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A Genetic Basis for Design of Biomaterials For *In Situ* Tissue Regeneration

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Abstract: Historically the function of biomaterials has been to replace diseased, damaged and aged tissues. First generation biomaterials, including bio ceramics, were selected to be as inert as possible in order to minimize the thickness of interfacial scar tissue. Bioactive glasses provided an alternative from the 1970's onward; second generation bioactive bonding of implants with tissues and no interfacial scar tissue. This chapter reviews the discovery that controlled release of biologically active Ca and Si ions from bioactive glasses leads to the up-regulation and activation of seven families of genes in osteoprogenitor cells that give rise to rapid bone regeneration. This finding offers the possibility of creating a new generation of gene activating bioceramics designed specially for tissue engineering and *in situ* regeneration of tissues.

Introduction

Recent cell and molecular biology studies of the reaction mechanisms of bioactive glasses provide the basis for genetic design of a new generation of biomaterials for use in tissue engineering (TE) and regenerative medicine. This chapter reviews these studies and discusses the potential to create new genetically active biomaterials molecularly tailored to treat individual patients. Until the early 1970's it was understood that placing any man-made material in the body would result in a foreign body reaction and formation of non-adherent scar tissue at the interface with the material. Thus, for many years the emphasis in biomaterials research and clinical application was on materials that were as inert as possible when exposed to a physiological environment. This approach to replacement of tissues was irreversibly altered when a special composition of soda-lime-phosphate-silicate glass was synthesised by the lead author and implanted in the femurs of rats in 1969. (1,2,3) This glass composition contained 45% SiO_2, in weight %. The network modifiers were 24.5% Na_2O and 24.5% CaO. In addition 6% P_2O_5 was added to the glass composition to simulate the Ca/P constituents of hydroxyapatite (HA), the inorganic mineral phase of bone. The glasses did **not** form interfacial scar tissue isolating them from the host femoral bone. Instead, the implants bonded to the living bone and could not be removed from their implant site. This discovery led to the development of a new class of materials, called *bioactive* materials, for use in implants or prostheses and repair or replacement of bones, joints and teeth.

Bioactive materials, including bioactive glasses (1-12) and glass-ceramics,(13-18) are special compositions made typically from the Na_2O-CaO-MgO-P_2O_5-SiO_2 system (Table 1). All form a

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mechanically strong bond with bone. Details are reviewed in references 2-8 and 18,19. The rate of bone bonding depends upon composition of the material. (1-3,7,15-17,20-25) Glass compositions with the fastest rates of bone bonding also bond to soft tissues. (12,26)

Bioactive materials are used as bulk implants to replace bones or teeth (27-35), coatings to anchor orthopaedic or dental devices (6) or in the form of powders to fill various types of bone defects. (28,36,37) When a particulate of bioactive glass, ceramic, or glass-ceramic is used to fill a bone defect, both the rate and quantity of bone regeneration depend on the material's composition. (38) Compositions such as 45S5 Bioglass that have the highest rates of bioactivity lead to rapid regeneration of trabecular bone with an amount, architecture and bio-mechanical quality of bone that matches that originally present in the site. The rapid regeneration of bone is due to a combination of processes called osteoproduction and osteoconduction.(41) Large differences in rates of *in vivo* bone regeneration and extent of bone repair indicate that there are two classes of bioactive materials (Table 1) (38,41) Class A bioactivity leads to both osteoconduction and osteoproduction (36,38) as a consequence of rapid reactions on the bioactive glass surface.(1-4,6,22-24,42-47) The surface reactions involve dissolution of critical concentrations of soluble Si and Ca ions that give rise to both intracellular and extracellular responses at the interface of the glass with its physiological environment. The intracellular and extracellular response of osteoprogenitor cells results in rapid formation of osteoid bridges between particles, followed by mineralization to produce mature bone structures. Rates of osteoproduction of various bioactive particulates have been quantified by Oonishi, et. al. that provide the fundamental *in vivo* comparisons of Class A vs. Class B bioactive materials. (39a,b)

Table 1. Composition and Properties of Bioactive Glasses and Glass-Ceramics Used Clinically for Medical and Dental Applications

Composition (wt%)	45S5 Bioglass (NovaBone)	S53P4 (AbminDent1)	A-W Glass-ceramic (Cerabone)
Na_2O	24.5	23	0
CaO	24.5	20	44.7
CaF_2	0	0	0.5
MgO	0	0	4.6
P_2O_5	6	4	16.2
SiO_2	45	53	34
Phases	Glass	Glass	Apatite Beta-wollastonite Glass
Class of Bioactivity	A*	A	B
Density (g/cc)	2.66		3.07
Vickers Hardness (HV)	458+/-9		680
Compressive Strength (MPa)			1080
Bending Strength (MPa)	42		215
Young's modulus (GPa)	35		218
Fracture toughness (MPa m1/2)	NA		2
Slow crack growth (n) (unitless)			33

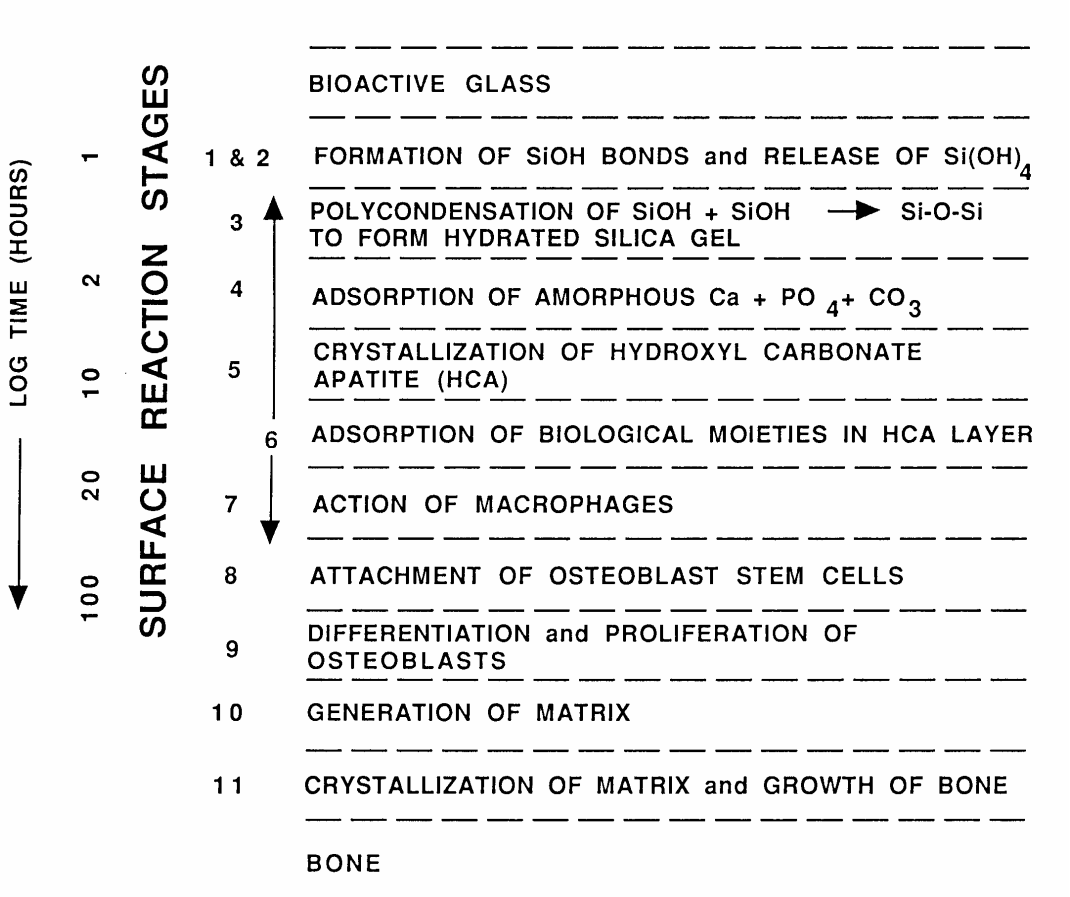

Figure 1. Sequence of interfacial reactions between bone and a Class A bioactive material.

Rates and Mechanisms of Bioactive Surface Reactions Involved in Control of Cell Cycles

Understanding the mechanisms and kinetics of surface reactions of bioactive materials in the presence of physiological solutions and cells is the key to design of new materials that activate genes required to regenerate tissues. There is a sequence of eleven reaction steps that occur at the surface of a Class A bioactive glass. Figure 1 indicates in the log time axis that the first five stages of surface reactions occur very rapidly and go to completion within 24 hours for the bioactive glasses with highest levels of bioactivity, e.g. 45S5 Bioglass. The effect of the surface reactions is rapid release of soluble ionic species from the glass into the interfacial solution. A high surface area hydrated silica and polycrystalline hydroxy carbonate apatite (HCA) bi-layer is formed on the glass surface within hours (Stages 1-5).(42-45) The reaction layers enhance adsorption and desorption of growth factors (Stage 6 and Table 2) and decrease greatly the length of time macrophages are required to prepare the implant site for tissue repair (Stage 7).(7)

Attachment of stem cells (Stage 8) and synchronised proliferation and differentiation of the cells, (Stage 9) rapidly occurs on the surface of Class A bioactive materials.(48-51) Several weeks are required for similar cellular events to occur on the surface of bio-inert and Class B bioactive materials. Differentiation of progenitor cells into a mature osteoblast phenotype does not occur on bio-inert materials and is rare on Class B bioactive materials because of the lack of ionic stimuli. In contrast, osteoprogenitor cells colonise the surface of Class A bioactive materials within 24-48 hours and begin production of various growth factors which stimulate cell division, mitosis, and production of extracellular matrix proteins, (Stage 10). Mineralisation of the matrix follows soon

thereafter and mature osteocytes, encased in a collagen-HCA matrix, are the final product by 6-12 days *in vitro* and *in vivo* (Stage 11).(39,48-57)

Formation of a surface HCA layer (Stages 4-5) is important but not an essential stage of reaction for bone regeneration. Studies of the molecular biological reactions of cells exposed to bioactive glasses demonstrate that Stages 8, 9 and 10 are required to achieve regeneration of bone and tissue engineering of bone.(48-53) Reaction Stages 8,9, and10 are all three controlled by the rate and concentration of soluble Ca and Si ions released from the surface of the bioactive glass. The seminal papers that led to this conclusion were that of Xynos et. al. where the effects of Class A bioactive glass (45S5 Bioglass®) on the cell cycle of human osteoblasts (hOBs) were compared with a bio-inert control (Thermanox® plastic).(48-51) The hOB cells were primary bone cell cultures obtained from excised femoral heads removed from patients, aged 50-70 years, undergoing total hip replacements. Details of the experimental procedures are given in references 48 to 51. Various assays described in references 48-51 were used to quantify the percentages of cells in specific segments of the cell cycle and the genes involved.

There are very few cells in the bones of older people that are capable of dividing and forming new bone. The few (1/100,000) osteoprogenitor cells that are present must receive the correct chemical stimuli from their local environment that instruct them to enter the active segments of the cell cycle leading to cell division (mitosis). Figure 2 summarises the sequence of cellular events that comprise a cell cycle for an individual cell (an osteoblast progenitor cell). Resting cells are in the Go phase and unless they are stimulated to enter into active phases of the cell cycle they will not lead to bone regeneration. A new cell cycle begins after a cell has completed mitosis. A key to regenerative repair of bone is to: 1) control the population of cells that are capable of entering into active phases of the cell cycle, 2) can complete mitosis, and 3) achieve differentiation into a phenotype capable of synthesizing a full complement of extracellular proteins that constitute a mature osteocyte. The series of studies reported by Xynos and the authors showed that such osteoblast cell cycle control is achieved by the controlled release of ionic dissolution products from 45S5 bioactive glass. (48-51) The cells colonise the surface of the bioactive glass; however, the concentration of soluble Si and Ca ions at the cell-solution interface is critical for controlling the cell cycle. Controlled rates of dissolution of the glass provide the critical concentration of the biologically active ions to the cells via the interfacial solution.

During step 1 in the cell cycle shown diagrammatically in Fig. 2, called the G1 phase, the cell grows and carries out its normal metabolism.(49) During the G1 phase osteoblasts are synthesising phenotypic specific cellular products that include alkaline phosphatase (ALP), an enzyme, which can be used as an osteoblast differentiation marker (Stage 9 in Fig. 1). ALP is a necessary extracellular marker for osteoblasts. Production of numerous proteins is required for full differentiation. For example, a differentiated, fully functional osteoblast also produces osteocalcin and tropocollagen macromolecules, which self assemble into type I collagen, the predominant collagenous molecule present in the bone matrix and numerous other extracellular matrix proteins, as shown in Table 2. It is especially important that more osteocalcin is being produced by the osteoblasts grown on the bioactive material. Osteocalcin is a bone extracellular matrix non-collagenous protein produced by mature osteoblasts and its synthesis correlates with the onset of mineralisation, the critical feature of new bone formation, Stage 11 in Figure 1. The Xynos et. al. studies showed that production of all these extracellular proteins was enhanced in the presence of the ionic dissolution products of bioactive glass.

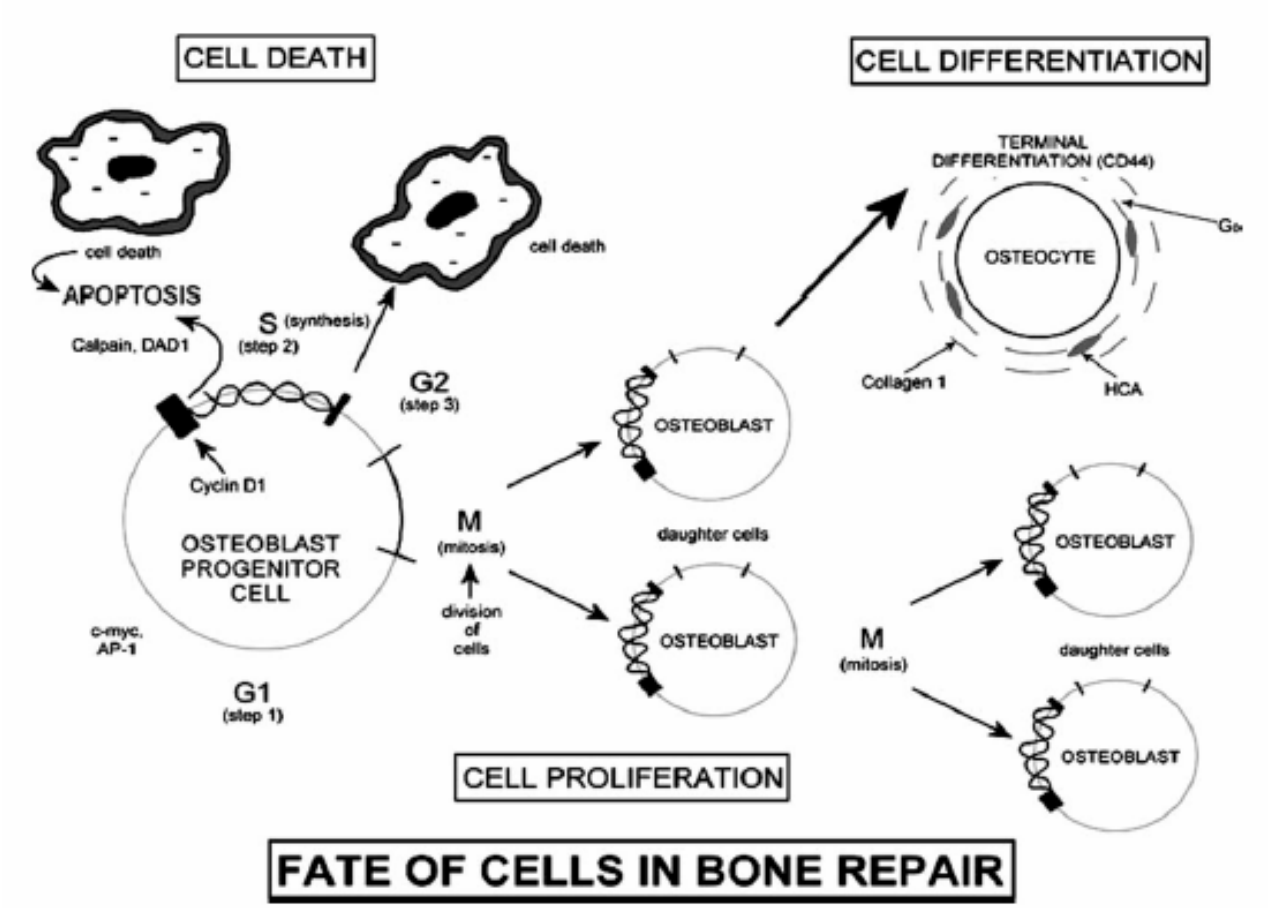

Figure 2. Schematic of Osteoblast Progenitor Cell Cycle leading to 1) Programmed Cell Death (Apoptosis), 2) Mitosis and Cell Proliferation or 3) Terminal Cell Differentiation Towards an Osteocyte

In order for cell proliferation and repair to occur there must be a critical period of growth in the G1 phase. Following that growth the cell enters the S phase (step 2 in Fig. 2), when DNA synthesis begins. The S phase eventually leads to duplication of all the chromosomes in the nucleus. Completion of the S phase requires synthesizing a complete genomic sequence of DNA and RNA. The chemical environment of the cell must be suitable to pass through the G1-S checkpoint to initiate the transcription of the host of proteins and nucleic acids required for duplication of the cell. Following DNA replication (step 3 in Fig. 2) the cell must prepare to undergo mitosis with a second phase of growth termed the G2 phase. During the G2 phase, as the cell prepares to undergo division, synthesis of additional proteins required for mitosis occurs. Also, prior to mitosis, replication accuracy is checked using DNA repair enzymes. A critical increase in cell mass is required and synthesis and activation of various growth factors is necessary for the G2-M transition. Details of the feedback controls and cell cycle checkpoints are reviewed in ref. 49. If the local chemical environment does not lead to the full completion of the G1 phase or the G2 phase then the cell proceeds to programmed cell death, apoptosis, as shown in Fig. 2. Apoptosis is essential to prevent proliferation of cells that are an incorrect phenotype for bone repair. The chemical environment surrounding bio-inert implants does not stimulate apoptosis. The consequence is rapid proliferation of cell types that are characteristic of non-adherent and non-mineralizing scar tissues. Bio-inert materials or Class B bioactive materials seldom enable the few osteoprogenitor cells present at their interface to pass through these cell cycle checkpoints and become fully differentiated osteoblasts. Only Class A bioactive materials that provide the biologically active ionic stimuli give rise to growth of mineralized bone nodules *in vitro* and rapid new bone formation *in vivo*.

Scanning electron microscopy (SEM) analysis of the human osteoblast cultures showed in the Xynos et.al. studies that osteoblasts growing on the Class A bioactive substrate as early as 6 days had already organised, in a process called self-assembly, into a three-dimensional structure composed of cells and mineralised extracellular matrix.(48) This 3-D structure is called a bone nodule with an organisational complexity similar to natural bone grown *in vivo*, although without a blood supply. The time for formation of collagen on bioactive substrates *in vitro* is similar to the kinetics of collagen formation *in vivo*, as discussed in ref. 19. The rate of forming mineralised bone nodules *in vitro* is also similar to the kinetics of bone growth *in vivo*, as reported by Oonishi et. al. (39) using a critical size defect model in the rabbit femoral condyle.

Additional confirmation of the 3-D structure of the bone nodules was obtained by Xynos et.al using confocal scanning laser microscopy.(48) The 3-D structure of the nodule was mapped to show the presence and organisation of the type I collagenous matrix and calcium deposition within the bone nodules. The results confirm that human osteoblasts growing in culture in the presence of a bioactive glass self-assemble into a three-dimensional architecture and create a mineralised matrix that is characteristic of mature osteocytes in living bone. In order for this architecture to be created by the osteoblasts there must be release of critical concentrations of the soluble ionic constituents of the bioactive glass. Approximately 17-21 ppm of soluble Si and 60-88 ppm of soluble Ca ions are required for primary bone cell cultures composed of cells from elderly humans. The ions can be provided by controlled dissolution of a bioactive glass substrate. It is also possible to partially dissolve bioactive glass powders in tissue culture medium and create the critical concentrations of soluble inorganic ions in the medium. When osteoblasts are grown in this ionically conditioned medium they differentiate and form a mineralised extracellular matrix and create bone nodules.

Genetic Control of Bone Regeneration

The life dependent consequence of the checkpoints in the osteoblast cell cycle described above is cell mitosis and formation of two daughter cells. The nuclei of both daughter cells each receive a complete and equivalent complement of genetic material (Fig. 2). However, the checkpoints in the cell cycle also result in fewer and fewer progenitor cells that can enter into the M phase unscathed. The built-in protective mechanism from multiplication of damaged genes means that fewer osteoprogenitor cells are available to replace diseased, damaged or dying bone cells of older people. The cumulative effect is a progressive decrease in bone density with age. Bone regeneration is much slower. In order for bone regeneration to occur at all it is also necessary for a large fraction of the daughter cells to undergo differentiation into the mature osteoblast phenotype capable of undergoing mineralization and formation of osteocytes, as illustrated in Fig. 2. The ionic dissolution products of biologically active Si and Ca released from Class A bioactive glasses stimulate the genes that control osteoblast differentiation as well as proliferation, as established in another set of molecular biology studies conducted by Xynos and the authors. (50,51) These findings have been subsequently confirmed and extended to include other progenitor cell types by Beilby, Christodoulou and the authors. (54-57)

Gene array analyses (50, 51) showed that within a few hours exposure of human primary osteoblasts to the soluble chemical extracts of 45S5 Bioglass, several families of genes were up-regulated or activated including: genes encoding nuclear transcription factors and potent growth factors, especially IGF-II along with IGF binding proteins and proteases that cleave IGF-II from their binding proteins. Table 2 shows that there was a 200 to 500% increase in the expression of these genes over those of the control cultures. Activation of several immediate early response genes and synthesis of growth factors is likely to modulate the cell cycle response of osteoblasts to Bioglass®. The conclusion is that Class A bioactive glasses enhance new bone formation

(osteogenesis) through a direct control over genes that regulate cell cycle induction and progression.

Other investigators have established that the entry of osteoblasts into the cell cycle (Go/G1 transition) and subsequent commencement of cell division is regulated by a family of transcription factors. (See refs. 49, 50 for discussion and details.) These molecules do not solely trigger the initiation of cell division but provide the specific stimuli needed for the development of cells that bear the osteoblast phenotype. These specific proteins must be transcripted and synthesized for a bone stem cell to become a bone-growing cell. The findings by Xynos et al. showed that treating human osteoblast cultures with the ionic products of bioactive glass dissolution for 48 hours activated expression of numerous transcription factors and cell cycle regulators (Table 2). The transcription factors that were activated include c-jun, fra-1 and c-myc, three well characterised osteoblast transcription factors.

The expression of AP-1 transcription factor by osteoblasts is correlated with osteogenesis *in vitro* and *in vivo*. Transcriptional regulation by AP-1 expression precedes osteogenic differentiation of cartilage cells in *vitro* and AP-1 expression appears to play a crucial role in the early regulation of endochondral osteogenesis in both bone formation and fracture healing. (See refs. 49, 50 for details and the relevant citations.) Osteoblast proliferation and phenotypic commitment is triggered by transcription factors c-Myc and AP-1 but depends on successful progression through the cell cycle, as described above. Certain cyclins are required for the progression from the G1 phase of the cell cycle to the synthesis (S) phase. These critical cyclins include cyclin D1 (G1/S specific cyclin) which phosphorylates the product of the retinoblastoma gene, resulting in the release of transcription factors important for the initiation of DNA replication. Cyclin D1 is up-regulated by 400% when osteoblasts are exposed to the ionic products of bioactive glass dissolution for 48 hours (Table 2).

This large increase in gene activation of cyclins demonstrates that the bioactive glass does not merely trigger the entry of osteoblasts into the cell cycle but also provides the vital stimulus needed for progression through the G1/S checkpoint, a crucial step for the successful completion of the cycle (Fig. 2). Two other important cell cycle regulators CDKN1A and cyclin K were also activated by the ionic dissolution products by 200% or more (Table 2). Both are involved in the regulation of the early stages of the mitotic cycle of the cells. Mistakes in the synthesis of proteins and nucleic acids are quite likely, especially in the mitosis of progenitor cells of older people. In order to avoid such mistakes being passed on during cell division the cell possesses an arsenal of mechanisms that can determine whether damage is present, evaluate its extent and correct it, if feasible. The up-regulation of DNA repair proteins by the ionic products of bioactive glass dissolution, listed in Table 2, indicates that these mechanisms are activated in human osteoblasts. At least four important genes involved in DNA synthesis, repair and recombination are differentially expressed at levels of >200% over the control osteoblast cultures. When the damage is beyond repair the cell voluntarily exits the mitotic cell cycle through death by apoptosis, programmed cell death.

Table 2. Families of Genes in Primary Human Osteoblasts Activated Or Up-Regulated By Ionic Dissolution Products of Bioactive Glasses

Transcription Factors and Cell Cycle Regulators	**Activation (%)**
RCL growth-related c-*myc*-responsive gene	500
G1/S-specific cyclin D1 (CCND1)	400
26S proteinase regulatory subunit 6A	400
Cyclin-dependent kinase inhibitor 1 (CDKN1A)	350
cAMP-dependent transcription factor ATF-4	240
Cyclin K	200
DNA Synthesis, Repair and Recombination	**Upregulation (%)**
DNA exclusion repair protein ERCC!	300
mutL protein homolog	300
High-mobility-group protein (HMG-1)	230
Replication factor C 38-kDa subunit (RFC38)	200
Apoptosis Regulators	**Upregulation (%)**
Defender against cell death 1 (DAD-1)	450
Ca-dependent proteinase small (regulatory) subunit; calpain	410
Deoxyribonuclease II (Dnase II)	160
Growth Factors and Cytokines	**Activation (%)**
Insulin-like growth factor II (IGF-II)	300
Macrophage-specific colony stimulating factor (CSF1; MCSF)	260
Bone-derived growth factor	200
Vascular endothelial growth factor precursor (VEGF)	200
Cell Surface Antigens and Receptors	**Activation (%)**
CD44 antigen hematopoetic form precursor	700
Fibronectin receptor beta subunit; integrin beta 1	600
N-sam; fibroblast growth factor receptor-1 precursor	300
Vascular cell adhesion protein-1 precursor (V-CAM1)	200
Signal Transduction Molecules	**Activation (%)**
MAP kinase-activated protein kinase 2 (MAPKAP kinase 2)	600
Dual specificity nitrogen-activated protein kinase 2	200
ADP-ribosylation factor 1	200
Extracellular Matrix Compounds	**Activation (%)**
Matrix metalloproteinase 14 precursor (MMP 14)	370
Matrix metalloproteinase 2 (MMP 2)	270
Metalloproteinase 1 inhibitor precursor (TIMP 1)	220
TIMP 2 (MI)	220
Bone proteoglycan II precursor; decorin	200

Apoptosis thereby prevents the creation of abnormal cells and represents a means to regulate the selection and proliferation of functional osteoblasts. The treatment of the osteoblast cultures with the bioactive glass stimuli induced the expression of several important genes involved in apoptosis, as summarised in Table 2. The up-regulated genes include calpain and defender against cell death (DAD1). Activation of the calpain system, a proteolytic mechanism, is thought to mediate apoptotic cell death. On the other hand, DAD1, a regulator of N-linked gyclosylation, is essential for cell survival since DAD1 mutation has been shown to induce embryonic apoptosis in mice. For a description of the role of these genes in apoptosis see refs.(49-51)

As discussed above, activation and completion of the osteoblast cell cycle does not merely provide the framework for cell proliferation but also determines to some extent cell commitment and differentiation. Bone cells cover a broad spectrum of phenotypes that include predominately the osteoblast, a cell capable of proliferating and synthesising bone cell specific products such as Type I collagen. However, in order for bone to be regenerated and repaired there must be a vital cellular population consisting of osteocytes. Osteocytes are terminally differentiated osteoblasts that are usually post-mitotic and not capable of cell division. Osteocytes are capable of synthesising and maintaining the mineralised bone matrix wherein they reside but subsequently do not divide. Thus, osteocytes represent the cell population responsible for extracellular matrix production and mineralisation, the final step in bone development and probably the most crucial one given the importance of collagen-hydroxyl carbonate apatite (HCA) bonding in determining the bio-mechanical properties of bone. Therefore, it is important to observe that the end result of the cell cycle activated by the ionic products of bioactive glass dissolution was the up-regulation of numerous genes that express growth factors and cytokines and extracellular matrix components (Table 2). An important finding was the 700% increase in the expression of CD44 (Table 2) a specific phenotypic marker of osteocytic differentiation.

The cDNA microarray analysis showed that expression of the potent osteoblast mitogenic growth factor, insulin-like growth factor II (IGF-II) was increased to 320% by exposure of the osteoblasts to the bioactive glass stimuli (Table 2). This is also an important finding because IGF-II is the most abundant growth factor in bone and is a known inducer of osteoblast proliferation *in vitro*. It is produced locally by bone cells and is considered to exert both autocrine and paracrine effects. IGF-II expression is relatively high in developing bone periosteum and growth plate, healing fracture callus tissue and developing ectopic bone tissue, as reviewed by Xynos et al., (50). Also, IGF-II can be used to augment collagen gel repair of facial osseous defects and bone formation induced by demineralised matrix. In addition, local administration of IGF-II directly stimulates bone formation in rats. Thus, these results demonstrate that biogenic stimulation of IGF-II by the ionic dissolution products is a key factor in enhanced osteogenesis.

Xynos et al., (50) confirmed the IGF-II mRNA up-regulation using quantitative real time PCR and also showed that the unbound IGF-II protein concentration was increased. The results indicate that the ionic dissolution products of Bioglass 45S5 may increase IGF-II availability in osteoblasts by inducing the transcription of the growth factor as well as its carrier protein and also by regulating the dissociation of this factor from its binding protein. The unbound IGF-II is likely to be responsible for the increase in cell proliferation observed in the cultures. Similar bioactive induction of the transcription of extracellular matrix components and their secretion and self-organisation into a mineralised matrix appears to be responsible for the rapid formation and growth of bone nodules and differentiation of the mature osteocyte phenotype.

Molecular Design of Bioactive Materials for Genetic Control of Bone Regeneration

Two developments make it possible to design a new generation of biomaterials that can control gene expression *in vitro* and *in vivo*. The first is the enhanced understanding of the role of controlled release of ionic dissolution products from bioactive glasses in controlling the molecular biology of osteoprogenitor cells, as reviewed above. The second is use of sol-gel processing of bioactive glasses to achieve additional control of the rates of ionic release of biologically active stimuli.

Compositions and textures of sol-gel derived glasses can be varied over wide ranges and thereby be used to control the rates and concentrations of soluble Si and Ca in the physiological solutions. Details of sol-gel processing of bioactive gel-glasses, textural analyses and bioactivity studies are presented in references. (58-70) Sol-gel processing makes it possible to produce hierarchical microstructures with nano-metre scale pores in the solid webs of 3-D scaffolds while creating an interconnected pore network with greater than 100 micro-metre passages between macro-pores of 100-300 micrometres in diameter.

A recent study by Jones, et.al.(70) has demonstrated that such bioactive 3-D scaffolds support osteoblast growth and induced differentiation of the cells without use of supplementary organic growth factors. Primary human osteoblasts (HOBs) were grown on 70S30C (70mol % SiO2, 30 mol% CaO) foam scaffolds made by the sol-gel process. The scaffolds had a modal interconnected pore diameter of 120 micrometers and a total porosity of 91%. Prior studies (58-61) showed that these unique materials resulted in a controlled release of soluble Si and Ca ions when exposed to simulated body fluids at 37C. Jones et.al (70) monitored cell viability and growth over a 3 week time period and the osteoblast marker of alkaline phosphatase enzymatic activity was measured at 4,7,14 and 21 days. Production of collagen type I, the extracellular matrix protein of fully differentiated osteoblasts, was measured at 7 and 14 days using an ELISA technique. (70) Cell attachment, distribution and morphology was measured by SEM. All cultures were compared statistically with primary osteoblasts grown on bio-inert Thermanox culture substrates. Four types of culture media were used to investigate the osteogenic potential of the bioactive, resorbable scaffolds. The results showed that the bioactive scaffolds stimulated formation of mineralized bone nodules within 2 weeks of *in vitro* culture of the primary HOBs without the presence of supplementary growth factors in the medium. Evidence of the complete sequence of bone formation, summarized in Fig. 2, occurs by growth of the osteoblasts on the bioactive 3-D scaffolds, including: cell attachment, cell growth, cell differentiation, extracellular matrix formation and matrix mineralization. Jones et.al. provided evidence that the cells completed differentiation into the mature osteoblast phenotype and proceeded towards self organization of bone architecture without the need of external organic supplements. These findings extend the conclusions of Bielby et al. (54, 55) and Christodoulou et. al. (56, 57) obtained from *in vitro* cultures of murine and human primary osteoblasts and embryonic stem cells. All of these investigations (54-57,64,70) show that the sol-gel derived bioactive gel-glasses provide controlled release of the ionic stimuli needed to control both proliferation and differentiation of cells of the osteoblast lineage.

The Beilby et.al. study (55) was especially significant because the cell source was embryonic stem (ES) cells. The results showed that the soluble Si and Ca ions released from 58S sol-gel derived glasses stimulated gene expression in the murine ES cells characteristic of a mature phenotype in primary osteoblasts. (55) Differentiation of the ES cells into osteogenic cells was characterized by alkaline phosphatase activity and the formation of multi-layered, mineralized bone nodules. The nodules contained cells expressing the transcription factor runx2/cbfa-1. Deposition of osteocalcin in the extracellular matrix was detected by use of immunostaining. The osteogenic effect of the bioactive gel-glass extracts was dose dependent. The study led to the important conclusion that the

bioactive gel-glass material was capable of stimulating differentiation of ES cells toward a lineage with therapeutic potential in tissue engineering. This conclusion extends the implications of the therapeutic use of the genetic findings of the studies of Xynos et. al, described above.

The study by Christodoulou and the authors (57) expanded even further the scientific basis for understanding the genetic effect of the dissolution products of bioactive gel-glasses on osteogenesis. The material studied was 58S bioactive gel-glass (58-69). The soluble Si and Ca dissolution products from the gel-glass were added to cultures of primary osteoblasts derived from human foetal long bone explants cultures (hFOBs). U133A human GeneChip oligonucelotide arrays were used to examine 22,283 transcripts and variants, which represent over 18,000 well-substantiated human genes. A 24 hour treatment with a single dosage of ionic products induced the differential expression of a number of genes important to differentiation of the osteoblast phenotype, including: IL-6 signal transducer/gp130, ISGF-3/STAQT1, HF-1 responsive RTP801, ERK1 p44 MAPK (MAPK3), MAPKAPK2, IGF-I and IGFBP-5. The over 200 % up-regulation of gp130 and MAPK3 and down-regulation of IGF-1 were confirmed by real-time RT-PCR analysis. These data suggest that 58S ionic dissolution products, Ca and Si, possibly mediate the bioactive effect of the gel-glass through components of the IGF system and MAPK signalling pathways. The results from human foetal osteoblasts confirm many of the findings reported earlier by Xynos et.al. (48-51) using primary human osteoblast cultures derived from excised femoral heads of elderly patients and thereby demonstrate the generality of the findings of genetic stimulation by the ionic dissolution products of bioactive glasses and gel-glasses. The findings are also consistent with prior investigations of the role of ionic dissolution products in stimulation of growth and especially mineralization of fetal long bones, as reported by Maroothynaden and Hench. (71)

The implications of the above studies is that it is now feasible to design the dissolution rates and architecture of bioactive, resorbable inorganic scaffolds to achieve specific biological effects *in vivo* that synchronize with the progenitor cell population present *in situ*, as discussed previously by the authors. (72) This offers for the first time the potential to design biomaterials for specific patients and their clinical needs.

Conclusions

Almost forty years ago it was discovered that bioactive glasses bond to bone. We now know that the same glass compositions will lead to regeneration and repair of bone in both young and old people. This unique material has been used clinically for more than 20 years with hundreds of thousands of successful cases. Research has shown that the mechanisms of bone bonding and bone regeneration and repair (osteogenesis) involve rapid ion exchange reactions on the glass surface, nucleation and growth of biologically active surface reaction layers and release of critical concentrations of ionic dissolution products composed of soluble silicon and calcium ions.

The molecular biological mechanisms involved in the behaviour of bioactive glasses are now understood with sufficient confidence that the results can be used to design a new generation of bioactive materials for tissue regeneration and tissue engineering. The bioactive response appears to be under genetic control. Class A bioactive glasses that are osteoproductive enhance osteogenesis through a direct control over genes that regulate cell cycle induction and progression towards a mature osteoblast phenotype. Cells that are not capable of forming new bone are eliminated from the cell population, a characteristic that is missing when osteoblasts are exposed to bio-inert or Class B bioactive materials. The biological consequence of genetic control of the cell cycle of osteoblast progenitor cells is the rapid proliferation and differentiation of osteoblasts. The result is

rapid regeneration of bone. The clinical consequence is rapid fill of bone defects with regenerated bone that is structurally and mechanically equivalent to normal, healthy bone. The chapter by Thompson and Hench in this volume illustrates the clinical advantages of use of Class A bioactive implants and powders for bone regeneration.

Perhaps of even more importance in the long term is the possibility that bioactive ionic dissolution products can be used to activate genes in a preventative treatment to maintain the health of our bones as we age. Only a few years ago this concept of using bioactive materials for preventative therapeutics would have seemed to be impossible. We need to remember that it was only forty years ago that the concept of a material that would not be rejected by living tissues was considered to be impossible. If we can activate genes by use of glasses to grow bone it is certainly possible that we may one day be able to use glasses to control genes to prevent the loss of bone.

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