 6% P_2O_5. The porosity was 20–30% and the pore size range 75–200 μm [El-Ghannam et al., 1993]. The glass surface was typically conditioned by immersion in 0.05 M "modified Tris" buffered at pH 6.8. After conditioning, the glass surface was analyzed by Auger electron spectroscopy (AES), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction analysis (XRD), and scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDAX). The glass surface was found to be covered by a calcium phosphate-rich layer.

After conditioning, the disks were placed in 60 mm diameter Petri dishes, wetted with tissue culture medium and inoculated with about 1×10^6 neonatal rat calvaria osteoblasts from a suspension containing 1.2×10^6 cells/ml [El-Ghannam et al., 1993]. To allow for cell attachment, the dishes were kept in the incubator for 1 h prior to flooding them with tissue culture medium supplemented with 20 mM Hepes (TCM). Two days later the TCM was exchanged by TCM supplemented with 3 mM β-glycerophosphate. This exchange was repeated at day 4. The dish contents were analyzed at day 7. Cell culture experiments with glass, but without cells and with cells, but without glass were run as control experiments.

After 1 week in culture, the porous specimens were found to be totally invaded by cells and the extracellular matrix they elaborated: Figures 1 and 2 are SEM micrographs illustrating this

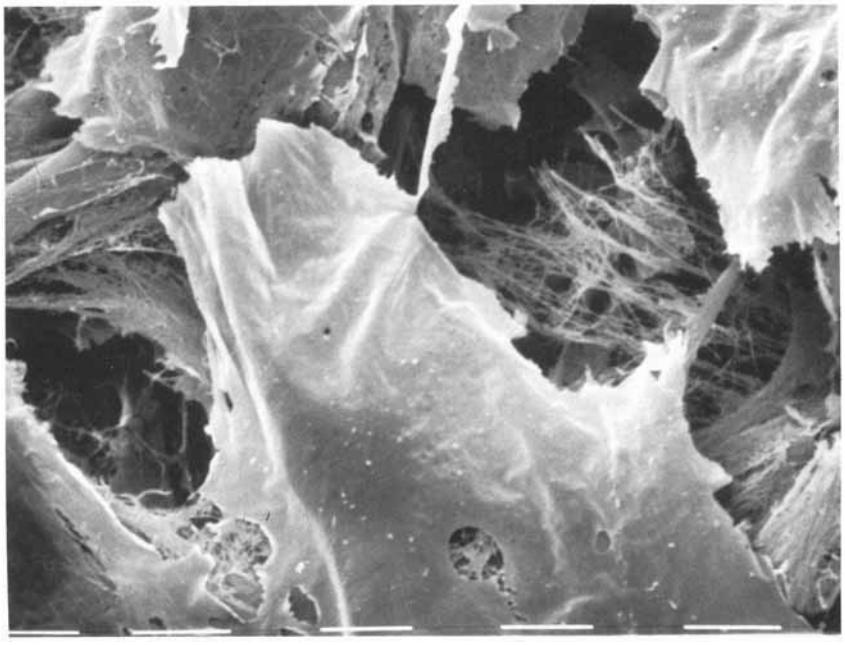

Fig. 1. Scanning electron micrograph of the bioactive glass surface inoculated with neonatal rat calvaria osteoblasts for 1 week. The glass surface was covered by collagen fibrils and bone-like tissue. ×1,000.

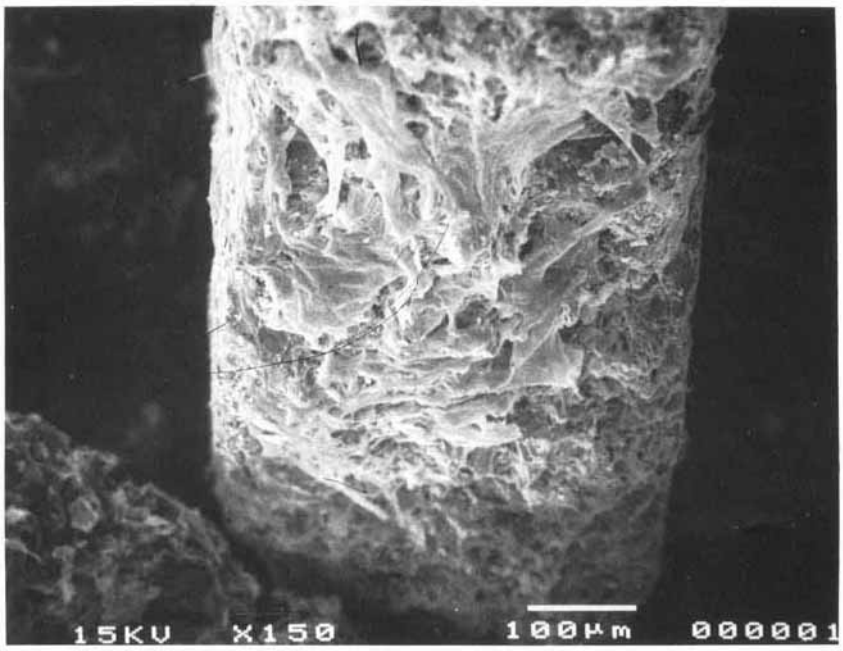

Fig. 2. Scanning electron micrograph of cross section through the porous bioactive glass after 1 week of incubation. Bone-like sheets grew throughout the whole thickness of the porous glass sample. ×150.

finding. The qualitative assessment of alkaline phosphatase activity revealed that the highest activity was with the cells on bioactive glass discs; there was only faint activity on the Petri dish bottoms. Quantitative alkaline phosphatase activity measurements were expressed as mean value per dish; the release of p-nitrophenol (pNP) from nitrophenyl phosphate was determined. The observed rate of 0.62 nmol pNP/min · µg DNA is a value typical of osteoblastic phenotype

expression measured in this laboratory [Brighton et al., 1991].

Morphologic observation of experimental vs. control samples suggested the following hypothetical sequence of events: 1) Foci of calcification were formed: small globules 1–2 μm in diameter appeared on the glass surface (EDAX confirmed the typical Ca/P ratio). 2) Collagen fibrils were produced and became attached to the calcified nodules (collagen I synthesis was confirmed [El-Ghannam et al., 1993]. 3) The synthesis of both calcified substance and intertwined fibrils continued, thereby leading to gradual formation of bundles that eventually coalesced into sheets of bone-like material. The calcification of the elaborated material was not the result of a physico-chemical phenomenon of precipitation from a solution supersaturated in Ca and P resulting from glass dissolution [El-Ghannam et al., 1993]. Control experiments with glass, but without inoculation of cells, or cell culture experiments without porous bioactive glass did not produce a measurable calcification. Standard thin section histological techniques can easily be applied to these samples to reveal the morphology of the tissue formed.

Cell culture studies with osteoprogenitor cells or cells of osteoblastic phenotype have been performed before, but have not yielded continuous sheets of bone-like tissue. As Davies et al. [1990] pointed out, differentiating bone-derived cells have been observed to produce interfacial extracellular matrix in vitro. They inoculated different material substrata with bone marrow cells from the femora of young adult male Wistar rats and maintained the cultures for a minimum of 2 weeks. Calcified matrix of globular accretions, containing also sulfur, was formed on the various materials. The authors compared this layer to the cementum layer typical for reversal lines in bone tissue. Furthermore, they provided evidence that formation of this calcified layer was dependent upon the expression of an osteoblastic phenotype by the cultured cells. Longer incubations yielded what they termed: "frank bone formation." The authors concluded that "this is the first time that such early interfacial matrix production (17 days) by differentiating bone-derived cells has been observed in vitro." In an earlier publication by Uchida et al. [1987] calcified tissue formation was not obtained. Our results [El-Ghannam et al., 1993] document not only formation of an interfacial layer much sooner, i.e., 7 days as opposed by 17, but also major formation of sheets of bone tissue-like material throughout the porous disks.

Two other sets of in vivo studies documenting materials dependent tissue response patterns are noteworthy. A series of experiments with porous hydroxyapatite and bone marrow cells was started by Ohgushi, Goldberg and Caplan [1989] and subsequently continued separately by Ohgushi and associates in Nara, Japan and by Caplan and associates in Cleveland. These experiments showed that the osteoprogenitor nature of the cells of a marrow cell suspension was activated more readily when the suspension was implanted in heterotopic sites, i.e., when the cells were infused into porous hydroxyapatite (Interpore) than when implanted in the same site by themselves. Okumora et al. [1993] have also applied this methodology of seeding marrow cells in porous materials to porous titanium. Marrow cells infused in porous titanium were also observed to lead to bone tissue formation in an ectopic site.

Glasses are known to produce a beneficial effect beyond what the calcium phosphate ceramics produce: Schepers et al. [1991] implanted bioactive glass granules of the same composition as in the cell culture experiment reported above. The granules were characterized by a critical narrow size range: 300–375 μm. Porous hydroxyapatite (Interpore R-200; interconnecting pore size: 190–230 μm) and dense hydroxyapatite (Calcitite: 400–800 μm in diameter) granules were used as intra animal controls. Each material exerted a beneficial effect on bone tissue formation. Bone tissue repair started from the bony defect wall and moved along the particles. Whereas each material was undoubtedly bioactive, there were significant differences, however, specifically with respect to i) the rate of resorption of the various particle types, ii) the rate of new bone formation which increases with increasing resorption rate of the particles, iii) the intensity and duration of any transient or sustained inflammatory response, and iv) mesenchymal cell differentiation to osteoblasts.

This fourth observation may be of particular significance. As early as 1 month after implantation, corrosion reaction penetrated into the glass particle core. The resulting pouches were connected to the surrounding fluids by small channels. Within these pouches of the glass particle, one could observe osteoid tissue, bone tissue and

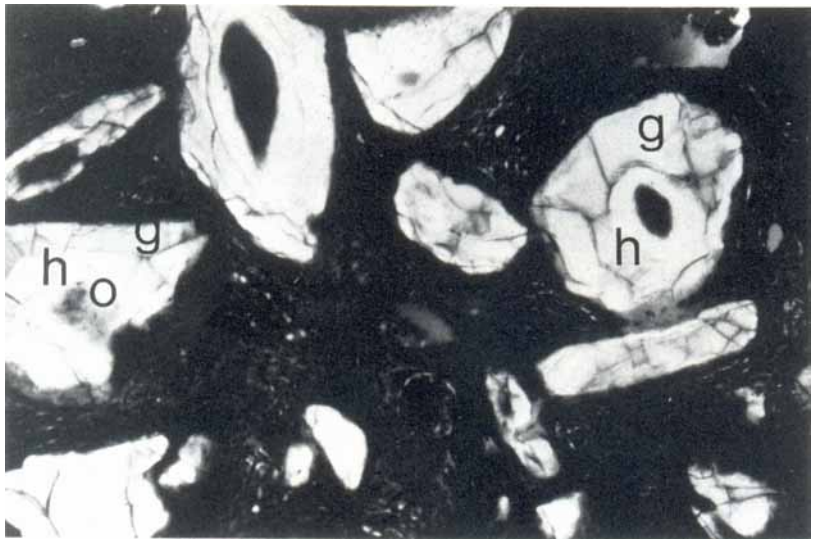

Fig. 3. Bioactive glass (g) particles which, at 3 months, have been hollowed out internally and now harbor osteoid tissue (h) and osteoblasts (o); there is no direct connection between the bone tissue in the particle and the bone of the defect walls. ×80.

a string of osteoblasts (see Fig. 3). This phenomenon occurred gradually and was present predominantly at three months. This newly formed bone tissue was not connected to the bone of the walls of the surgically created defect. None of these observations could be made for either of the hydroxyapatites.

Other recent culture studies [Elgendy et al., 1993; Vrouwenvelder et al., 1993] with cells expressing the osteoblastic phenotype seeded onto synthetic material templates also achieved bone-like tissue formation in vitro, but again the extent was markedly less than with our approach described here. Aside from differences in experimental objectives, we suggest that the more thorough osteogenesis observed in our experiments derives from integrating material science and biochemical insights to produce a result neither achieved by either approach alone. Thus, to optimize in vitro synthesis of bone tissue one must use or achieve i) cells with osteogenic capacity, ii) proper tissue culture medium composition, iii) optimal incubation conditions including flow conditions, and iv) optimal substrate material including type of material, porosity and surface conditioning. Although it should be apparent that materials affect the biological pathways to bone tissue formation, a mechanistic explanation for the materials effect has not been achieved.

EDITOR'S NOTE

This contribution was selected by the Editors to compare an approach using biomolecular material (Reddi) with an approach using nonbiologic material, in this case bioactive glass as a template for stimulating osteoblast proliferation and formation of bone-like tissue.

REFERENCES

Albrektsson T, Branemark PI, Hansson HA, Kasemo B, Larsson K, Lundstrom I, McQueen DH, Skalak R (1983): The interface zone of inorganic implants in vivo: Titanium implants in bone. Ann Biomed Eng 11:1–27.

Aoki A, Kato K, Tabato T, Ogiso M (1979): "Orthopaedic and Dental Implant Ceramic Composition and Process for Preparing Same." US Patent Number 4,149,393.

Brighton CT, Schaffer JW, Shapiro DB, Tang JJS, Clovek CC (1991): Proliferation and macromolecular synthesis by rat calvarial bone cells growing in various oxygen tensions. J Orthop Res 9:847–851.

Cheung HS, Haak MH (1989): Growth of osteoblasts on porous calcium phosphate ceramic: an in vitro model for biocompatibility study. Biomaterials 10:63–67.

Cheung HS (1985): In vitro cartilage formation on porous hydroxyapatite ceramic granules. In Vitro Cell Dev Biol 21:353–357.

DeGroot K (1980): Bioceramics consisting of calcium phosphate salts. Biomaterials 1:47–50.

Davies JE, Ottensmeyer P, Shen X, Hashimoto M, Peal AF (1990): "Early Extracellular Matrix Synthesis by Bone Cells," Bone-Biomaterials workshop. JE Davies (ed). Toronto: University of Toronto Press, pp 214–228.

Ducheyne P, Hench LL, Kagan A, Martens M, Bursens A, Mulier JC (1980): The effect of hydroxyapatite impregna-

tion on skeletal bonding of porous coated implants. J Biomed Mater Res 14:225–237.

Elgendy HM, Norman ME, Keaton AR, Laurencin CT (1993): Osteoblast-like cell (MC3T3-E1) proliferation on bioerodible polymers: An approach towards the development of a bone-bioerodible polymer composite material. Biomaterials 14:4.

El-Ghannam A, Ducheyne P, Shapiro I (in press): Bioactive material template for synthesis of a bone-like tissue in vitro. J Biomed Mater Res.

El-Ghannam A, Ducheyne P, Shapiro I (1993): A new bioactive glass template for the synthesis of bone tissue in vitro. In: Ducheyne P, Christiansen D (eds). "Bioceramics 6." Oxford, U.K.: Butterworth and Hienemann, Ltd., pp 143–149.

Gregoire M, Orly I, Menanteau J (1990): The influence of calcium phosphate biomaterials on human bone cell activities: An in vitro approach. J Biomed Mater Res 24:165–177.

Hench LL, Splinter RJ, Allen WC, Greenlee TK Jr (1972): Bonding mechanisms at the interface of ceramic prosthetic materials. J Biomed Mater Res Symp 2:117–141.

Hyakuna K, Takao Y, Yoshihiko K, Yoshiaki K, Tochiaki K, Haruki T, Masanori O, Tadashi K (1989): The influence of calcium phosphate ceramics and glass ceramics on cultured cells and their surrounding media. J Biomed Mater Res 23:1049–1066.

Jarcho M, Bolen H, Thomas MB, Bobick J, Kay JF, Doremus RH (1976): Hydroxyapatite synthesis and characterization in dense polycrystalline form. J Mater Sci 11:2027–2035.

Maniatopoulos C, Sodek J, Melcher AH (1988): Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res 254:317–330.

Ohgushi H, Golalberg VM, Caplan A (1989): Heterotopic osteogenesis in porous ceramics induced by marrow cells. J Ortho Res 7:568–578.

Okumura M, Ohgushi H, Ducheyne P (1993): Porous titanium felt as a carrier of osteogenic cells. Ducheyne P, Christiansen D (eds). "Bioceramics 6." Oxford, U.K.: Butterworth & Hienemann, Ltd.

Orly I, Gregoire M, Menanteau J, Dard M (1989): Effect of synthetic calcium phosphate on the 3H-thymidine incorporation and alkaline phosphatase activity of human fibroblasts in culture. J Biomed Mater Res 23:1433–1440.

Puleo DA, Holleran LA, Doremus RH, Bizios R (1991): Osteoblast responses to orthopaedic implant materials in vitro. J Biomed Mater Res 25:711–723.

Schepers E, De Clercq M, Ducheyne P, Kempeneers R (1991): Bioactive glass template material as a filler for bone lesions. J Oral Rehab 18:439–452.

Uchida A, Nade S, McCartney E, Ching W (1987): Growth of bone marrow cells on porous ceramics in vitro. J Biomed Mater Res 21:1–10.

Vrouwenvelder WCA, Groot CG, de Groot K (1993): Histological and biochemical evaluation of osteoblasts cultured on bioactive glass, hydroxyapatite, titanium alloy, and stainless steel. J Biomed Mater Res 27:465–475.

Yamamuro T, Nakamura T, Higashi T, Kassai R, Kakutani Y, Kitsugi T, Kokubo T (1984): An artificial bone for use as a bone prosthesis. In Atumi K, Maekawa M, and Ota K (eds): "Progress in Artificial Organs—1983," Vol. 2. Cleveland: ISAO Press, p 810.