Cellular Responses to Metal Ions Released From Implants

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In the process of calcified tissue formation, cells secrete a protein-rich matrix into which they add a metal ion that nucleates in the presence of phosphorus to form an inorganic salt (usually calcium hydroxyapatite). Cellular and tissue responses to metal ions—released from implants, for example—can therefore be considered from the perspective of how cells handle calcium ions. A critical factor in determining cellular toxicity will be free ion concentrations and the competitive interactions that occur in a physicochemical manner. Three of the parameters used to assess the biocompatibility of implant materials are (1) the ability to influence mitotic activity, (2) intercellular adhesion, and (3) promotion of cell death. A spectrum of responses to free intracellular calcium ions can be identified, ranging from presence of the ion being essential for cell division through to an excess of the free ion that results in cell death (apoptosis). In between these extremes, cells may become postmitotic and express phenotypic variations as they adapt to their environment and establish equilibrium to maintain intracellular calcium homeostasis. The response of cells to implants can be linked to ions released and interactions between these and other ions and/or molecules present in the tissues, similar to the manner in which cells handle calcium ions.

Key Words: metal ions, calcium, titanium, bone formation, bone growth

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INTRODUCTION

Since Branemark coined the term "osseointegration" for the tissue responses to placement of a dental implant, there has been intensive research into the development of biocompatible materials for a wide range of biomedical applications. The definition of the term osseointegration has, however, been modified since its introduction to broadly encompass clinical success. There is general agreement that the process of osseointegration depends on several factors, and clinical success is well documented for titanium and titanium-based alloys. Critical to the success of osseointegration is the response of cells that results in the formation of hard tissues, either through the processes of bone formation (osseoinduction—matrix secretion and initiation of mineralization de novo) and/or bone growth (osseoconduction—matrix secretion and addition or removal of mineral to or from an existing mineralized tissue). However, concerns have been raised over potential adverse effects of some of these implanted materials—in particular, metals—arising from their release of ions into the tissues.[1] As all biomaterials will evoke biological responses, a variety of studies have been undertaken to determine significant biological properties of materials. These studies have shown that surface morphology, topography, roughness, chemical composition, surface energy, chemical potential, strain hardening, the presence of impurities, thickness of titanium oxide layer, and the presence of metal and nonmetal composites have a significant influence on responses within the tissues, as reviewed by Elias and Meirelles.[1]

In an attempt to closely match the physical properties of the implants with those of host bone, a number of titanium alloy scaffolds have been developed to provide "bone-mimicking" properties.[2] Commonly used metallic elements are tantalum (Ta), niobium (Nb), zirconium (Zr), tin (Sn), molybdenum (Mo), and the semi-metal silicon (Si). The cytotoxicity of these elements has been recently investigated and "safe" ion concentrations determined using a range of cell culture assays.[2] In these studies, the metallic elements were considered biocompatible if the cell viability in the

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presence of the metal ions was equivalent or greater than that of a "media-only" control group. Parameters investigated included mitotic activity, cell morphology and cell attachment, and extracellular matrix synthesis (including local cytokine production).

Tissue Responses to Calcium Ions

Calcium is chemically a very reactive metal classified with the alkaline earth group of metals (M.P. 810°C, S.G. 1.53,) that forms divalent cations, Ca^{2+}, in solution. The free ion concentration in the extracellular matrix $[Ca^{2+}]_e$ is 10^{-3}M. The intracellular free ion concentration $[Ca^{2+}]_i$ is in the range 10^{-7}M, representing a 1000 x gradient across the plasma membrane. $[Ca^{2+}]_i$ is maintained at a low concentration by the establishment of ion gradients across membranes, either the plasma membrane or the membranes of intracellular organelles.[3] By keeping intracellular free ion concentrations low, cells have been able to utilize calcium and phosphorus for many metabolic events,[4] regulating a large number of cellular processes, either directly or indirectly, through interactions with calcium binding proteins.[5] Disruption of intracellular Ca^{2+} homeostasis is frequently associated with the early development of cell injury[6,7] that has led to the proposal that disruption of intracellular Ca^{2+} homeostasis may be a common step in the development of cytotoxicity.[8] A steady-state situation where a cell expresses a stable phenotype would represent the presence of an equilibrium across the plasma membrane, whereas an imbalance in intracellular Ca^{2+} homeostasis will show variations in phenotype as long as these are within the cell's adaptive capacity. If the adaptive capacity is exceeded, cell death will ensue.

The extracellular Ca^{2+} concentration is also closely regulated through a range of cellular activities, and free ions may be bound to organic molecules or form inorganic salts, such as calcium hydroxyapatite. Serum calcium homeostasis has been extensively investigated and provides an example of the interactions involving a wide range of cells; their products achieve a relatively constant physiological level. Central to maintaining serum levels is bone, with its unique cells, osteoblasts, osteocytes, and osteoclasts playing a pivotal role.

While the plasma membrane is relatively impermeable, ions may move across it due to the electrochemical gradient, acting as first messengers.[9] Free calcium ions may enter the cell either by "leaking" across the membrane or following activation of a receptor, due to specific Ca^{2+} transport systems in the membrane.[10] Maintenance of $[Ca^{2+}]_i$ is an active event that requires free Ca^{2+} to be actively transported out of the cytosol and across a membrane utilizing adenosine triphosphate (ATP)-dependent ion pumps. Within a cell, free calcium ions are compartmentalized, with most being bound to proteins or negatively charged molecules in the cytosol. An influx or increase in free ions in the cytosol, either from release of intracellular stores or influx across the plasma membrane, can result in a variety of signaling events, ranging from mitosis,[11–13] repair of the plasma membrane,[13] modifications to cell-cell adhesion,[14] or cell death.[15] Following stimulation of a receptor-operated calcium ion channel, for example, the free $[Ca^{2+}]$ in the cytosol undergoes a transient increase that may be utilized for signaling pathways, leading to changes in gene expression[16,17] or buffering by Ca^{2+}-binding proteins. Activated calcium-binding proteins may in turn modify the dynamics of Ca^{2+} release or removal. These events of Ca^{2+} release, Ca^{2+} influx, and Ca^{2+} binding are closely coupled, and prolonged elevation of intracellular Ca^{2+} levels above the adaptive capacity of the cell will result in cell death.[18,19] The ability of a cell to adapt to variations in free ion concentration can be seen in the range of morphological and biochemical changes that occur during the differentiation of cells, in particular those associated with calcified tissues. The requisite for cell survival is an ability to maintain low intracellular Ca^{2+} levels.[20]

There are differences in the energy requirements for active membrane transport across the membranes of organelles or the plasma membrane. The energy requirements for active transport of Ca^{2+} out of the cytosol and across the plasma membrane are greater than that for transport across the membrane of organelles; hence, calcium ions may be "temporarily" transported into an organelle, potentially to be later exported from that organelle. For example, calcium ions may be moved across the membrane of the endoplasmic reticulum and subsequently exported into the extracellular matrix along with negatively charged molecules, such as proteins.[21] Calcium ions may also be moved across the mitochondrial membrane into an inorganic

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phosphate-rich environment, and studies have suggested that mitochondrial loading could be a mechanism for the transport of calcium within a cell[22]; however, this mechanism could be potentially death-threatening due to the formation of inorganic calcium phosphate salts within the mitochondria. Nucleation of an inorganic salt within the mitochondrion would lead to cell death due to the continued attraction of ions to the inorganic salt.

Bone Formation and Bone Growth

The processes of bone formation and bone growth demonstrate the diversity of mechanisms used by cells to adapt to the toxic metal ion calcium. The cells upregulate protein synthesis and add calcium ions into the matrix. The two patterns of cellular response have been extensively described: The processes of bone formation, where cells add mineral *de novo*, and bone growth where cells utilize a calcified substrate, either adding ions to or removing them from the substrate. The cellular responses can be demonstrated in the development of bones by either endochondral ossification or intramembranous ossification (bone formation), followed by bone growth, appositional and resorptive activity by osteoblasts and osteoclasts, respectively.

Cells initiate mineralization within the extracellular matrix through the release of membrane-limited particles, matrix vesicles, in which nucleation occurs. The release of matrix vesicles occurs by budding-off or blebbing[23,24] from the plasma membrane; once released, the vesicles are no longer under cellular control. Their manner of release or budding off from the plasma membrane is such that the Ca^{2+} pumps in the membrane of the vesicle are orientated so as to pump ions from within the vesicle to the outside.[25] This process is similar to that which occurs during the early stages of apoptosis.[26,27] In the absence of cell organelles within the vesicle, energy-dependent processes—such as the activity of the Ca^{2+}ATPase—terminate and, further, the vesicle is no longer able to maintain the integrity of its plasma membrane. Mineralization may be initiated within a vesicle prior to complete membrane breakdown,[25] and first evidence of crystal growth is commonly seen adjacent to the inner aspect of the bilaminar plasma membrane of the matrix vesicle.[28,29] Once released, cessation of Ca^{2+}ATPase activity in the vesicle membrane will inevitably occur and lead to an ionic equilibrium being established between $[Ca^{2+}]_i$ and $[Ca^{2+}]_e$ across the vesicle's plasma membrane. Additional free ions appear to be necessary to nucleate the inorganic phase, as establishing an equilibrium between the free ion concentration in the extracellular matrix and within the vesicle alone does not lead to nucleation.[25] The source of these additional ions is not known; however, the location of the initial inorganic salt suggests that bound ions released from phospholipids and phosphoproteins within the inner part of the plasma membrane as it breaks down could be the source of additional free ions.

The dynamics of the processes in bone formation are well demonstrated in cartilage during the development of long bones, where the chondroblasts undergo a series of key cellular events[28] with changes in cell shape accompanying their growth, gene expression, protein secretion, differentiation, and apoptosis. The chondroblasts become postmitotic, hypertrophic, secretory, and, in the latter stages, release matrix vesicles and die—leaving behind a calcified substrate, calcified cartilage. This calcified substrate may be utilized by a second population of cells that carry out either appositional activity (osteoblasts) or resorptive activity (osteoclasts). These two latter activities represent the subsequent process of bone growth (apposition/remodeling). Any such second population of cells is able to adapt to and survive on the calcified substrate, due to the utilization of a variety of mechanisms, usually described as the process of differentiation. Upregulation of the expression of a variety of genes will in turn establish equilibria between these cells and any changes in $[Ca^{2+}]_i$, provided that the adaptive capacity of the cells has not been exceeded. Similar changes are observed during intramembranous ossification; however, some of the cells responsible for the initiation of mineralization and the formation of woven bone die, whereas others may adapt to their environment and express the phenotype of osteoblasts.[30]

Dental tissues

The differentiation of odontoblasts shows a pattern similar to that seen in cartilage; however, the dental papilla cells adapt to the changes in $[Ca^{2+}]_i$ by

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becoming postmitotic, releasing matrix vesicles and the secretion of an extracellular matrix called mantle dentin. Following the release of matrix vesicles and subsequent mineralization of mantle dentin, these cells adapt to a new equilibrium and, through continued appositional activity, form circumpulpal (primary) dentin throughout life.[31,32]

Interactions of metal ions from implants

Interactions of cells with the surface of the newly formed calcified tissue are critical in determining their ability to adapt to their new environment, and similar changes would occur adjacent to implant surfaces.[33,34] Similar patterns of response can therefore be anticipated in response to other metal ions—particularly those with a similar ionic radius and charge to calcium—and further the effect of these changes on intracellular calcium homeostasis. An overriding principle, however, would remain: If the intracellular Ca^{2+} homeostatic mechanisms within a cell are overcome, the cell will die.

In assessing osseointegration of an implant, it is not always clear how the implant surface promotes or inhibits osteogenesis. A number of authors, reviewed by Elias and Meirelles,[1] have proposed that the success of an implant depends on the ability of osteoblasts to attach to a solid substrate and upregulate bone apposition (bone growth or osseoconduction), resulting in the production of lamellar bone. An alternative, similarly favorable, outcome would be that the connective tissue cells adjacent to the implant would initiate mineralization of the matrix *de novo* (bone formation or osseoinduction). During the process of adaption (cell differentiation), cells can be considered to be responding to free metal ion concentrations or gradients; therefore, cells adjacent to implants will show a range of responses similar to those to calcium ions. While most movements across the plasma membrane are ion specific, nonspecific ion fluxes can occur with ions of similar properties, such as titanium and cadmium ions. Titanium and cadmium have a similar ionic radius and charge to calcium and therefore will have the ability to compete for Ca^{2+} binding sites within the membrane, cytosol, and nucleus. Binding of these ions can have a concentration-dependent impact on the cells associated with bone metabolism, in addition to affecting many other cell populations, though perhaps less obviously.[35] Titanium ions appear to have a similar binding pattern to Ca^{2+}, where ions may be bound in a rapid transient manner; by contrast, Cd^{2+} has different binding properties in what appears to be a more permanent manner. Toxic effects for Cd^{2+} are readily apparent with metal ion concentrations lower than for Ca^{2+}.[36,37]

From a cell's perspective, the differences between calcium and titanium ions in low concentrations are unlikely to be discriminated, and the uptake of calcium and phosphorus from the environment is consistent with that of hard-tissue formation.[38] However, in higher concentrations, titanium ions may be a potent inhibitor of the differentiation of osteoblasts and their ability to mineralize their matrix, resulting in an imbalance in bone metabolism.[39] "Abnormal" bone resorption—described as occurring at the interface between tissues and titanium based dental implants—has been attributed to a number of factors, including infection. The interface between the implant and tissues, however, also acts as a substrate and cells, such as osteoclasts, will also respond to the presence of high ion concentrations in a manner similar to that seen in calcified tissues.[40] As with cells forming calcified tissues, the response of cells to titanium ions will occur in a concentration-dependent manner, excepting that the effect of extracellular divalent metal cations on intracellular Ca^{2+} homeostasis will show variations among cells of different phenotypes due to the mechanisms used to achieve intracellular calcium homeostasis.

Conclusion

A requisite for the development of new alloys for dental or medical applications is consideration of the homeostatic mechanisms that mammals have evolved to monitor, regulate, and respond to a ubiquitous metal, calcium. The use of metals will inevitably result in ions being released into adjacent tissues and these will have a physiological role, as they compete with or act synergistically with other ions that are present. The emphasis presented here has been on responses to the metal ion calcium that is utilized by cells for a wide range of biological activities. Although other divalent cations also play critical roles in intermediary metabolism, maintenance of intracellular calcium homeostasis is a high level requirement for cell survival. The addition of metal ions, such as those in metal implants and with

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similar chemical properties to those of calcium, will result in competition with binding sites used in many biochemical pathways. The "safety" of implants will be determined by a wide variety of factors, some of which can be directly linked to the released ions themselves and others to interactions between these and other ions and molecules present in the tissues.[41]

Abbreviation

ATP: adenosine triphosphate

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