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Genetic design of bioactive glass

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Abstract

This paper 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 glasses designed specially for tissue engineering and *in situ* regeneration of tissues. Recent findings also indicate that controlled release of lower concentrations of ionic dissolution products from bioactive glasses can be used to induce angiogenesis and thereby offer potential for design of gene activating glasses for soft tissue regeneration.

Keywords: Bioactive glass; Genes; Gene expression; Osteogenesis; Osteoblast; Bone; Osteoconduction; Osteoproduction; Regenerative medicine; Tissue engineering; Cell culture; Alkaline phosphatase; Collagen; Osteocalcin; Mineralization; Angiogenesis; Soft tissues

1. Introduction

During the 20th century a revolution in public health resulted in a new challenge for society: maintenance of quality of life in an aging population. Few children die in birth or in their early years in developed countries, unlike previous centuries. As a consequence, in the 21st century tens of millions of people aged between 60 and 100 years old are alive. All desire a high quality of life. Achieving this desire is difficult. All tissues begin a progressive path of deterioration from the age of 30 years onwards. Replacement of aged, diseased or damaged tissues has become routine as a result of the development of reliable and affordable biomaterials and perfection of surgical procedures for implantation of prostheses and subsequent rehabilitation of patients. However, all biomaterials in use today are a compromise compared to the natural tissues they replace. All have limitations. Mismatches in elastic moduli, breakdown of the tissue-material interface, fatigue, wear or other factors lead to 15–50% failures of prostheses over a 15–30 year life time. More and more patients outlive their replacement parts. Thirty years of research and development of new materials and designs has had limited success in extending the lifetime of most prostheses. An alternative approach to the present emphasis on replacement of body parts and tissues is needed. A roadmap for the use of glass in medicine for 2020 needs to consider an alternative: regeneration of tissues. A key to achieving regeneration of tissues is to activate the body's own repair processes. To do so it is necessary to control both the proliferation and the differentiation of progenitor, stem cells that are present in tissues, even for older patients. This paper reviews the evidence that supports a hypothesis that underlies development of a new approach to maintain quality of life in an aging population of 2020.

Hypothesis *(:)*. The ionic dissolution products released from bioactive glasses stimulate the genes of cells towards a path of regeneration and self-repair.

2. Background

An extensive body of work now confirms this hypothesis for regeneration of bone. The evidence is based upon the osteogenic response to bioactive glasses. The original discovery of bioactive glasses and emphasis of research for many years was concerned with the mechanisms of interfacial bonding of bone to bioactive glasses, as described in numerous reviews.[1–5] The seminal step to shift thinking from bioactive bonding to bone regeneration occurred in the paper by Wilson et al. when it was reported for the first time that new bone had colonized the surface of an array of 45S5 Bioglass particulate placed in a surgically created site in the jaw of monkeys, mimicking bone resorbtion from periodontal disease.[6] New bone was formed around the particles and created a regenerated architecture of bone that bridged the bioactive glass particles. The new phenomenon was labelled as

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osteoproduction. It is also called osteostimulation. The concept of osteostimulation was quantified by Oonishi et al. a few years later.[7,8]

The second key finding in developing a genetic basis for bone regeneration was the finding of Xynos et al. that it was not only the glass but it was the ionic dissolution products released from 45S5 Bioglass that influenced and controlled the cell cycle of osteogenic precursor cells and ultimately controlled the differentiated cell population.[9,10] Cells that were not capable of achieving a fully differentiated phenotype characteristic of mature osteocytes were eliminated from the *in vitro* cultures by programmed cell death, apoptosis. The shift in cell population towards mature osteoblasts occurred rapidly, in hours, and led to mineralized bone nodules in culture, without addition of organic bone growth factors, such as bone morphogenetic proteins (BMP).

Another key finding from this work was that the effective ionic dissolution products released at slow rates from 45S5 Bioglass were biologically active soluble Si and Ca ions. The ions produced osteostimulation when present at a particular ratio of ions and at a particular concentration range of 15–30 ppm Si and 60–90 ppm Ca.

These findings provide an understanding of the clinical success of use of 45S5 Bioglass particles, NovaBone and PerioGlas, in a wide range of dental and orthopaedic applications in use for 15 years, as discussed in (Refs. [2,5]). The bio active glass particles dissolve slowly and release the critical concentrations of Si and Ca over many weeks, as needed for progressive regeneration of bone to fill the defect. The initial effect is proliferation of osteogenic precursor cells at the periphery of the particles. These cells undergo mitosis and lead to an expanded population of mature osteoblasts that generate extracellular matrix proteins, especially type I collagen that mineralizes to form regenerated bone.

Oonishi et al. used a critical size defect model in a rabbit femoral condyle model to quantify the histological sequence of osteostimulation by 45S5 Bioglass particulate.[7,8] The studies show that there is both more rapid bone formation in the presence of the osteostimulation particles and regeneration of a more highly mineralized quality of bone in the defect, in comparison with synthetic hydroxyapatite (HA) particles of AW glass–ceramic particles. The rate of bone regeneration in the Oonishi model is related to the rate of release of the soluble Si and Ca ions from the particulates tested.

The third key step in developing a genetic basis for tissue regeneration was the discovery that critical concentrations of ionic dissolution products (soluble Si and Ca ions) activate or up-regulate seven families of genes in osteogenic cells.[11–14] The genes encode transcription of numerous proteins that control the cell cycle, proliferation and ultimately the differentiation of the cells towards the mature osteoblastic phenotype. The seminal paper leading to identification of the genetic response to bioactive dissolution products was published by Xynos et al. in 2001[12] and resulted from a collaboration at Imperial College London of biomaterials investigators (the author's group) and the cell and molecular biology groups of Professor Dame Julia Polak.

Table 1
Cell and organ culture models used to establish genetic basis for osteostimulation by bioactive glass ionic dissolution products

(1) Primary human osteoblasts (pHOBs)
(2) Foetal human osteoblasts (fHOBs)
(3) Murine embryonic stem cells (mES)
(4) Human embryonic stem cells (hES)
(5) Murine foetal long bones (mFLBs)

3. Confirmation of the hypothesis

Several studies have confirmed the results of the early Xynos et al. research and extended the generality to include several types of precursor cells and differing sources of ionic stimuli. Gene array analyses of five different *in vitro* models using five different sources of inorganic ions provide the experimental evidence for a genetic theory of osteogenic stimulation.[16–26]

The cell and organ culture models are summarized in Table 1. Sources of the ionic stimuli are given in Table 2.

4. Description of the experiments

The composition of the melt-derived 45S5 bioactive glass culture discs (A) and particulate (B) was 45% (by weight) SiO_2, 24.5% CaO, 24.5% Na_2O, and 6% P_2O_5.[1]

Samples of (A) were obtained from US Biomaterials Corp., Alachua, FL from a certified batch. Commercial powders of (B) with a particle size of 90–710 ppm were obtained from NovaBone Products, LLC, Alachua, FL.

The 58S sol–gel derived particulate (C) composition (58% SiO_2, 36% CaO, 6% P_20_5) and the 70/30 sol–gel sample (D) composition (70% SiO_2, 30% CaO) were made by the Dept. of Materials, Imperial College London.[27]

Sample (E), the ionic dissolution products of (B)–(D) were obtained by immersing particulates of (B)–(D) in simulated body fluid solution at 37 °C for various times to achieve concentrations of 15–30 ppm of soluble Si ions and 60–90 ppm of soluble Ca ions.[20] A prior study of dose dependence of ionic dissolution products showed this range of concentrations led to enhanced proliferation of osteoblasts.

Human primary osteoblasts were obtained from excised femoral heads of total hip arthroplasty patients aged 50–70 years.[9] The first cell cycle and gene array experiments compared samples (A) with Thermanox plastic controls; the 2nd experiment compared ionic dissolution products of (B) with Thermanox controls[12]; experiment 3 used PCR methods to confirm effects of the ionic dissolution products of (B) on expression of specific genes from osteoblasts obtained from excised femoral

Table 2
Source of ionic dissolution products used in studies of osteostimulation by gene activation

(A) 45S5 bioactive glass culture discs
(B) 4555 bioactive glass particulate (NovaBone)
(C) 58S bioactive gel-glass
(D) 70/30 bioactive gel-glass 3D porous tissue engineering scaffolds
(E) Ionic dissolution products of B–D.

heads of five individual patients.[11] Student's *t*-tests were used to determine statistical significance of the results.

The 4th and 5th experiments tested the effects of sample (E) on fHOBS[15] and hES cells. The 6th and 7th experiments confirmed the findings of experiments 1–5 by comparing dosage effects of samples A and E on murine foetal metatarsals grown for 4 days in organ culture post-day 14 gestation,[28] and growth of primary hOBs within 3D scaffolds (sample D).[24]

5. Results

All seven experiments showed enhanced proliferation and differentiation of osteoblasts towards a mature, mineralizing phenotype without the presence of any added bone growth proteins, such as dexamethasone or BMP. Shifts in osteoblast cell cycles were observed as early as 6 h, with elimination (by apoptosis) of cells incapable of differentiation. The remaining cells exhibited enhanced synthesis and mitosis. The cells quickly committed to generation of extracellular matrix (ECM) proteins and mineralization of the matrix. Gene array analyses at 48 h showed early up-regulation or activation of seven families of genes that favoured both proliferation and differentiation of the mature osteoblast phenotypes, including: transcription factors and cell cycle regulators (6 with increases of 200–500%); apoptosis regulators (3 at 160–450% increases); DNA synthesis, repair and recombination (4 at 200–300%); growth factors (4 at 200–300%) including IGF-I1 and VEG F); cell surface antigens and receptors (4 at 200–700%, especially CD44); signal transduction molecules (3 at 200–600%); and ECM compounds (5 at 200–370%).

A summary of the seven families of genes activated or up-regulated from the experiments is given in Table 3.

Fig. 1 illustrates the changes that occur in the osteoblast progenitor cell population in the presence of the biologically active ionic dissolution products. 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). Fig. 1 summarizes the sequence of cellular events that comprise a cell cycle for an individual cell (an osteoblast progenitor cell). Resting cells are in the G0 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. Such osteoblast cell cycle control is achieved by the controlled release of ionic dissolution products from 45S5 bioactive glass.[9–12] Cells colonize 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. 1, called the G1 phase, the cell grows and carries out its normal metabolism.[10] During the G1 phase osteoblasts are synthesizing phenotypic specific cellular products. 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 3. It is especially important that more osteocalcin is being produced by 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 mineralization, the critical feature of new bone formation. Production of all these extracellular proteins is enhanced in the presence of the ionic dissolution products of bioactive glass.

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. 1), 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. 1) 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. [9]. 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. 1. 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*. Details of differences in surface chemistry and cellular interactions between Class A and Class B bioactive materials are given in Refs. [2,29].

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 organized, in a process called self-assembly, into a three-dimensional structure composed of cells and miner-

Table 3
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	**Up-regulation (%)**
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	**Up-regulation (%)**
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

alized extracellular matrix.[9] This 3D structure is called a bone nodule with an organizational 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. [29]. The rate of forming mineralized bone nodules *in vitro* is also similar to the kinetics of bone growth *in vivo*, as reported by Oonishi et al.[8] using a critical size defect model in the rabbit femoral condyle.

Additional confirmation of the 3D structure of the bone nodules was obtained by Xynos et al. using confocal scanning laser microscopy.[9] The 3D structure of the nodule was mapped to show the presence and organization 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 mineralized 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

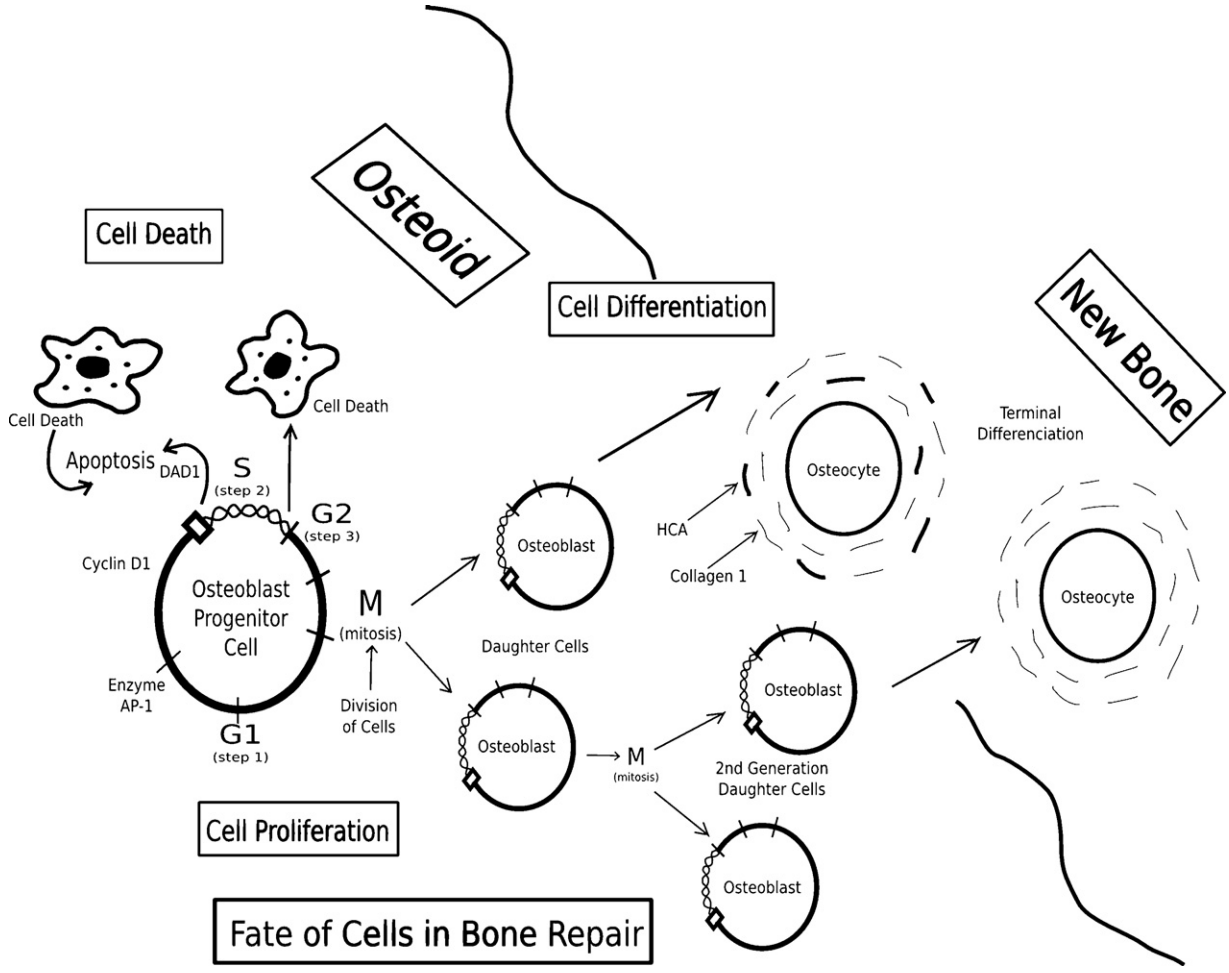

Fig. 1. Schematic of osteogenic progenitor cell cycle leading to (1) programmed cell death (apoptosis); (2) mitosis and cell proliferation; or (3) terminal differentiation and formation of a mineralized osteocyte (mature bone).

differentiate and form a mineralized extracellular matrix and create bone nodules.

When the checkpoints in the osteoblast cell cycle described above have been satisfied cell mitosis and formation of two daughter cells occurs. The nuclei of both daughter cells each receive a complete and equivalent complement of genetic material (Fig. 1). 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. 1. 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.[11–14] These findings have been subsequently confirmed and extended to include other progenitor cell types by Beilby, Christodoulou and the authors.[15–18]

Gene array analyses 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.[11–14] Table 3 shows that there was a 200–500% 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.

Entry of osteoblasts into the cell cycle (G0/G1 transition) and subsequent commencement of cell division is regulated by a family of transcription factors. 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 syn-

thesized 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 h activated expression of numerous transcription factors and cell cycle regulators (Table 3). The transcription factors that were activated include c-jun, fra-1 and c-myc, three well characterized osteoblast transcription factors.

The expression of AP-1 transcription factor by osteoblasts is correlated with osteogenesis *in vitro* and *in vivo*. 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. Cyclin D1 is up-regulated by 400% when osteoblasts are exposed to the ionic products of bioactive glass dissolution for 48 h (Table 3).

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. 1). Two other important cell cycle regulators CDKN1A and cyclin K were also activated by the ionic dissolution products by 200% or more (Table 3). 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 3, 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.

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 summarized in Table 3. The up-regulated genes include calpain and defender against cell death (DAD1). For a description of the role of these genes in apoptosis see Refs. [9–13].

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 synthesizing 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 synthesizing and maintaining the mineralized bone matrix wherein they reside but subsequently do not divide. Thus, osteocytes represent the cell population responsible for extracellular matrix production and mineralization, 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 3). An important finding was the 700% increase in the expression of CD44 (Table 3) 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 3). 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*. These results demonstrate that biogenic stimulation of IGF-II by the ionic dissolution products is a key factor in enhanced osteogenesis.

Xynos et al. confirmed the IGF-II mRNA up-regulation using quantitative real-time PCR and also showed that the unbound IGF-II protein concentration was increased.[11] 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. Bioactive induction of the transcription of extracellular matrix components and their secretion and self-organization into a mineralized matrix appears to be responsible for the rapid formation and growth of bone nodules and differentiation of the mature osteocyte phenotype.

5.1. Design concepts 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.[19–32] Sol–gel processing makes it possible to produce hierarchical microstructures with nano-metre scale pores in the solid webs of 3D scaffolds while creating an interconnected pore network with greater than 100 μm passages between macro-pores of 100–300 μm in diameter.[33]

Jones et al. demonstrated that such bioactive 3D scaffolds support osteoblast growth and induced differentiation of the cells without use of supplementary organic growth factors.[24] Primary human osteoblasts (HOBs) were grown on 70S30C (70 mol% SiO_2, 30 mol% CaO) foam scaffolds made by the sol–gel process. The scaffolds had a modal interconnected pore diameter of 120 μm and a total porosity of 91%. Prior studies 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. 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.[24] 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. 1, occurs by growth of the osteoblasts on the bioactive 3D scaffolds, including: cell attachment, cell growth, cell differentiation, extracellular matrix formation and matrix mineralization. This study shows 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.[15,16] and Christodoulou et al.[17,18] obtained from *in vitro* cultures of murine and human primary osteoblasts and embryonic stem cells, shown in Table 1. All of these investigations[15–18,21,24] 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[16] was especially significant because the cell source was embryonic stem (ES) cells. 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. Differentiation of the ES cells into osteogenic cells was characterized by alkaline phosphatase (ALP) 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 conclusion was 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 (Table 3) where the cell primary human osteoblasts were from older people.

The study by Christodoulou et al.[18] expanded even further the scientific basis for understanding the genetic effect of the dissolution products of bioactive gel-glasses on osteogenesis (Table 3). The material studied was 58S bioactive gel-glass.[30–33] 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-h 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 reviewed above (Table 3) 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 foetal long bones, as reported by Maroothynaden and Hench.[25]

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.[26] This offers for the first time the potential to design biomaterials for specific patients and their clinical needs.

All seven experiments summarized above showed enhanced rates of collagen I production and mineralization of bone modules. The murine foetal long bone cultures (Exp. #6) showed that sequential dosages of the inorganic osteostimulation were most effective.[25] This effect is achieved *in vivo*[2,7,8] by use of a range of particle sizes of bioactive particles (Sample B) where the rate of release is controlled by the radius of curvature (r) of the particles, i.e.:

$$[\mathrm{Ca},\ \ \mathrm{Si}] = (l/r)[-k_1 t^{0.5} - k_2 t^{1.0}]. \quad (1)$$

5.2. Proof of the hypothesis for use of ionic dissolution products to control osteogenesis by gene activation

The findings of the seven experiments summarized above demonstrate that the full range of cell sources of the osteoblast lineage (ES cells, foetal cells and adult primary cells) are stimulated at a genetic level by critical dosages of Ca and Si ionic dissolution products. The up-regulated or activated genes control the osteoblast cell cycle to favour proliferation and subsequent differentiation of only the cells that can lead towards creation of a mineralized extracellular matrix (ECM), mature osteocytes, and new bone.

The critical dosages and kinetics of release of the ionic osteostimulation can be achieved by controlling the particle size range, composition, processing method or nano-structure of Ca- and Si-containing materials.

5.3. Control of vascularisation of tissues by ionic dissolution products

Clinical use of bioactive glass particulate for dental, maxillofacial and orthopaedic applications has been successful in part because fully vascularized bone is regenerated *in situ*. The gene array studies summarized above show that VEGF (vascular endothelial growth factor) is one of the important growth factors up-regulated. Healing of wounds in soft tissues, and soft tissue engineering also requires establishment of a viable blood supply; i.e., vascularisation. Recent findings of Leu and Leach,[34] expanding upon prior work of Day et al.,[35] show that ionic dissolution products released from 45S5 Bioglass particulate are effective in promoting angiogenesis in an endothelial cell module and tubular formation in a co-culture. Their experiments confirm that there is up-regulation of VEGF production from human micro-vascular endothelial cells (HMVEC). The stimulation of angiogenesis depends upon the concentration of ions present in the cultures which was controlled by using differing quantities of 45S5 Bioglass in collagen sponges. They found that when there were too few ions, there was no effect; too many ions also had no effect. Leu and Leach also showed that larger concentrations of the ionic dissolution products led to osteogenesis, as described in the seven experiments reviewed above.

There are important implications from these findings. At present most treatment modalities for chronic wounds are at best palliative. There is great need for bioactive wound dressings that can counter the negative stimuli that prevent healing of chronic wounds. Combining the anti-inflammatory characteristics of 45S5 Bioglass particles with their proangiogenic potential, shown above, offers great promise for design of wound dressings that stimulate keratinogenesis and angiogenesis required to achieve a rapid regeneration of the skin.

6. Conclusions

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. 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. This process is termed osteostimulation. 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 use of bioactive glass particulate to release smaller concentrations of ionic dissolution products from a polymer sponge offers the possibility of designing a new generation of wound care dressings and soft tissue engineering constructs.

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 40 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 and stimulate repair of soft connective tissues it is certainly possible that we may one day be able to use glasses to control genes to prevent the loss of tissues.

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