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Focal adhesions in osteoneogenesis

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Abstract

As materials technology and the field of tissue engineering advances, the role of cellular adhesive mechanisms, in particular the interactions with implantable devices, becomes more relevant in both research and clinical practice. A key tenet of medical device technology is to use the exquisite ability of biological systems to respond to the material surface or chemical stimuli in order to help develop next-generation biomaterials. The focus of this review is on recent studies and developments concerning focal adhesion formation in osteoneogenesis, with an emphasis on the influence of synthetic constructs on integrin mediated cellular adhesion and function.

Keywords

focal adhesions; biomaterials; differentiation; cell signalling

1.1. Introduction

This review highlights the importance/development of the biomechanical processes that regulate early cell/biomaterial interaction and the influence of integrin mediated cellular adhesion in bone regeneration. As materials technology and the field of tissue engineering advances, the role of cellular adhesive mechanisms, in particular the interactions with implantable devices, becomes more relevant in both research and clinical practice.

Biomaterials are never truly inert, being at best biotolerable. The cell-substratum interface functions as more than a simple boundary of definition between the host and an implanted device, rather it presents primary cues for cellular adhesion and subsequent induction and tissue neogenesis. Indeed, the function and cytocompatibility of a construct can be assessed *in vitro* by observing the viability and adhesion of cells at the substratum interface. The range of materials currently in use within biomedical applications and their lack of biofunctionality reflects an increasing need for biomimetic constructs but also indicates the challenges present within the field, i.e. to ultimately control the interactions that occur at the cell-substratum interface.

A key tenet of medical device technology is to use the exquisite ability of biological systems to respond to the material surface or chemical stimuli in order to help develop next-generation biomaterials. Recently published in Science are the prerequisites for third-generation biomaterials; not only should they support the healing site (as first generation),

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but they should be bioactive and possibly biodegradable (as second generation and they should influence cell behaviour in a defined manner at the molecular level [1].

In order to investigate the reaction elicited by a material *in vivo* an understanding is required of the roles played by the cytoskeleton, cellular membranes, and the extracellular matrix (ECM) following implantation of a foreign material. An increased knowledge of the extracellular environment, topographical and chemical cues present at the cellular level and how cells react to these stimuli has resulted in the development of advanced orthopedic materials with an aim to regulate cell attachment and subsequent cellular function. The focus of this review is on recent studies and developments concerning focal adhesion formation in osteoneogenesis, with an emphasis on the influence of synthetic constructs on integrin mediated differential cellular function.

1.2. Cell–Biomaterial Interactions

Cell-substrate interactions can be regarded as the defining factors of the long-term performance and biofunctionality of an orthopedic device *in situ*. It can be reasoned that the integration of exogenous materials can be regulated by controlling the associated interfacial reactions, in an attempt to minimise non-functional tissue generation or aseptic loosening. Materials that promote osteoblast specific adhesion may enhance functional differentiation [2] resulting in the neogenesis of mineralised matrix, bony tissue formation and deposition. Fibrous encapsulation is known to occur with both metal [3] and polymeric orthopaedic constructs [4], usually with the presence of a fluid filled void between the tissue and implant. This reduced biocompatibility may have many causative origins; however, a frequent outcome is diminished device integration followed by destabilisation alongside an inhibition of tissue regeneration and repair as well as an increase in the potential for infection [5].

Conversely, many functional biomaterials require minimal protein and/or cellular interaction *in vivo* for optimal device function or to facilitate future device removal. For example a body of research suggests that permanent device retention following orthopaedic fixation is not ideal and may present future implant site morbidity. Increased osteoblast adhesion and bony tissue mineralisation can, for example, complicate the removal of plating systems, increasing removal torque and predisposing screw damage and bone refracture during the removal procedure [6,7]. It follows that selective adhesion of specific cellular phenotypes is crucial to regulate optimal tissue specific integration while preventing inflammatory cell adhesion and scar tissue formation.

Adherent cells are complex, self-sustaining units [8] that require ECM anchorage in order to proliferate and undergo differential function [9]. Modern implants make use of chemical and topographical modification to regulate cellular adhesion [10,11], differentiation and *de novo* tissue deposition [12] [13,14]. Following implantation ECM proteins undergo rapid adsorption to a material surface in response to surface free energy [15,16]. In adherent cells a network of dynamic contractile machinery facilitates both cellular motility and the formation of protrusions termed ‘lamellipodia’, structures essential for cellular spreading, polarisation and spreading *in vitro* [17-19]. Lamellipodia are associated with fine hair-like protrusions termed ‘filopodia’ (fig. 1), which contain a core of extended actin filament bundles and actively probe the external environment to gather spatial, topographical and chemical information from the ECM and/or material surface.

Initial cell tethering and filopodia exploration is followed by lamellipodia ruffling [20], periodic membrane activity and cellular spreading. With time, endogenous matrix is secreted by the cells and matrix assembly sites form on the ventral plasma membrane. Once cells locate specific ECM protein motifs, a signalling feedback pathway initiates integrin receptor

clustering at the plasma membrane and focal adhesion associated protein recruitment [21]. It can be reasoned that this reduction in cellular migration, the formation of mature adhesion sites and the onset of mineralised ECM synthesis are processes indicative of osteospecific differentiation and osteoneogenesis.

At present the science of fracture fixation and orthopedic construct fabrication is being advanced by functional modification technologies, which aim to regulate osteoblast adhesion and osteoneogenesis both on the implanted device and at the peri-implant site. Osteoconductive cement [22], topographical modification [17] and immobilisation of bioactive molecules at the substrate surface [10] have all been employed successfully to reduce giant cell recruitment, osteolysis and fibrous capsule formation. Of particular importance for the clinical success of load bearing orthopedic constructs however is the adhesion of osteospecific cellular populations, a process mediated by focal adhesion formation and reinforcement.

1.3. The Integrins

Osteoprogenitor cells, osteoblasts and osteoblast derived osteocytic cells interact with the extracellular environment via single or multiple chain transmembrane proteins termed integrins [23]. These receptors are composed of non-covalently linked α and β subunits which bind specifically to motifs located on ECM molecules i.e. the prototypic integrin ligand fibronectin, which contains the amino acid sequence RGD [24] and the GFOGER motif present in collagen type I [25]. First cloned in 1986 [26], integrin proteins are a fundamental initiator of cell and tissue organisation and are preserved throughout evolution, even in the most primitive of metazoan organisms [27,28]. Integrin-mediated adhesion is a highly regulated and complex process involving receptor-ligand binding as well as post-ligation interactions with multiple intracellular binding partners [29].

The function of integrins in cell-matrix adhesion can be divided into three mechanochemical processes. First, ECM-integrin binding forces must surpass a critical threshold in order to withstand the high forces [30,31] required for adhesion reinforcement, which lie in the nanonewton range. Second, integrins must mechanically couple the ECM to the cytoskeleton, enabling extracellular transmission of forces to the cell interior. Finally, these forces must be translated into biochemical signals (mechanotransduction) promoting an integrated cellular response. A recent study by Roca-Cusachs et al indicated the differential function of integrin species in these processes. High matrix forces were found to be primarily resisted by clustered $\alpha_5\beta_1$ integrins, while less stable $\alpha_v\beta_3$ integrin binding was shown to initiate mechanotransduction, resulting in a reinforcement of the integrin-cytoskeleton interactions [32], indeed these integrins have been identified as key regulators of osteoblast proliferation [33] and differentiation [34].

Although the RGD and GFOGER binding integrins rapidly associate with these motif sequences *in vivo*, their ability to recognize these sequences is dependent on the fibrillar status and accessibility of the interactive domains in fibronectin and the fibrillar collagens. Thus osteospecific adhesion at the implant surface is critically regulated by the composition as well as orientation and spacing of the adsorbed proteins [35]. Recent studies have exploited the specificity of the collagen GFOGER sequence in an attempt to enhance the osteospecific adhesive potential of orthopedic materials while preventing inflammatory cell adhesion [36]. GFOGER-modified implants have been shown to significantly enhanced osseointegration relative to surfaces modified with full-length type I collagen, highlighting the importance of presenting specific biofunctional domains within the native ligand [37].

The crystallization of a soluble integrin heterodimer has made clear that integrins exist in a compact bent conformation [38]. Later research has shown that this state represents an

inactive conformation [39]. Integrin ligation and strengthening is thought to involve the formation of specialized catch bonds which appears to involve force-assisted activation of the headpiece [40]. For a full review of integrin structure, function and their ligand-binding properties see [41]. Ligand binding in itself alters integrin conformation and affinity and, in the case of multivalent ligands, integrin clustering. Upon activation, integrins rapidly associate with motif sequences via their globular head domains and are reinforced intracellularly to form discrete supramolecular complexes at the lamellipodium leading edge that contain structural adaptor proteins, such as vinculin, talin, and paxillin [20,42,43]. These transient complexes are observed to have a substrate distance at closest approach of 10 nm [44], and by reinforcement, undergo anisotropic growth in response to increased intracellular and/or extracellular tension to form anchoring focal adhesions.

1.4. The Focal Adhesion

The regulation of focal adhesion formation in adherent cells is highly complex and involves both the turnover of single contacts and the reinforcement of the adhesion plaque by protein recruitment. Focal adhesions emerge as diverse protein networks that provide structural integrity and dynamically link the ECM to the intracellular actin cytoskeleton (fig. 2), directly facilitating cell migration and spreading through continuous regulation and dynamic reinforcement. Furthermore, in combination with transmembrane growth factor receptors, these adhesive clusters activate signalling pathways crucial to cell survival, cell growth and differentiation as will be discussed below.

Ward and Hammer developed a model of adhesion strengthening [45], which predicts large increases in adhesion strength following increased receptor clustering and adhesion size, marked by an elongation of the adhesion plaque. This process is believed to be due to an increase in tension at the adhesion site as focal adhesion size has been shown to be proportional to the force applied to it by the cell [46] indicating that adhesion sites act as mechanosensors [8] that form additional contact points with the underlying substratum in response to internal tension.

This force must exceed a critical value for adhesion reinforcement, but when growth occurs it occurs preferentially in an anisotropic manner in the direction of the force [31]. When a cell adheres to a surface, it exerts traction forces to balance the internal forces generated by cytoskeletal tension [47]. These forces have been measured, and most cells appear to generate traction forces in the range of tens to hundreds of nanonewtons [30,48]. This fixes a minimal value for the force that enables the growth of the focal adhesion by anisotropic elongation and indicates the role of substrate stiffness and motif density in affecting the size and strength of focal adhesions [49]. This force threshold depends on the precise biochemical nature of the adhesion, however there is an upper limit to the force: very large stresses or a decrease in substrate rigidity prevent adhesion elongation consistent with the observation of the transformation of focal adhesions into fibrillar adhesions [50].

1.5. The Fibrillar Adhesion

Focal complexes originate as dot-like structures of 0.5 μm in length, which are regulated by the GTPase Rac and precede larger focal adhesions that are regulated by the small G-protein Rho [51]. Mature focal adhesions are typically dash shaped, 1 – 5 μm in length (fig. 3A) and contain vinculin, paxillin and talin signalling complexes and mediate integrin function to regulate cellular behaviors such as cell migration [52]. Fibrillar type adhesions specifically contain α_5 and β_1 integrins and tensin [51] and play a major role in fibronectin organisation [53].

When intracellular force per unit length exceeds a critical threshold at the integrin substrate interface the displacement of the anchoring integrins is sufficient enough to induce a stick-slip motion where the adhesion complex moves rather than deforms. In this scenario changes in protein density around the adhesion plaque are negligible and no further adsorption of proteins to the focal adhesion occurs [50]. This progressive recruitment of integrins in the absence of focal adhesion proteins results in the formation of tension-insensitive fibrillar adhesions, structures intimately associated with extracellular fibronectin (fig. 3B). The assembly of a fibronectin matrix, a structure essential for cell migration during embryogenesis and wound healing is dependent on integrin clustering and fibrillar adhesion formation [54,55]. Whilst these adhesions are involved in fibronectin organising, fibrillar adhesions are relatively weak contacts, which are morphologically fibrillar by co-alignment with actin stress fibres. However fibrillar adhesions have been shown to play an important role in osteoblast assembly of the extracellular matrix [56] and may be fundamental in the deposition of an ECM architecture favorable to osteoblast and osteoprogenitor adhesion.

1.6. Bone Repair and Osteogenesis

Osteogenesis is an active process tightly regulated to ultimately generate a normal vascularised bone structure. Bone formation depends on the cooperation of several factors, namely: (i) the genesis of specific cell types such as progenitor cells and osteoblasts; (ii) a mineralised extracellular matrix scaffold; (iii) soluble bioactive molecules (cytokines, growth factors, hormones, ions, vitamins); and (iv) mechanical stimuli.

In the adult, the osteoblast is derived from a bone marrow stromal fibroblastic stem cell termed the mesenchymal stem cell (MSC), a non-hematopoietic multipotent stem-like cell vital for the osteogenic process and capable of differentiating into both osteoblastic and non-osteoblastic lineages (fig. 4). The adult stem cell, first described in the haematopoietic system following an investigation sparked by the detonation of atomic devices in Nagasaki and Hiroshima has been isolated from virtually every tissue of the body [57]. Of greater interest however is the apparent phenomenon that these adult cells, arising from different sources have the inherent potential to spontaneously trans-differentiate into other tissue progenitor cells [58].

Following the implantation of exogenous materials inflammation factors and cytokines attract regenerative cells, which expand and differentiate in order to build up a bone highly similar to that before injury. Bone marrow derived MSCs as skeletal stem cells; in conjunction with endothelial progenitors are at the origin of such reparation mechanisms. Stem cells form uncommitted populations, capable of both self-renewal, differentiation into multiple cell lineages and the capacity for maintained proliferation. Thus a stem cell can be defined as maintaining a supply of undifferentiated progenitor cells (self-renewal) in spite of continual differentiation. Due to difficulties in MSC isolation and maintenance as well as the ethical issues associated with cell harvesting recent studies have begun to focus on the molecular circuitry of pluripotency and self-renewal. Genomic reprogramming, i.e. the process of resetting the epigenetic modifications that are characteristic of the adult cell nucleus to ones that approximate those of the embryonic cell indicate that somatic cells might be reprogrammed back to the pluripotent state [59], it is envisaged that such technology will be instrumental in revolutionising the field of regenerative medicine.

1.7. Osteospecific Differentiation Through Focal Adhesions

The major event that triggers osteogenesis is the transition of MSCs into bone-forming, differentiated osteoblast cells and is controlled by sequential activation of diverse transcription factors that regulate the expression of specific genes. In addition to their

adhesive functions, integrins mediate bi-directional signalling between the ECM and the cell interior and are crucial in cell survival and differentiation [60-62]. Although integrin molecules provide a platform for intracellular signalling, they do not have intrinsic enzymatic activities in their cytoplasmic domains [63]. Therefore, downstream signalling is mediated by non-receptor tyrosine kinases [64]. It is becoming increasingly clear that cellular interaction at the substrate interface and the induction of focal adhesion formation plays a central role in the process of osteospecific differentiation and subsequent osteoneogenesis. However little is known regarding the influence of osteospecific biomaterials on integrin-mediated adhesion and the activation of osteospecific signalling pathways [65]. The process of integrin-mediated mechanotransduction relies on the ability of proteins of the focal adhesion to change chemical activity state when physically distorted, converting mechanical energy into biochemical energy by modulating the kinetics of intracellular protein-protein or protein-ligand interactions within the cell. The ability of proteins to translate the mechanical forces observed at the site of focal adhesion to nuclear activity facilitates the process of focal adhesion mediated cellular function.

The exact signaling mechanisms linking integrin clustering with the commitment of MSC to the osteogenic lineage are essentially unknown. However several studies suggest that the FAK→ERK→Runx2 signalling pathway constitutes an important element of the transduction machinery controlling this process. How this process may be regulated *in vivo* by osteoinductive biomaterials is currently of much interest and represents a major challenge in field of orthopedic device design. Below, this pathway is described within the context of osteoneogenesis following material implantation, a process that translates the dynamics of focal adhesion initiation and reinforcement to the regulation of differential processes in adherent progenitor cells.

1.8. Focal Adhesion Kinase

With reference to focal adhesion mediated mechanotransduction, integrin-dependant signalling pathways are mediated by non-receptor tyrosine kinases [64,66], most notable of these is focal adhesion kinase (FAK) a molecule constitutively associated with the β -integrin subunit [67]. the predominant adhesion receptor subfamily utilised by stromal precursor cells for adhesion formation *in vitro*. FAK plays central roles in adhesive interactions by functioning as a scaffold for focal adhesion components [68] as well as an important initiator of multiple signaling cascades via its many binding partners including Src [69], Cas [70], and paxillin [71].

Upon integrin ligation FAK is activated to bind to Src which then phosphorylates FAK on multiple residues [72], moreover FAK activation is highly regulated by integrin ligation to specific ECM motifs [29]. This phosphorylation increases FAK activity and can influence cellular transcriptional events through FAK phosphorylation of downstream signaling molecules rich in the SH2 domain [68]. In osteoblasts FAK directly influences the survival signalling pathway, and is responsible for negating mitochondria dependant apoptosis (programmed cell death), resulting in the inactivation of caspase 9 and the inhibition of cellular anoikis (apoptosis resulting from insufficient adhesion) [60].

Further to the role of FAK in adhesion mediated cellular survival recent studies indicate the role of FAK in promoting osteoblast focal adhesion formation on orthopedic constructs [73,74] and suggest that its expression is upregulated during osteospecific differentiation [75], acting to phosphorylate downstream signaling targets that regulate the synthesis of osteospecific proteins. Of these functional cascades, the best studied is FAK activation of the extracellular signal-related kinase 1 and 2 (ERK 1/2) pathways, important mediators of osteodifferentiation in mesenchymal progenitor cells [76,77].

1.9. Focal Adhesion Kinase Mediated ERK 1/2 Signalling

It is well known that the mitogen-activated protein kinases (MAPKs) play important roles in cellular response to environmental stimuli. There are at least three distinctly regulated groups of MAPKs: ERK 1/2, c-Jun N-terminal kinases (JNK1/2/3), and p38 MAPKs (p38).

Preliminary studies indicate that the 44 and 42 kDa ERK proteins participate in early osteodifferentiation [76,78-81], proceeding to phosphorylate a multitude of downstream target substrates [82]. It has also been shown that an increase in the distribution and reinforcement of focal adhesions in both osteoblasts [83] and MSC [84] results in the activation of ERK 1/2 by the Grb2-Sos-Ras pathway (fig. 5). Further to this, MSCs cells cultured on bioactive materials such as hydroxyapatite have been shown to up-regulate osteospecific genes and osteoinductive proteins through the activation of ERK 1/2 [85]. As might be expected, activation of ERK by progenitor cell adhesion to an orthopedic construct and the onset of integrin clustering, results in the increased activity of osteospecific transcription factors – the mediators of osteospecific differentiation and early function [86].

1.10. Focal Adhesion Regulation of Runx2 mediated transcription

The central regulation of bone differentiation and formation in osteoprogenitor cells is controlled by the transcriptional activity of Runx2, a factor subject to a number of post-transcriptional controls including selective proteolysis and phosphorylation [87]. Runx2 is a Runt-related transcription factor, characterised as a heterodimeric protein with a DNA-binding α subunit and a non-DNA binding β subunit. Examination of different truncations of the RUNX2 protein showed that the C-terminal proline/serine/threonine region of RUNX2 is required for both ERK 1/2 responsiveness and phosphorylation [88]. Runx2 expression is highly restricted to bone and its activity during early embryonic development acts as a master regulator in the commitment of these cells to the osteoblastic lineage [77], indeed homozygous deletion of Runx2 in mice results in the complete absence of osteoblasts and bone formation [89,90], while the extent of mineralisation in trabecular bone is higher in transgenic mice that have been modified to overexpress Runx2 [94]. Further to this, Runx2 is shown to regulate several osteoblast-specific genes including alkaline phosphatase, Osteopontin, Osteocalcin and matrix metalloproteinase 13 [90-93] vital mediators of bone homeostasis.

In a biomaterials setting, mesenchymal stem cells have been shown to undergo osteospecific differentiation and functional tissue formation when cultured on topographies that increase focal adhesion frequency and reinforcement [95,96]. Further to this Runx2 expression has been directly correlated with focal adhesion reinforcement [77,97] and is shown to be increased in mesenchymal populations cultured on a variety of next generation materials including nanostructures [98], three-dimensional fibrous scaffolds [99], biofunctionalised titanium [100] and polymers [101] and hydroxyapatite/tricalcium phosphate scaffolds [102].

1.11. Conclusions

It is predicted that the percentage of persons over 50 years of age affected by bone diseases will double by 2020 [103]. Clearly this represents a need for permanent, temporary or biodegradable orthopedic devices that are designed to substitute or guide bone repair. Orthopedic biomaterials should be designed with optimal physical and chemical properties to promote tissue regeneration as well as bioactivity to induce specific cellular responses at the molecular level and modulate cellular function. It is known that enhanced osteoneogenesis can be induced by surface chemical or topographical functionalisation,

however the ideal materials for the induction of osteoadhesion and osteoneogenesis are still under investigation.

It has previously been observed that focal adhesion maturation rather than frequency is important in osteospecific differentiation [84,104] and that with focal adhesion reinforcement, increased FAK is activated to initiate downstream signaling cascades. Conversely, when integrin mediated adhesion is primarily restricted to sparse focal complexes these mechanosensitive signaling events are reduced. This balance between mature focal adhesion formation and related cell signaling appear to be critical in mesenchymal stem cell differentiation.

Because such a wide variety of signals affect bone marrow stromal cells differentiation, it is very likely that no single signaling pathway is responsible for the regulation of early osteoprogenitor differentiation. Rather, a network of signaling pathways is likely at work, and FAK is highly suited to integrate these signaling activities. These pathways are intimately related and activation may be mediated by adhesive mechanisms or soluble signaling factors. There is growing appreciation that simultaneous activation and repression of gene expression is a feature of multiple developmental signalling pathways and importantly it is this crosstalk between mechanotransductive and other developmental pathways that regulates transcriptional events.

It can be hypothesised that third generation biomaterials, may be modified to present osteoconductive elements with a view to controlling the cellular processes [105] specific to bone regeneration. The next stage in the evolution of orthopedic biomaterials may rely on a highly specific regime of topographical modification coupled with bioactive eluting properties with an aim to regulating cellular adhesion and differentiation through specific osteoinductive pathways. Specifically, manipulation of MAPK signaling could offer interesting opportunities for enhanced bone repair and device integration. However, the participation of this pathway in numerous biological processes, notably inflammation, may restrict the therapeutic use of modified materials, which should strongly depend on the indication or stage of bone repair.

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Fig. 1. Filopodia formation in endothelial cells on a nanogroove substrate
Endothelial cells probe the underlying grooved substratum with fine filopodial extensions (arrows). These extend from the leading and trailing free edge.

Fig. 2. A simplified overview of the molecular composition of the focal adhesion

Focal adhesions are dynamic supramolecular structures that function as anchoring complexes as well as mechanomediated modulators of cellular function. These complexes effectively couple the extracellular matrix to the cytoskeletal filamentous actin (F-actin) through the dynamic interactions that exist between the component proteins, in particular talin and vinculin. Focal adhesion kinase (FAK) acts as a modulator of protein recruitment to the focal adhesion as well as an important initiator of downstream signalling pathways.

Fig. 3. Focal adhesions are distinct from fibrillar adhesions

FA elongation is either associated with (A) mature adhesion formation (vinculin label) or (B) fibrillar adhesion formation (tensin label). These structures are morphologically and biochemically distinct and play individual roles in cellular adhesion and tissue formation respectively.

Fig. 4. The differentiation pathway of MSCs

MSCs undergo differential functions following cellular adhesion and in response to external stimuli, i.e. mechanical loading, substrate topography and secreted growth factors.

Fig. 5. Integrin mediated regulation of the ERK 1/2 signalling pathway

Extracellular forces and cellular adhesion are translated into differential cellular function through the regulatory effects of ERK 1/2 on the nuclear machinery of cellular transcription. FAK recruitment and subsequent activation at the focal adhesion site results in Runx2 modulation of cellular transcription. This process is mediated primarily by Grb2-Sos-Ras activation of the ERK 1/2 pathway.