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Gene activation by bioactive glasses

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Abstract Bioactive glasses have been shown to regulate gene expression in both hard and soft tissue repair. New resorbable bioactive glass constructs are now being developed that can influence gene expression in the local environment by manipulating material properties such as the surface chemistry, topography and the release of dissolution ions. The success of these scaffolds, however, may depend upon a greater understanding of the bioactive glass stimulated gene expression pathways. This will allow the construction of tissue specific scaffolds with tailored surface chemistry, topography and ion release rates. This paper summarises the advances made in understanding gene expression in response to bioactive glasses and discusses the future steps required for further insights into these molecular mechanisms.

General introduction

In 1969 Hench and colleagues in Florida established a specific compositional range of soda lime phosposilicate glasses that did not become surrounded by fibrous (scar) tissue when implanted and instead bonded intimately to bone [1]. This bone bonding melt derived glass was trademarked as Bioglass 45S5® (45% SiO_2, 24.5% Na_2O, 24.5% CaO and 6% P_2O_2 (wt%)), and generated a family of melt derived and sol-gel derived glasses collectively known as bioactive glasses.

Since 1969 Bioglass 45S5® has obtained FDA approval for middle ear prosthesis (1985) and endosseous ridge maintenance implants (1986). Numerous *in vivo* [2–4] and *in vitro* [5–9] studies have also shown that both Bioglass 45S5® and other bioactive glass formulations stimulate bone regeneration. It is only relatively recently, however, that the molecular process governing the cellular response to bioactive glasses has begun to be unravelled [10–13] using a variety of methods (Tables 1 and 2). Greater understanding of the mechanism of bioactive glass activated gene expression will enable greater regulation and/or manipulation of gene expression and consequently control of cell behavior by bioactive scaffolds. This paper will discuss the progress made in understanding the molecular response to bioactive glasses and the exciting potential of these materials in tissue engineering with the creation of resorbable bioactive glass composite tissue scaffolds.

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Design of bioactive glass composites for tailored gene expression

An important goal in tissue engineering is to design constructs capable of orchestrating cellular behaviour in the *in vivo* environment. Bioactive glasses have been shown to influence cellular behaviour and thereby the production of extra-cellular matrix (ECM) through the release of dissolution ions, surface chemistry and possibly topography. It is now possible to design bioactive glass constructs with specific chemical compositions, ion release rates, topography and pore sizes, permitting the tailoring of constructs for both soft and hard tissue repair [14]. The sol-gel process appears to be particularly suitable for manipulating and controlling pore size and dissolution ion release rates for specific applications [14]. The challenge that remains is to determine what material properties (topography, pore size, dissolution rates) are optimal for regulating gene activation for tailored tissue regeneration. Indeed, ionic release by Bioglass 45S5® has been shown to be critical to bioactivity and consequently

Table 1 Analytical techniques used to detect gene expression in response to bioactive glasses

Technique	Description	Advantages	Disadvantages	Studies
RT-PCR	Rapid amplification of pre-determined regions of DNA using a polymerase enzyme. cDNA is made from RNA via reverse transcription.	Small amount of RNA required. Sensitive tool for detection of a particular gene.	Only semi-quantitative at best	6, 11, 17, 20, 22, 25
Northern blotting	RNA is isolated and separated by gel electrophoresis prior to transfer to nitrocellulose paper and detection with a labelled RNA probe.	No amplification required. Quantitative by radio-active counting or band intensity.	Large amounts of RNA necessary. Band intensity determination often lacks sensitivity.	18, 21, 23, 27, 28
In-situ hybridisation	Localizes specific nucleic acid sequences within cells through the use of specific nucleic acid-labelled probes.	Allows in situ location of gene expression in specific tissue regions, semi quantitative.	Sensitivity and specificity variable depending upon tissue tissue and probe quality.	19, 26
Real-time RT-PCR	Fluorescent reporter molecules monitor the production of amplification products during each cycle of the PCR reaction.	Simple, sensitive, quick, specific and quantitative.	Equipment cost	12, 15, 18
Micro-array analysis	Based on Northern blotting and measures the expression levels of a large numbers of genes simultaneously.	Quantitative and allows comparison of a vast array of gene upregulation or down-regulation.	Expensive, currently requires considerable amounts of RNA	17

gene response [10, 13]. The sensitivity of the gene response to these ionic products is such that the manufacturing process needs to ensure the exact reproducibility of bioactive glass constructs to ensure the precise dissolution release rates for the required gene expression.

Gene response to Bioglass 45S5®—the story so far

Since the pioneering paper by Hench et al. [1] over 500 original research articles have been published on bioactive glasses but only a small proportion of these papers focus on the molecular response of cells [5, 6, 10–12, 15–28] (Table 2). The interest in the gene response to bioactive glasses has, however, recently increased dramatically with approximately 28% of the total peer reviewed papers on this subject having been published within the last year (2005) [15–19]. The vast majority of papers published on gene activation by Bioglass 45S5® relate to bone formation [6, 10, 15, 17–23, 25–28], with others concerning cartilage formation [12], the inflammatory response [11, 20], vascularity [16, 24] and gene expression in embryonic stem cell differentiation into osteogenic cells [17]. Of particular importance in wound healing and bone formation is the physiologically vital process of new blood vessel formation (angiogenesis) which has only recently gained recognition in the tissue engineering field [16, 29]. Uncontrolled or persistent angiogenesis, however, can have various pathological consequences including chronic inflammation [29] and therefore requires the correct gene activation and deactivation signalling pathways. The potential of bioactive glasses in wound healing strategies, bone formation and soft tissue repair would greatly benefit from further understanding of the molecular response of a variety of cells (endothelial cells, fibroblasts, macrophages, keratinocytes) to bioactive glass compositions.

Mechanism of bioactive glass induced gene expression

Cells continually interact with their environment through receptors that detect cytokines, chemokines, mechanical stress, gases and physiologically important ions. Cell surface receptors such as integrins interact with the ECM causing cascades of intracellular cell signalling molecules that ultimately, via transcription factors (such as Cbfa1 in osteoblasts), activate or deactivate gene expression. Bioactive glass stimulated genes (genes previously reported to be upregulated or down-regulated by Bioglass 45S5® are summarised in Table 2) are unwound from DNA, transcribed to mRNA and translated to proteins (Fig. 1). These proteins determine the cell phenotype and thereby the response to the initial stimuli i.e. proliferation, differentiation, matrix formation or cell death. Bioactive glass composites determine gene expression by four main mechanisms, namely surface chemistry, topography, rate and type of dissolution ions released and shear

Table 2 Gene expression response to Bioglass 45S5®

Genes	Function/s	Cell Type	Expression
Bone			
Alkaline phosphatase	Makes phosphate available for calcification.	FOB[15], HOB[19],[20] HOB cell line[23], Rat OB[25].	+[15], ++[19], +[20] +[23], +[25] +[23], +[25]
Bone Sialoprotein (BSP)	Almost exclusively found in mineralized connective tissues, may function in the nucleation of hydroxyapatite crystals.	Rat OB[25] HOB[19]	+[25], +[19]
Collagen I	The major organic component of bone matrix, produced by osteoblasts.	FOB [15], HOB[19;20;20], HOB cell line[23]	+[15], ++[19], ++[23], +[20]
Osteopontin	Anchors the bone cells via their $\alpha V\beta 3$ integrin to the mineralized bone surface.	FOB[15], HOB[19], Rat OB[25]	+[15], ++[19], +[25]
Osteocalcin	Activates both osteoclasts and osteoblasts during early bone formation.	FOB[15] HOB[19], Rat OB[25]	+1[15], ++[19], +[25]
Osteonectin	A glycoprotein that is present at high concentration in bone.	FOB[15] HOB[19], Rat OB[25]	+[15], ++[19] , +[25]
Cbfa1/Runx2	The "master gene" in osteogenesis. No bone formation in Cbfa1 deficient mice.	FOB[15], ES[17], preOB cell line[22]	+[15], +[17], +[22]
Bone morphogenetic protein (BMP-2)	A potent inducer of bone formation.	HOB cell line[23]	++[23]
Cartilage			
SOX9	Sox9 binds to essential sequences in the Col2a1 and collagen $\alpha 2$(XI) gene (Col11a2) chondrocyte-specific enhancers.	FC	+[12]
Cbfa2/Runx1	Transcription factor present in matured chondrocytes.	FC	+[12]
Collagen II	Principal component of non-calcified cartilage extracellular matrix.	FC	+[12]
Collagen X	Produced by hypertrophic chondrocytes undergoing endochondral ossification.	FC	++[12]
Indian hedgehog	A critical mediator transducing mechanical signals to stimulate chondrocyte proliferation.	FC	++[12]
Inflammation			
Nitric oxide synthase (iNOS)	Disputed- may cause cellular protective "stress" response and down-regulate pro-inflammatory response.	Synoviocyte	0[20]
Interleukin-1β (IL-1β)	Acute and chronic inflammatory response stimulator.	Synoviocyte	0[20]
Tumour necrosis factor (TNFα)	Potent inflammatory stimulator.	Macrophage (mouse)	++[11]
VEGF	Promotes angiogenesis.	Fibroblasts	++[16]

Gene expression: 0 no expression, + expressed, ++ up-regulated expression. Abbreviations- OB = Osteoblast, FOB = foetal osteoblasts, HOB = Human osteoblasts, FC = Foetal chondrocyte, VEGF = Vascular endothelial growth factor.

stress at implant interfaces (mechanical properties) (Fig. 1). The intracellular signalling pathways, however, remain uncertain.

Which bioactive glass dissolution product/s causes gene activation?

Bioactive glasses have been shown to activate genes associated with bone formation within a few hours of exposure to Bioglass 45S5® dissolution products in a concentration dependant manner [10, 15]. The importance of the various dissolution product/s responsible remains disputed. It has been previously suggested that the presence of phosphate ions is vital for osteoblasts to form calcium phosphate deposition and extracellular mineralised matrix [30, 31]. Indeed the mechanism of β-glycerophosphate (βGP), the commonly used bone mineralising agent, is believed to be due to rapid hydrolization by alkaline phosphatase (ALP) to produce high levels of local phosphate ions thereby providing the chemical conditions for mineral deposition [30, 32]. Valerio et al. (2004), however, reported that biphasic calcium phosphate dissolution products caused decreased osteoblast viability and collagen I production compared to Bioglass 45S5® despite higher ionic phosphate concentrations. This suggests that the other ionic products of Bioglass 45S5® dissolution, such as Si, may also be important in osteoblast maturation. Indeed Si dietary supplements have been associated with increased bone mineral density [33], prevention of osteopenia [34] and Si-doped hydroxyapatite materials have recently

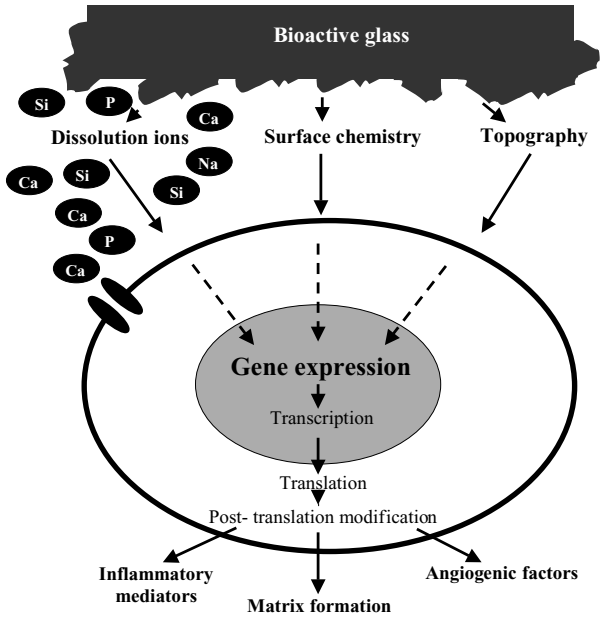

Fig. 1 Gene expression regulation mechanisms by bioactive glasses

been explored as biomedical coatings and bone tissue scaffolds [35].

How to determine gene expression in response to bioactive glasses

Various methods have been used to determine gene response to bioactive glasses (or dissolution ions) both *in vitro* [10, 15, 22] and *in vivo* [18, 19, 21] (Table 1). An important consideration when reviewing literature or designing experiments is whether the methods are (or are required to be) qualitative (Northern blot, RT-PCR (reverse transcription polymerase chain reaction [20, 25], semi-quantitative (*in-situ* hybridisation [19, 26], image analysis of RT-PCR band intensity [11, 22, 23]), or truly quantitative (Real time RT-PCR [12, 13, 15], gene micro-array analysis [10]). Each method has its advantage and disadvantages (Table 1), (these techniques and other emerging ones are reviewed in more detail by Ahmed (2002) and Baak et al. (2005)[36, 37]. Qualitative methods can be used to confirm the expression of genes known to be specific to a certain cell type and thereby determine the phenotypic characterisation of a cell population following exposure to bioactive glasses. Caution must clearly be extended using this method to ensure that the gene expression specific markers (or more commonly the combination of markers) are truly specific to that particular cell type. For example ALP and collagen type I are regularly used as markers of osteoblasts, but these markers have also been reported be expressed by endothelial cells [38, 39], fibroblasts [40, 41], smooth muscle cells [42, 43], hepatocytes [44, 45] and chondrocytes [46]. Qualitative methods do not enable the temporal determination of gene up or down-regulation, although semi-quantitative analysis of band intensity in comparison with controls is possible (if often subjective).

In accordance with recent technological advances, enabling cheaper and faster measurements, the majority of recent papers determine bioactive glass induced gene expression with real-time RT-PCR [12, 13, 15]. This technique allows for the relative quantification of the gene of interest and in turn, with the appropriate experimental design determine temporal gene up regulation or down regulation in response bioactive glasses relative to control.

Gene micro-array analysis has also been used to quantitatively determine bioactive glass activated gene expression [10]. This technique allows quantification of a number of genes simultaneously (up to 30,000 genes in humans). Gene micro-array analysis, however, requires a large amount of quality (fresh) RNA and a number of repeats to determine statistical significance, which makes it a relatively expensive technique. Caution should also be extended when interpreting the confusing and often conflicting plethora of either up-regulated or down-regulated genes obtained from micro-arrays. For example in the pioneering paper by Xynos et al. (2001) it is difficult to explain why both matrix metalloproteinase-2 and its inhibitor (tissue inhibitor of matrix metalloproteinase-2) are up-regulating in response to Bioglass 45S5® dissolution ions or the significance of Sp2 protein or c-jun N-terminal kinase 2 (JNK2) down-regulation [10]. JNK2 down-regulation also illustrates the point that many genes have multiple complex functions, JNK2 expression has been associated with (amongst other things) both the pro- or the anti-apoptotic pathway depending upon cell type, stage of cell differentiation and type of apoptotic stress [47]. Clearly the interpretation of gene arrays is restricted by our limited understanding of the complex interaction of signalling pathways. An additional problem is that most current commercially available microarrays correspond to a single "exon" or coding area of a gene, when actually most genes are composed of many exons interspersed with non-coding elements "introns". Investigators are therefore only looking at part of the total gene, which can lead to misleading results where different splice variants (isotypes) of the same gene with often opposing functions, share the same exon [48]. New micro-array technologies that overcome these problems have been recently been developed [49].

The non-invasive Bio Raman spectroscopy method has also been used to monitor the RNA content of cells and has reported a decrease in the relative amount of RNA during both murine embryonic stem cell and more recently foetal osteoblast differentiation [50, 51]. Non-invasive determination of gene expression is an exciting prospect in tissue engineering [52].

An important consideration when interpreting quantitative gene expression is whether the differences are not only

statistically significant but also physiological relevant. For example what is the physiological impact of a small but significant increase in vascular endothelial growth factor (VEGF) or collagen-I gene expression by osteoblasts in response to bioactive glasses in vitro [10, 19] in the multifactoral, multicellular *in vivo* environment? Clearly this question is extremely difficult to answer and may be important in terms of cytokine gradients *in vivo*. This example also highlights the point that gene expression is relative to the base levels of the control or inactivated cells. The increased gene expression in one cell type (i.e. VEGF expression by osteoblasts) may only be equal to protein production in the resting state of another cell type (i.e. VEGF expression by macrophages). An additional complication is that increased gene expression does not necessarily correlate to protein produced, due to various post-translational mechanisms mentioned below.

Limitations of gene expression and the importance of proteomics

Whilst mRNA provides the blue print for all proteins, it is not the last chemical step in protein formation. Proteins far out number genes, which is the result of the way genes are transcribed (gene splicing) and post-translation modification [53]. Most proteins undergo post-translational modifications (PTMs) which may alter the chemical properties, folding, stability, activity and consequently the function of the proteins. PTMs are implicated as a source of protein mutations in a number of diseases. For example, one or more combinations of the 18 known PTMs of p53 are believed to be involved in a mutation associated with tumour formation [54]. The post-genomic era (i.e. post sequencing of the human genome) has been characterised by the realisation of the importance and complexity of gene transcription and PTM. This point is clearly illustrated in a recent study concerning osteoblast differentiation using both mass spectrometry based proteomics and real time RT-PCR where, with the exception of ALP, no correlation was detected between the changes in the levels of gene and protein expression during differentiation [55]. This has led to a rapid increase in protein research or proteomics. Investigating gene expression in response to bioactive glasses, however, is still an important part of understanding the complex signalling process. When combined with proteomic technologies, mRNA analysis will help develop an even clearer picture of the cellular response to bioactive glass.

Conclusion

Bioactive glasses have been shown to activate a number of genes involved in bone formation, cartilage formation and wound repair. Further insights into the genes affected by bioactive glasses and the gene activation pathways will allow the development of resorbable bioactive scaffolds which cause controlled and sequenced gene activation events. Caution should be extended in interpreting results from molecular biology techniques and should be complemented with proteomic technologies to ensure a more detailed understanding of cellular responses to bioactive glasses.

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