

A novel hypothesis based on clinical, radiological, and histological data to explain the Dentinogenesis Imperfecta Type II phenotype

Hakan Turkkahraman^{ab}, Fernando Galindo^c, Ustun Serdar Tulu^a, and Jill A. Helms^a

^a Division of Plastic and Reconstructive Surgery, Department of Surgery, Stanford University School of Medicine, Stanford, CA, USA.

^b Department of Orthodontics and Oral Facial Genetics, School of Dentistry, Indiana University, Indianapolis, IN, USA

^c Invited professor, Postgraduate program of Periodontics, School of Dentistry, Javeriana University, Bogotá. Emeritus member, Medical Staff, Fundación Santa Fé de Bogotá Hospital, Bogotá. Private practice, Bogotá.

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Corresponding author: Jill A. Helms

Stanford University, 1651 Page Mill Drive, Palo Alto, CA 94304, USA.

jhelms@stanford.edu

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ABSTRACT

Purpose/Aim: The aim of this study was to explore whether dentinogenesis imperfecta (DGI)-related aberrations are detectable in odontogenic tissues.

Materials and Methods: Morphological and histological analyses were carried out on 3 teeth (two maxillary 1st molars, one maxillary central incisor) extracted from a patient with DGI Type II. A maxillary 2nd molar teeth extracted from a healthy patient was used as control. A micro-computed tomographic (μ CT) data-acquisition system was used to scan and reconstruct samples. Pentachrome and picosirius red histologic stains were used to analyze odontogenic tissues and their collagenous matrices.

Results: Our findings corroborate DGI effects on molar and incisor root elongation, and the hypomineralized state of DGI dentin. In addition to these findings, we discovered changes to the DGI pulp cavity: Reactionary dentin formation, which we theorize is exacerbated by the early loss of enamel, nearly obliterated an acellular but still-vascularized DGI pulp cavity. We also discovered an accumulation of lamellated cellular cementum at the root apices, which we hypothesize compensates for the severe and rapid attrition of the DGI tooth.

Conclusions: Based on imaging and histological data, we propose a novel hypothesis to explain the complex dental phenotypes observed in patients with DGI Type II.

Introduction

Dentinogenesis imperfecta (DGI) is a rare autosomal dominant disease which affects an estimated 1 in 6,000 to 8,000 people (1-3). DGI Type II usually occurs in people without other inherited disorders like osteogenesis imperfecta, and it is primarily characterized by morphologic variations of the teeth and severe hypomineralization of the dentin; these characteristics are frequently accompanied by an obliterated pulp cavity (4). It is generally believed that because of the defective dentin there is a sub-optimal integration of enamel; consequently, the enamel usually chips away easily and exposes the underlying hypomineralized dentin, leading to its rapid abrasion.

Dentin extracellular matrix is secreted by odontoblasts and is composed of 90% collagen type I and 10% non-collagenous proteins including dentin sialoprotein (DSP), dentin phosphoprotein (DPP) and dentin glycoprotein (DGP). These non-collagenous proteins convert pre-dentin into mineralized dentin (4-7). All of these proteins are encoded by a single gene: dentin sialophosphoprotein (DSPP) (8). DSPP belongs to a family of Small Integrin-Binding Ligand N-linked Glycoproteins, which includes DSPP, osteopontin, bone sialoprotein, dentin matrix protein 1, and matrix extracellular phosphoglycoprotein (9, 10).

Mutations in the DSPP gene, and a resultant deficiency of DSPP, is accepted as a causative factor in DGI Type II (11, 12). More than 40 different mutations in the DSPP gene have been identified (13) and even within a single affected family, patients can exhibit radically different phenotypes. It has been suggested that this extreme phenotypic variation is related to the varying genotypes (6, 7), although this relationship has not been established. For example, in a recent study, Taleb et al (7) assessed genotype-phenotype findings in three families with DGI-II. Their analyses revealed three distinct mutations, that caused disturbances in the primary and permanent dentition but how those mutations affected protein translation and/or processing was not clear, nor was it established that any particular mutation was responsible for a specific phenotype (7). Some mutations in the signal peptide of DSPP are thought to affect intracellular trafficking of the protein (7, 14), while other mutations are conjectured to result in aberrant post-translational modifications that lead to inappropriate trafficking or arrest. In any case, most mutations are thought to result in a reduced amount of DSP and DPP in the extracellular matrix of the dentin.

This hypothesis is supported by analyses of DSPP “knockout” mice (5) and analyses of dTGF-B1 animals where the DSPP gene is down-regulated (15).

Although DSPP was initially thought to be a protein specific to dentin, recent studies have clearly demonstrated that its expression is more widely distributed; DSPP is expressed in bone (8, 16), cementum (8), and even in non-mineralized tissues including the kidneys and lungs (17). For example, Baba and colleagues (8) showed the expression of DSPP in osteoblasts of alveolar bone, fibroblasts of the periodontal ligament and cementoblasts in cellular cementum. These data strongly suggest that DSPP is involved in the formation of the periodontium, as well as the formation of dentin.

To date, most histomorphological studies have focused on the effects of DGI Type II on dentin phenotypes (2, 3, 7, 18) but fail to connect other distinguishing features of the disease, such as the fact that the pulp is typically obliterated (3, 7, 12, 18-22). Why this is the case is not known, but one theory suggests that the failure of the enamel to remain attached to the dentin results in trauma to the pulp, and the tooth responds by secreting more dentin (23, 24). We took a closer look at multiple permanent teeth from a patient with DGI Type II. In addition to a clearly hypomineralized dentin we also discovered changes to the pulp cavity, the cellular and acellular cementum, and to Sharpey’s fibers embedded in cementum. Based on our clinical, radiographical and histological data, we proposed a novel hypothesis that explains how, DGI hypomineralized dentin acts as a poor insulator and in response to noxious stimuli, DGI odontoblasts rapidly secrete a type of reactionary dentin with an atubular appearance. The loss of enamel may compound this reaction to external stimuli, thereby producing the unique characteristics of the DGI Type II phenotype.

Materials and methods

A male aged 5 presented to a dental practice with a chief complaint from his parents of an abnormal coloration to his primary dentition and abnormal erosion of the enamel. The primary dentition was affected, as illustrated in Supplemental Figure 1. Genetic testing indicated that neither parent carried a known mutation associated with DGI; the boy had no siblings. The genetic report indicated a de novo mutation, leading to a diagnosis of DGI Type II. Based on imaging data

from aged 17, it was determined that maxillary and mandibular alveolar ridges had undergone collapse, secondary to the absence of tooth roots, and the presence of torus mandibularis located in the anterior (see Supplemental Figure 2). At this time, the chief complaint of the patient and his parents was a fear of losing all of the patient's teeth because of their mobility, and compromised esthetics. The associated chronic inflammation and the lack of supporting bone eventually necessitated the removal of the remaining dentition, followed by reconstruction (Supplemental Figure 3).

Morphological and histological analyses were carried out on 3 teeth (2 maxillary 1st molars, maxillary central incisor) extracted from this DGI Type II patient. A maxillary 2nd molar teeth extracted from a healthy patient for periodontal problems was used as control for morphological analyses.

Samples obtained from healthy wild type mice were used as control for histological analyses. Data from a rodent pulp exposure model are also shown. In this case, healthy adult mice (2-3 months old) were anesthetized with an IP injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). To produce pulp exposures, a 0.3 mm diameter round bur (E0123, Dentsply Maillefer, Switzerland) was used to generate cavities on maxillary molars, then a sterilized endodontic explorer (DG16, Hu-Friedy, USA) was advanced until the pulp cavity was encountered. After pulp exposure, Ketac™ Cem Easy Mix Glass Ionomer Cement (3M ESPE, China) was used to cover the cavity. Mice were sacrificed at 28 days post-pulp exposure and tissues were processed for analysis.

Tissue preparation

Immediately following extraction, teeth were kept in formaldehyde for 24 hours, then placed in a buffered saline solution. Samples were decalcified in 10% ethylene diamine tetra-acetic acid (EDTA), dehydrated using an ascending graded ethanol series, and embedded into paraffin for sectioning.

Photographic imaging

One of the DGI tooth was sectioned with a cutting carbide disc. Sections were smoothed with sand paper until they were transparent then placed over an illuminated screen. Images were obtained with a micro Nikkor 105 mm, Nikon digital camera 7200 (Nikon Corporation, Tokyo, Japan) in automatic mode.

Micro-computed tomography (μ CT)

Samples were transferred to 70% ethanol for μ CT scanning. A μ CT data-acquisition system (VivaCT 40, Scanco, Brüttisellen, Switzerland) at 10.5 μ m voxel size (70kV, 115 μ A, 300 ms integration time) was used for scanning and reconstruction of maxillary molar and central incisor with DGI, and the maxillary second molar tooth extracted from a healthy patient.

Histology

DGI samples were paraffin embedded and all analyses were conducted on tissue sections arising from this material. Control samples were both paraffin- and cryo-embedded. Histologic analyses were carried out on paraffin-embedded samples; DAPI/TUNEL analyses were carried out tissue sections from cryo-embedded materials. Tissue sections were generated at an 8- μ m thickness. Before staining or other histological/cellular activity analysis, all sections were de-paraffinized in Citrisolv (#1601, Decon Labs Inc. PA), and hydrated via a descending graded ethanol series. After staining, sections were dehydrated in a graded series of ethanol and Citrisolv, and subsequently cover-slipped with Permount (#SP15, Fisher Scientific) mounting media.

Pentachrome staining was performed as described (25). In brief, after dehydration, slides were stained with 1% Alcian Blue (#A5268, Sigma), Verhoeffs Hematoxylin (#S71299, Fisher Scientific), Sodium Thiosulfate (#14518, Alfa Aesar, MA), Crocein-Scarlet-Acid Fuchsin solution (#22914, Chem Impex International, IL; #F8129, Sigma), 5% Phosphotungstic Acid (#P4006, Sigma) and Saffron (#3801, Harlecon), with washing steps between each stain using ethanol, acetic acid and distilled water. In pentachrome stained tissues, nuclei stain blue to black, cytoplasm stains red, collagen stains yellow to greenish yellow, and fibrous tissue stains an intense red (25).

Picrosirius red staining was used to detect collagenous matrix. Tissues were stained with picrosirius red solution (0.5 g Sirius red (#35780, Pfaltz & Bauer, Inc., CT) dissolved in 500 mL saturated picric acid solution), and then viewed under polarized light. Tightly-aligned fibrillary collagen molecules appear red compared to less organized collagen fibrils that show colors of shorter (green-yellow) wavelengths.

The distribution of viable cells was determined using a nuclear stain, DAPI. Slides were mounted with ProLong Gold Antifade Mountant containing DAPI (#P36935, Life Sciences) then viewed under

fluorescent light. To detect cell death, TUNEL staining (#11684795910, Roche, Indianapolis, IN) was performed as described by the manufacturer.

Results

DGI affects pulp morphology and molar and incisor root elongation

The clinical appearance of our DGI patient's teeth align with previous descriptions, where the crowns of the teeth were opalescent, with a color ranging from light grey to brown (Figure 1A). Panoramic radiographs revealed that relative to the short, thick roots, the crowns appeared bulbous with cervical constrictions (Figure 1B). Radiographs suggested that the pulp space was obliterated in most teeth (Figure 1B). No apparent periapical radiolucencies were detected.

Morphological characteristics of the extracted DGI teeth were analyzed by μ CT scanning (Figure 1C, D). In keeping with radiographic data, the roots of the teeth were deformed (Figure 1C, D). Most notably in the molars, roots were thicker and shorter than normal (compare Figure 1D with 1E) and, in the incisors, the roots were thinner and shorter than normal (Figure 1C).

By increasing the transparency of tooth surface reconstructed from μ CT data, a lucent pulp cavity was detectable in all DGI teeth; this cavity occupied space in both the crown and roots (Figure 1C', D'). The cavity was notably malformed (compare Figure 1C', D'): in general, the DGI pulps occupied a larger area of the crown and roots but the volume was much smaller than normal (Figure 1D'). There was no obvious pulp chamber; rather, the cavity was primarily comprised of a network of thin extensions projecting from slightly larger vacuoles (Figure 1D'). Therefore, contrary to conclusions based on radiographic data, more sensitive imaging methods revealed that these DGI teeth retained a pulp cavity- albeit a misshaped one. Consequently, the next series of analyses focused on understanding the likely basis for this pulp malformation.

Atubular reactionary dentin dominates the majority of the DGI tooth structure

In keeping with some theories (26, 27), we hypothesized that the malformed DGI pulp was a secondary consequence of abnormally accelerated dentin deposition. Therefore, we examined the dentin in detail, first using illuminated screen photography and then using histology and polarized light microscopy. When photographed over an illuminated screen, the dentin was clearly heterogeneous: for example, underneath the enamel-covered cusps, coronal dentin appeared light-colored and obviously tubular (asterisk, Figure 2A). More laterally, coronal dentin had a

darker appearance and was clearly atubular (arrows, Figure 2A). Root dentin, on the other hand, appeared transparent compared to coronal dentin (arrowheads, Figure 2A), suggesting that the mineral content was lower. Thus, from a mutated DSPP gene in one patient, three different types of dentin were produced.

We proceeded to microscopic examination of the dentin, first using Pentachrome staining. This histologic assessment readily revealed hypomineralized regions throughout the dentin matrix (red areas, Figure 2B). Some vacuoles were identifiable throughout the hypomineralized dentin (arrows, Figure 2B), which likely corresponded to the thin extensions and projections of the malformed pulp cavity observed by μ CT. Closer examination of the hypomineralized dentin revealed enormous organizational variation: compared to normal tubular dentin (Figure 2C), few tubules existed in the lateral coronal DGI dentin, which was occasionally interrupted by small pulp cavities (Figure 2D).

To better examine the organization of collagen in the dentin, staining with picrosirius red was employed. When viewed under polarized light, the normal dentin can be easily sub-divided into immature pre-dentin (appearing red, Figure 2E), tubular dentin (appearing green, Figure 2E), and the lines of von Ebner, which represent the cyclic activity of odontoblasts during dentin formation (appearing yellow, Figure 2E). In sharp contrast, picrosirius red staining of DGI tissue samples revealed an irregular and disorganized collagen structure (Figure 2F). In the majority, an organized pre-dentin layer was undetectable (Figure 2F). Instead, the collagen fibers were criss-crossed without any apparent bundle formation or preferable direction (Figure 2F). The same atubular pattern was obvious in root dentin. Apoptotic odontoblast cells were also detected with TUNEL staining (Figure 2G and 2H), leading us to speculate that DGI dentin resembled the structure of reactionary dentin (28), which itself has an atubular morphology. We therefore analyzed pulp odontoblasts for clues as to how and why the DGI dentin was atubular.

Reactionary dentin nearly obliterates an acellular but obviously vascularized DGI pulp cavity

Since the DGI teeth under examination were collected at a single time point, we were unable to assess the rate of dentin secretion in our DGI patient. Instead, we compared the phenotype of a normal pulp with a DGI pulp, then included an analysis of a pulp traumatized by exposure. For example, in an intact tooth the pulp cavity is characterized by a dense packing of odontoblastic

cell bodies lined up against the pre-dentin (Figure 3A). In the DGI tissues, the pulp cavity was nearly devoid of all cell types (Figure 3B) and in place of odontoblasts, there were thick (20 μm) connective tissue bands that spanned the space between the pulp cavity and the pre-dentin (Figure 3B). Very few pulp cells were detectable (Figure 3B). Picrosirius red staining and visualization with polarized light demonstrated that these connective tissue bands were comprised of collagen and was a finding unique to the DGI pulp (compare control, Figure 3C with D). These structures located at the interface between the dentin and the pulp (Figure 3D), resembled von Korff fibers, which are thought to be located in either the mantle dentin or on the circumpulpal pre-dentin surface (29-31).

As noted previously, a healthy intact pulp is densely cellular, which is readily visualized using DAPI staining (Figure 3E). Minimal cellular apoptosis, detected by TUNEL staining, is evident in a healthy intact pulp (Figure 3E). The DGI pulp cavity, however, was nearly acellular, as shown by the paucity of DAPI⁺ cells (Figure 3F). This lack of cellularity was not due to the pulp being avascular, as large vascular spaces were evident in all teeth examined (Figure 3F). Some of the endothelial cells were apoptotic, however, as indicated by their TUNEL⁺ status (Figure 3F).

A normal response to injury (in this case, pulp exposure) involves the rapid secretion of dentin by odontoblasts; this dentin typically has an atubular structure (dotted line, Figure 3G). The DGI tooth in question was not subjected to a pulp exposure; nonetheless, the DGI hypo-mineralized, circumpulpal dentin had an atubular structure (Figure 3H). It remains a speculation whether the majority of hypomineralized DGI dentin is atubular because it is rapidly deposited.

Lamellated cellular cementum formation at the apex compensates severe and rapid attrition

Similar to the acellular cementum in a control tooth (Figure 4A), the DGI acellular cementum had distinct and clear Sharpey's fiber formation (Figure 4B). Unlike the control, however, we failed to detect an apical foramen in the DGI teeth (compare Figure 4C with 4D). Like the dentin, the DGI cellular cementum was also hypo-mineralized (Figure 4D). An increase in the amount of cellular cementum in the DGI teeth was noteworthy. Unlike the normal amorphous accumulation of cellular cementum at the root apices, in this DGI case the cellular cementum had a distinct lamellar

organization, suggesting that at the root apex (Figure 4D), periods of active new cementum deposition alternated with periods of rest.

Discussion

DGI is an autosomal dominant condition that affects both primary and permanent dentitions. DGI teeth have short roots and bell-shaped crowns, and the genetic mutation affecting the DSPP gene leads to a hypomineralized state of dentin. The genetics of DGI are well described in the literature, but there is a gap between our understanding of the DSPP genotype and the phenotypic characteristics of this disease. Here, we addressed this knowledge gap by presenting a detailed molecular/cellular analysis of the manifestations of DGI in permanent teeth of a patient. Based on these data, we propose a model that provides a logical sequence of pathophysiological events that may ultimately contribute to the DGI dental phenotype.

DSPP is expressed in odontoblasts (32) but the actual secretion of dentin matrix is unaffected by DSPP mutations. In this DGI patient, the first-formed (e.g., mantle and primary) dentin is tubular, and appears to have undergone normal mineralization. These histologic features suggest that the loss of enamel may be attributed to a primary defect in amelogenesis (3, 7, 12, 33) and not exclusively to a defect in the mantle dentin-enamel interface as suggested by others (4). It is also formerly possible that a difference in microhardness of DGI hypomineralized dentin and enamel creates stresses at the dentinoenamel interface, which then result in enamel loss. Some data from animal studies, however, argue against this theory (34). Whatever the basis for enamel loss in DGI teeth, its absence may exacerbate a cascade of events that ultimately affect the dentin, cementum, and pulp.

A normal, healthy tooth responds to excessively high or low temperatures, or excessive masticatory forces, by secreting dentin that insulates the pulp from the noxious stimuli. In our proposed model, DGI teeth also respond to noxious stimuli by secreting dentin but the stimuli themselves may not be “excessive”: because enamel is rapidly lost in DGI teeth, even normal occlusal forces may be sufficient to trigger the secretion of new dentin. In the DGI tooth, however, hypomineralized dentin is a poor insulator and thus may fail to adequately protect the pulp from thermal/mechanical damage.

In our model, this inadequate insulation stimulates accelerated dentin secretion (Figure 5). In an unremitting cycle, normal external stimuli trigger secretion of intertubular dentin from the DGI dentition but because of the hypomineralized state of the dentin, it is an ineffective shield for the pulp. DGI odontoblasts eventually become entrapped in this reactionary intertubular dentin matrix. We speculate that this rapid deposition of a poorly mineralized dentin matrix leads to a progressive reduction in the size of the pulp cavity. The fact that a web-like pulp cavity still exists in the DGI pulp is in keeping with this theory (22).

In our DGI samples, the first-formed dentin appeared normal (see (7, 22)) and this dentin is deposited before occlusion begins. Dentin that is secreted after occlusion begins, however, is affected in our DGI Type II patient: for example, dentin closest to the pulp (i.e., circumpulpal dentin) was malformed and atubular (see (6, 7, 18, 35)). We speculate that this atubular dentin is the result of DGI odontoblasts rapidly producing a matrix in response to trauma that is, despite its mass, an ineffective insulator of the pulp.

Most studies report that DGI teeth initially exhibit large pulp cavities that quickly become obliterated (3, 7, 18, 36-38). Our DGI samples were from a 17-year old patient and all teeth showed evidence of a pulp cavity. The pulps, however, were clearly malformed in that they consisted of a void with web-like projections from a very small central cavity. Additionally, there were few viable cells within the cavity itself, despite the fact that blood vessels were present. We speculate that the DGI pulp responds to occlusal trauma as does a normal pulp: for example, according to Goldberg et al. (27) when a pulp is irritated or exposed to a carious attack, odontoblasts and/or subjacent cells in Höehl's layer secrete a reactionary dentin. The primary difference is the DGI dentin is a poor insulator of the pulp. Analyses of the dentition in patients with hypoplastic Amelogenesis Imperfecta (AI) support this interpretation: AI cases frequently exhibit crown attrition accompanied by a drastic reduction in pulp volume and pulp calcification (39-42).

Why are the roots of DGI teeth smaller and narrower compared to healthy teeth? We speculate that problems associated with defective secondary dentinogenesis may also perturb cementogenesis and consequently, root formation. Cementum deposition is similar to dentin secretion in that both are physiologic processes that continue throughout life. Both dentin secretion and cementum deposition are accelerated in response to noxious stimuli. In the case of

cementum deposition, the process is accelerated in response to enamel attrition, presumably so as to maintain occlusal contact (43-45). The increased amounts of cellular cementum at the apex of DGI roots may be indicator of another adaptive response to the loss of enamel that occurs as a consequence of the disease. Severe attrition is a hallmark of DGI and our analyses revealed the presence of hypomineralized bands in DGI cementum, strongly suggesting that the compensatory mechanisms that result in atubular dentin may be responsible for the laminar cementum observed in the DGI teeth.

In conclusion, DSPP mutations result in the secretion of dentin that is hypomineralized, and these same mutations may directly affect other mineralized tissues including enamel and cementum. The imaging analyses and histologic data shown here support a model whereby several pathophysiological events of DGI, including early loss of enamel, may exacerbate the resultant dentin pathology and pulp obliteration. While some of this model remains speculative, analyses of DGI teeth from patients of different ages should be able to address remaining knowledge gaps. Further, the proposed model has direct clinical implications: if DGI teeth can be protected from external stimuli that trigger excessive secretion of dentin then many of the pathological features of this disease- especially the obliteration of the pulp- could be prevented.

Declaration of interests

The authors declare that they have no conflicts of interest with the contents of this article.

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