In vitro and *in vivo* methods to determine the interactions of osteogenic cells with biomaterials

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To assess new biomaterials for possible use as bone graft substitutes, a number of techniques allow interactions with osteoblastic cells to be studied, with respect to effects on proliferation and differentiation of osteoprogenitors. *In vitro* models include the use of bone explant cultures, fetal rat calvarial-derived osteoblast cells, primary stromal populations, transformed and non-transformed cell lines and immortalized osteoblast cell lines. However, these assessments are limited by the extent of osteogenic differentiation and bone formation that can be observed *in vitro*, species differences and phenotypic drift of cells cultured *in vitro*. The use of *in vivo* experimental systems such as the segmental/calvarial bone defect model, the subcutaneous implant model and the diffusion chamber implantation model circumvent some of these issues and, in the appropriate model, provide data on efficacy, biocompatibility and osteointegration of a biomaterial. The combination of *in vitro* and *in vivo* approaches together with the development of new cell labeling techniques, in particular the ability to genetically mark and select specific human bone cell populations provides new avenues for their potential evaluation in combination with appropriate biomaterials for clinical use. These *in vitro* and *in vivo* techniques are reviewed and those recently developed for assessment of human osteogenic cells should be applicable to many other cell systems where knowledge of specific human tissue or cell interactions with biomaterials is required.

1. Introduction

How cells interact with their environment is a crucial aspect of cell biology. The extensive use of biomaterials for human tissue restructuring and tissue and organ regeneration procedures demands extensive knowledge about the cellular interactions with these materials. This will enable future sound development of highly compatible medical devices. Furthermore, the many different proliferation and differentiation responses exhibited by cells derived from different species dictates that human cells should be used for optimal assessment. This is relatively easily accomplished with respect to any *in vitro* test proposed, and there are many examples using a variety of currently used methods in the literature [1–6] and some will be mentioned here. With regard to *in vivo* procedures, any final assessments must await licensing for human implantations. Nevertheless, before this occurs there are a number of ways which may be useful to assess important interactions of human cells with biomaterials in neo-physiological situations that may give important information concerning the cellular responses and the behavior of the biomaterials in *in vivo* systems. Despite obvious limitations of these models, their use has the potential to give additional information on cell–biomaterial responses and such methods will also be described here. The techniques to assess these interactions are expanding rapidly as new morphological and analytical methods of cell and molecular biology become applied to this extended area of interactions of cells with the surfaces of materials.

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2. *In vitro* approaches

To function within a skeletal site, issues of (i) biocompatibility, (ii) bone apposition, (iii) mechanical integrity and (iv) maintenance of skeletal function need to be addressed for any potential biomaterial for clinical use. To this purpose, progress in the evaluation of biomaterial interaction with cells of the osteogenic lineage has been facilitated by the development of tissue culture techniques for the isolation and growth of cell populations with osteogenic potential [reviewed in 1]. These include: (i) *bone explant cultures* [7–10]; (ii) *fetal rat calvarial derived osteoblast populations* [2, 11–12]; (iii) *primary stromal populations* [3, 13–14]; (iv) *transformed and non-transformed cell lines* [15–18] and (v) *immortalized osteoblast cell lines* [6, 19–20]. These model in combination with mechanical and material analysis, morphological (light/electron/transmitted/scanning microscopy), histochemical, biochemical (enzyme assays, radio-immunoassays, receptor binding assays,

immunocytochemistry) and molecular biology approaches (*in situ* hybridization, reverse transcriptase polymerase chain reaction, northern blot analysis), have allowed an evaluation of the cytotoxicity or biocompatibility of biomaterials and to improved assessments of materials for use *in vivo*.

2.1. Bone explant cultures

The use of bone-derived cells from explant cultures has gained wide acceptance over the last decade. Early reports of the culture of human bone cells in *in vitro* culture came from Bard *et al.* [7], over 25 years ago and from Cheung *et al.* [21] who utilized bone explant cultures from Pagetic bone. However, a number of investigators in the mid-1980s developed protocols, currently employed in the field, for the generation of populations of human bone-derived cells, from trabecular explants of bone placed in defined culture conditions [8–10, 22–23]. The fibroblastic cells derived from these explants express many of the characteristics of the osteoblast phenotype including alkaline phosphatase activity, an ability to synthesize collagenous and non-collagenous proteins as well as secretion of the osteoblast-specific matrix protein osteocalcin [reviewed in 23]. Bone-derived cells form mineralized bone-like nodules *in vitro* but do not form cartilage and, significantly, *in vivo* studies indicate these cells are osteogenic [24]. The heterogeneity of cell populations obtained and the potential loss of osteoblast phenotype with sequential passaging or subculture of the osteoblast-like cells may limit the use of this model. However, the latter issue of phenotypic drift can be partially circumvented by replating the trabecular explants at the end of primary culture into new flasks to give additional cell populations [23].

2.2. Fetal rat calvarial-derived populations

The development and establishment of protocols for the culture of fetal rat calvarial-derived osteoblast populations has provided a significant aid in the elucidation of the temporal sequence of gene and protein expression in the development of the osteoblast phenotype. Work from Stein and Lian's group elegantly demonstrated, using *in situ* hybridization, that primary cultures of calvarial derived osteoblasts undergo a developmental expression of genes reflecting growth, extracellular matrix maturation, and mineralization [11, 12]. Furthermore, these cultures form multilayered nodules with a bone-tissue-like organization and thus this model system appears to mimic *in vivo* bone formation. However, variation in preparations from fresh tissues between studies, practical limitations in the numbers of cells available and, significantly, species differences including any variation in responses from fetal versus mature osteoblasts, are all limitations of this model.

2.3. Primary stromal preparations

Adherent marrow stromal cell preparations can be generated *in vitro* from single-cell suspensions of marrow cells which proliferate to form colonies derived from a single colony-forming unit-fibroblastic (CFU-F) whilst the majority of the haemopoietic cells remain in the supernatant [reviewed in 3]. These CFU-F can differentiate into cells of osteogenic, adipogenic, fibroblastic and reticular lineages. Thus, preparations of adherent marrow stromal fibroblasts allow examination of their normal differentiation and proliferation in *in vitro* culture and provide a useful model for the assessment of the effects of growth factors, hormones and biomaterials on these cells [5, 25–28]. Using this system, a number of agents have been shown to induce the osteoblast phenotype including dexamethasone and 1,25 dihydroxyvitamin $D_3(1,25(OH)_2D_3)$, both of which have been shown to increase total colony number as well as the number of colonies expressing the osteogenic marker alkaline phosphatase [3], suggesting a similar approach for the examination of the effects of new and established biomaterials. We have recently examined the growth and differentiation of human bone marrow cells on three novel calcium-deficient, hydroxyapatite-type, calcium phosphate ceramics and an amorphous calcium phosphate cement using morphological, histochemical and biochemical approaches, as outlined above [29–30]. Cell proliferation was significantly reduced and cell differentiation, assessed by alkaline phosphatase activity and collagen production, increased in the presence of the ceramics compared to cells cultured on plastic. Light microscopic examination showed close integration of the bone marrow cells and the ceramics and marked toxicity was observed on cells grown on an amorphous calcium phosphate. Thus, calcium-deficient hydroxyapatite type calcium phosphate ceramics, which approximate bone hydroxyapatite in composition, support the growth and differentiation of human bone marrow cells, indicating the potential use of this *in vitro* model for the evaluation of new biomaterials.

2.4. Non-transformed and transformed cell lines

The use of bone-derived cells, fetal rat calvaria and adherent marrow stromal cultures has provided a wealth of information on the proliferation and differentiation of osteoblasts *in vitro*. However, these models all contain heterogeneous cell populations and, with subculture, cells with greater proliferative ability will dominate the culture altering the original properties of the primary culture. The development of established clonal osteoblast-like cell lines from rat osteosarcomata (UMR series and ROS series) provided cell lines that were homogeneous, phenotypically stable, easy to propagate and maintain in culture and which expressed many of the properties of their non-transformed osteoblast counterparts, and this has helped circumvent some of these problems [15–18]. However, as these cells are (i) tumor derived, (ii) transformed and display an aberrant genotype, (iii) have an uncoupled proliferation/differentiation relationship and, (iv) exhibit phenotypic instability in long-term culture, these osteoblast-like cells may not reflect the true phenotype of the non-transformed osteoblast population. An alternative approach has been the generation of clonal osteoblast-like cells from neonatal mouse (MC3T3E1) and fetal rat

calvarial populations (RCJ) by limiting dilution, which express many of the characteristics of the osteoblast phenotype including alkaline phosphatase activity, collagen type I production and nodule formation [31–33]. However, the fetal rat calvarial populations have not proved phenotypically stable and murine cells require culture under defined and controlled conditions to avoid phenotypic drift limiting the usefulness of these cell lines.

2.5. Immortalized cell populations

The phenotypic instability observed in many of the rat and mouse clonal cell lines and the obvious concerns over species differences have led to the development of conditionally transformed adult human osteoblast cell lines [6, 19, 20]. Houghton and coworkers [6] have reported on the generation of a human stromal cell line, which contains bipotential precursor cells able to differentiate into either an adipocytic or osteoblastic lineage, by immortalization with a temperature-sensitive oncogene (SV40 large T antigen). This, and other cell lines produced using similar techniques, should prove useful tools for biologist and material scientist alike.

3. Morphological and biochemical assessment

A number of markers are available to characterize osteoblast populations and enable the evaluation of the effects of growth factors, hormones and materials on the differentiation and proliferation of osteoblast populations including: type I collagen, osteocalcin, bone-gla protein, osteonectin, osteopontin, bone sialoprotein, and a variety of matrix glycoproteins (reviewed in [3]). Perhaps the most widely evaluated marker of the osteoblast is the membrane bound enzyme, alkaline phosphatase, which is expressed at relatively high levels in the osteoblast, has long been recognized as a marker of osteoblastic differentiation [34], and, has been implicated in the mineralization process. Expression of alkaline phosphatase, collagen and the other osteoblast proteins has been shown to be induced in a highly co-ordinated and temporal sequence [12], with type I collagen and alkaline phosphatase expressed by the early osteoprogenitors, whereas osteopontin and osteocalcin are expressed later in the osteoblast differentiation pathway.

Thus alkaline phosphatase expression together with type I collagen expression (which constitutes over 90% of the organic material in the bone matrix), and osteocalcin expression (a 49 amino acid non-collagenous protein specifically expressed by differentiated osteoblasts), a profile of the modulation and activity of osteoblasts and the interaction of biomaterials on the expression of the differentiated phenotype can be followed.

Until recently, identification of the earliest osteoprogenitors was hampered by the absence of appropriate markers. The generation of monoclonal antibodies against early osteoblastic cell surface antigens by Bruder *et al*. [35] (monoclonal antibody SB10) and ourselves [36] (monoclonal antibody HOP-26) provides new tools for the isolation of specific subpopulations of early osteoprogenitors using appropriate cell-sorting techniques for the promotion and evaluation of biomaterials in the modulation of osteogenesis and cellular differentiation. In an extension of these studies we have developed protocols for the marking of cells of the osteogenic lineage using retroviral-mediated gene transfer [37]. This has allowed the generation of genetically labeled populations of human and rabbit osteogenic precursor cells using a murine leukaemia virus encoding a reporter gene *(lacZ)* and a selective marker gene (neo^r), and these marked cells have been shown to form bone in diffusion chamber culture. These populations will provide a useful tool for the evaluation of human bone cell differentiation and this method has the potential to provide direct information on the interaction of biomaterials with human bone cell populations in the osteogenic process in neo-physiological situations.

Studies on the expression of proteins in bone *in vivo* following material implants have been hampered by the hard nature of bone tissue and, until relatively recently, the absence of appropriate protocols of sufficient sensitivity to detect these proteins. However, a number of molecular probes are now available for the bone proteins which will provide further tools for the evaluation of materials on the modulation of the osteogenic process [11, 12, 35, 36, 38]. Neo *et al.* [38] in a recent study demonstrated the power of *in situ* hybridization to investigate in a longitudinal study, the effects of β-tricalcium phosphate (β-TCP) implanted into the distal epiphysis of rabbit femurs on bone formation *in vivo*. In normal bone sections, procollagen α-1(1) RNA was observed in periosteal osteoblasts and in osteoblasts in the mineralizing zone. Similarly, in the β-TCP implants the temporal expression of procollagen α-1 (I) RNA was comparable to that observed in controls suggesting no altered modulation of bone-forming activity of osteoblasts by β-TCP, which is consistent with osteoconductive nature of this biomaterial. Furthermore, the application of reverse-transcriptase polymerase chain reaction to analyze samples following implant retrieval or material analysis *ex vivo* will allow the determination of changes in protein/growth factor/cytokine expression from limited tissue samples.

4. *In vivo* approaches

Modulation and delineation of the osteogenic lineage and the control of bone cell proliferation and differentiation has been aided by the extensive *in vitro* studies available in the literature. From this work, the pluripotentiality of stem cells derived from marrow stroma and their ability to differentiate into fibroblastic, osteogenic, adipogenic and reticular cells with an apparent degree of plasticity or interconversion has been demonstrated. Furthermore, it is clear that osteogenic differentiation is a precisely co-ordinated and tightly regulated temporal sequence and it is important, therefore, that any potential biomaterial for clinical use should not interfere with the differentiation of osteoprogenitors or the subsequent development of osteogenic tissue *in vivo*. It is crucial that any new biomaterial for use in the treatment of bone defects and for implantation must demonstrate biocompatibility and

osseointegration within the bone micro-environment and the maintenance of normal marrow stromal cell development *in vivo*. *In vitro* assays are not without their limitations: (i) species differences encountered in the various osteoblast culture models; (ii) phenotype drift of bone cell cultures *in vitro* with time; (iii) the loss of a three-dimensional matrix organization and the complexity of factors and mechanical forces observed *in vivo;* and (iv) *in vitro* observations may not extrapolate to the *in vivo* situation. All these drawbacks have necessitated the development of *in vivo* models. *In vivo* models allow the evaluation of toxicity of a biomaterial and, if an appropriate model is used, the efficacy of a biomaterial (for example in the induction of any newly formed bone) within a therapeutic application. Furthermore *in vivo* studies allow the examination of osteointegration, that is, the close apposition of bone to the implant/material surface, essential for clinical application and this further enables analysis of the effects of the biomaterial on the expression of the osteoblast phenotype *in vivo*.

In vivo experimental systems include: (i) the segmental/calvarial bone defect; (ii) subcutaneous implantation of demineralized bone matrix/material; and (iii) the diffusion chamber implantation model. The segmental bone defect and the subcutaneous implant model have been used extensively in the evaluation of the efficacy of osteoinductive matrices [39–44, reviewed in 45]. Ohgushi *et al*. [43] using rodents and Ripamonti and coworkers [45–47], using primates, have shown the value of these models to evaluate the bioactivity and biocompatibility of a number of hydroxyapatites *in vivo*. In studies using hydroxyapatite impregnated with rat marrow stromal cell populations the former authors observed extensive new bone formation after subcutaneous implantation in the rat. In adult primates, the latter authors observed that adsorption of a bone morphogenetic protein on to hydroxyapatite induced rapid bone differentiation in calvarial defects of these animals [45–47].

The diffusion chamber model provides an enclosed environment to investigate the effect of biomaterials on the differentiation of skeletally derived cells when implanted in an appropriate syngeneic or allogeneic animal host. Using this model, the formation of bone from isolated bone marrow cell suspensions and cultured marrow preparations has been observed from a number of different species including rat, rabbit porcine and human. The key advantage of this animal model is the unequivocal identification of the new bone tissue generated within the chamber, to the donor cells. Furthermore, bone formation within this system has been shown to follow the temporal pattern of bone differentiation observed in normal embryonic and adult development [48]. The evaluation of human osteoblast cells in this model and the interaction of biomaterials such as hydroxyapatite on the osteogenic differentiation and capacity of these cells has important therapeutic implications. Indeed, the increase in hip arthroplasties, skeletal reconstructions for bone defects and joint replacement has led to the need for synthetic biomaterial bone substitutes and model systems for their evaluation. A number of calcium phosphate ceramics including hydroxyapatite, tricalcium phosphate or chemical mixtures of the two agents have been developed to this end. These compounds exhibit excellent osteoconductivity, biocompatibility, are non-carcinogenic and bind directly to bone tissues *in vivo* [43, 47, 49, 50]. These studies indicate porous ceramic provides a scaffolding for bone ingrowth with ultimately the substitution of the implant with new bone to form a functional skeletal element, although there is no evidence that porous hydroxyapatite is osteoinductive. In studies using the diffusion chamber model Gundle *et al*. [24] confirmed the osteoconductive nature of hydroxyapatite. Human osteoprogenitor cells derived either from trabecular bone explant cultures or marrow suspensions cultured in the presence of dexamethasone and impregnated into porous hydroxyapatite (Triosite) form bone following implantation in xenogeneic hosts. These observations validate the diffusion chamber as an experimental system to evaluate the potential osteoconductivity of new and established biomaterials. Furthermore, our use and development of new techniques for producing genetically marked human osteoprogenitor cells and incorporation of these cells into biomaterials with subsequent implantation into xenogeneic immunocompromised hosts, gives the possibility of further assessing important interactions of human osteogenic cells with biomaterials in neophysiological situations.

5. Summary

In conclusion, a variety of *in vitro* and *in vivo* techniques have been developed over the last two decades to study bone cell differentiation and the effect of biomaterials on this process. The advent of molecular techniques, in particular the ability to mark and select specific subpopulations of osteoprogenitor cells with enhanced osteogenic capacities provides new avenues for their potential evaluation in combination with appropriate biomaterials for clinical use in a number of therapeutic areas. The challenge for the millennium will be the integration of clinicians, scientists and tissue engineering to deliver therapeutic applications from the plethora of biomaterials available for clinical use.

Acknowledgments

The authors would like to acknowledge the contribution of various past and present members of the group to the work presented including Roger Gundle, Clive Joyner, Herve Petite, Lorna Smith and Giovanni Zambonin. Due to limitations of space, many key references have been omitted and recent reviews cited in their place. The authors acknowledge the crucial contributions made by other investigators to the work discussed. The support of the Medical Research Council is gratefully acknowledged.

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Received 1 December 1998
and accepted 16 February 1999