A Physiologically Relevant Model of Collagen Biomineralization

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Introduction:

Collagen biomineralization is a complex process and the controlling factors, at the molecular level, are not well understood. The periodontium, the set of tissues involved in tooth anchorage, is an excellent environment for the investigation of the controlling mechanisms of mineralization, as there are both mineralized and non-mineralized tissues in close proximity. More specifically, the periodontal ligament (PDL) becomes mineralized along a sharp front of about 200nm at the cementum/PDL junction. It is thought that this fine spatial control over mineralization must be due to extracellular matrix (ECM) components, specifically, highly acidic/anionic non-collagenous proteins. However, the role of these proteins in collagen biomineralization is not known. Here, we present a model of collagen biomineralization, which employs mouse periodontium, to display ECM control over mineralization. Remineralized periodontium retains spatial control over mineralization, and thus we are able to probe the controlling factors. Using enzymatic digestions we can show how specific ECM components are involved in directing physiological mineralization at the PDL/cementum junction. Understanding the mechanisms involved in selective mineralization can be used in the development of bioactive scaffolds for bone implants, or for the treatment of periodontitis and other mineralization diseases.

Materials and Methods:

Male CD1 mice are sacrificed by cervical dislocation in accordance with University of Toronto animal use protocol 20008386. Mandibles are removed and immersed in a solution of glutaraldehyde, paraformaldehyde, and EDTA to simultaneously fix and demineralize the tissue. Ultra-thin sections of demineralized mandible are sectioned under cryo-conditions to show bone, ligament and dentin. Sections are exposed to solutions, which are supersaturated with respect to hydroxyapatite, at physiological conditions. Enzymatic digestions are conducted at 37°C prior to exposure to mineralizing solutions.

Results:

When exposed to mineralizing solutions, histology and transmission electron microscopy have shown that the sections demonstrate selective remineralization. The bone and dentin remineralize preferentially to ligament, which remains unmineralized. However, simulated body fluids (SBF) and calcium phosphate solutions with polymeric additives (mimicking soluble proteins) show different remineralization behaviors. The latter has demonstrated oriented crystalline mineral in all three mineralized tissues as shown by electron diffraction, where as SBF solutions produce amorphous calcium phosphate (ACP). These behaviors suggest that the

tissue retains sufficient information to guide selective remineralization and that the soluble protein content (which is lost during demineralization) is required to direct oriented hydroxyapatite formation. This model allows nanoscale characterization of the mineral formation within the collagen matrix, showing mineral nucleating in the peritubular dentin and in the collagen matrix, and growing along collagen fibers. Furthermore, banding patterns are visible in the remineralized collagen, suggestive of intrafibrillar mineral.

We employ this *in vitro* model to elucidate the controlling mechanisms of mineralization from a top down approach, which involves the enzymatic removal of non-collagenous proteins and/or proteoglycans in order to evaluate their role in governing mineralization. We have developed digestion protocols for the removal of all non-collagenous proteins (trypsin); the removal of all proteoglycans (chondroitinase, hyaluronidase); and complete dephosphorylation (alkaline phosphatase), as shown by histology and transmission electron microscopy. Currently, we have shown that remineralization is significantly retarded in all naturally mineralized tissues after digestion with alkaline phosphatase. These results support the hypothesis that proteins can promote mineralization of tissues, in a phosphorylation dependent manner.

Discussion:

This novel model of collagen mineralization shows, for the first time, well-defined regions of mineralized and non-mineralized collagenous tissue, allowing a direct comparison between hard and soft tissues. Further, the use of natural tissue allows for a high physiological relevance. Simultaneous demineralization and fixation ensures the tissue is as close to the native condition as possible. The unmodified tissue ensures both insoluble protein content and collagen structure of both mineralized and unmineralized tissues remain intact. However, it is inevitable that some soluble proteins will be removed with the mineral. In addition, fixation introduces crosslinks into the tissue, and can modify the structure of proteins. However, despite these limitations, the results clearly show that ECM can direct selective mineralization, to a fine degree, without cellular or enzymatic activity.

The use of polyaspartic acid, as a solution additive, was required in order to achieve intrafibrillar, oriented mineral formation. This implies that the retained NCP content is not sufficient to guide mineral phase. However, even with SBF solutions (no additives) the spatial, tissue level control over mineralization is retained. Soluble proteins, and polyAsp, must play a role in the formation of a less stable ACP, which allows for a transformation to oriented hydroxyapatite.

Understanding biological control over collagen mineralization can aide in the engineering of hard/soft tissue interfaces. Periodontitis is an inflammatory disease, which is characterized by the degradation of the ligament/cementum junction, due to bacterial invasion. This condition is the leading cause of tooth loss worldwide. Current attempts at periodontal regeneration have been unsuccessful at predictably regenerating tooth attachment. Bioactive scaffolds have the potential for the directed regeneration of this interface and subsequent tooth reattachment.