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Cytomegalovirus inhibition of embryonic mouse tooth development: A model of the human amelogenesis imperfecta phenocopy

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ABSTRACT

Objective: Cytomegalovirus (CMV) is one of the most common causes of major birth defects in humans. Of the approximately 8400 children born each year in the U.S. with CMV-induced birth defects, more than 1/3 of these children exhibit hypoplasia and hypocalcification of tooth enamel. Our objective was to initiate the investigation of the pathogenesis of CMV-induced tooth defects.

Design: Mouse Cap stage mandibular first molars were infected with mouse CMV (mCMV) *in vitro* in a chemically-defined organ culture system and analysed utilising histological and immunolocalisation methodologies. The antiviral, acyclovir, was used to inhibit mCMV replication and comparisons made between mCMV-infected and acyclovir-treated, mCMV-infected teeth.

Results: Active infection of Cap stage molars for up to 15 days *in vitro* results in smaller, developmentally-delayed and dysmorphic molars characterised by shallow, broad and misshapen cusps, infected and affected dental papilla mesenchyme, poorly differentiated odontoblasts and ameloblasts, and no dentin matrix. Initial protein localisation studies suggest that the pathogenesis is mediated through NF-κB signaling and that there appears to be an unusual interaction between abnormal mesenchymal cells and surrounding matrix. Rescue with acyclovir indicates that mCMV replication is necessary to initiate and sustain progressive tooth dysmorphogenesis.

Conclusions: Our results indicate that mCMV-induced changes in signaling pathways severely delays, but does not completely interrupt, tooth morphogenesis. Importantly, our results demonstrate that this well-defined embryonic mouse organ culture system can be utilised to delineate the molecular mechanism underlying the CMV-induced tooth defects that characterise the amelogenesis imperfecta phenocopy seen in many CMV-infected children.

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1. Introduction

Human clinical studies and mouse models clearly demonstrate that cytomegalovirus (CMV) is dysmorphogenic to organ and tissue development. CMV, an enveloped, double-stranded DNA betaherpesvirus, is species-specific and has a slow replication cycle. In infected newborns, CMV establishes a long-lasting persistence in salivary glands and the virus is shed in saliva for months to years before termination of productive infection and establishment of latency.[1]

It is established that about 2% of live born infants are congenitally infected with active CMV. About 10% of this group has newborn symptoms, and most of these infants will exhibit subsequent abnormalities of the central nervous system (CNS): microcephaly, mental retardation, deafness and blindness.[2,3] Thus, at least 1 in every 500 newborns will exhibit major CMV-induced congenital pathology, making CMV one of the most common causes of major birth defects in humans (http://www.cdc.gov/cmv).[2,3]

It has been established that about 36% of children with CMV-induced birth defects and 5% of CMV-infected asymptomatic infants also exhibit enamel hypoplasia and hypocalcification of the teeth.[4–7] In many cases, the enamel is absent and affected teeth tend to wear down rapidly or to fracture. These distinct defects of amelogenesis are mostly reported in primary teeth. However, given the persistence of active CMV infection in some infants for 6–18 months postnatal, enamel defects may be expected in the permanent dentition as well. It can be estimated that each year in the U.S. there are an additional 3000 children with CMV-induced amelogenesis imperfecta (AI), and that the prevalence to age 12 is more than 30,000. This is a significant problem in that children have incisal and cuspal attrition, as well as rampant dental caries.[6] Further, these children may require orthodontic therapy due to caries-induced loss of primary teeth, as well as abnormalities of growth and development of the oral–facial complex secondary to microcephaly and growth retardation.[4]

Since mouse CMV (mCMV) has many features in common with human CMV (hCMV), the mouse model has been widely employed for studying the pathogenesis associated with acute, latent, and recurrent infections.[8] Baskar et al.[9–11] have extensively investigated the effects of CMV on embryonic development. They consistently observed substantial foetal loss, foetal growth retardation, and foetal dysmorphogenesis, particularly of the craniofacial complex (brain and branchial arches). Subsequently, Tsutsui[12] reported that viral-antigen positive cells were abundant in the mesenchyme of the oral and nasal cavities, and in the mesenchyme of the brain. He postulated that mesenchymal infection is the critical step in disrupting organogenesis. If so, oral–facial organogenesis, which is highly dependent on mesenchymal integrity and epithelial–mesenchymal interactions, would be particularly vulnerable to CMV infection. Indeed, recent studies in our laboratory has demonstrated that first branchial arch derivatives [submandibular salivary gland (SMG)[13] and mandible (unpublished)] are vulnerable to CMV infection during critical stages of their organogenesis, and that CMV has a particular tropism for neural-crest-derived ectomesenchyme (EM).

Active mCMV infection of embryonic day 15 (E15) mouse SMGs for up to 12 days *in vitro*[13] results in a remarkable pathology, characterised by significantly smaller SMGs, atypical ductal epithelial hyperplasia, apparent epithelial–mesenchymal transformation, and oncocytic-like stromal cell metaplasia. Expression studies indicate that molecular pathogenesis centers around the activation of the canonical and noncanonical NF-κB pathways. At the cellular level, there appears to be a consequential interplay between the transformed SMG cells and the surrounding extracellular matrix, resulting in significant changes in fibronectin and β-catenin distribution. While much is still unknown regarding CMV-induced embryopathology, these experimental results served as a framework for investigating mCMV-induced AI.

The objective of the present research was to begin to delineate the pathogenesis of CMV-induced tooth defects. Using a chemically-defined organ culture system, we investigated the effect of mCMV infection on mouse Cap stage mandibular first molar morphogenesis and differentiation. We also determined whether mCMV infection induces changes in cell proliferation and components of relevant signal transduction pathways.

2. Materials and methods

2.1. *Embryonic culture system and mCMV infection*

Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, ME), were maintained under standard laboratory conditions and mated as previously described;[13,14] plug day = day 0 of gestation. Timed-pregnant females were sacrificed on gestation day 15 (E15) by carbon dioxide narcosis and cervical dislocation. Embryos were dissected in cold phosphate-buffered saline (PBS). All procedures are performed in accordance with the Institutional Animal Care and Use Committee of USC in accordance with the Panel on Euthanasia of the American Veterinary Medical Association. E15 mandibular molar regions were cultured under chemically-defined conditions using a modified Trowell method as previously described for up to 15 days in culture.[15,16]

2.1.1. *mCMV infection*

On day 0, E15 tooth organs were incubated with 50,000 or 100,000 plaque-forming units (PFU)/ml of *lacZ*-tagged mCMV RM427^{+}[17] for 24 h and then cultured in virus-free BGJb-defined media for a total of 12 (E15 + 12) and 15 (E15 + 15) days; controls consisted of E15 mandibular molar organs cultured in BGJb-defined media for the entire culture period. Explants were collected and processed for whole mount morphology, routine histology, viral expression, and immunolocalisation as previously described.[13] For each experimental protocol, 3–20 tooth organs/treatment/day were analysed for each assay. Since no marked difference was seen in tooth organs infected with 50,000 or 100,000 PFU mCMV, tooth organs were routinely cultured in 100,000 PFU except where noted. For histological analyses, tooth organs were fixed for 4 h in Carnoy's fixative at 4 °C or overnight in 10% neutral buffered formalin at room temperature, embedded in paraffin, serially-sectioned at 8 μm and stained with haematoxylin and eosin as previously described.[13]