

Ionic Products of Bioactive Glass Dissolution Increase Proliferation of Human Osteoblasts and Induce Insulin-like Growth Factor II mRNA Expression and Protein Synthesis

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Bioglass 45S5 is an osteoproliferative material, which resorbs by releasing its constitutive ions into solution. Treatment with the ionic products of Bioglass 45S5 dissolution in DMEM for 4 days increased human osteoblast proliferation to 155% of control. Two days after treatment, differential gene expression was analyzed by cDNA microarrays. Expression of a potent osteoblast mitogenic growth factor, insulin-like growth factor II (IGF-II), was increased to 290%. Additionally, there was a 168% increase in the concentration of unbound IGF-II protein in the conditioned media of treated osteoblasts. Expression levels of IGF-BP-3, an IGF-II carrier protein, metalloproteinase-2 and cathepsin-D were also increased to 200, 340, and 310% of control levels, respectively. Metalloproteinase-2 and cathepsin-D are proteases that cleave IGF-II from its carrier proteins, resulting in the release of the unbound biologically active IGF-II. We suggest that the stimulatory effect of the ionic products of Bioglass 45S5 dissolution on osteoblast proliferation may be mediated by IGF-II. © 2000 Academic Press

Key Words: human osteoblasts; osteogenesis; bioactive glass; silicon; IGF-II; IGF-BP-3; MMP-2; cathepsin-D.

Abbreviations used: Ca, calcium; C_T, threshold cycle; EIA, enzyme immunoassay; ICP, inductively coupled plasma analysis; IGF-BP, insulin-like growth factor binding protein; IGF-II, insulin like growth factor 2; MMP, matrix metalloproteinase; Na, sodium; P, phosphorus; PCR, polymerase chain reaction; RT, reverse transcription; Si, silicon.

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Recent strategies to repair bone utilize resorbable material matrices as scaffolds for the *in vitro* synthesis of bone tissue or, alternatively, as conduits for new bone tissue formation *in vivo* (1). Current scaffold materials can support new tissue growth by providing structural support for the initial phases of bone tissue invasion and growth (2). Nevertheless, these scaffolds often lack the additional soluble factors required to stimulate or guide the desired cell responses in terms of cell proliferation and tissue-specific differentiation (3). Soluble factors capable of inducing bone formation both *in vitro* and *in vivo* include growth factors such as bone morphogenetic proteins, transforming growth factor- β , and insulin like growth factors (4). Many current therapeutic bone repair strategies are based at the delivery of bone inducing molecules to skeletal defect sites. These agents could be delivered either directly (5, 6) or conjugated to implantable inert polymer materials (7). These delivery techniques, however, suffer from pharmacokinetic loss of protein due to a combination of a physical and biological degradation mechanisms.

Such considerations have led to the use of bioactive materials in tissue engineering strategies for the repair and regeneration of bone. Bioactivity indicates the ability of these materials to interact with cells and tissues and stimulate repair and regeneration. Bioactive glasses, in particular, are synthetic materials made of calcium (Ca), phosphorus (P), silicon (Si) and sodium (Na) oxides. Those containing 45–52 per 8% SiO₂ are osteoproliferative, thus they can induce the osteogenic differentiation of osteoblasts and stimulate the formation of bone tissue both *in vitro* and *in vivo* (8–10). Although the osteogenic ability of these materials is well documented, their mode of action on cells and tissues is largely unknown. It is anticipated that bone induction by bioactive glasses may occur through

direct contact between substrate and cells as well as through soluble ions released by these materials during their resorption. In this paper, we investigate the possible stimulatory effects of the ionic products of Bioglass 45S5 dissolution on human osteoblast proliferation and provide evidence that simple inorganic chemical solutions can deliver mitogenic stimuli.

MATERIALS AND METHODS

Cell culture and treatments. Human osteoblasts were isolated from trabecular bone of femoral heads taken during total hip arthroplasty, as described by Beresford *et al.* (11). The average donor age was 64 ± 8 years with a male: female ratio of 1:1. Cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin G, 50 μ g/ml streptomycin B and 0.3 μ g/ml amphotericin B (complete medium) at 37°C, in 95% air humidity and 5% CO₂. The bioactive glass-conditioned medium containing the dissolution products of Bioglass 45S5 (an osteoproliferative glass containing 45% SiO₂ w/w) was prepared by incubating 1% w/v Bioglass 45S5 particulate (710–300 μ m in diameter, US Biomaterials Inc., U.S.A.) in DMEM for 24 h at 37°C. Particulates were removed by filtration through a 0.20- μ m filter (Sartorius, UK) and the collected medium supplemented as described above for the complete medium. The elementary content of calcium (Ca), silicon (Si), phosphorus (P) and sodium (Na) in this solution were determined by inductively coupled plasma (ICP) analysis (12).

Osteoblasts were used at passages 2 and 3. For the evaluation of cell number, cells were seeded on to a 24-well plate at a density of 2.5×10^4 cells/cm². Forty-eight hours following seeding, cells were treated with bioactive glass-conditioned complete DMEM and control complete DMEM. After 4 days in culture, cells were released by trypsin, stained with trypan blue, and counted using a hemocytometer. For gene expression analysis, cultures at approximately 75% confluence were treated with bioactive glass-conditioned complete DMEM and control complete DMEM. After 48 h, cells were released by trypsin, centrifuged and snap frozen in liquid N₂. For studies of IGF-II protein synthesis, cells were seeded on to a 24-well plate at a density of 2.5×10^4 cells/cm². Forty-eight hours later, cells were treated with bioactive glass-conditioned DMEM and control DMEM, which were not supplemented with fetal bovine serum. Conditioned media and cells were harvested after 48 h.

Extraction of RNA. Total RNA was extracted using a phenol/chloroform method (Clontech Laboratories, Inc., Palo Alto, CA), precipitated with isopropanol and centrifuged at 15000g at 4°C. The RNA pellet was washed with 80% ethanol and resuspended in diethylpyrocarbonate-treated water. To remove genomic DNA, the RNA samples were treated with DNase (0.10 U/ μ l of DNase I, in DNase I buffer). The concentration and purity of total RNA in each sample were determined by light absorbance at 260 nm and by calculating the A₂₆₀/A₂₈₀ ratio, respectively. RNA integrity was confirmed by electrophoresis on a denaturing agarose/formaldehyde/ethidium bromide gel.

Analysis of gene expression using cDNA microarrays. Differential gene expression analysis of treated and untreated osteoblasts was performed using the ATLAS cDNA microarray Platform (Atlas 1.2 Human array, Clontech), which allowed the simultaneous screening of 1176 genes on paired nylon membranes. Osteoblasts cultures from four donors were compared in order to verify that the observed differences in gene expression were not due to differences between individuals. Briefly, gene specific primers were used for the synthesis of labeled cDNA using MMLV Reverse transcriptase which incorporated [³²P]-dATP (Amersham, UK). Labeled cDNA was purified from unincorporated nucleotides by gel filtration through Chroma Spin-200 columns (Clontech). The incorporation of ³²P in the probe was

determined by scintillation counting. Each filter was hybridized with equal amounts of radioactive probe. Pre-hybridization and hybridization were done at 68°C for 30 min and 16 h, respectively. Arrays were washed at 68°C in low stringency buffer (2 \times SSC, 1% SDS, 3 \times 30 min) followed by high stringency buffer (0.1 \times SSC, 0.5% SDS, 3 \times 30 min) and scanned using a Molecular Dynamics 445 SI PhosphorImager. Data analysis was performed using the AtlasImage 1.1 software package (Clontech). Gene expression was normalized using the expression of the genes encoding 40S ribosomal protein S9 and 23 kDa highly basic protein (housekeeping genes).

Analysis of gene expression using reverse transcription real time quantitative PCR. To confirm the validity of microarray data, a moderately upregulated gene, IGF-II, was chosen for analysis by real time PCR. Reverse transcription (RT) reactions were carried out for each RNA sample using the ThermoScript RT-PCR system (Life Technologies, UK), according to manufacturer's protocol. Each reaction tube contained 1 μ g of total RNA (DNA free) in a total volume of 20 μ l containing 1 \times cDNA Synthesis Buffer, 5 mM DTT, 40 U RNASEOUT, 1 mM dNTP mix, 15 U reverse transcriptase and 2.5 μ M oligo (dT)₂₀ primer. RT reaction was incubated at 50°C for 60 min and terminated by heat inactivating the reverse transcriptase at 85°C for 5 min. The RNA template, was removed by RNase H treatment at 37°C. PCR primers and a TaqMan probe to amplify the human IGF-II cDNA sequence (13) were designed with the aid of Primer Express 1.0 Software program (PE Biosystems, UK). The forward primer, located on exon 4 (residues 584–601), was 5'-GTGCTA-CCCCGCCAAGT-3' and the reverse primer, located on exon 5 (residues 696–674), was 5'-CTGCTTCCAGGTGTCATATTGGA-3'. The probe was designed to span an exon/intron boundary to avoid genomic DNA amplification. The TaqMan sense probe sequence was 5'-CTCCGACCGTGCTTCCGGACAAC-3' which spanned the exon 4/exon 5 boundary (residues 623–646) and was labeled with the reporter fluorescent dye FAM (6-carboxyfluorescein) at the 5' end and the fluorescent dye quencher TAMRA (6-carboxytetramethylrhodamine) at the 3' end. Equal amounts of reaction mixture were amplified by PCR in 1 \times TaqMan Universal Master Mix (PE Biosystems, UK), 300 nM forward primer, 300 nM reverse primer and 50 nM probe. TaqMan 18S ribosomal RNA endogenous control reagent (VIC fluorescent-labeled probe and appropriate primers) was used as an internal amplification control. Each sample was run in quadruplicate. Real time PCR was carried out using a PE-ABI 7700 sequence detector (PE Biosystems, UK). Cycling parameters were: 50°C for 5 min, 95°C for 10 min followed by 40 cycles of a two-stage temperature profile of 95°C for 15 s and 60°C for 1 min. Data points collected following primer extension were analyzed at the end of thermal cycling. A threshold value was determined as 10 SD above the mean of the background fluorescence emission for all wells between cycle 1 and 15. The cycle number at which the fluorescence signal from a positive sample crossed this threshold was recorded. Serial dilutions of human primary osteoblast cDNA were analyzed for each target, IGF-II and 18S ribosomal RNA, and threshold Cycle (C_T) was plotted versus the log of the initial amount of cDNA to give a standard curve. C_Ts for IGF-II and 18S ribosomal RNA were adjusted using the appropriate standard curves. Then IGF-II-adjusted C_T was normalized to 18S-adjusted C_T to correct for differences in the RT efficiency.

Measurement of IGF-II protein. Cells were seeded and stimulated as described earlier. Conditioned medium was collected after 48 h following stimulation and stored at 20°C until assayed. Cells were washed with PBS (3 \times 5 min) and then lysed in 250 μ l lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 30 μ l/ml aprotinin and 1 mM sodium orthovanadate) under continuous mixing at 4°C. The cell lysates were stored at -20°C until assayed. Unbound IGF-II, which represents the fraction of the molecule which is not bound to binding proteins, was assayed using an enzyme immunoassay (EIA) kit (Diagnostic Systems Laboratories, Inc., Webster, USA) following the manufacturer's protocol. All samples were assayed in duplicate and

TABLE 1

Inductively Coupled Plasma Analysis of Control DMEM and Bioglass 45S5-Conditioned DMEM Containing the Ionic Products of Bioactive Glass Dissolution

	Si	Ca	P	Na
Control DMEM	0.19 ± 0.01	76.33 ± 0.96	33.48 ± 0.41	2885 ± 42.72
Bioglass 45S5-conditioned DMEM	16.58 ± 1.78	88.35 ± 2.32	30.45 ± 0.64	2938 ± 24.62

Note. The concentration of Si in the bioactive glass conditioned DMEM solution was 8800% of control ($P < 0.005$), Ca concentration was 110% ($P < 0.05$), and P concentration was 90% of control ($P < 0.001$). No significant differences in Na content were observed. Units are expressed in parts per million.

IGF-II levels were referred to total protein concentration to correct for any differences in cell number per well. Protein concentration of the cell lysates was assayed by a dye binding method (14) using bovine serum albumin as a standard.

Statistical analysis. The effect of stimulation with Bioactive glass-conditioned medium was compared with controls by Student's two tailed paired t test. A $P < 0.5$ was accepted as statistically significant. Values are expressed as mean ± standard error of the mean ($M \pm SEM$). Correlation analysis was performed using the Pearson's correlation analysis test.

RESULTS

Inductively coupled plasma analysis. In order to characterize the outcome of ion leaching from the material into solution, we analyzed the elementary composition of the culture medium incubated with Bioglass 45S5 particulate and control medium in Si, Ca, P, and Na by ICP. The concentration of Si in the bioactive glass conditioned DMEM solution was 8,800% of control, Ca concentration was 110% and P concentration was 90% of control, whereas Na concentration remained unchanged (Table 1).

Evaluation of cell number. Osteoblast number was increased to $155.1\% \pm 6.45$ ($M \pm SEM$, $n = 5$, $P < 0.001$) of control, following four days of stimulation with the bioactive glass-conditioned medium containing the ionic products of Bioglass 45S5 dissolution. Increase in osteoblast number implies increase in cell proliferation.

Gene profiling using cDNA microarrays. Treatment with the bioactive glass conditioned medium induced changes in the gene expression profile of osteoblasts that were reproducible in all four cases examined. 5.5% of the genes examined were shown to be either upregulated or downregulated greater than twofold (data not shown). Included in the group of upregulated genes were those encoding IGF-II, a known bone mitogenic growth factor, and insulin-like growth factor binding protein-3 (IGFBP-3), an IGF-II carrier protein. Specifically, IGF-II m-RNA expression level in the stimulated cultures was 290% of control and IGFBP-3 mRNA was 200% of control (Fig. 1). Two proteases (15–17) that have been shown to cleave IGF-II from its carrier proteins were also upregulated. The mRNA

levels of MMP-2 and cathepsin-D were increased to 340 and 310% of control, respectively (Fig. 1). Expression of the IGF-II receptor was unaffected by the stimulus.

Reverse transcriptase quantitative real time PCR for IGF-II. Reverse transcriptase quantitative real time PCR showed the IGF-II m-RNA expression level in stimulated cultures to be 390% of control. The normalized expression of IGF-II mRNA in individual donor cultures, as determined by microarray analysis when compared with the normalized C_t values obtained from the reverse transcription quantitative real time PCR analysis, showed a strong positive correlation ($R = 0.9468$, $P < 0.0004$) (Fig. 2).

Measurement of secreted 'unbound' IGF-II. A large proportion of the secreted IGF-II is bound to IGFBPs. Unbound IGF-II, which represents the biologically active form, was measured by EIA. The bioactive glass-conditioned medium increased the concentration of unbound IGF-II to 168% of control, from 9.99 ± 0.56 to 16.83 ± 0.91 fg/ μ g of total cell protein ($M \pm SEM$, $n = 7$, $P < 0.0001$) (Fig. 3).

DISCUSSION

Our earlier findings demonstrated that the Bioglass 45S5 substrate induces osteoblast proliferation by stimulating the entry of cells into the S phase of the

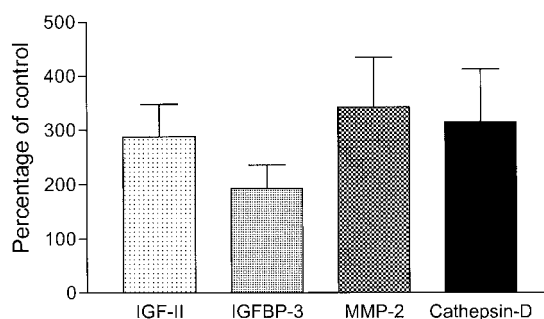


FIG. 1. Analysis of IGF-II, IGFBP-3, MMP-2, and cathepsin-D gene expression using cDNA microarrays. The mRNA expression of these genes was increased in human osteoblasts following treatment the ionic products of Bioglass 45S5 dissolution.

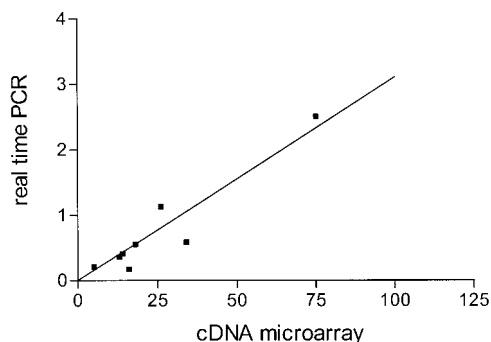


FIG. 2. The expression of IGF-II mRNA in individual donor cultures was analyzed by both microarray analysis (normalized to the expressions of 40S ribosomal protein S9 and 23-kDa highly basic protein) and reverse transcriptase real time PCR C_t values (normalized to the expression of 18S ribosomal RNA). Data comparison using the Pearson's correlation analysis test showed a strong positive correlation between the two data sets ($R = 0.9468$, $P < 0.0004$).

cell cycle (10, 18). Here we showed that the ionic products of bioactive glass dissolution alone can increase osteoblast number implying marked increase in proliferative rate. Thus the data presented here suggest that the presence of the substrate might not be required for the delivery of the mitogenic stimulus. Instead, the ionic products of bioactive glass dissolution could provide an adequate stimulus for cell proliferation. The changes in the elementary composition of solutions exposed to bioactive glasses are due to an ion exchange mechanism at the material-solution interface (8). Ion exchange is followed by network dissolution and this process contributes to the materials resorbability. Elementary analysis of the bioactive glass-conditioned medium demonstrated a 88 fold higher Si concentration and, to a much lesser extend, changes in Ca and P concentrations relative to control. These data support an earlier report by Keeting *et al.* (19) showing that solutions containing high Si concentrations are mitogenic for bone cells.

On the basis of these findings, we proceeded to investigate the molecular mechanisms underlying the mitogenic effect of the ionic dissolution products of bioactive glass on the human osteoblasts. Using cDNA microarrays, we showed that a number of genes were differentially expressed in human osteoblasts as a result of treatment. As one of the upregulated genes was IGF-II, a known inducer of osteoblast proliferation *in vitro* (20, 21), we studied its expression in more detail. We confirmed IGF-II mRNA upregulation using quantitative real time PCR and we also showed that the secreted unbound IGF-II protein concentration was increased. IGF-II is an anabolic peptide of the insulin family and constitutes the most abundant growth factor in bone (22, 23). It is produced locally by bone cells and is considered to exert mostly autocrine or paracrine effects. IGF-II expression is relatively high in developing bone periosteum and growth plate (24),

healing fracture callus tissue (25–27) and developing ectopic bone tissue (28). Also, IGF-II has been reported to augment collagen gel repair of facial osseous defects (29) and bone formation induced by demineralized matrix (30) and local IGF-II administration directly stimulates bone formation in rats (31).

As the majority of secreted IGF-II is bound to IGF binding proteins (IGFBPs) (32), IGF-II activity is influenced not only by the level of expression of the IGF-II polypeptide but also by the type and concentration of the IGFBPs present locally. We found that the mRNA expression of one IGFBP was induced also. IGFBP-3 has been shown to regulate the biological activity of IGF-II by forming storage complexes with IGF-II and stabilizing IGF-II for slow release into the tissues (32, 33). We anticipate that the increase in IGFBP-3 mRNA synthesis in treated osteoblasts reflects an increase in IGFBP-3 protein synthesis, which could subsequently contribute to the biological effectiveness of secreted IGF-II.

The bioavailability of IGF-II at the local level is regulated by proteases, including MMP-2 (15, 16) and cathepsin-D (17). Limited proteolysis of IGFBPs by these proteases results in IGF-II cleavage from its binding protein and release of the unbound, biologically active form (32). Both MMP-2 and cathepsin-D were transcriptionally induced by the ionic products of bioactive glass dissolution. The increase in MMP-2 and cathepsin-D mRNA expression may result in the increase secretion of these proteases. Increased activity of these proteases would, therefore, lead to an increase in unbound IGF-II, as shown by enzyme immunoassay.

In conclusion, our data suggest that the ionic dissolution products of Bioglass 45S5 may increase IGF-II availability in osteoblasts by inducing the transcription of the growth factor and its carrier protein and by regulating the dissociation of this factor from its binding protein. This unbound IGF-II is likely to be responsible for the increase in cell proliferation observed. This is in agreement with earlier reports describing

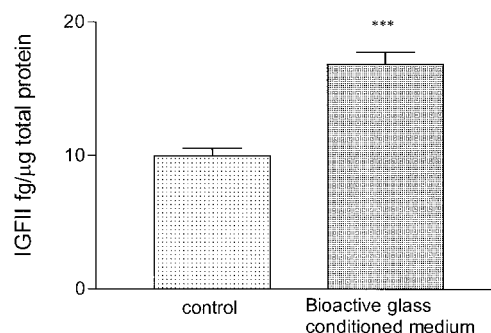


FIG. 3. The concentration of secreted unbound IGF-II was measured by EIA. Unbound IGF-II was increased ($P < 0.0001$) in osteoblasts following treatment with the ionic products of Bioglass 45S5 dissolution.

IGF-II as a potent autocrine and/or paracrine mitogenic stimulus for osteoblasts (20, 21).

In the field of tissue engineering and of repair and regeneration an ideal material scaffold would be one that resorbs by releasing by-products with appropriate growth promoting properties. Here we show that synthetic material could disintegrate into simple inorganic chemical solutions that are not merely biocompatible but can carry specific morphogenic cues. This property can be achieved by designing inorganic material matrices of specific chemical compositions with controlled rates of dissolution and surface chemistries. This is now possible with a new generation of low temperature sol-gel-processed bioactive materials, which allow molecular control of texture over six orders of magnitude, from scales of nanometers to millimeters (34). Therefore, the field is open for the design of resorbable biomaterials and tissue engineering scaffolds that can stimulate tissue growth, repair and regeneration.

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