OSTEOBLAST AND GINGIVAL FIBROBLAST MARKERS IN DENTAL IMPLANT STUDIES

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Background. Dental implants are a suitable option for the replacement of some or all missing teeth. Their main function is to secure the stability of the artificial tooth. The implant material interacts with several cell types including osteoblasts, gingival fibroblasts, periodontal ligament fibroblasts and monocytes. The most common material used is pure titanium which is corrosion resistant and has an elasticity modulus similar to that of bone. In recent years, diverse modified titanium surfaces have also been developed. The wound healing around the implant is a complex process that determines how well the host can heal and accept the implanted material. For this reason, search for markers of the biocompatibility of these new materials is paramount. To identify markers found to be suitable for studying the biocompatibility of dental implants.

Methods. Review of Pubmed and Web of Science databases for the years 1958-2010.

Conclusions. The surface of dental implant material should enhance firm attachment of the implant to junctional epithelium, soft connective tissue and bone. For the purposes of dental implant biocompatibility studies, a number of markers produced by osteoblasts or by cells of periodontal ligament have been proposed. In general, the most typical markers for osteoblasts and fibroblasts are alkaline phosphatase and collagen I, respectively. The involvement of both cell types in the inflammatory response is primarily evaluated by determination of tumour necrosis factor α and proinflammatory interleukins.

INTRODUCTION

Dental implants are a widely used alternative to some conventional dental treatments. However for a successful implant, biocompatibility and bioadhesiveness at the implant-tissue interface are crucial as the implant is in contact with soft tissue and bone. For this reason, the proteins expressed by the gingival fibroblasts and osteoblasts are important in the study of implant biocompatibility. Many of these proteins are secreted into the extracellular matrix (ECM). The durability of an implant depends on stable connection between implant surface and surrounding tissue¹. It is known that the surface properties of implants impact the interaction with the biological system with bone formation being determined by four interrelated surface properties: composition, surface energy, topography and roughness².

The most common dental implant material is titanium which is resistant to corrosion and has an elasticity modulus similar to that of bone. In recent years, diverse modified titanium surfaces have also been developed. Osseointegration is facilitated using hydroxyapatite coatings, higher surface roughness and surface-free energy. Increased surface roughness is achieved by blasting the surface with titanium oxide, analytical etching, sand blasting and acid etching³. One of the main aims of materials research for implantology is study and development of

surface modification techniques to improve the bone integration of titanium-based dental implants^{4,6}.

There are three types of dental implantations. The first, immediate implantation is based on placing the implant in the periodontium before the site of the missing tooth has healed. The second type is early implantation, which is 4-8 weeks after extraction of tooth. In the third type, the implant is inserted into healed bone. Hence, knowledge of the histology and structure of the periodontium and bone is relevant to study of the biocompatibility and bioadhesiveness of dental implants. The success of a material after implantation depends on the outcome of a number of complex processes. Initially the implantation induces an acute inflammatory response and this is followed by repair processes resulting in wound healing.

THE PERIODONTIUM AND THE BONE

The periodontium

The periodontium is the tissue which surrounds and supports the teeth. It consists of the periodontal ligament (PDL), the gingiva, the root cementum and the alveolar bone. The mature periodontal ligament includes periodontal ligament fibroblasts, neurovascular elements, epithelial cells, cementoblasts, osteoblasts and their precursors. The PDL also contains a large number of matrix

macromolecules, such as collagens, glycosaminoglycans and various non-collagenous proteins⁷⁻⁹.

An important function of the root cementum is to invest and anchor the principal periodontal ligament fibres which spin like a meshwork between the root and alveolar bone, to the root. In addition, the cementum participates in the maintenance of occlusal adaptation, repair of root defects after resorption or fracture and, protection of the pulp¹⁰.

Periodontal tissue is mainly inhabited by gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF)(ref.¹¹). Gingival fibroblasts have an important role in the development, function and regeneration of the tooth-support structures^{12,13}. They are also involved in the production and maintenance of the connective tissue matrix^{13,14}. It is these cells that are responsible for the overall production and turnover of the extracellular matrix. Gingival fibroblasts maintain the synthesis and integrity of the gingival connective tissues. However, the fibroblasts have additional specialized functions as they are involved in the repair, remodeling and regeneration of the adjacent alveolar bone and cementum¹⁵.

The bone

Bone is a dense tissue, containing three types of cells, i.e. osteoblasts, osteocytes, and osteoclasts¹⁶. Osteoblasts are responsible for bone formation¹⁷ and osteocytes are the matured osteoblasts that have become entrapped in the deposited bone matrix. The function of osteoclasts is to break down the bone matrix¹⁶. Polarized mature osteoblasts secrete and deposit most bone matrix proteins. After implantation, osteoblasts adhere to the surface of the implant and mediate its strong fixture in a process called osseointegration. Osseointegration provides a direct structural and functional connection between ordered, living bone and the surface of a load carrying implant¹⁷. Therefore, *in vitro* studies with osteoblasts isolated from bone can offer insight into the biological performance of bone implant materials².

Cell models for *in vitro* studies

The biocompatibility of dental implants can be studied using various cell models. Well-established primary cultures include human gingival fibroblasts, alveolar fibroblasts and human osteoblasts. Besides the primary cultures, *in vitro* studies often utilize cell lines such as human osteoblast-like cells SaOS-2 or human osteosarcoma cell line MG-63. In addition, more complex *in vitro* models consisting of two cell types are used. These co-cultures combine, for instance, certain types of bone cells, such as MG-63 cells or osteoprogenitor cells, with human umbilical vein endothelial cells or other types of endothelial cells¹⁸.

MARKERS FOR DENTAL IMPLANT STUDIES

Cell-matrix interactions depend on cytoskeletal organization, transmembrane integrin receptor expression and, most importantly, on the nature of the extracellular

matrix. In bone and in periodontium, the extracellular matrix is composed of proteins such as collagen, fibronectin, laminin, osteopontin, osteonectin, alkaline phosphatase and other specific glycoproteins. The extracellular matrix is crucial in mediating cell adhesion to implanted materials since its organization and production modulates the degree of cell attachment to the materials. The success of non-biodegradable implants will depend primarily on biocompatibility, followed by the capacity of the surface topography of the implants to evince desired cell matrix, surface-cell matrix interactions^{19,21}.

Proteins involved in the healing process

Tenascin

Tenascin is an extracellular matrix glycoprotein whose expression is regulated by growth factors such as TGF- β (ref.^{22,23}) and by mechanical stress^{24,25}. It is expressed during normal processes such as wound healing, nerve regeneration, tissue involution and in pathological states including vascular diseases and tumorigenesis²⁶. In dental tissues, tenascin is involved in the differentiation of odontoblasts²⁷. These cells form the inside surface of the tooth and separate it from the cavum pulpa. Tenascin may thus also be associated with secondary dentine elaboration when pulp cells differentiate into odontoblasts in response to physical stimuli²⁷⁻³⁰.

Fibronectin (FN)

Fibronectins are heterodimeric adhesive glycoproteins (220 kDa) composed of two large subunits³¹. They are expressed by many cell types, epithelial, endothelial and mesenchymal³². The soluble form of fibronectin circulates in the blood and other body fluids. All the other forms assemble on the surface of cells and are deposited in the ECM as the highly insoluble FN³³. Fibronectin has been implicated in a variety of cell functions, including adhesion, migration, growth and differentiation^{32,34}. It is also found in association with dental basement membrane during tooth formation, polarization and differentiation of odontoblasts^{30,35}. Fibronectin is considered to play important roles in the maintenance of normal tissue order and in interface interactions, since it mediates cell-matrix interactions, recognizing different cell types as well as bacteria^{36,37}. Fibronectin is also organized in focal adhesions which participate in cell locomotion and in cell adhesion³⁸⁻⁴¹.

Collagens

The production of collagen is a key part of the formation of new connective tissue, a process critical to the durable performance of the implanted device. Periodontal ligament, gingival fibroblasts and osteoblasts produce collagens I and III (ref.²⁶). Collagen I, one of the earliest osteoblast markers found, is upregulated in committed osteoprogenitors and its deposition as extracellular matrix appears to be a relatively early event in the osteoblast differentiation pathway³⁹. Collagen I is one of the major components of gingival connective tissue. Collagen I is known to contribute to rapid regeneration of bone defects.

Collagen III that constitutes 43% of total collagen⁴² may have an important function in the elasticity of the tissues⁴³, whereas collagen I is believed to maintain tissue architecture⁴⁴. Although collagens are the main organic components of dentine 80-90% (ref.⁴⁵), no collagen III is found in dentine^{30,46,47}.

Osteonectin (ONEC) and osteopontin

Osteonectin and osteopontin are adhesive glycoproteins specifically localized in the mineralized ECM of bone^{48,49} and synthesized primarily by osteoblasts, endothelial cells and megakaryocytes. Osteonectin and osteopontin are major noncollagenous proteins of bone⁵⁰. Both are responsible for the regulation of bone mineralization, since they promote the deposition of calcium phosphate^{30,51,52} and inhibit the growth of hydroxyapatite crystals⁵³⁻⁵⁶.

Alkaline phosphatase (ALP)

Alkaline phosphatase, a glycoprotein belonging to a family of proteins anchored to the plasma membrane via glycosylphosphatidylinositol linkage⁵⁷⁻⁵⁹, catalyses the hydrolysis of phosphate esters in alkaline pH. ALP is a common biochemical marker used to assess osteoblast differentiation and is considered to be involved in skeletal mineralization^{60,61}. ALP is abundant in matrix vesicles which play a role in extracellular matrix processing and calcification of bone. The levels of ALP are increased just before mineralization is initiated. This aside, the precise role of ALP in mineralization remains unclear. It may be related to its calcium-binding action, generation of free phosphate, or degradation of mineralization inhibitors⁶². Increased levels of alkaline phosphatase activity have also been reported as one of the phenotypic changes of fibroblasts in wounds and inflammation^{59,63, 64}.

Receptor activator of nuclear factor- κ B (RANK), receptor activator of nuclear factor- κ B ligand (RANKL), parathyroid hormone (PTH) receptor, osteoprotegerin

Receptor activator of nuclear factor κ B ligand (RANKL) is a member of the TNF receptor family and functions as a specific receptor for RANK. Osteoblasts express RANKL as a membrane-associated factor, while RANK is expressed by osteoclast progenitors and matured osteoclasts. Interaction of RANK with RANKL induces differentiation of pre-osteoclasts to osteoclasts^{65, 66}. In contrast, soluble RANK, also called osteoprotegerin (OPG), strongly inhibits osteoclast formation. The RANKL/OPG ratio is critical in the pathogenesis of bone diseases that result from increased bone resorption⁶⁷. Expression of RANKL by osteoblasts arises during the action of osteotropic factors including parathyroid hormone (PTH), prostaglandin E2 and interleukin-11. It has been shown in various models that the PTH receptor increases bone mass when given intermittently but reduces bone mass when infused continuously. Consistently, PTH plays an important role in the regulation of osteoblast number and bone volume, presumably by decreasing osteoblast apoptosis^{39, 55, 68}.

Laminin-5

Laminin-5 plays an important structural role in the formation of hemidesmosomes, junction structures that serve to attach cells to underlying substrate. Hemidesmosomes ensure efficient attachment of epithelial cells to a variety of natural substances, including the enamel of the tooth and to implant material such as titanium and ceramics. *In vitro*, epithelial cells adhere more readily to laminin-5 than to other extracellular matrix elements, including laminin-1, laminin-2 and fibronectin, presumably due to enhanced hemidesmosome formation. Increased attachment, spreading and hemidesmosome formation of epithelial cells on laminin-5 passivated titanium indicate enhanced integration of tissue and metal and thus predict significant utility of this molecule for the long-term stabilization of implants⁶⁹.

Bone sialoprotein (BSP)

Bone sialoprotein is one of the major calcium binding, non-collagenous glycosylated phosphoproteins in the extracellular matrix of mineralized tissues such as bone and dentine. BSP belongs to the small family of integrin-binding ligand N-linked glycoproteins. It is expressed by several cell types associated with mineralized tissues but is produced in abundance by osteoblasts. The molecule of BSP is linear with little secondary structure. However, the protein is highly flexible, containing spatially segmented motifs that can bind several ECM constituents with diverse biological roles, including collagen, matrix metalloproteinases, hydroxyapatite, as well as integrins present in numerous cell types. *In vitro* experiments with bone derived osteoblasts and bone marrow cells have revealed that BSP not only stimulates calcification of newly synthesized organic matrix but also promotes cell proliferation and expression of osteoblastic phenotypes, suggesting that BSP may play a role in osteoblast differentiation and the onset of mineral formation^{70,71}.

Adhesion proteins

Local stabilization of the implanted material requires strong adhesion of cells on the material surface. Cell adhesion to the extracellular matrix plays a fundamental role in regulation, cell differentiation, growth, and survival. Cell adhesion to both extracellular matrix and synthetic surfaces is dependent on integrin-mediated signal transduction and cytoskeletal proteins that form complexes known as focal adhesions. These complexes couple focal adhesion kinase (FAK), vinculin, integrins and actin filaments. Thus, focal contacts are sites of more or less stable connections between intra- and extracellular fibre systems^{1,72,73}.

Integrins

Integrins are one of the major families of the cell adhesion receptors⁷⁴. Each integrin is a heterodimer consisting of an independent α subunit and β subunit⁷⁵ (see Fig. 1).

Both subunits are transmembrane proteins with their cytoplasmic domains attached to the cytoskeleton⁷⁶. To date, 16 α subunits and 9 β subunits are recognized.

However, only specific combinations of α and β subunits may form integrin heterodimers^{13,75}.

Integrins interact with the ECM through their extracellular domains and with components of the cytoskeleton and various signaling molecules through their intracellular domains. Through these interactions, integrins can regulate many cellular functions such as cell adhesion, motility, shape, growth and differentiation¹⁶.

The expression of integrin in osteoblastic cells has been intensively studied. Osteoblasts have been shown to express a wide variety of integrins, including integrin subunits α_1 , α_2 , α_3 , α_4 , α_5 , α_6 , α_v , β_1 , β_3 , and β_5 (ref.¹⁶).

A characteristic feature of most integrin receptors is their ability to bind an array of ligands. Moreover, many extracellular matrix and cell surface adhesion proteins bind to multiple integrin receptors^{13,37,77-80}.

Fig. 1. The basic structure of the integrin.

Vinculin

Vinculin is a membrane-cytoskeletal protein. It forms a linkage between integrin adhesion molecules and actin cytoskeleton in focal adhesion plaques, and thus participates in regulation of both cell-cell and cell-extracellular matrix junctions. Vinculin contains a “head” domain, a “tail” and a proline-rich hinge region. The head domain binds to actin-binding proteins, talin and α -actinin, whereas the tail domain binds to F-actin and paxillin (see Fig. 2). Moreover, intramolecular head-tail interactions may occur in the vinculin molecule. However, another molecule of vinculin enables the interactions between N- and C-terminal domains, and the resulting conformational changes activate vinculin and allow it to bind to F-actin. The importance of vinculin for the structure and function of focal adhesions was confirmed by micro-injection of the vinculin binding site. This targeted the vinculin within the cells, disrupted vinculin interactions with talin and α -actinin and thus disassembled focal adhesions⁸¹⁻⁸⁴.

Fig. 2. The domain structure of vinculin and interacting partners (modified according to ref.⁸⁵).

Proteins associated with the inflammatory response

Periodontal disease is a disorder of the oral connective tissues affecting the gingiva, periodontal ligament and alveolar bone. Gingivitis is characterized by inflammation of the gingival tissue around the teeth, while periodontitis includes loss of connective tissue, including the periodontal ligament and alveolar bone⁸⁶. Inflammatory markers i.e. cytokines, matrix metalloproteinase and growth factors serve for determination of inflammation.

Cytokines

Cytokines are proteins secreted by various cells and mediate many cellular functions. Among others, they function as intercellular messengers essential for the pathogenesis of many diseases including peri-implantitis. In periodontology and implantology, cytokines are involved in inflammation-related alteration and repair of periodontal or peri-implant tissues⁸⁷.

Tumor necrosis factor-alpha (TNF- α)

TNF- α is a potent osteoclastogenesis agent secreted by various cells, gingival fibroblasts and fibroblasts of the periodontal ligament⁸⁸⁻⁹⁰. It has been implicated in the bone loss and connective tissue destruction associated with periodontal disease^{88,91,92}. This aside, TNF- α functions as a pro-inflammatory mediator stimulating production of matrix metalloproteinases (MMPs)(ref.^{93,94}) and contributing to the development of such diseases as rheumatoid arthritis, periodontitis and multiple sclerosis⁹⁵⁻⁹⁹. TNF- α is also the main cytokine mediating response to gram-negative bacteria where the concentration of TNF- α reflects the amount of bacteria and stage of inflammation⁸⁷.

Interleukins and chemokines

IL-6 is produced by fibroblasts and its expression is increased at diseased periodontal sites including inflamed gingiva. IL-6 stimulates bone resorption presumably by

stimulating osteoclast formation. IL-8 is produced by a variety of cells including monocytes, epithelial cells, endothelial cells as well as gingival fibroblasts in response to inflammatory mediators such as interleukin-1 β , and functions as a potent chemotactic factor for neutrophil granulocytes. Levels of IL-8 mRNA and protein have been found elevated in chronically inflamed gingival tissue as well as in gingival crevicular fluid from patients with periodontitis. Through the production of cytokines, such as IL-6 and IL-8, fibroblasts and epithelial cells act as accessory immune cells and contribute to periodontal destruction^{86,87,100}.

Matrix metalloproteinases (MMPs)

MMPs are produced by the cells of the connective tissue, e.g. fibroblasts, osteoblasts and odontoblasts synthesize and secrete MMPs into the ECM. In the ECM, MMPs are responsible for the degradation of extracellular matrix components during physiological processes such as morphogenesis, wound healing and cell migration¹⁰¹. The remodeling of ECM by MMPs generally occurs at neutral pH. Under normal physiological conditions, the MMPs are expressed only when needed for tissue remodeling. In contrast, their aberrant expression is associated with the tissue destruction observed in many pathological conditions such as rheumatoid arthritis, periodontitis, cancer, tissue ulcers and fibrosis¹⁰².

MMPs are classified into five groups based on their structural homology and their substrate specificity (Table 1)(ref.¹⁰²).

MMP-1, also called fibroblast or interstitial collagenase, is produced by several cells including fibroblasts, keratinocytes, chondrocytes, lymphocytes and monocytes. It preferentially degrades collagen molecules by enzymatic activity against triple helical collagen¹⁰¹. In general, collagens can be also degraded by other human interstitial collagenases such as MMP-8 and MMP-13 which release 3/4- to 1/4-length peptides. These peptides lose the triple-helical conformation and can be further degraded by the gelatinases MMP-2 and MMP-9(ref.¹⁰²).

In periodontitis, the increased levels of MMP-1 detected in gingival tissue and in gingival fluid may be derived from fibroblasts and monocytes/macrophages. Gingival fibroblasts are in close contact with monocytes/macrophages that are heavily distributed in the gingival tissue during periodontitis and these cell-to-cell contacts stimulate the expression of MMP-1(ref.¹⁰¹). One particular MMP, matrilysin (PUMP-1, MMP-7), which is expressed during normal processes such as wound healing and bone differentiation, has been shown to degrade many ECM proteins including proteoglycans, fibronectin, laminin and others¹⁰³.

Table 1. Characteristics of all known MMPs (ref.¹⁰²).

MMP	Characteristics
MMP-1, -8, -13, -18	collagenases
MMP-2, -9	gelatinases
MMP-3, -10, -11	stromelysins
MMP-14, -15, -16, -17, -24, -25	transmembrane
MMP-7, -26	matrilysins

Growth factors

Regeneration of periodontal tissue is regulated by transforming growth factor- α (TGF- α) and transforming growth factor- β (TGF- β). TGF- α regulates angiogenesis, while TGF- β stimulates collagen I, fibronectin and osteonectin synthesis as well as bone matrix formation and basic fibroblast growth factor (bFGF). TGF- β is known to be expressed during the implantation process and contributes to implant osseointegration¹⁰⁴⁻¹⁰⁸. TGF- β has been also implicated in reparative dentinogenesis in the pulp¹⁰⁹.

Epidermal growth factor (EGF) stimulates a variety of biological actions including proliferation and differentiation of epithelial and mesenchymal cells and plays a central role in inflammatory and immunological responses. In the periodontal tissues it is expressed by human periodontal ligament and gingival fibroblasts, junctional epithelial cells and alveolar bone cells. The expression of EGF might be different in periods of periodontal destruction quiescence and wound repair. EGF increases the expression of both MMP-1 and -3 in gingival fibroblasts^{100,110}.

CONCLUSION

Dental implants are an ideal option for people in overall good oral health who have lost a tooth or teeth due to periodontal disease, injury or for some other reason. The success of dental implants, i.e. the ability to support a dental restoration, is critically dependent upon how much bone is available at the implant site¹¹¹. The biocompatibility of the implant itself is determined by both the physical and chemical characteristics of the material and particular features of implant surface, such as the thickness of the oxide layer, microstructure and porosity. The surface of the dental implant material should enhance firm attachment of the implant to junction epithelium, soft connective tissue and bone^{38,112}. In summary, a number of markers produced by osteoblasts or by cells of periodontal ligament have been proposed for testing the dental implant biocompatibility, e.g. integrins, vinculin, alkaline phosphatase, collagen I and inflammatory cytokines. The most typical markers for osteoblasts and fibroblasts are alkaline phosphatase and collagen I, respectively. The involvement of both cell types in the inflammatory response is evaluated by determination of tumour necrosis factor α and proinflammatory interleukins.

ABBREVIATIONS

ECM, Extracellular matrix; PDL, Periodontal ligament; PDLF, Periodontal ligament fibroblasts; MG-63, Human osteosarcoma cell line; FN, Fibronectin; Col, Collagen; ALP, Alkaline phosphatase; RANKL, Receptor activator of nuclear factor; PTH, Parathyroid hormone; OPG, Osteoprotegerin; BSP, Bone sialoprotein; IL, Interleukine; TNF- α , Tumor necrosis factor alpha; MMPs, Metalloproteinases; TGF, Transforming growth factor; bFGF, Basic fibroblasts growth factor

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