Generalizations Regarding the Process and Phenomenon of Osseointegration. Part II. In Vitro Studies

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In this review, the appropriate use of cell culture to evaluate substrate effects on osteoblast behavior during the process of osseointegration has been considered in the context of existing reports. The interactions of osteoblasts with different substrates can be measured in terms of cytotoxicity, attachment, proliferation, and differentiation. The osteoblast culture systems that produce an osteoblast matrix opposing implant material substrates provide one model for evaluating the implant-bone interface. Alterations in osteoblast behavior at different culture substrates may reflect clinical determinants of bone formation at these substrates in vivo; however, cell responses in vitro have not been compared or correlated with in vivo outcomes. Legitimate interpretations of in vitro experiments are discussed in terms of practical, technical, and biologic limitations presented by the cell culture approach. Cell culture provides access to molecular and cellular information that fosters nanostructural engineering approaches to implant design and significant hypotheses to be tested in vivo. In this way, cell culture offers unique insights into the process and phenomenon of osseointegration.
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In Part I of this review,[1] the bone-implant interface was considered in terms of information provided from in vivo studies. The molecular character of this interface and its precise role in maintaining bone at implant surfaces has not been fully revealed. During osseointegration, the osteoblast is the major tissue-forming cell.[2,3] Many of the individual biologic events associated with osteoblast-mediated healing of bone at implant surfaces—stem cell recruitment, cellular proliferation and differentiation, and the production and mineralization of extracellular matrix at a surface—can be investigated at the level of the isolated osteoblastic cell. This second review addresses the features of different culture systems, consolidates observations regarding the effect of implant material substrates on cultured osteoblast behavior, and suggests how these observations may be appropriately viewed in the context of in vivo phenomena.

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Models to Investigate Aspects of Osseointegration In Vitro

In vitro models of bone formation have been fostered by the success of osteoblast cell culture.[4,5] While the state-of-the-art falls short of recapitulating all aspects of bone cell biology in the laboratory, cell culture offers unique opportunities to investigate aspects of bone formation. One or another system may be suited for investigating implant effects on cell attachment,

Table 1 Summary of Cell Culture Models of Osteoblast Activity at Implant Surfaces

Cells cultured	Surfaces	Culture period	Morphologic analysis	Biochemical/ molecular finding	Reference
Rat bone marrow–derived osteoblast	cpTi	2–3 wk	LM, SEM, TEM		33,34
	0–100% HA on plastic	1,2,4,8 wk	LM, SEM,TEM	AP	36–38
	TCP; glass	3,11,12 d	FM, SEM	OC, OP, FN, CS, Col I, Col III	42
	Δ Ca-P coatings	18 d	LM, SEM		31
	Ca-P coated & uncoated cpTi	3 h; 3,6,9,12,15 d		Protein, cell number	93
	HA, cpTi, glass-ceramic, TCP	7,14 d		DNA, AP, Ca, OP, BSP, Col I	101
Rodent calvaria–derived osteoblast	HA, TCP; Al (r & p), bone	0.5,1.5,2 h	SEM		35
	cpTi Δ plasma cleanings	15,30,60,120 min	SEM	Attachment assays	39
	SS, aTi, PMMA, HA, BS glass, TCP, CoCrMo	2 h; 1,4,7 d	LM, von Kossa, phase contrast microscopy, TEM	Col I, AP, OC, ON, OP, proliferation	46
	Ti (s & mm)	30 d	SEM		26
	Bioactive, normal, quartz, & coverslip glass	2,6,12,20 d	Transmitted LM, SEM, FM	DNA, Col I/II, OC, AP	40
	Coral, HA, cytodex	9,12 d	TEM		48
	SS, aTi, HA, Al, BS glass CoCrMo	1,12,24,36 h	FM	Actin, vinculin	55
	HA, cpTi, TCP	35 d		OC, ON, OP,	54
	HA (r & p), glass	1,3,7,14,21 d	TEM		49
	TCP, aTi, cpTi	17–20 d	TEM, FM	FN	44
	cpTi (p & g & m + various sterilizations)	4,6,8,10,12 d		OC, AP, Col I, Ca, DNA	51
Chick embryonic calvarial osteoblast	ATi (s, r, po), TCP, glass	6 d	LM, von Kossa, SEM	AP, Col I, Ca	30
Bovine osteoblast	IC, TCP, PLA	1,4,7,11,24 h; 14,21 d	LM, SEM	AP, OC, OP, Col I, Col III, protein, DNA	41
	cpTi, HA, TCP, glass	1,3,5,7,10,14 d	LM, SEM, TEM	OP, BSP, OC	45,103
Rabbit calvarial osteoblast	Ca-P ceramics	2 d; 5,10 wk	LM, SEM	AP, Col I	26
Human osteoblast	CoCr alloy (r & g & p & sp)	1,3,6,9,15,21,27 d	SEM, FM	Proliferation, protein, AP	20
	aTi (r & s), CoCr alloy (r & s)	4 wk	Epifluorescence	Integrin expression	74
	aTi (r & s), CoCr alloy (r & s)	4 wk	Epifluorescence	Attachment no. cytoskeleton	77

Continued on next page

proliferation, differentiation, and matrix production or matrix mineralization.

Culture systems have been categorized as *(1)* primary cultures (eg, bone marrow stromal cells,[6,7] intramembranous bone,[8–10] or trabecular long bone[11,12]); *(2)* nontransformed clonal cell lines (eg, MC3T3-E1[13]); *(3)* osteosarcoma cell lines (eg, UMR-106,[14] ROS17/2.8,[15] or MG63[16]); and *(4)* intentionally immortalized cell lines (eg, RCT-1[17] or HOBIT[18]). This classification reflects the origin of cell lines, but not the features of each model.

Osteosarcoma cell lines may display patterns of gene expression, modes of attachment, or signal transduction pathways that reflect a particular stage of differentiation, but most do not demonstrate a complete pattern of in vitro differentiation and, as

Table 1 continued

Organs cultured	Surfaces	Culture period	Morphologic analysis	Biochemical/ molecular finding	Reference
Rodent calvaria	HA	1–13 d	LM, TEM		24
	GL-IO, HA, TP ceramic	2 wk	SEM, TEM		27
	Ti (s & mm)	30 d	SEM		26
	Bioactive & quartz glass	4 wk	LM, SEM, TEM		25
Chick, embryonic and human	Thermanox, SS	14 d	FM, LM, SEM	AP, FN, BSP, OP, OC, Col I, Col III	21
Clonal MC3T3-E1	cpTi, glass	1–2 h	FM	FN, VN, actin, integrin ß1 & ß3, PT	70
	Protein-coated cpTi, Col I gel, TCP	0–4 h		Attachment assays	29
	HA, TP; bioglass, In-Ceram, feldspar glass, ceramic	1,4 h	FM, SEM	Actin, FN	43
	cpTi, Pt, various Pt coatings on cpTi, A1, PD, TCP	1–25 d	von Kossa	DNA, protein, AP, Ca	65,66
	Dense & porous HA	4–60 d	SEM		32
Clonal MG63	cpTi with five different surface treatments	24,48,72 h	LM, SEM laser confocal SEM	Proliferation, DNA, RNA, protein, AP, cytokines	60,80,87, 97

Abbreviations used: cpTi = commercially pure titanium, LM = light microscopy, SEM = scanning electron microscopy, TEM = transmission electron microscopy, Δ = different, HA = hydroxyapatite, AP = alkaline phosphatase, TCP = tissue culture plastic, FM = fluorescence microscopy, OC = osteocalcin, OP = osteopontin, FN = fibronectin, CS = chondrotin sulfate, Col I = type I collagen, Col III = type III collagen, BSP = bone sialoprotein, r = rough, p = polished, SS = stainless steel, aTi = Ti-6A1-4V, PMMA = polymethylmethacrylate, BS = borosilicate, s = smooth, mm = micromachined, p = polished (< 1 µm) surface, Pt = platinum, g = 50-µm sandblasted surface, m = 600-grit polished surface, pc = porous coated, IC = ionomeric cement, TCP = tricalcium-phosphate poly-L-lactic acid composite foil, PLA = poly-L-lactic-polycitric acid composite foil, sp-RF = sputtered, GL-IO = glass ionomer, TP = tricalcium phosphate, VN = vitronectin, PT = phosphotyrosine, Al = aluminum oxide, PD = silver palladium alloy.

with cancer cells, proliferation could represent disregulation. Cell lines intentionally immortalized by oncogenes acquire unique phenotypes, some bearing more useful (akin to normal osteoblasts) molecular attributes than others.[19] Compared to some primary culture models that must be established for individual experiments, immortalized cells represent relatively stable, consistent, and homogeneous biologic systems. The usefulness and validity of each system depends on the question to be addressed and the biochemical and molecular characteristics of the system.

Nontransformed and primary cultured osteoblasts display a well-defined inverse relationship of proliferation and differentiation. Measures of osteoblast-specific matrix protein expression define valuable reference points used in observations of regulated osteoblast physiology (Fig 1).

The inherent character of primary cultured osteoblasts is dependent on several factors. Both the species and age of the donor tissue affect the culture system behavior. Primary cultured osteoblasts have been obtained from rodent, avian, bovine, and human bone (Table 1). Compared to cultures derived from neonatal rodent bone, human osteoblast cul-

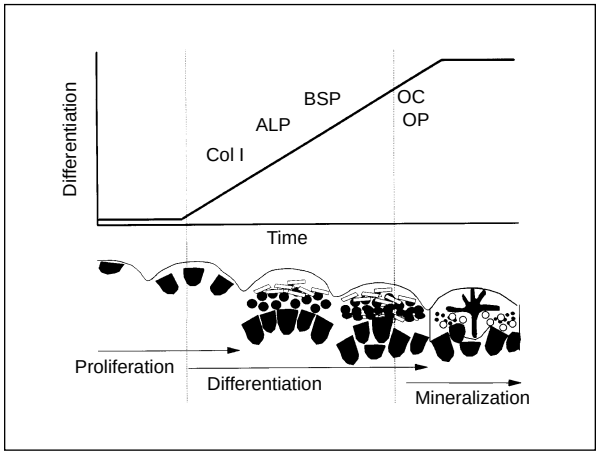

Fig 1 Formation of bone matrix at any surface requires the coordinated proliferation and differentiation of osteoblasts to osteocytes.[73] During this process, a temporal pattern of bone matrix protein expression is revealed. Collagen (Col I) and alkaline phosphatase (ALP) are expressed earlier, while bone sialoprotein (BSP) and osteocalcin (OC) are expressed later. Osteopontin (OP) is expressed later in culture and is also present at the implant-bone interface. Studying this process may guide the engineering of osteogenic or osteoconductive implant surfaces.

tures (typically from adult surgical donor tissues) grow relatively slowly.[20–22] Primary cultured osteoblastic cells can be derived from two sources: committed cells from the osteoblastic cells lining the trabecular surfaces of cortical bone, and the osteocytes within this bone or by differentiation of uncommitted stromal stem cells. In light of these variables, the most powerful tools may be the most carefully defined osteoblast culture models.

Organ culture represents an alternative to cell culture that has an advantage of providing multiple cell types within an established matrix. Organ culture can be adapted to mechanical testing strategies[23] and investigating interface formation at artificial surfaces.[21,24–27] In the context of vascular prosthesis design, Duval and coworkers[28] indicated that organ culture provided for cell-substrate relationships that reflect cytocompatibility. Most recently, Chehroudi et al[26] used calvaria explant cultures to examine the effect of titanium surface morphology on extracellular matrix formation and confirmed in vivo observations that surface topography could affect mineralization. Organ culture models represent another in vitro alternative for assessing osseous responses to implant surfaces. Yet, organ culture has not been widely adopted for studies of osseointegration.

Advantages of Osteoblast Cell Culture in Implant Research

The obvious experimental advantages of in vitro studies of osteoblast behavior include the isolated and homogeneous nature of the osteoblastic system, a defined temporal course of events, relatively limited expenses, the reproducible growth of multiple cultures, and the reduced animal morbidity and mortality, especially when clonal cell lines are used. Experimental variables can be directly controlled, and cell culture studies are amenable to detailed biochemical and molecular analyses. Cells can be grown on a relatively large surface area to yield abundant interfacial material for analysis. Analytic methods, including scanning electron microscope (SEM) morphologic descriptions of cells and matrix relationships with different surfaces,[20,29–35] light microscopic methods including immunohistochemistry,[20,44,45] and transmission electron microscopy (TEM) analyses[33,34,36–38,44–59] can extend the precise characterization of the cell layer and interface. Molecular and biochemical assays are based on a growing library of reagents that reflect specific aspects of osteoblast attachment, proliferation, and differentiation.[50] Sensitive biochemical tests can determine substrate effects on osteoblast production of growth factors and cytokines associated with osteogenesis.[60] Metabolic labeling techniques[30,51] provide access to novel biochemical and physiologic data, and newer methods to examine gene expression, such as in situ hybridization[52,53] and the polymerase chain reaction,[54] enhance both the quantitative value and the resolution of cell culture studies. These same techniques are readily transferable to in vivo applications.

Limitations of Osteoblast Cell Culture in Implant-Related Research

Osseointegration, by definition, cannot be modeled by osteoblast cultures. Cell and matrix interactions with alloplastic surfaces can be investigated in detail. Processes that include cell attachment and motility, proliferation, differentiation, and protein biosynthesis can be measured. Interpretation requires consideration of several inherent limitations.

Technical difficulties are encountered in analysis of cells grown on implant surfaces. Because bulk implant substrates are opaque and not amenable to transmitted light microscopy, current examinations use alternate methods, including epifluorescence microscopy[40,43,55] and SEM.[20,25–27,30] Multilayering, mineralizing cultures may be examined in cross-section by light microscopic techniques[20,44,45] and TEM.[24–27,33,36–38,44–47] Separation of cultures from substrates is not without risk of artifactual damage to the interface. A number of methods have been used to remove cell and matrix layers from the culture substrate, including simple mechanical dissection,[21] pneumatic reflection,[34] spontaneous reflection,[34,45] selective dissolution,[44,47,49] and freeze fracture.[34,44,53] Alternately, cultures may be grown on coated dishes. Advocates of sputter-coated surfaces suggest that the coatings are precisely controlled, pore-free, transparent, sterile, and support cell adhesion. Direct comparison of sputter-coated chromium cobalt alloys with blasted, machined, or as-cast bulk substrates led Naji and Harmand[20] to state that alloys coated onto plastic films are not good models for biocompatibility studies because the physical and chemical states are quite different.

Cell culture requires media supplements, which may alter cell behavior; and the impact of these supplements on results must be considered. For example, media calcium concentration that may alter proliferative behavior varies among different mineralizing systems. Culture differentiation and mineralization is further supported by supplementing media with ascorbic acid and an organic source of phosphate, typically ß-glycerophosphate. The potential for calcium phosphate transformation of culture substrates has not been adequately considered. Serum-derived protein (eg, serum fibronectin, vitronectin, or albumin) adsorption to substrates may be a deter-

minant of culture behavior. The potential interactions of substrates and these supplements must be reconciled before any one surface, on the basis of in vitro data, may be interpreted as osteogenic.

Davies et al[56] indicated that one limitation of using cell culture to create "reference" bone-biomaterial interfaces is the absence of biomechanical forces experienced in vivo. Further interpretation of such a "reference" interface should acknowledge that interface synthesis in vitro occurs in the additional absence of the complex interplay of the inflammatory response, neovascularization, platelet interactions, and the role of stromal stem cells and other cell types. This importance of heterologous cellular influence on cultured osteoblast behavior has been demonstrated using primary cultured osteoblastic cells, periodontal ligament cells, and peripheral blood cells.[57] Thus, in addition to technical limitations, the isolated and homogeneous character that is so valued in cell culture must be reconciled with the interplay of many cell types in a complex in vivo environment.

Applications of Cell Culture for Implant-Related Research

Several biologic issues pertaining to implant-tissue reactions include cytocompatibility, cell attachment, proliferation, protein biosynthesis associated with osteogenesis and interface formation. Existing alloplastic concerns center around composition and topographic features of implant surface design. Cell culture models are employed in examining the effects of many alloplastic features on all of the biologic issues indicated above. In addition, cell culture models are used to gain further information about the bone-implant interface.

Cytocompatibility. Whether or not a material is cytotoxic is an important first question to address in developing alloplastic materials. Cell culture is an important method to assess the effects of surface changes that occur as a result of manufacturing or sterilization procedures, material selection, or topography alterations. Cell attachment, proliferation, and viability are common measures of substrate cytotoxicity. These experimental outcomes are dependent on cell type and material status.

Cells may be used to investigate cytocompatibility in two ways: basal cytocompatibility, which relates to common functions (attachment, viability, proliferation, protein synthesis), and specific cytocompatibility, which relates to specific function of each cell type (eg, bone-specific gene expression or mineralization).[20] Cytocompatibility may also be measured by the reaction of matrix components with substrates (discussed below).[58] Restricting endosseous implant-related cytotoxicity studies to the use of osteoblasts instead of fibroblast cultures is supported by observations that osteoblasts and fibroblasts respond differently to culture conditions and that osteoblasts may be more sensitive than fibroblasts to some surface modifications.[20,59] However, many aspects of foreign body responses to oxidized surface substrates or to particulate debris are presently attributed to multinucleated giant cells and macrophages[61,62] and will not be measured by osteoblast culture.

Cytocompatibility can be measured at the level of soluble ions, small or large particles, and as intact surfaces of varying topography. Material status remarkably impacts cell viability and physiology. For example, titanium and hydroxyapatite surfaces are cytocompatible, but titanium or hydroxyapatite particles of defined size (which may be present at the implant-bone interface following surgery[63]) cause cell death when applied to osteoblast cultures.[64]

The physicochemical reactivity of surfaces with cell culture media is also important. For example, the ion implantation of platinum or palladium alloy surfaces diminished cellular activity and toxic reactions at experimental implant surfaces.[65,66] It is not clear if ion-mediated events reflect in vivo conditions and, thus, cell responses to surfaces in vivo. In this regard, Gross and Strunz[67] showed that implants release ions that may influence differentiation of cells in the peri-implant compartment in vivo.

Cell Attachment. Many of the in vitro studies that attempt to understand alloplastic surface–bone interactions are based on cell attachment phenomena. Three important issues are raised by review of this body of literature: *(a)* What is the nature and significance of cell attachment to implant substrates? *(b)* Do substrate parameters affect cell attachment in predictable ways? *(c)* Do observations from cell attachment studies predict the behavior of bone at implants?

Cell attachment is a phenomenon of growing importance to cell biology in general,[68] and the process of attachment is well defined in terms of adhesive extracellular matrix proteins (the biologic substrates for attachment) and a family of transmembrane cellular receptors—termed integrins[69]—that mediate cell attachment to this substrate (Fig 2).

Based on investigations at the molecular level, implant substrate–osteoblast interactions may be characterized as specific, protein-mediated (indirect), dynamic, and signal-generating events. Osteoblasts use integrin receptors to bind specific proteins adsorbed on implant surfaces.[43,55,70] Some of these proteins, including fibronectin and osteopontin, are present in serum or are expressed by osteoblasts.[42,71,72] This process is specific in two ways: first, adhesive proteins mediating attachment may be spe-

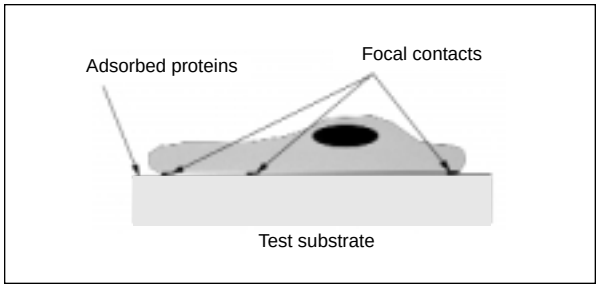

Fig 2 Cell attachment to implant surfaces is adhesive protein–mediated (eg, osteopontin, fibronectin). Focal contacts represent contact points of cells to the adhesive proteins. They contain transmembrane receptors (integrins) that transduce important signals to the adherent cells and inform the cell of its structural environment.

cific for bone or a particular implant surface; and second, the osteoblast display of integrins is differentiation- and matrix protein–specific. Compelling evidence for this is provided by the observation that cultured osteoblasts use different receptors for attachment to polystyrene, titanium, and cobalt chrome alloy substrates.[74] The dynamic nature of this protein-mediated attachment is suggested by observations of adsorbed serum-derived protein replacement by cellular fibronectin produced by the adherent, immature osteoblastic cell.[70] A dynamic state may be important in the development of the implant-bone interface. This attachment engenders important signals, informing the cell of the local environment. Signals transduced through integrin receptors may influence motility, proliferation, and differentiation. Cell interactions with adhesive extracellular matrix proteins contribute to processes that direct tissue formation and differentiation.[75] It is apparent that the recapitulation of biologic modes of cell attachment occur during osteoblast attachment to artificial surfaces in culture.[43,55,70,74]

Regardless of the mechanism and relevance of cell attachment phenomena in culture, it is now well established that physical or chemical changes in test substrate parameters affect cell attachment. Cell attachment studies have directed attention to three main issues: composition effects, topography effects, and procedural effects.

Fundamental compositional parameters that might affect cell attachment include physicochemical properties such as pH, pI, van der Waal's forces, ionic forces, wetability, and so forth (see, for example, Grinnell[76]). Instead of characterizing cell responses to physically defined parameters, implant substrate effects have been investigated at a level of clinical description (eg, titanium, hydroxyapatite, cobalt chrome, sandblasted, autoclaved, plasma-sprayed, or plasma-cleaned).

The description and measurement of cell interactions with different clinical implant materials have shown that, in the presence of serum, cells adhere to and spread on many test substrates, unless the surface is intentionally contaminated.[29] Comparison of cell attachment to various implant materials, including cobalt-chromium, stainless steel, titanium, titanium alloy, aluminous ceramics, and hydroxyapatites, indicates this general cellular behavior at all test surfaces.[35,46] Existing comparative data are inconsistent; human osteoblast adherence was greater on titanium than on cobalt chrome,[77] but rat calvarial osteoblast adherence was greater on cobalt chrome than on titanium.[46]

Surface modification caused by pH alteration, cleaning, and sterilization was examined at the level of cell attachment.[78–80] Plasma-cleaning studies suggest that surface-wetting phenomenon alone does not affect cell attachment.[39] In contrast, Meyer et al[41] argue that wetability remains an important parameter. The chemical or physical state of the surface may affect the adsorption of ions and proteins that support cell attachment.[81] Manufacturing processing, cleaning, and sterilizing may further influence the physicochemical behavior of substrates.[81,82] Thus, cell attachment can be affected by altered surface chemistry.

Surface roughness is another facet of implant surface design that has been aggressively studied using cell culture techniques. Brunette[83] has categorized surfaces as smooth (eg, electropolished), porous, rough and/or sharp-edged (eg, etched, sandblasted, plasma-sprayed), and machined surfaces. For the cellular responses to be meaningful, the surfaces must be characterized in physical, not clinical, terms. Cells do not discern a "sandblasted" surface from a "machined" surface; surface parameters should be defined with respect to the molecular responses of adhering osteoblasts or responding tissues. Toward this end, roughness factors have recently been defined in no fewer than eight measurable parameters.[84]

Brunette also identified cellular responses occurring at the implant surface.[83] The types of contacts observed in culture (focal contacts, focal adhesions, extracellular matrix contacts, and hemidesmosomes) and the principles that govern cell behavior (categorized as contact guidance, rugophilia, the two-center effect, and heptotaxis) must be studied in the context of physically defined implant surface characteristics. This level of fundamental concern is required for the rational design and engineering of implant surfaces.

Surface roughness impacts osteoblast attachment and spreading; however, studies of osteoblast attachment and spreading on substrates of differing topography reveal little consistency. Rat calvaria–derived

osteoblast attachment increased as a function of surface roughness in studies by Bowers and coworkers.[85] A second study comparing cpTi and Ti alloy surfaces further indicated increased attachment as a function of surface roughness.[86] In contrast, other studies of rat calvaria–derived osteoblasts,[35,41] MG63 osteosarcoma cells,[87] ROS17/2.8 osteosarcoma cells,[88] and human osteoblasts[89] indicate that attachment favors smooth surfaces. Care must be taken in comparing these data since measurements represent unique culture conditions and cell types and disparate time courses. It may be further concluded that studies indicating that rough surfaces enhance osteoblast adherence are distinct from studies using gingival fibroblastic cells, in which smooth surfaces consistently promote greater attachment and spreading.[90–92]

Obvious or subtle variations in surface topography, as well as differences in surface-preparation techniques, make interpretations based on different investigations difficult. For example, the cited differences in surface topography were not considered in a report which concluded that, compared to cobalt chrome or polystyrene, titanium substrates were capable of supporting greater osteoblast attachment and spreading.[77] A confounding factor in the analyses of surfaces of differing composition but of similar roughness is the similar physicochemical attributes of carefully polished disks or sputter-coated culture dishes. A recent comparison of cell attachment to sputter-coated HA and Ti surfaces indicated no significant difference in the attachment of bone marrow–derived rat osteoblasts to the two surfaces. Ong et al[93] indicated that changing surface conditions and the similar presence of calcium phosphate compounds on the HA and Ti surfaces might account for this result. Additional caution regarding surface topography relates to the various methods of processing. Könönen et al[90] provide a succinct description of the variety of complications caused by machining, grit-blasting, acid-etching, and sputter-coating.[90]

While cell attachment is required for osteoblast survival in culture, is cell attachment to implant substrates requisite to the process of osseointegration? First, despite the studies of and interest in cell attachment to various implant substrates, there is a lack of evidence that cell attachment data predict the degree of bone formation or osseointegration. Second, histologic representations of implant-bone interfaces associated with osseointegration fail to demonstrate abundant cell-implant interactions. Well-integrated surfaces typically oppose relatively acellular mineralized bone matrix via an organic interfacial zone devoid of abundant cell attachment.[1]

It may be argued that successfully integrated alloplastic surfaces are not necessarily designed to optimally support (nor are they fortuitously congruent with) cell attachment. Findings from cell culture attachment assays are not generally congruent with observations from in vivo studies. Regarding cpTi or Ti alloy implants, some in vivo studies indicate that increasing surface roughness is associated with enhanced bone formation at implant surfaces.[94,95] Conversely, electropolished surfaces, to which cultured cells avidly bind, do not support abundant bone formation.[96] In culture, smooth surfaces have been shown to promote attachment and spreading. The incongruent outcomes of in vivo and in vitro studies suggest that osseointegration may not be wholly dependent on cell attachment phenomena.

Cell Proliferation and Protein Biosynthesis

An important aspect of osseointegration is the rapid formation of woven bone following surgical implant placement. This process requires sufficient numbers of cells and high levels of biosynthetic activity to produce a protein matrix for mineralization. Immediate and short-term cell culture can measure proliferative capacity and biosynthetic ability. These parameters are affected by culture surface variables (again, the principal experimental variables include clinically defined composition and topography parameters). The work of Stanford et al[51] represents a carefully controlled analysis of the effect of implant surface roughness and sterilization-related titanium surface modification on rat calvaria–derived osteoblast cultures. Smoother surfaces supported greater osteoblastic osteocalcin and alkaline phosphatase synthesis. Using the MG63 osteosarcoma cell line, Martin and colleagues[87] have shown that surface roughness enhanced osteoblast matrix production. In subsequent studies, rough surfaces enhanced cytokine expression.[97] Similar studies indicate that surface roughness promoted the phenotypic traits of primary cultured chondroblasts.[98] Studies of the effect of different clinical implant materials, including stainless steel, titanium, and hydroxyapatite, suggest modest changes in phenotype as a function of surface composition.[21,25,27,99] On sputter-coated surfaces (titanium, titanium dioxide, zirconium, zirconium dioxide, hydroxyapatite, and aluminum oxide), the expression of collagen, fibronectin, and alkaline phosphatase varied most notably in cell-line specific ways.[99] This implicates genotypic control as a major determinant of these responses. In other work using rat calvaria–derived cells, proliferation, alkaline phosphatase activity, and collagen synthesis were deemed similar for all nonapatite substrates.[46] Apatite surfaces promote phenotypic protein expression.[32,36,49,89]

Insight into apatite surface–mediated effects was recently provided by observations that high calcium ion concentrations in media as a consequence of substrate reactivity could account for cell responses.[100] An earlier report attributed similar changes to the degree of apatite crystallinity and the degradation rate of the hydroxyapatite.[38]

It is tempting to interpret surface-related effects such as the enhanced alkaline phosphatase expression and collagen synthesis on HA versus Ti surfaces[35,46,101] as predictive of enhanced bone formation at HA endosseous implants. It must be reiterated that the outcomes of culture experiments have rarely been correlated or directly compared to in vivo results (see, for example, Bagambisa et al[32]). In this context, the analysis of osteoblast behavior at the level of matrix vesicle formation and content may be the most predictive measure currently presented.[98]

An important generalization that can be made regarding the many different in vitro studies of cell proliferation and protein biosynthesis at implant surfaces is that implant surfaces lack a significant mitogenic role in osteogenesis. This review has not revealed observations of surface-related multifold or order-of-magnitude changes in proliferation rates or cell numbers. Regarding the effects of surface parameters on protein biosynthesis, it appears that the inverse relationship of proliferation to collagen and alkaline phosphatase expression is maintained on different substrates. At least two detailed investigations of osteoblast responses to altered titanium surfaces indicate the potential to modulate protein biosynthesis at culture interfaces.[51,87] However, marked alterations in the genetic program of protein biosynthesis (eg, promiscuous expression of osteocalcin or bone sialoprotein) have not been reported.

Mineralizing Matrix and Interface Formation in Cell Culture. Attempts to create a culture microenvironment to support the synthesis of a mineralizing extracellular matrix by osteoblasts have been globally successful. The essential features shared by all of these mineralizing matrix models include: *(a)* initial cell attachment, *(b)* a proliferative phase, followed by *(c)* a process of osteoblast differentiation defined by the expression of bone matrix proteins, and *(d)* eventual matrix mineralization. Two current uses for mineralizing osteoblast cultures include investigating substrate effects on osteoblast differentiation and investigating the composition of the implant-bone interface.

Alterations in implant surface chemistry may have long-term biologic consequences.[102] Mineralizing osteoblast cultures have been used to examine the potential effect of substrate characteristics on mineralizing matrix formation in culture. Both HA and Ti surfaces support differentiation of bone marrow–derived and calvaria-derived rat osteoblasts.[33,49] The direct comparison of differentiation-associated gene expression by osteoblasts grown on Ti and HA surfaces indicated that HA supported more rapid differentiation.[101] The enhanced differentiation of rat osteoblasts grown on bioactive glass was attributed, in part, to the calcium phosphate layer that forms on these substrates.[25] Using chick osteoblasts, rough and porous-coated titanium surfaces supported greater alkaline phosphatase expression and calcium accumulation than smooth surfaces.[30] Morphodifferentiation of bovine mandibular osteoblasts was qualitatively similar on ceramic HA and Ti surfaces.[103] Because differentiation of osteoblastic cells is generally observed in all of these reports and occurs in a qualitatively similar pattern as cultures formed on tissue culture plastic or glass coverslips, the conclusion that one or another implant-material surface is specifically osteoinductive or osteogenic cannot be drawn.

Mineralizing osteoblast cultures have also been used to examine the formation of a bone like matrix-implant surface interface.[58] An interesting analogy of the bone-implant interface with the reversal lines of bone has been made by molecular analyses performed in vitro. The expression of osteopontin occurred at the surface-attached cellular pseudopodia in early cultures; fibronectin was present at an intracellular location and not at the interface; and collagen was observed in the interfacial zone following at least 3 days in culture.[42] For the most part, these observations concur with recent molecular analyses of interfaces formed between bone and implants in vivo.[72] A previous review that considered the interfacial morphology from both in vivo and in vitro studies provided a well-diagrammed description of variable experimental outcomes.[104] Since then, other cell culture models with further variations in the interface morphology have been reported.[45,49]

The morphology of substrate-culture interfaces is culture system dependent. In models where a matrix apposes the alloplastic surface,[33,34,36] an afibrillar region with calcium phosphate globular accretions was formed, and the elaboration and eventual mineralization of a collagen-rich matrix was observed. The presence of proteoglycans in the interfacial region has been defined by ruthenium red staining.[33,34] The relative thickness of these in vitro–formed interfaces (approaching 1 μm) may indicate a relatively immature state when compared to evaluations of in vivo–formed interfaces. In models where cells appose the alloplastic surface, an intervening fine reticular matrix largely devoid of collagen fibrils separates the cell layer from a multilaminated electron-dense structure reminiscent of the implant-bone

interface formed in vivo.[32,34,45,49] Both systems permit analysis of the constituents of osteoblast-derived materials adsorbed to supporting substrates. There are many reagents for bone matrix proteins and glycoproteins that can advance the molecular characterization of in vitro–formed interfaces.[50,105,106] In this way, cell culture may be an incubator of significant interface-related hypotheses to be tested in vivo.

Conclusions

Bone formation that occurs during the process of osseointegration may represent osteoblast activity affected by the implant surface. Osteoblast culture methods are used to investigate this effect. Although osteoblasts have been successfully grown on a wide variety of substrates, osteoblast culture systems may be used to study cytocompatibility. Cell attachment to implant substrates is a specific, indirect, dynamic, and signal-generating event; its relevance to osseointegration should be investigated in detail. Subsequent cellular responses at implant substrates recapitulate osteoblast responses observed in other environments, making it difficult to ascribe osteoinductive or osteogenic attributes to one or another surface characteristic. Investigations of cell proliferation and protein biosynthesis and differentiation are of limited meaning without direct correlations to in vivo outcomes. The interfaces formed by cultured osteoblasts may be used to identify components of the implant-bone interface that exists in vivo. Careful application of osteoblast culture methods and cautious interpretations of results support the formulation of significant hypotheses to be evaluated in vivo.

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