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Odontogenic Keratocysts

Gene Therapy

Cariogenic Virulence

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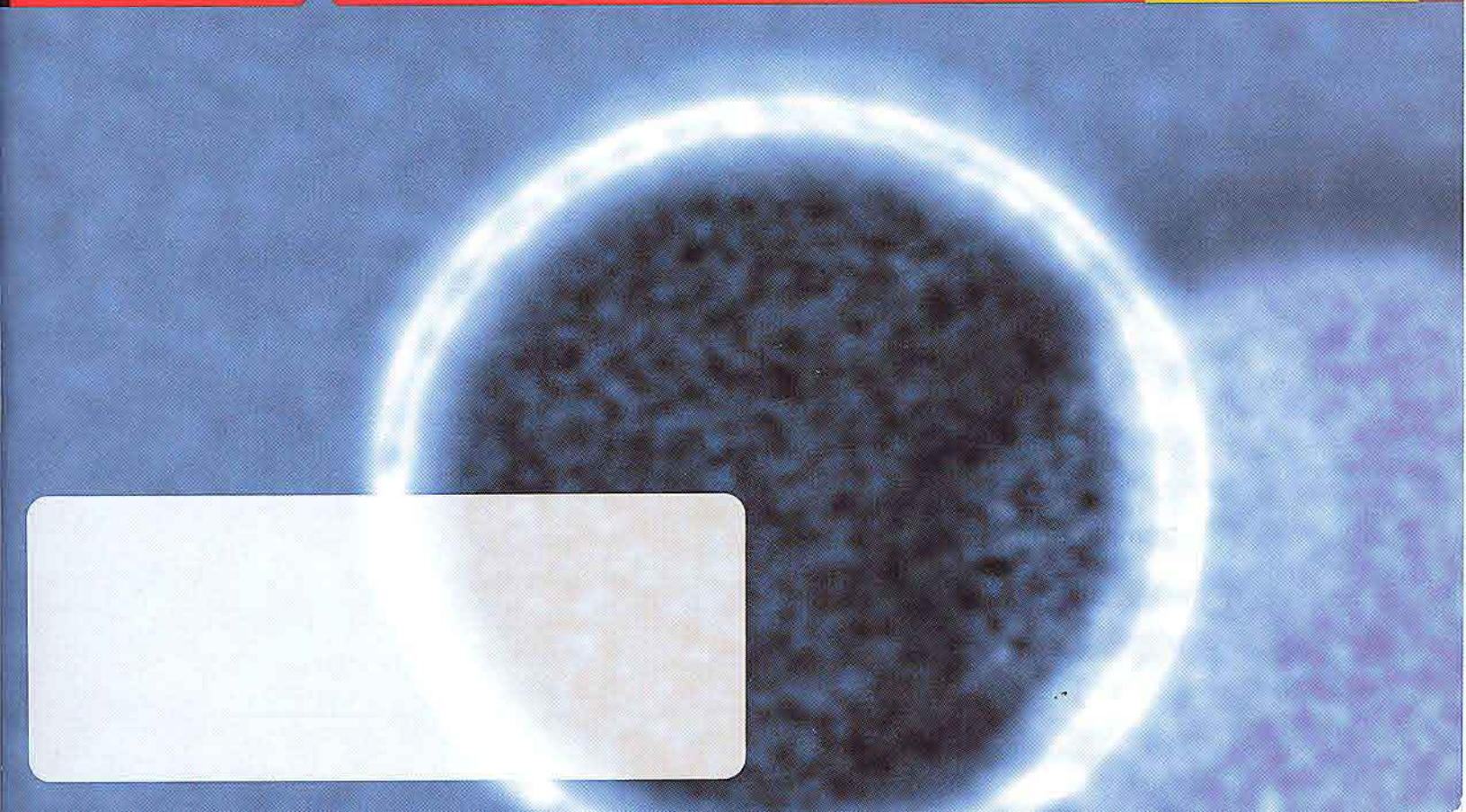
STUDENT

» Harold C. Slavkin, DDS

RESEARCH



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DEPARTMENTS

FEATURES

Alan L. Felsenfeld, DDS

A Tale of Two States

Rivalries between universities are an ongoing "bragging rights" institution in our country. Army-Navy, UCLA-USC, and Cal-Stanford are a few of the well-established competitive conflicts we monitor on a continuing basis. On a culinary note, there is continual competition as to who has the best oranges: Florida or California? And while the question of oranges may never be resolved, there are some very significant differences we can see in these two states.

Florida recently enacted a constitutional amendment similar to our own Three Strikes Law. Unfortunately, it did not deal with the punishment of criminals. Rather, it provided that if a physician loses three medical malpractice suits, they automatically lose their license to practice. This amendment does not apply to dentists, but consider the implications of such regulation. Malpractice suits are not necessarily based on malfeasance or incompetence but may occur when there is a bad result and the patient seeks someone to blame. Additionally, the loss of a lawsuit by the practitioner does not reflect on their competence or ability necessarily but may be a result of trial by "peers," who rarely are peers at all. Couple this with the fact that certain practitioners (specialists and those in teaching institutions) tend to treat the more difficult patients and might inure a higher tendency to be sued, and a significant problem is created. If an individual has a loss or two on their record, they become cannon fodder for the plaintiff attorneys, who will be more likely to sue for minimal cause or justification in that the individual will be quick to settle even the most egregious of inappropriate lawsuits to avoid an additional loss. To ponder the financial implications for the insurers and the purchasers of insurance boggles the mind.

On our coast, we have the Medical Injury Compensation Reform Act, enacted in 1975, to combat difficulty in obtaining malpractice insurance at reasonable rates. The insurance companies were driving premiums in an upward spiral in response to large jury awards and other factors, and many physicians, notably obstetricians and neurosurgeons, were leaving the state for more friendly locales. While dental malpractice premiums generally pale when compared to our medical colleagues, the implications of the law have far-reaching impact on our practices. Noneconomic damages, the "pain and suffering" part of any alleged malpractice, are limited to a maximum of $250,000. The law specifies a limit on contingency fees that plaintiff attorneys could collect. That the plaintiff attorneys should be enjoined in the financial gains of their clients and not charge by the hour or on a case-by-case basis is counterintuitive. After all, the allegation is that the plaintiff, not the attorney, suffered the alleged damage and seeks remedies.

Other portions of the law require advance notice on filing of claims, allow for payout over a long-term schedule, provide a

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Support of the activities of CAPP, on both the administrative and financial ends, is an excellent member benefit that is not always clearly understood.

statute of limitation on claims and several other user-friendly stipulations. The law has been in effect for 30 years and works well with relative stabilization of malpractice premiums and helps to create a better environment in which we can practice. It is estimated that federal health insurance spending would be reduced by $14 billion, and state and local spending would be reduced by $6 billion over 10 years if similar laws were enacted nationally.[1] President Bush recently used the California model as an example of how the pervasive medical malpractice problem can be managed appropriately.

Periodically, the trial lawyers seek to raise the limit for noneconomic damages to greater than the $250,000 limit ostensibly to help their clients but clearly to increase their earnings on a successful suit. Health care providers have fought these attempts successfully over the years and must continue to do so. In an effort to provide collaboration and unification of these groups to deal with trial attorneys, a consortium of health care providers including physicians, dentists, hospitals, and other facilities formed Californians Allied for Patient Protection. The group is funded from each of the interested organizations and continually monitors legal and legislative efforts aimed at altering MICRA as it presently exists.

The CDA is fortunate to be a participant in CAPP as it represents all of our members. We have contributed to the costs of enabling the activities of CAPP and should continue to do so. Next year, Peter DuBois, our executive director, will chair the group putting dentistry to be in the forefront of this important organization and activity. Support of the activities of CAPP, on both the administrative and financial ends, is an excellent member benefit that is not always clearly understood.

While the controversy as to who has the best oranges may never and need not ever be resolved, it is clear that in the medical malpractice arena, the two states have an apples and oranges relationship. The thought process of the people of Florida is far from clear, though. On a positive note, a judge has temporarily enjoined the law pending additional legislative investigation. The bad news is that the Legislature is crafting enabling language to put the amendment into effect. It is apparent that we have through the provisions of MICRA, a controlled practice environment conducive to good patient care without undue fear of lawsuits.

The continuation of the MICRA program, as it has been designed and is functioning, is critical to a healthy practice location. The MICRA legislation in our state is an equitable means of assuring an environment for user-friendly health care delivery. Our participation in CAPP is essential to continue the efforts to maintain a crucial law to our practices. We must contribute the resources necessary to sustain our involvement in this worthwhile organization and endeavor, and we must continue to support such legislation because it is good for our membership. CDA

References / 1. Congressional Budget Office estimate, the Health Act, 2003.

Comments, letters and questions can be addressed to the editor at alan.felsenfeld@cda.org.

Providing Care for Uninsured Children

I read with great interest the letter by Dr. Oscar E. Valenzuela, published in the September 2005 issue of the *Journal of the California Dental Association*, about "remembering the children" and the problem of addressing health care needs for poor and uninsured people in our communities.

I'm writing this letter to let Dr. Valenzuela and other health care providers know of an organization I've been involved with for the past 11 years. In 1994, Glendale Memorial Hospital, Glendale Adventist Medical Center, and Verdugo Hills Hospital (the three hospitals in the Glendale area) funded the startup of Glendale Healthy Kids. Glendale Healthy Kids helps provide medical, dental, and mental health services to low-income, uninsured children living in the Glendale area, and health education to families in Glendale.

There are approximately 60 dentists and 80 physicians who provide services in their offices at no cost to children who are referred through school nurses to Glendale Healthy Kids. We also staff and train an annual in-school dental hygiene education program. Glendale Healthy Kids is staffed by two full-time administrators and utilizes the services of a part-time grant writer.

The neighboring cities of Burbank and Pasadena have similar programs in place to help provide care for uninsured children. Glendale Healthy Kids has a large, about 25 members, board of directors and raises funds throughout the year via community fund raisers and grants. Parents of children referred to GHK are required to complete a financial statement to confirm they qualify for the program.

I would urge all communities to consider a similar organization to help meet the health care needs of the poor.

Gary S. Finer, DDS
President-elect
Glendale Healthy Kids Board of Directors

Glendale Healthy Kids helps provide medical, dental, and mental health services to low-income, uninsured children living in the Glendale area, and health education to families in Glendale.

Dental Student Research: Helping to Shape the Future

Harold C. Slavkin, DDS

We are living in an extraordinary time in human history. The convergence of the digital and biological revolutions of the last 50 years is yielding significant benefits for the human condition. Arguably, California is one of the most scientifically productive "places" on Earth.

The nature of science in California is shaped and reflected by research from our great universities. California's university research scientists produce more than 20 percent of America's science and technology. The five California dental schools — University of the Pacific; University of California, San Francisco; University of California, Los Angeles; Loma Linda University; and University of Southern California — collectively receive more than 20 percent of federally sponsored and peer-reviewed research support from the National Institutes of Health. These federal research dollars are leveraged with additional research support from nonprofit foundations, state research agencies, and private industry. The consequence is that California dental schools within great universities continue to shape what is thought, what is taught, and what is practiced in the oral health professions.

The energy that perpetuates this extraordinary human intellectual progress can be found within our dental students, residents in the various dental specialty programs, graduate students pursuing their education and training in various PhD programs, postdoctoral fellows learning and training in our laboratories/clinics/hospitals, and, of course, the remarkable faculty which constitute California's five dental schools.

I have the special pleasure of serving as guest editor for this issue of the *Journal of the California Dental Association*, an issue that provides you with a glimpse into dental student research within each of the five dental schools of California. As you read each of these scientific papers, you will discover remarkably innovative approaches to the management of oral infections such as molecular controls of *Streptococcus mutans* from UCLA; discoveries that are resulting in new ways to control root resorption related to orthodontic movement from USC; improving our understanding of the highly aggressive odontogenic keratocyst as intraosseous lesions from LLU; highly innovative approaches to use gene therapy to manage intraoral squamous cell carcinoma from UOP; and scientific studies that compare aciduricity, acidogenicity, and intracellular production of polysaccharides of *S. mutans* strains isolated from caries-active versus caries-free adults from UCSF. All of these scientific papers are a delight to read. Each reflects the superb faculty mentoring of the dental students from our five dental schools. These published papers remind us all that science is the fuel that drives the technology that continues to improve oral health care in California and beyond. CDA

Guest Editor / Harold C. Slavkin, DDS, is dean of the University of Southern California School of Dentistry, and G. Donald and Marian James Montgomery Professor of Dentistry.

Dental Caries and Chemical Warfare Within the Mouth

Trang Nguyen; Phoebe Tsang, DDS, PhD; Wenyuan Shi, PhD; and Fengxia Qi, PhD

ABSTRACT

To date, it appears that the dentists' war against dental caries has no end in sight due to the fact that dentists lack any genuine offensive firepower. Make no mistake, the defense has drastically improved as dentists have shifted the focus more toward the preventive aspects of dental care. But defense by itself cannot defeat the enemy; at best, it can maintain the status quo. In order to defeat the enemy, one must study and understand the enemy, to know its strengths and weaknesses, and to strike at those points of vulnerability. The aim of this research is to identify and characterize genes that are responsible for observed virulence factors in *Streptococcus mutans*, which is the primary pathogen involved in the development of dental caries. Once there is a more defined understanding of the many virulence factors of *S. mutans*, there will be a much more valuable insight into its role in the ecology of the oral cavity. Eventually, this knowledge could enable dentists to convert the bacterial "weaponry" into its own arsenal, which could then be innovatively employed as preventive, diagnostic, and therapeutic agents to treat oral bacterial-related diseases.

Dental caries is a direct result of the localized destruction of tissues of the teeth by acids produced from fermentation of dietary carbohydrates by plaque bacteria on the tooth surface. Teeth are made of calcium phosphate hydroxyapetite together with some organic constituents. Under prolonged and repeated acidic conditions, the hard crystals of the tooth enamel slowly dissolve, resulting in cavitation, which is the clinical manifestation of dental caries.

It is well known that dental caries is one of the most common infectious dis-




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eases afflicting humans. The fact that it tends to remain untreated, particularly in underdeveloped parts of the world, leads to considerable levels of suffering that are often eradicated only by extraction or exfoliation of the infected teeth. The significance of dental caries is well exemplified by the fact that annual expenditures of the United States population on dental services exceed $65 billion, with more than half of these costs attributable to dental caries.[1]

Streptococcus mutans is considered as a primary agent in cariogenesis.[2] Its abilities to adhere to and to form biofilms on the tooth surface, to metabolize carbohydrates to produce acids, and to survive low pH and other environmental insults are believed to be critical in its persistence, and eventual becoming dominant in the dental plaque. The dental plaque consists of a complex bacterial community of more than 500 different species of bacteria.[3] Thus, in addition to the previously mentioned virulence properties, *S. mutans* also possesses the ability to kill other competing species in the dental plaque. This killing ability is conferred by the production of proteinaceous antibiotics called mutacin.[4] Mutacin production gives *S. mutans* an edge over its competitors when nutrients become limited in the dental plaque, as is often the case between meals. Thus, mutacin is, in essence, a "weapon" that *S. mutans* uses in this "chemical warfare" in order to prevent the growth of other competing bacteria and to facilitate its dominance within the dental plaque.[5,6]

Mutacin production is controlled by many factors, some of which are genetic, and others of which are environmental.[7] Environmental factors usually need to interact with genetic factors (regulatory genes) to exert their effects. These environmental factors may range from temperature and pH levels to the presence of certain minerals, buffers, and nutrients within the oral cavity. It is apparent that nutritional availability plays a critical role in modulating the levels of mutacin production. One of the nutritional factors is phosphate, which is present in large quantities in the saliva.[8] Our preliminary studies have demonstrated that in the presence of 5 mM phosphate, mutacin production is severely impaired. This study aims to understand how phosphate regulates mutacin production. Specifically, the authors wanted to find the genes that are involved in this phosphate inhibition of mutacin production. Genetic and molecular techniques were used to carry out this study.

Research Materials and Methods

Bacterial Strains and Culture Conditions

Escherichia coli strain DH5α was used for cloning as well as plasmid amplifications. *E. coli* cells were grown in Luria-Bertani (LB, Fisher) medium aerobically at 37°C. *E. coli* strains carrying plasmids were grown in LB medium containing spectinomycin (300 µg/ml). *S. mutans* wild-type strain UA140 was cultured in brain heart infusion (BHI, Difco) medium or on Todd-Hewitt (TH, Difco) agar plates. For selection of antibiotic resistant colonies, BHI medium was supplemented with spectinomycin (800 µg/ml). TH medium supplemented with potassium phosphate buffer (5 mM) was used to screen for mutants that have overcome phosphate inhibition of mutacin production.

Library Screening

The deferred antagonism assay was employed to screen a random insertional mutagenesis library for mutations that produce mutacin in the presence of inhibitory amounts of phosphate.[9] Briefly, the mutant colonies, which were grown in 96-well plates, were stabbed onto potassium phosphate plates and grown anaerobically for 72 hours. Each plate was then overlaid with a thin layer of soft agar mixed with overnight culture of the mutacin-sensitive indicator strain, *S. sobrinus*. The zone of inhibition was inspected after an overnight incubation under anaerobic conditions. The presence of "zones of growth inhibition" around any of the colonies was considered to be a positive indicator of the presence of a mutant that has overcome inhibition by phosphate (**Figure 1**).

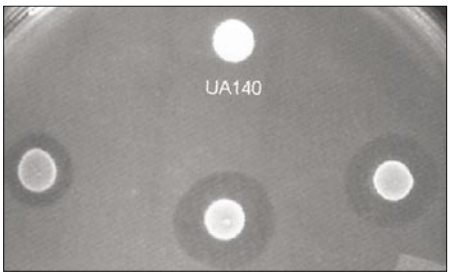

Figure 1. Deferred antagonism assay of mutacin. Mutacin-producing mutants can be identified by the presence of "halos" or zones of inhibition which circumscribe the colonies. The colony labeled UA140 is the wild-type (non-mutant) strain, which the authors would expect to produce no mutacin under experimental conditions. Note the absence of a "halo" around this colony.

Identification of Mutated Genes

Chromosomal DNA from each selected mutant was prepared from 10 ml of cell culture at OD_{600} of 0.8 by standard DNA extraction protocol.[10] Ten µg of chromosomal DNA from each mutant was digested with one of the following restriction enzymes: *Xmn*I, *Sca*I, *Acl*I and *Bst*BI. All of these restriction enzymes do not cut within the p0SC plasmid that was used for constructing the library. DNA fragments

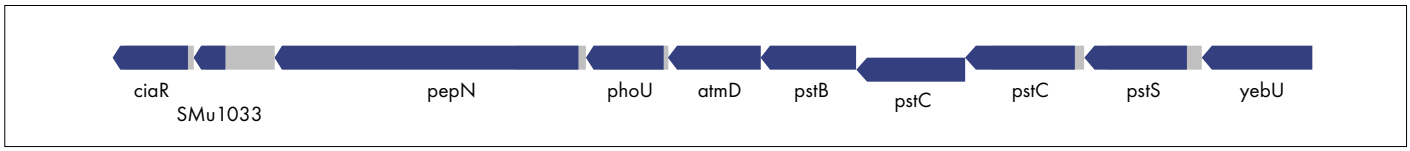

Figure 2. Schematic diagram of the Smu1034 region of the *S. mutans* UA159 chromosome. The operon consists of seven genes in the order of *pstS, pstC, pstB, atmD, phoU,* and *pepN* (Smu1034). *pstS* encodes the phosphate ABC transporter, a periplasmic phosphate-binding protein. *pstC* encodes the permease protein of the phosphate ABC transporter system. *pstB* and *atmD* encode the ATP binding proteins. *phoU* encodes the phosphate transport regulatory protein, and *pepN* is annotated as the peptidase N gene.

were circularized by self-ligation with T4 DNA ligase (Promega). The recircularization of DNA fragments allowed for direct sequencing of the inserts by using M13 primers. The ligated DNA fragments were transformed into *E. coli*, and plasmid was isolated from positive clones. The insert was sequenced by the University of California, Los Angeles Core DNA Sequencing Facility. The sequences obtained were compared to the genomic sequences of *S. mutans* UA159 available at the Los Alamos Oral Pathogen Sequence Databases (http://www.oralgen.lanl.gov) via BLAST.

Results

Screening for Mutants that Produce Mutacin on Phosphate Plates

A random insertional mutagenesis library of 12,000 clones was generated via random insertions of a suicide plasmid into the chromosome of *S. mutans* strain UA140.[11] This library was screened for mutants which demonstrated mutacin production in the presence of phosphate. Screenings for each mutant within the library were performed in triplicates. Overall, 48 mutants were isolated which consistently produced mutacin in the presence of 5 mM phosphate.

Identification of the Mutated Genes

A portion of the 48 mutants was sequenced and their mutated genes were identified as described in Materials and Methods. From these sequenced clones, three unique genes were identified (**Table 1**). Two of these genes (Smu1848 and 1713) are classified as conserved hypothetical proteins, which means they currently have no known function. The third gene, which is of particular interest, is Smu1034, which is in the same transcription unit (an operon) as the phosphate transporter genes.

Table 1

Summary of genes involved in phosphate regulation of mutacin production

Organism	Gene	Function of protein
S. mutans UA 159	Smu1034	Aminopeptidase
S. mutans UA 159	Smu1713	Hypothetical membrane protein
S. mutans UA 159	Smu1848	Hypothetical membrane protein

Discussion and Summary

In this paper, the authors reported the identification of three genes that are possibly involved in phosphate regulation of mutacin production: Smu1034, Smu1848, and Smu1713.

Smu1034 is also known as *pepN*, which is a gene that codes for an aminopeptidase (**Figure 2**). It is involved with other peptidases in the degradation of peptides generated during intracellular protein breakdown.[12] As mentioned previously, *pepN* is in the same transcription unit as the phosphate transporter genes, and the authors speculate that a mutation in this gene could affect the phosphate transporters. The *pepN* gene may be an integral component required for the process of the transporter protein themselves. In this situation, a mutation in *pepN* may result in a loss of high-affinity phosphate transport, thus reducing the intracellular concentration of phosphate. This would consequentially alleviate the toxic effect of excess phosphate on mutacin production.

The other two genes (Smu1848 and Smu1713) are classified as conserved hypothetical proteins and currently have no known function. Nevertheless, it is important to note that both proteins are membrane proteins. Thus, it can be speculated that their inactivation may have disrupted the normal membrane structure, which then indirectly affected phosphate transport. Alternatively, these proteins may be part of a signaling pathway for phosphate mediated gene

regulation. More studies are required to determine their function.

In summary, the authors screened a random mutation library of 12,000 clones and isolated 48 mutants that rescued mutacin production on 5 mM phosphate plates. Some of the clones were sequenced and three unique genes were identified. One of the genes may be involved in phosphate transport, which explains why its mutation alleviated the toxic effect of phosphate on mutacin production. The other genes encode membrane proteins, but their function is currently unknown. In the continuing investigations, the authors will complete sequencing all 48 mutants, which would allow the opportunity to identify all genes involved in phosphate regulation of mutacin production. This information will hopefully help future studies aimed at finding new target or strategies to curb dental caries.

Conclusion

Dentists seem to do a lot of "rescuing" of patients, saving them from the pain and agony of tooth decay and caries caused by *S. mutans*. But the philosophy behind this research is simple: Dentists must fix the problem at its source. Once there is a more defined understanding of the intricate weaponry of the enemy, *S. mutans*, dentists will have much more valuable insight into its role in the ecology of the oral cavity.

Eventually, this knowledge could enable dentists to develop "counterattack" measures that could depress these aggressors for good. On the other hand, this offensive weapon of *S. mutans* can be used for the benefit of the host. For example, a genetically engineered effector strain of *S. mutans* has been constructed, which produces elevated amounts of mutacin but diminished amounts of acids.[13] This strain is currently being used in a limited clinical trial for the prevention of dental caries. This is just one of an infinite number of possible uses of mutacin that could be innovatively employed as preventive, diagnostic and therapeutic agents to treat oral bacterial-related diseases. This may change the face of dentistry as it is known today. CDA

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Celebrex Offers a Small Protection From Root Resorption Associated With Orthodontic Movement

John Jerome; Timothy Brunson, DDS; Gerald Takeoka; Chad Foster; Hong B. Moon, DDS, MS; Enrique Grageda, DDS, MS; and Maggie Zeichner-David, PhD

ABSTRACT

Tooth movement results from alveolar bone resorption/deposition following application of orthodontic forces, and root resorption can be an undesirable complication associated with this process. No treatment for external root resorption is available to date.

Objective: To determine if COX-2 inhibitors like Celebrex are effective in protecting root resorption associated with orthodontic forces.

Methods: A force of 80 grams was applied to the left maxillary first molars of 7-week-old female Wistar rats using nickel titanium closed coil springs attached to the cervical area of the incisors with 0.010 stainless-steel ligature wires. Twenty animals were divided into three experimental groups: one receiving no treatment, the second receiving 25mg/kg, and the third receiving 50 mg/kg of celecoxib (Celebrex) in their drinking water. Rats were maintained on a soft diet and euthanized two weeks after initial placement of the force. Paraffin-embedded sections of the right (control) and left (experimental) maxillae were stained with H&E and the areas of root resorption were examined by counting the number of lacunaes in the roots.

Results: No difference in the distance of tooth movement (0.5 mm/two weeks) was seen in all three groups. The rats that received the low dose of Celebrex showed no statistically significant difference in root resorption than that of the rats that received no dose. The rats that received the high dose of Celebrex showed a lower number of lacunaes (mean = 3.5) than that of the control group (mean 10.2; p=0.02).

Conclusions: Administration of Celebrex during the application of orthodontic forces does not interfere with tooth movement and appears to offer some slight protection against root resorption.

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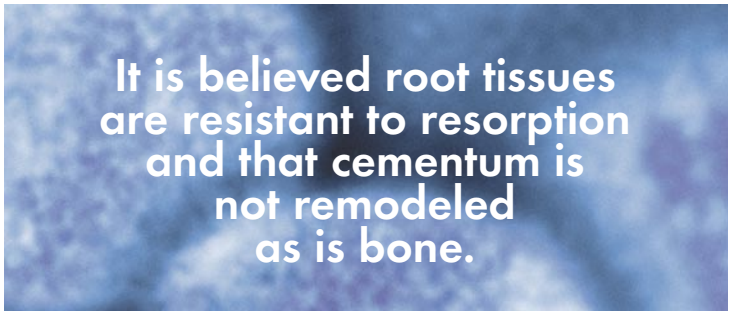
Acknowledgment / This study was supported by NIH/NIDCR grant DE15148.

Orthodontic treatment is reliant on the resorption and apposition of bone adjacent to the root structure with none or minimum resorption of the root. It is believed root tissues are resistant to resorption and that cementum is not remodeled as is bone.[1,2] The resistance of cementum to resorption appears to be derived from the cellular layer known as precementum, and the etiology and pathogenesis of root resorption would depend on the amount of damage caused to the periodontal ligament, PDL. Thus, when there is limited mechanical damage to the precementum, superficial resorption will occur. This type of resorption is reversible and not detectable radiographically. On the other hand, inflammatory resorption is present when larger and deeper resorption lacunaes reach dentinal tubules, which leads to an infected leukocyte zone and consequently destruction of the root.[3,4] Replacement resorption or ankylosis occurs after extensive necrosis of the PDL and the root is incorporated in the alveolar bone.[3]

Clinical observations in patients with apical hypercementosis, where Sharpey's fibers are more spaced and an increase in cementum turnover is found, suggest the PDL could be involved in this resistant process, and that Sharpey's fibers lack recognition for osteoclasts. This idea is supported by studies in which, after the removal of periodontal tissue from deciduous teeth, osteoclasts got easy access to the root surface.[4] Some other investigators support the idea that cementum and dentine are more resistant to resorption than bone because of differences in vascularity. While relative abundance of blood vessels is present in bone, few are close to the tooth side of the periodontal ligament.[4,5] The presence of proteinase inhibitors in cementum and PDL has also been suggested as being responsible for the resistance of roots to resorption.[1] More recent studies suggest that when an orthodontic force is applied, there is remodeling of the adjacent bone and cementum, with the later repair occurring at a relative passive rate after force levels decline. Therefore, root remodeling appears to be also a constant feature in orthodontic tooth movement, and permanent loss of root structure will occur only if repair does not replace the initial resorbed cementum.[6,7]



It is a common belief the use of strong orthodontic forces will increase the risk of apical root resorption. It has been shown that in cases where compression of the PDL is strong and of long duration, root resorption tends to increase.[8-11] However, other studies suggest the force magnitude is probably not as decisive for root resorption as much as the duration of the force application. The more teeth are displaced, the more root resorption will occur and therefore intermittent forces will cause less severe root resorption than continuous forces.[12] A recent meta-analysis of the literature suggests the treatment-related causes of root resorption appear to be the total distance the apex moved and the time it took.[13-15] Root shortening has also been associated with age, agenesis, duration of contraction period, treatment group, trauma, application of continuous forces, etc.[16,17] Apical root resorption appears to be influenced also by root approximation to the palatal cortical plate during orthodontic treatment.[18] Larger teeth appear to have a greater amount of root resorption, but the amount of alveolar bone, thickness of cortical bone, density of the trabecular network, and fractal dimension have no correlation with apical root resorption.[19] The idea that root resorption of the upper first molars by the use of nighttime extraoral traction has been rejected and no relationship between root resorption and the number of activations has been found.[9,20] In other studies, it has been found that adults experienced more resorption than children, but only in the mandibular anterior segment; that Caucasian and Hispanics experienced more root resorption, that an increased overjet, but not overbite, was significantly associated with greater root resorption. Duration of treatment and the horizontal (but not vertical) displacement of the incisor apices were also significantly associated with root resorption. No difference between male and female patients was found.[21,22] It has been suggested that genetic factors account for at least 50 percent of the variation in external apical root resorption.[23] Amongst the genes implicated are variations in the Interleukin 1-beta gene and a member of the tumor necrosis factor (TNFRSF11A) family.[24-26]

Treatment for Root Resorption

Successful treatment, depending upon the degree and position (internal or external) of the resorption, when the resorption is mild and internal, can be treated by interrupting the treatment and/or root canal therapy.[27] However, when the resorption is external and has penetrated the dentin layer, there is no known successful treatment. Several studies are now being focused at finding ways to either prevent or treat root resorption. Amongst them is a recent study which tested the effect of clodronate, a type of bisphosphonate that strongly inhibits bone resorption and has anti-inflammatory properties, as treatment for root resorption using a rat animal model. Although the local injection of clodronate was somehow successful in reducing root resorption, it had to be injected every third day, and caused a significant reduction in tooth movement.[28] Other studies tested the effect of a noninvasive method like low-intensity pulsed ultrasound (LIPUS) in reducing root resorption in human studies. It was shown that LIPUS can enhance healing of various types of traumatized connective tissues and stimulate dental tissue formation. These studies are more promising since LIPUS-exposed human premolars had a significant decrease in the areas of resorption, the number of resorption lacunae, and showed healing of the resorbed root surface by hypercementosis.[29]

Objective of This Study

Since for the most part, the process of resorption results from inflammation, in this study the authors wanted to test if administration of nonsteroid anti-inflammatory, NSAIDS, had a protective effect on root resorption without altering the rate of tooth movement. There are more than 50 NSAIDs on the market, all with varying degrees of therapeutic and side effects. Traditional NSAIDs such as aspirin, ibuprofen, Aleve Vioxx, Bextra, Celebrex, and other prescription drugs, act by interfering with the synthesis of prostaglandins, chemical mediators released when tissue is injured. NSAIDs provide analgesia and suppress inflammation by inhibiting the action of the enzyme cyclooxygenase, resulting in decreased prostaglandin synthesis. Cyclooxygenase exists in two isoenzymatic forms, cyclooxygenase-1, COX-1, and cyclooxygenase-2, COX-2. COX-1 appears to be constitutively expressed in many tissues and produces prostaglandins, which regulate normal cellular functions like the synthesis of eicosanoids that have important homeostatic functions, for example, in the gastric mucosa and platelets.[30]

However, COX-2 activity is induced by proinflammatory cytokines and produces prostaglandins that mediate the inflammatory response and pain signaling transmission. The suppression of prostaglandin synthesis can also produce gastric and renal toxicity, as well as impair normal platelet function. Thus, NSAIDs are associated with potentially harmful side effects. Traditional nonspecific NSAIDs inhibit both COX-1 and COX-2, and in doing so, not only decrease inflammation and pain, but also promote gastrointestinal tract damage and bleeding. A class of anti-inflammatory medications has been developed that primarily inhibits COX-2 while sparing the enzymatic activity of COX-1 at therapeutic dosages like rofecoxib and celecoxib.[31] The authors selected celecoxib, Celebrex, for these studies and a rat animal model previously established in the laboratory, in which root resorption consistently resulted after the application of orthodontic forces of 80 grams or greater.

Methods and Materials

Twenty 7-week-old female Wistar rats were used in this study. All animals were treated under the most humane conditions according to protocols approved by the University of Southern California Institutional Animal Care and Use Committee. The average weight of the rats was 105-115 grams, and the rats were anesthetized by intraperitoneal injection of phenobarbital (0.1 mg/gm of body weight). A modified technique described by Brudvik and Rygh was used.[32] A nickel titanium closed coil spring with an additional spring eyelet was attached to the cervical area of the incisors and left molar of the maxilla with 0.010 stainless-steel ligature wire. A continuous force of 80 gm using a nickel titanium (Sentalloy) closed coil spring (GAC, N.Y.; 10-00-02 NiTi with M hooks) was applied to the left maxillary first molar with the right side being the control. The force applied was measured using a dynamometer (Dentaurum No 040711, Newtown, Pa). After the appliance was tied on

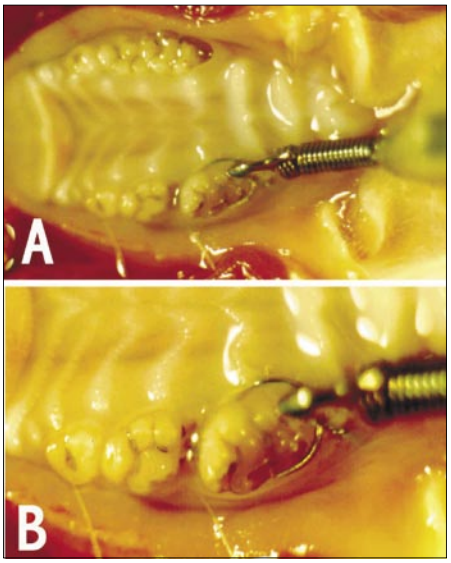


Figure 1. (A) Tooth movement on rat maxillary first molar after 14 days of force application. (B) High magnification of A.

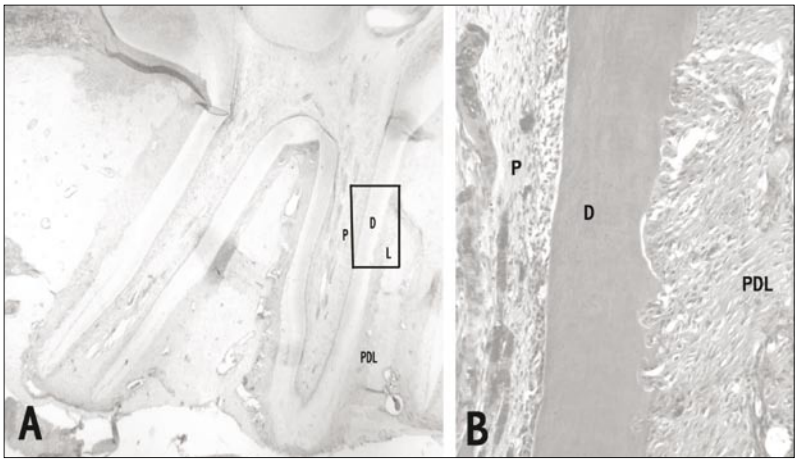


Figure 2. (A) Sagittal section of the rat maxillary first molar after two weeks of application of an orthodontic force of 80g and (B) the area of lacunaes showed at a 20x magnification. P=Pulp, D=Dentin, PDL=Periodontal ligament, L=Lacunaes.

the molar, the coil spring was stretched until its mesial eyelet touched the distolingual surface of the anterior teeth, and the force registered by the dynamometer indicated 80g. The force applied was measured twice to guarantee equal force delivery in all rats. A notch in the middle third of the distal surfaces of the incisors was placed with a low-speed carbide bur to prevent occlusal migration of the ligature. In order to avoid possible breakage of the wire, the surface of the maxillary incisors was etched for 20 seconds with 37 percent phosphoric acid and then covered with self-cured orthodontic composite (Ormco orthodontic bonding kit), the incisal edge of the mandibular incisors was reduced, and the rats were maintained on a soft food diet under standard conditions in the central animal care facilities. All animals were housed in facilities maintained by the University's Vivaria. Rats were divided into three groups:

- **Group No. 1 or Control:** Rats were fed a soft diet and regular drinking water.
- **Group No. 2 or Low Dose:** Rats were fed a soft diet, drinking water with Celebrex (25 mg per kg of body weight), and a little sugar to induce them to drink the water. The water was changed daily and the amount of water consumed was monitored by measuring the level of water remaining in the bottle after 24 hours.
- **Group No. 3 or High Dose:** Rats were fed a soft diet, drinking water with Celebrex (50 mg per kg of body weight), and a little sugar to induce them to drink the water. The water was changed daily and the amount of water consumed was monitored by measuring the level of water remaining in the bottle after 24 hours.

Doses of Celebrex were extrapolated from doses recommended for human use by the manufacturers. All animals were euthanized with CO_2 after two weeks of initial placement of the orthodontic forces. The rats were decapitated, the appliances were removed, and the tooth movement was measured as the distance between the first and second molars. Whole palate samples were cut in half and fixed in 10 percent neutral buffered formalin, NBF, solution for 24 hours. Tissues were then washed with distilled water in vials for one to two hours with four changes and decalcified with EDTA at 4°C for six weeks. Tissues were processed for paraffin embedding procedures and sagittal 5 microns sections were prepared using a microtome and stained with hematoxylin and eosin or Mallory, and analyzed under light microscopy.

The data was analyzed using histomorphometric measurements of root resorption. The numbers of lacunae and the resorption surface area were checked and recorded in those cases where signs of root resorption were present. Each section was independently evaluated by

Table 1

Analysis of the number of lacunaes, intensity of lacunaes and position

Control (right) no drug											
Rat#	**Lacunaes**	**Total score**	**MM**	**MD**	**DM**	**DD**	**Cervical**	**Middle**	**Apical**	**Comp**	**Tension**
C1	3	3	3	0	0	0	0	3	0	3	0
C2	2	3	0	0	0	2	2	0	0	0	2
C3	3	3	0	0	0	3	0	3	0	0	3
C5	1	3	0	1	0	0	0	1	0	1	0
C6	2	3	2	0	0	0	2	0	0	2	0
Avg	2.2	3	1	0.2	0	1	0.8	1.4	0	1.2	1
STDE	0.836660027	0									

Experimental (left) no drug											
Rat#	**Lacunaes**	**Total score**	**MM**	**MD**	**DM**	**DD**	**Cervical**	**Middle**	**Apical**	**Comp**	**Tension**
C1	8	10	4	2	2	0	1	7	0	6	2
C2	10	13	4	2	2	2	1	9	0	6	4
C3	10	17	3	2	1	4	1	9	0	4	6
C5	9	11	3	1	3	2	1	5	3	6	3
C6	12	15	4	4	2	2	2	10	0	6	6
Avg	9.8	13.2	3.6	2.2	2	2	1.2	8	0.6	5.6	4.2
STDE	1.483239697	2.863564213									

Control (right) low dose											
Rat#	**Lacunaes**	**Total score**	**MM**	**MD**	**DM**	**DD**	**Cervical**	**Middle**	**Apical**	**Comp**	**Tension**
L1	3	4	3	0	0	0	3	0	0	3	0
L2	2	3	0	1	0	1	0	1	1	1	1
L3	3	5	3	0	0	0	0	3	0	3	0
L4	3	5	3	0	0	0	1	2	0	3	0
Avg	2.75	4.25	2.25	0.25	0	0.25	1	1.5	0.25	2.5	0.25
STDE	0.5	0.957427108									

Experimental (left) low dose											
Rat#	**Lacunaes**	**Total score**	**MM**	**MD**	**DM**	**DD**	**Cervical**	**Middle**	**Apical**	**Comp**	**Tension**
L2	8	12	4	0	0	4	1	6	1	4	4
L3	6	11	3	0	2	1	1	4	1	5	1
L4	6	10	3	3	0	0	1	5	0	3	3
L6	10	14	3	4	3	0	1	7	2	6	4
Avg	7.5	11.75	3.25	1.75	1.25	1.25	1	5.5	1	4.5	3
STDE	1.914854216	1.707825128									

Control (right) high dose											
Rat#	**Lacunaes**	**Total score**	**MM**	**MD**	**DM**	**DD**	**Cervical**	**Middle**	**Apical**	**Comp**	**Tension**
H1	5	5	1	0	4	0	1	0	4	5	0
H4	3	3	3	0	0	0	1	2	0	3	0
H5	1	2	1	0	0	0	1	0	0	1	0
H6	4	6	4	0	0	0	4	0	0	4	0
Avg	3.25	4	2.25	0	1	0	1.75	0.5	1	3.25	0
STDE	1.707825128	1.825741858									

Experimental (left) high dose											
Rat#	**Lacunaes**	**Total score**	**MM**	**MD**	**DM**	**DD**	**Cervical**	**Middle**	**Apical**	**Comp**	**Tension**
H1	8	11	0	4	3	1	1	4	1	3	5
H3	5	9	3	0	2	0	1	2	1	5	0
H4	6	10	0	4	0	2	1	4	1	0	6
H6	6	11	3	0	3	0	3	3	0	6	0
Avg	6.25	10.25	1.5	2	2	0.75	1.5	3.25	0.75	3.5	2.75
STDE	1.258305739	0.957427108									

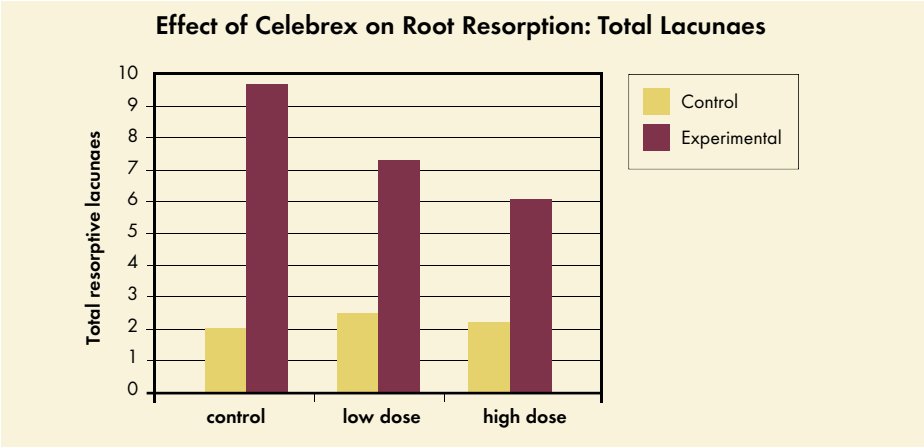


Figure 3. Effect of Celebrex on the number of lacunaes formed as consequence of orthodontic forces.

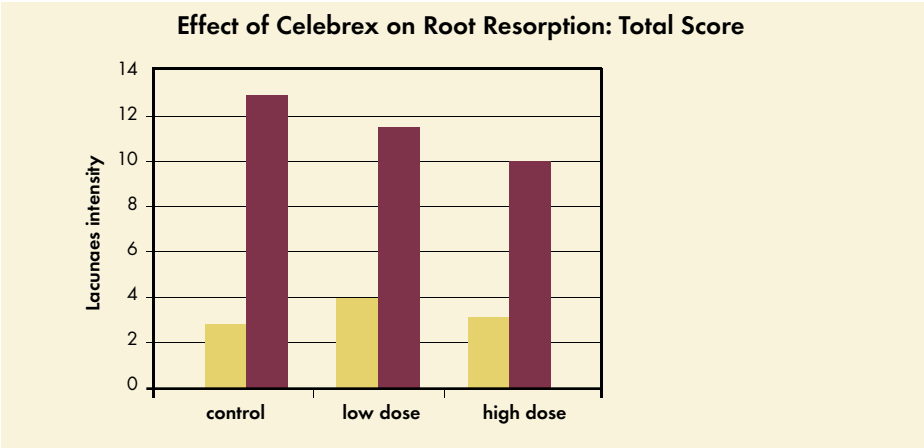


Figure 4. Effect of Celebrex on the number and intensity of lacunaes produced as consequence of application of orthodontic forces.

two investigators, each evaluating the resorption two times. The total number of lacunaes was determined (lacunaes) and the extent of the resorption was scored in a scale of 1 to 5, depending on the severity of the resorption, "1" being minimal severity and "5" being maximum severity. The sum of all the lacunae scores per histological section was the "total score." The location of the resorption was noted as apical, middle or cervical third of the root; and, the root surface the resorption occurred was noted as mesial root mesial aspect, mesial root distal aspect, distal root mesial aspect, or distal root distal aspect. Lacunaes in the compression vs. tension sites were also noted. A T-test was performed to determine the statistical significance of the data.

Results

Analysis of the "gap" created between the first and second molars was used as an indication of the amount of tooth movement resulting from the application of the orthodontic forces (**Figure 1**). No differences were found between the three groups of rats. A representative histological section of a first molar showing the resorption lacunaes can be seen in **Figure 2**. The analysis of the data can be seen in **Table 1**. When the total number of lacunaes in the three different groups was plotted, the number of "natural" resorption in the right molars, where no appliance was placed remained constant. However, the number of lacunaes decreased in the rats treated with both low and high doses, with the last one decreasing even more (**Figure 3**). When the total score (number and deepness of the lacunaes) was plotted, the results were very similar, although the differences appeared to be slightly less (**Figure 4**). Application of a T-test indicated that the difference between the low dose and the control has a p-value of 0.07, and does not represent a statistically significant difference. However, the difference between the high dose and the control has a p-value of 0.02, and indicated that the results were statistically significant, suggesting that Celebrex does provide some protection from root resorption.

Analysis of the distribution of resorption lacunaes by site (**Figure 5**) indicated that in the control, the majority of the lacunaes were present in the middle portion of the root, whereas only 6 percent were present in the apical portion, and 12 percent in the cervical portion. In the low dose group, this distribution changed slightly with an increase in the apical resorption while the proportion of lacunae in the middle portion diminished. In the high dose

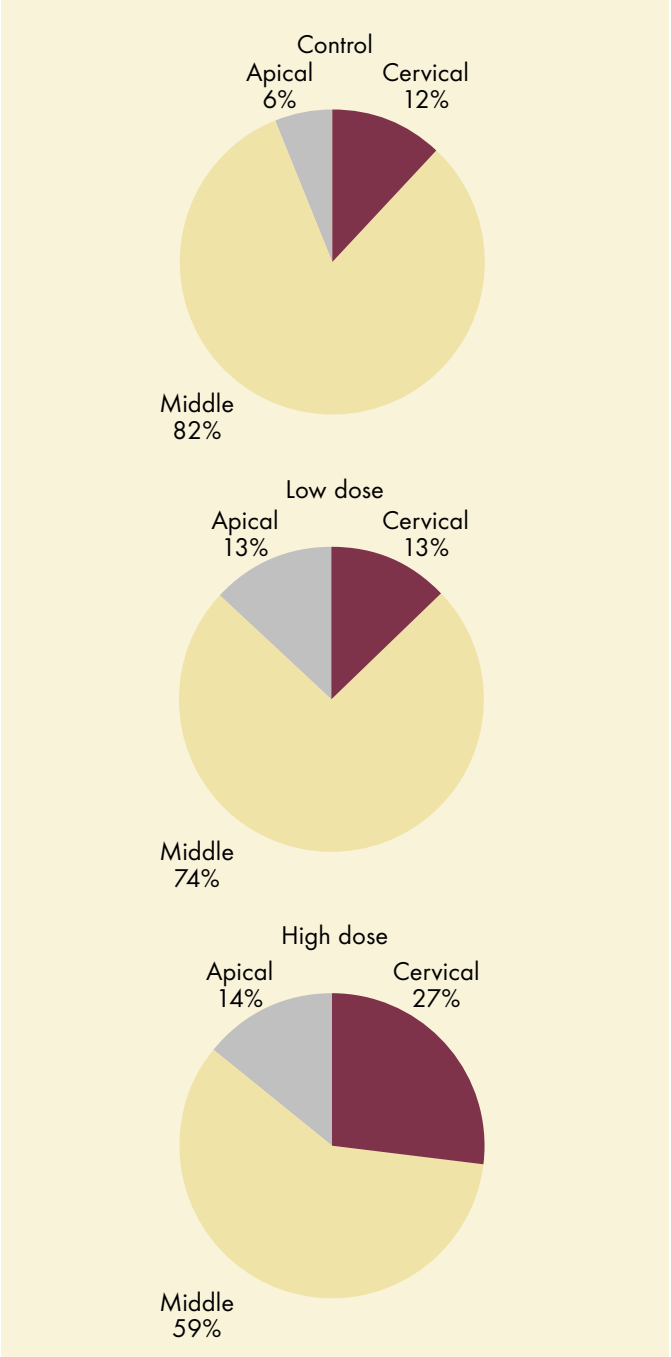


Figure 5. Distribution percentage of lacunaes in cervical, middle, and apical regions of the experimental molars.

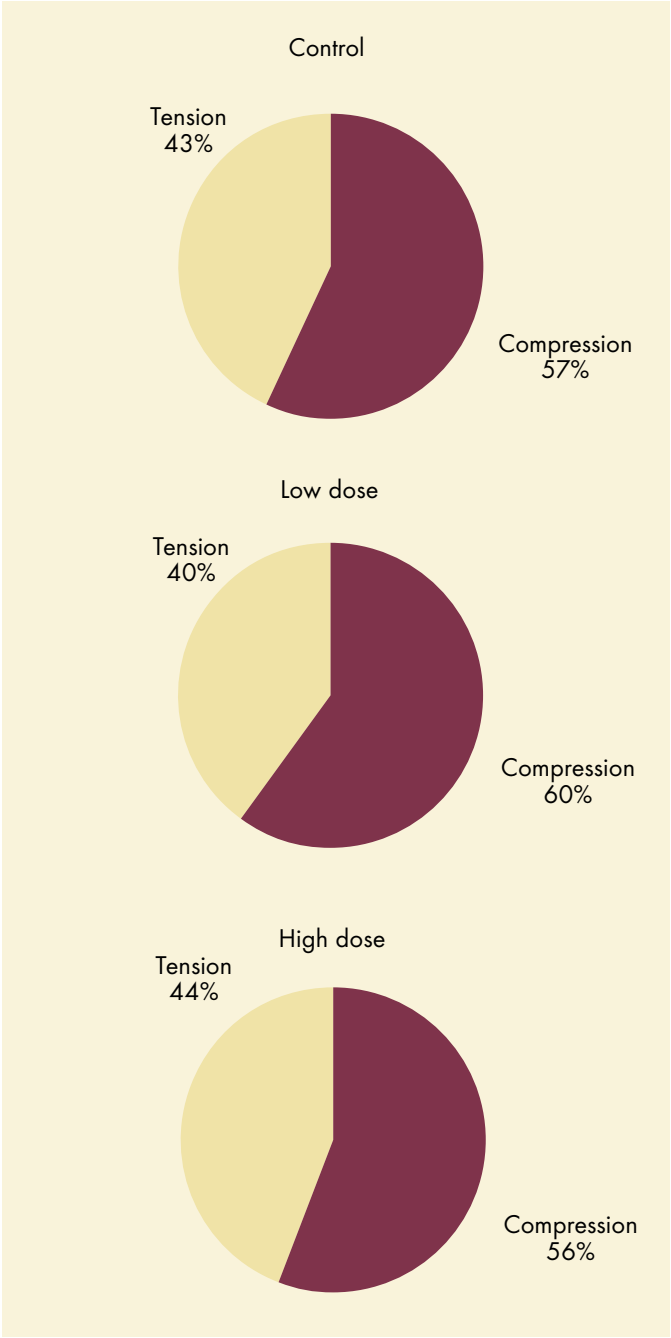


Figure 6. Distribution of lacunaes in the compression and tension sites of experimental molars.

group, there was an increase in the cervical resorption, while the apical resorption did not appear to change (**Figure 4**). When the presence of lacunaes was plotted by tension or compression sites, all three groups appeared to have had similar distribution, with the resorption being more prevalent (56 percent to 70 percent) in the compression site that in the tension site (40 percent to 44 percent).

Discussion

The results presented in this study suggest that the use of the NSAID Celebrex concomitant with the application of orthodontic forces might offer not only pain relief but also some mild protection from root resorption associated with this process. Although extrapolation from animal studies to humans is not always perfect or completely accurate, animal models offer several advantages as compared to human studies that can provide good insights as to possible mechanisms that may well work also in humans. Animal studies offer several advantages that make these type of studies possible; for example, a reliable system where we know that application of an orthodontic force of 80g will result in root resorption in a relatively brief period of time that can be detected early and accurately and can be measured. Therefore, although taken with caution, the authors' results suggest that the use of NSAIDS may be of benefit for patients undergoing orthodontic treatment.

The effectiveness of NSAIDS like ibuprofen in relieving the pain associated with orthodontic force activation in human studies has been reported.[33] However, one animal study suggested that administration of NSAIDS (or prostaglandin inhibitors) will interfere with tooth movement and therefore will slow down progress in treatment.[34] The authors did not find this to be the case in their studies. Pain relievers like acetaminophen have been recommended for patients undergoing orthodontic treatment since they do not interfere with tooth movement.[35] Unfortunately, acetaminophen does not have anti-inflammatory properties and will not offer much protection against inflammatory root resorption.

The use of a COX-2 inhibitor, rather than a COX-1 and COX-2, has the advantage that it protects the GI tract from the side effects of other NSAIDS.[36] Since this study was initiated, the use of COX-2 inhibitors has been questioned due to their potential to produce cardiovascular problems, although the evidence for Celebrex is still being evaluated.[37] Perhaps the use of other NSAIDS might be as, or more effective than Celebrex in protecting from root resorption associated with orthodontic treatment. CDA

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The Multifocal Nature of Odontogenic Keratocysts

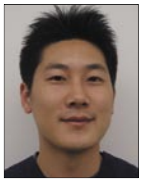
Philip J. Boyne, DMD, MS, DSc; David Hou, BS; Carlos Moretta, DDS; and Tyler Pritchard, BA

ABSTRACT

The odontogenic keratocyst, OKC, is a very aggressive intraosseos lesion with a recurrence rate of approximately 25 percent to 60 percent.[1] The tendency for this lesion to "return" after surgical treatment has prompted studies to obtain more information concerning the inherent nature of the lesion. The OKC lesions are usually treated with enucleation of the soft tissue lining, curettage and ostectomy of the bony margins, or with more aggressive block resection. The purpose of this study was to characterize the multifocal aspect of the OKC and to demonstrate the presence of cystic lesions remote from the margins of the primarily diagnosed cyst itself. A retrospective chart review was conducted of seven patients who had sustained a long history of recurrent OKCs. Three types of documentation were reviewed for each patient:

- Orthopantomograms, cephalograms, and CT scans, which had been taken over the long-term course of the disease,
- Detailed operation reports of surgical procedures to treat the OKC lesions, and
- Large histologic specimens from the six patients who received total resection of the involved mandibular bodies.

These hemimandibulectomy slides offered a unique opportunity to observe OKC activity throughout a wide osseous area. All patients had been operated multiple times over a period of 10 to 21 years, coming eventually to mandibular resection. The operating surgeon in all of the cases was one of the authors, Philip J. Boyne, DMD, MS, DSc. All patients exhibited the multifocal nature of OKCs with demonstrable cyst formation at distant sites in the mandible. Two patients had local recurrences at the margins of the primary lesion in addition to cyst formation at distant sites. The authors concluded that clinicians should respect the multifocal nature of OKCs. The "recurrences" observed in OKCs may not necessarily be due to the degree of skill of the surgeon or the technique used to eradicate the primary cyst, but instead are probably a reflection of the multifocal nature of the pathologic lesion itself. The OKC is a very aggressive intraosseos lesion of the jaws, which not infrequently clinicians detect in the process of routine oral examination.[2]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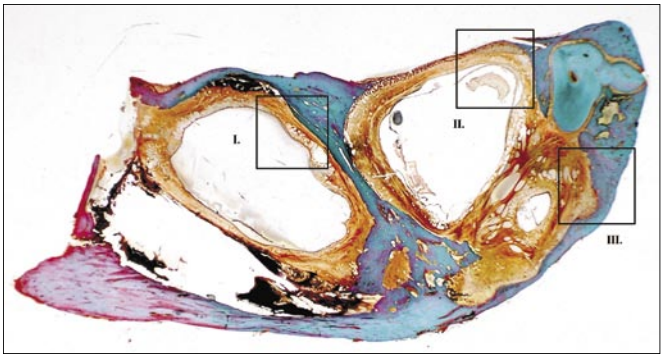

Figure 1.

Box I. Shows the lining of the large primarily diagnosed OKC lesion.

Box II. Shows the area of an additional secondary proliferating OKC separate from the primary lesion.

Box III. Shows an area containing small early proliferating OKCs, again separate from the primarily diagnosed OKC lesion.

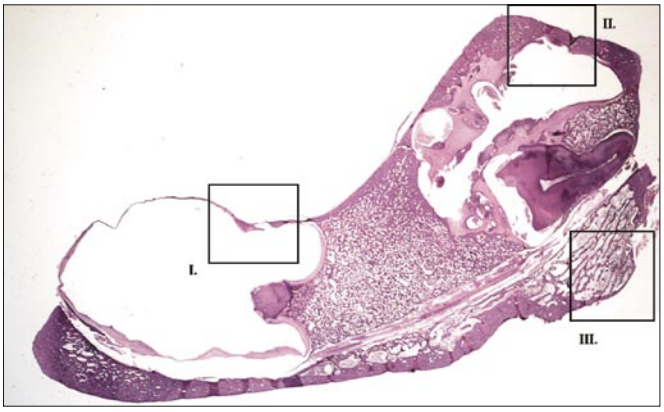

Figure 2a.

Box I. The lining of a large primarily diagnosed OKC is shown.

Box II. The lining of large mature secondarily diagnosed OKC is shown. (The space surrounding the crown of the impacted tooth represents a dentigerous cyst, which is separate from the surrounding OKCs.)

Box III. An area of additional epithelial cell nests and small developing OKCs separate from the other larger OKCs is shown at the angle of the mandible.

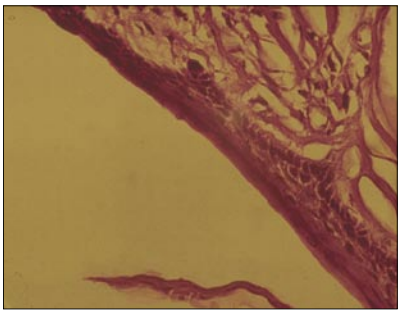

Figure 2b. The thin epithelial lining of the primary OKC lesion shown in Figure 2a Box I at a higher magnification. The existence of a basal cell layer with high mitotic activity is characteristic of an OKC, along with a marked sloughing of keratin into the lumen of the cyst.

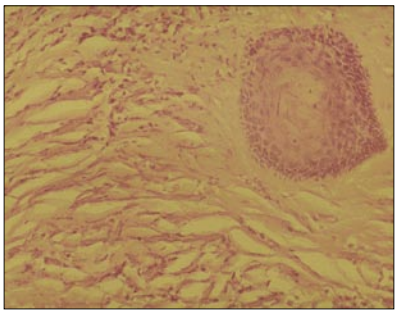

Figure 2c. This view shows a magnified view of Figure 2a Box III, where a small cyst is developing into a characteristic OKC. The existence of a basal layer with high mitotic activity is shown.

There are two types of OKCs: nonsyndromic or sporadic, and syndromic Nevoid Basal Cell Carcinoma, NBCCs. The NBCCs Gorlin-Goltz syndrome is characterized by multiple OKCs, nevoid basal cell carcinomas of the skin, bifid ribs, calcification of the fax cerebri, and other findings.[1]

The most commonly involved areas for occurrence of OKCs are the angle of the mandible and the ascending ramus.[3] The symphyseal area is also frequently a locus for this lesion. The current treatment modalities of OKCs range from conservative enucleation to more radical partial en bloc resection. The cause of recurrence has been attributed in some reports to the type of surgical treatment of the bony margins as well as the skill and experience of the surgeon.[4]

The epithelial lining of OKCs is thought to be a source of the recurrence. "Disruption of the epithelial lining was documented in 50 percent of the cases of recurrence and 45 percent of all cysts, leading to the belief that new cysts form from remaining fragments of cyst walls."[5]

More recent research has shown that "differences in proliferative activity in OKCs suggest an alteration of the cell's cycle control" producing "an increase in cell proliferation that could explain the biological behavior of OKCs."[6]

The goal of this study was to demonstrate the presence or absence of additional cysts proliferating in areas not necessarily adjacent to the primary cystic lesion. This study is felt to be of clinical importance since OKC patients are usually treated to "prevent" recurrence at the margins of the initially presenting lesion. If there are additional cysts present at some distance from the original lesion, then drastic treatment of the original cyst's bony margin may not be helpful in preventing the persistence of the lesion.

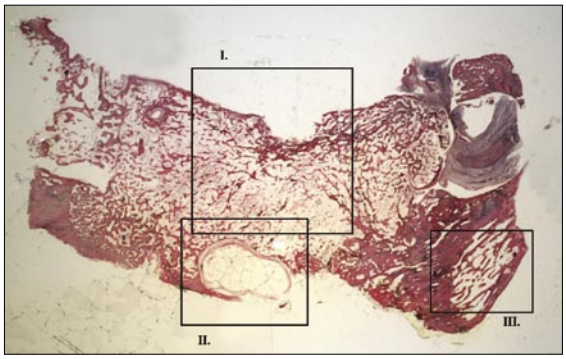

Figure 3a.

Box I. The lines indicate the borders of a previous block resection for an OKC, followed by a bone graft. There is no OKC recurrence in the bone grafted area.

Box II. This area shows the presence of a secondarily occurring OKC at the inferior border of the mandible.

Box III. The presence of additional developing OKCs is shown in the MV spaces at the angle of the mandible.

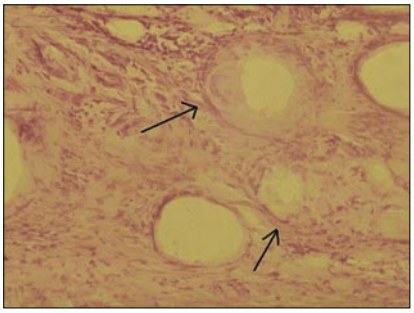

Figure 3b. This is a magnified view of Figure 3a Box III showing the presence of young developing OKCs.

The authors' study is unique because of the availability in the cases of histologic single-sectioned slides of the entire horizontal mandible. These specimens were procured from the resected hemimandibles of six of the seven patients. These large slides offer a view of the entire mandible not usually obtained from routine pathologic specimens, which usually are multisectioned cuts through the lesion's margins. A literature search revealed no other investigation has presented such histologic material.

Materials and Methods

A retrospective chart review was completed of seven patients having a long history of recurrent OKCs, and who eventually came to resection of the mandible because of continual recurrence of the lesions. Three types of documentation were studied and reviewed for each patient:

- Orthopantomograms, cephalograms, and CT scans, which had been taken over the long-term course of the disease,
- Reports of the patients' history and physical examination, and surgical procedures throughout the entire treatment of the OKC lesions, and
- A review of large pathologic specimens from the hemisections of the mandibular body.

Results

It was found that all of the patients in the study experienced multiple surgical operations for treatment of OKCs over a period of 10 to 21 years. Operations included curettage of bony margins, enucleation of cysts, block resection, and hemisection of the mandible.

In treating the lesion, six patients came to complete surgical unilateral resection of their horizontal mandible due to continual recurrences of aggressive cysts. These resections were performed after multiple failed attempts to control the recurrences. In all patients, the osseous defect created by the surgery was restored by an autogenous iliac crest bone graft. In these patients receiving resection, no recurrences of OKCs were noted in the bone grafted areas or in the surrounding native bone.

All of the patients exhibited the multifocal nature of OKCs. Initially, a single cyst was discovered and surgically treated, followed by "secondary" cyst formations at distant sites in the mandible (**Figures 4a-b; Figures 5a-d**). The authors' study showed that all the patients had evidence of multifocal OKCs (**Figures 1-5**). These histologic findings revealed collections of epithelial cells at distant sites in the marrow vascular spaces of the mandible. Some of these epithelial cells demonstrated a central lumen, while others showed actual cyst formation with characteristic OKC keratin desquamation (**Figures 2a-c; Figures 3a-b**). Two patients had local recurrences at the margins of the primary lesion in addition to cyst formations at distant sites (**Figure 5d**).

Discussion

Successful treatment of OKCs is usually attributed to the use of techniques which address the margins of the initially presenting cyst, with the objective of removing all of the epithelial lining.[7-9] In the background literature review, the term "recurrence" was used to describe cysts that recurred near the bony margin of the originally treated cyst.[10] It could be proposed that rather than "recurrences" of the original cystic lesion, many OKC patients are actually experiencing separate "occurrences" at distant sites. Although this study was only based upon seven patients, the availability of histopathologic review of the entire body of the mandible, on the involved side, has given interesting information on this problem area. It could be assumed from this review that aggressive surgical treatment of the bony margins of the primary lesion would not necessarily preclude a second occurrence.

It is of interest that the observations made in this paper may corre-

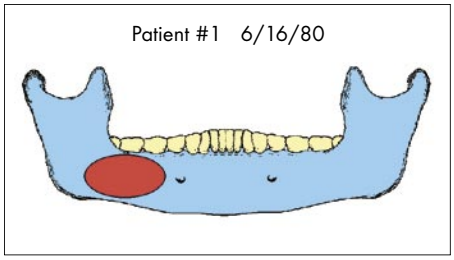

Figure 4a. Patient No. 1 presented with a large OKC lesion near the right angle of the mandible. The lesion was treated with enucleation, peripheral ostectomy, and iliac crest autogenous particulate bone graft. The area healed completely.

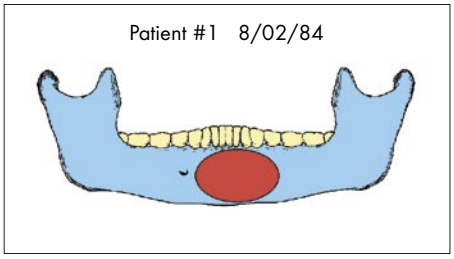

Figure 4b. Four years later, Patient No. 1 showed healed bone at the right angle of the mandible. However, the patient now presents with a large OKC lesion toward the left symphysis area. This OKC is separate and distinct from the primary lesion that was treated four years previously. This new lesion clearly illustrates the multifocal nature of OKCs.

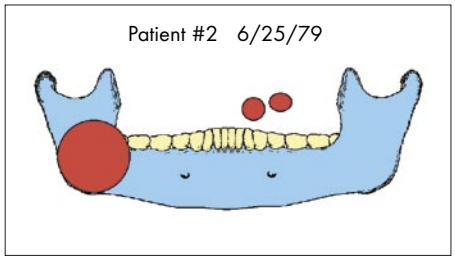

Figure 5a. Patient No. 2 presented with a large OKC lesion at the right angle of the mandible, along with two other OKCs in the left maxillary incisor and canine areas.

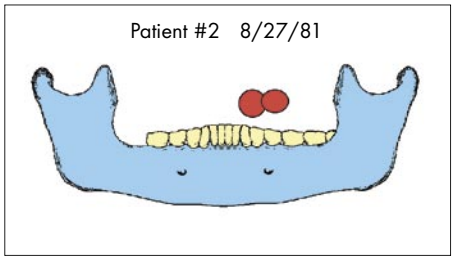

Figure 5b. Patient No. 2 was treated by curettage for the large OKC lesion at the right angle of the mandible. This depiction of the radiographs, taken two years later, showed osseous healing in that area.

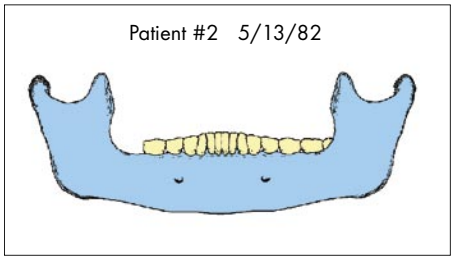

Figure 5c. Patient No. 2 was treated for the maxillary OKCs. Radiographs taken a year later show good postoperative bone healing.

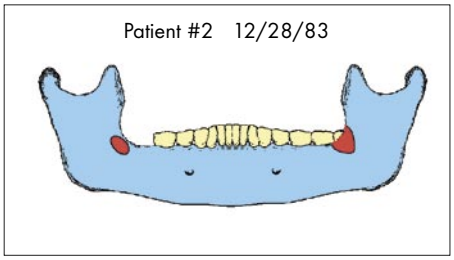

Figure 5d. Four years after initial treatment of the mandible OKC, the patient presents with a local OKC recurrence in the right mandibular area, along with a new OKC lesion at the left third molar area of the mandible. The new lesion in the left mandible is representative of the multifocal nature of this lesion.

Figures 4a and b and **5a-d** are two-dimensional representations of the radiographs that were reviewed for these two patients. The presence of OKCs (depicted in red) were derived from orthopantomograms, cephalograms, and CT scans.

late with the results of recent genetic research. A tumor suppressor gene has been shown to be associated with the nevoid basal cell carcinoma syndrome or NBCCS. Alterations in this tumor suppressor gene, which is also called the patched gene or PTCH, may be the first important step in the pathogenesis of the OKC. It is possible that OKC epithelial cells remain in a "resting state" in the mandible for an indeterminate amount of time, under the protective influence of the PTCH tumor suppressor gene. Any mutations in the favorable influence of this tumor suppressor gene, could lead to rapid OKC cell proliferation and a change from a dormant state to a more aggressive form. Recent immunocytochemical studies have shown that the PTCH gene is also present and exerting its protective influence in the case of sporadic OKCs, as well as in the case of the syndromic NBCCS cysts. This dramatic mutation of the PTCH gene in sporadic OKCs, as well as the syndromic OKCs, is very important in understanding the nature of this aggressive lesion. If the PTCH gene undergoes mutation, its protective influence may be lost through a subsequent immunologic "hit" and the OKC resting cells may become neoplastically aggressive.[11-13]

This genetic change could help to explain the aggressive behavior of the OKC cells just as the loss of the tumor suppression produces aggressive behavior in other forms of neoplastic disease. In fact, Shear, in 2002, stated that the "loss of tumor suppressor genes supports the view that OKC is a benign neoplasm."[11]

The histological sections, which demonstrated the presence of clusters of epithelial cells and secondary cysts, show the multifocal characteristics of OKCs. In fact, many of these distant cysts were too small to be shown by

radiographic imaging. Clinicians should be aware that these small cystic areas can exist in OKC patients without detection.

Literature has shown that patients sustaining large resections of the mandible have the lowest recurrence rate of OKCs compared with other surgical techniques.[14,15] The authors' study illustrated a possible reason for this observation. These patients demonstrated the presence of additional cysts and epithelial clusters at distant sites in the large mandibular histological sections. The reported low OKC recurrence rate following mandibular resections could possibly be explained by the fact that the multifocal lesions have become part of the large resected specimen.

In the authors' study, two of the seven patients were syndromic. It was reported that a characteristic of syndromic patients, NBCCS, was a high multifocal occurrence of OKCs, along with a high recurrence rate.[16] The authors showed that nonsyndromic or sporadic OKCs also can exhibit multifocal OKCs. Additionally, the authors' sporadic OKC cases also had recurrences that were highly destructive and aggressive (**Figure 1**).

The distant lesions were seen to be in various stages of maturation from single collections of epithelial cells to lumen development and cyst formation. Pathologic specimens viewed microscopically showed that the remote cysts tended to begin around a keratin pearl leading to lumen formation.

Conclusions

■ Clinicians should respect the multifocal characteristics of OKCS.

■ OKCS exhibit highly aggressive behavior, and persistence of the lesion can lead to mandibular resections.

■ In all of the mandibular resected patients, no "recurrences" were noted to date in the bone-grafted surgical site or elsewhere in the native bone. The one patient who did not receive hemiresection experienced "recurrence" of the OKCs.

■ Syndromic patients, along with nonsyndromic or sporadic patients, tend to present with multifocal lesions.

■ The recurrence of OKCs may not necessarily be due to the degree of skill of the surgeon or the technique used to eradicate the primarily diagnosed cyst, but instead is probably a reflection of the multifocal nature of the lesion itself. CDA

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UNIVERSITY OF THE PACIFIC
Arthur A. Dugoni School of Dentistry

Gene Therapy for Oral Cancer: Efficient Delivery of a 'Suicide Gene' to Murine Oral Cancer Cells in Physiological Milieu

Mark Young; Nathan Overlid; Krystyna Konopka, PhD, MD; and Nejat Düzgünes, PhD

ABSTRACT

Gene therapy is a new therapeutic modality in which defective genes are replaced with functional ones, or genes are delivered that can specifically kill cancer cells. Efficient gene delivery is an important component of gene therapy approaches. Potential safety problems with viral vectors necessitate the development of efficient nonviral vectors. DNA complexes with synthetic cationic liposomes or polymers constitute a simple means of transferring DNA into target cells. Gene delivery mediated by many nonviral vectors, however, is inhibited by serum components, and this is expected to limit the efficiency of gene delivery in vivo. In this study, the authors examined two novel gene transfection reagents, Metafectene and GeneJammer, for their ability to deliver a reporter gene to SCCVII murine squamous cell carcinoma cells in the presence of high concentrations of mouse serum. After establishing conditions that achieved significant gene delivery, the authors introduced the Herpes Simplex Virus Thymidine kinase (HSV-tk) gene into the cells using the cationic liposome reagent, Metafectene, followed by the administration of ganciclovir. After seven days of incubation, 90 percent and 82 percent cytotoxicity was observed in 0 percent and 60 percent mouse serum, respectively. The authors' observations suggest that Metafectene may be useful for the gene therapy of oral squamous cell carcinoma in a murine model involving the induction of oral tumors by SCCVII cells.

Head and neck cancers constitute about 3 percent to 5 percent of all cancers in the United States and are more common in persons over age 50.[1] About 39,000 individuals are predicted to develop head and neck cancer in 2005.[1] The five-year survival rate in patients with oral and pharyngeal cancers has remained at 53 percent between 1974 and 1994, the rates for African Americans and whites being 32 percent and 55 percent, respectively.[2] Current treatments for head and neck cancers include surgery, radiation therapy, and chemotherapy, all of which have severe side effects. The genetic approach to the treatment of HNSCC is based on the hypothesis that expression of therapeutic genes in target cells will cause a cytotoxic effect or mediate apoptosis, or

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that the defective genes can be replaced with normal ones.[3] Oral cancer is a particularly appropriate target for gene therapy, since direct injection of genes into most primary and recurrent lesions is possible. The gene therapy approach is also amenable to cancer cell-specific gene delivery and expression, which will alleviate the problem of destroying normal cells during therapy.

SCCVII is an aggressive squamous cell carcinoma (SCC) cell line established from a carcinoma that developed spontaneously in C3H/HeJ mice. Injection of SCCVII cells into the floor of the mouth in C3H/HeJ mice results in the development of oral tumors, serving as a useful immunocompetent animal model of HNSCC.[4] Successful suicide gene therapy in this system, via direct injection of the HSV-tk gene into the tumor, followed by GCV treatment, would be expected to reduce the tumor size. This approach could be used in conjunction with immunostimulatory gene therapy.

Although generally efficient in transducing cells, viral vectors suffer from problems of immunogenicity, toxicity, limits in the size of exogenous DNA, and the risk of inducing tumorigenic mutations and generating active viral particles through recombination. Synthetic cationic polymer-DNA complexes (polyplexes) and cationic liposome-DNA complexes (lipoplexes) constitute a promising alternative to the use of viral vectors and provide a simple means of transferring DNA into target cells ("transfection").[5-8] One of the limitations of transfection mediated by nonviral vectors is that it is usually inhibited by serum components.[8-14] Thus, gene delivery in vivo is expected to be far from efficient. It is therefore important to identify nonviral vectors that are resistant to the inhibitory effects of serum. Previous studies in the authors' laboratory had indicated that both the cationic polymer, GeneJammer, and the cationic liposome, Metafectene, are effective in transfecting human SCC cells even in the presence of high concentrations of fetal bovine serum.[15]

In this study, the authors examined the effect of mouse serum on the delivery of the genes encoding luciferase and the Herpes Simplex Virus thymidine kinase (HSV-tk), to SCCVII cells, by Metafectene and GeneJammer. The luciferase gene is expressed from the pCMV.Luc plasmid under the control of the cytomegalovirus promoter, and is used as a reporter gene to monitor the efficiency of gene transfer. Delivery of HSV-tk to these cells, followed by ganciclovir (GCV) treatment is expected to cause cell death, in an approach termed "suicide gene therapy."[3,16-18] In previous studies, the authors demonstrated that nonviral vectors, including Fugene and transferrin-lipoplexes, could mediate the delivery of HSV-tk to human SCC cells in serum-free medium, thereby causing extensive cytotoxicity in the presence of the prodrug GCV.[19]

Oral cancer is a particularly appropriate target for gene therapy, since direct injection of genes into most primary and recurrent lesions is possible.

Materials and Methods

Cells. SCCVII murine squamous cell carcinoma cells were a gift of Dr. D. Li and Dr. B. O'Malley (University of Pennsylvania). They were propagated in Dulbecco's modified Eagle's MEM medium (DMEM; Irvine Scientific, Santa Ana, Calif.), supplemented with 10 percent (v/v) fetal bovine serum (FBS) (Sigma, St. Louis, Mo.), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (4 mM) (DMEM/10). Mouse serum was obtained from Equitech-Bio, Inc. (Kerrville, Texas).

Plasmids and reagents. Metafectene, a polycationic transfection reagent based on liposome technology, containing a polyamino-lipid and dioleoylphosphatidylethanolamine (DOPE) was obtained from Biontex Laboratories GmbH (Munich, Germany). GeneJammer, a proprietary formulation of polyamine and other components in 80 percent ethanol, was purchased from Stratagene (La Jolla, Calif). The plasmids pCMV.Luc (VR-1216), encoding luciferase was a gift of Dr. P. Felgner (Vical, San Diego, Calif). The plasmid pCMV.lacZ, encoding ß-galactosidase was from Clontech (Palo Alto, Calif.), and pCMV.HSV-tk (pNGVL1-tk), expressing the Herpes Simplex Virus thymidine kinase was obtained from the National Gene Vector Laboratory (University of Michigan, Ann Arbor, Mich.).

Transfection. For transfection, 2 x 10^5 cells were seeded in 1 ml of DMEM in 48-well culture plates one day before transfection, and used at approximately 80 percent confluence. Metafectene- and GeneJammer-mediated transfection procedures were performed according to the manufacturers' recommendations. Cells were prewashed with serum-free DMEM medium and then covered with 0.4 ml of the same medium. Complexes were prepared by mixing Metafectene or GeneJammer with 0.1 ml of serum-free DMEM medium, followed by the addition of plasmid DNA. The mixture

was incubated for 15 min at room temperature after the addition of the transfection reagent, and another 15 min after addition of DNA. Lipid/DNA complexes were added in a volume of 0.1 ml per well, the cells were incubated for 4 h at 37°C, and then 0.5 ml of serum-containing medium was added. Luciferase activity was assayed 48 hours after transfection, using the Luciferase Assay System (Promega, Madison, Wisc.), and a TD-20/20 luminometer (Turner Designs, Sunnyvale, Calif.). The data were expressed as relative light units (RLU) per ml of cell lysate. These values are designated "transfection activity." Transfection efficiency, i.e. the percentage of transfected cells in the culture, was examined by transfecting pCMV.lacZ followed by staining for ß-galactosidase, using the X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) reagent as a substrate for the expressed enzyme.[19,20]

HSV-tk + ganciclovir treatment and cytotoxicity. Cells transfected with the HSV-tk plasmid (pCMV.HSV-tk) were incubated in the absence or the presence of GCV (20 µg/ml) for the indicated periods of time. Ganciclovir was a gift from Hoffmann-La Roche, Inc. (Nutley, N.J.). GCV-mediated cytotoxicity was assessed by the Alamar Blue (Accumed International Companies, Westlake, Ohio) assay, as described by Konopka et al.[21]

Results

To achieve the highest levels of gene expression without causing significant toxicity to the cells when using nonviral vectors, it is essential to determine the optimal ratio of the transfection reagent to plasmid DNA, and the amount of DNA for every cell line.[22] Therefore, in preliminary experiments, transfection conditions were optimized using different ratios of reagent:pCMV.Luc. The optimal conditions for SCCVII cells were 2 µl Metafectene:1 µg DNA per well, and 3 µl GeneJammer:0.5 µg DNA per well (data not shown).

While luciferase expression, using the pCMV.Luc plasmid, indicates the level of transgene expression in the entire culture, the pCMV.lacZ plasmid can be used to evaluate the percentage of cells that can be visibly transfected. With the latter plasmid, cells expressing ß-galactosidase turn blue after the addition of the specific substrate, X-gal. SCCVII cells were transfected using Metafectene. **Figure 1** shows that approximately 60 percent to 70 percent of the cells stained positive for ß-gal.

To mimic the effect of physiological, or in vivo, conditions on gene transfer and expression in SCCVII cells mediated by nonviral vectors, cells were transfected with Metafectene-pCMV.Luc complexes in the presence of varying concentrations of mouse serum. At 20 percent serum, transfection was inhibited by 70 percent, but the inhibitory effect of serum was not as pronounced at 40 percent, and at 60 percent serum luciferase expression was inhibited by only about 23 percent (**Figure 2**). GeneJammer-mediated luciferase expression was reduced by about 56 percent in 20 percent and 40 percent serum, and about 67 percent in 60 percent serum (**Figure 3**).

To establish whether the relatively serum-resistant nonviral vector Metafectene could also mediate the delivery of a therapeutic gene to murine SCC cells, the pCMV.HSV-tk plasmid was complexed with this reagent and incubated with SCCVII cells in the absence or presence of 60 percent mouse serum. Treatment with GCV for three days, resulted in 36 percent and 0 percent cytotoxicity, respectively. After seven days of GCV treatment, however, 90 percent and 82 percent cytotoxicity was observed in the presence of 0 percent and 60 percent mouse serum, respectively (**Figure 4**). No significant nonspecific cytotoxicity was observed in the absence of GCV.

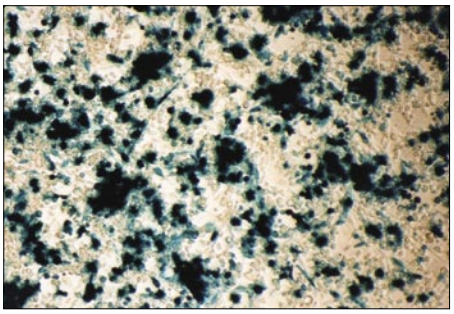

Figure 1. Gene expression in SCCVII cells transfected with the pCMV.lacZ plasmid using Metafectene. Cells expressing ß-galactosidase turn blue after the addition of the specific substrate, X-gal.

Discussion

The inhibitory effect of serum on transfection by lipoplexes is most likely mediated by negatively charged proteins that bind to the cationic components of the vector, resulting in the inhibition of electrostatic interactions with cellular membranes and release of the DNA. Serum nucleases can also degrade the exposed segments of plasmid DNA. Most transfection protocols involving cultured cells require that the reagents are added either in the absence of serum, or with only 10 percent serum normally used in culture media, the latter only with a few reagents. In vivo applications of nonviral vectors include intravenous or intratumoral injection, both of which involve exposure to physiological milieu. To identify transfection reagents that are likely to be effective when delivered in vivo, particularly in murine models of oral or other cancers, the effect of high concentrations of mouse serum was investigated.

Two transfection reagents that have

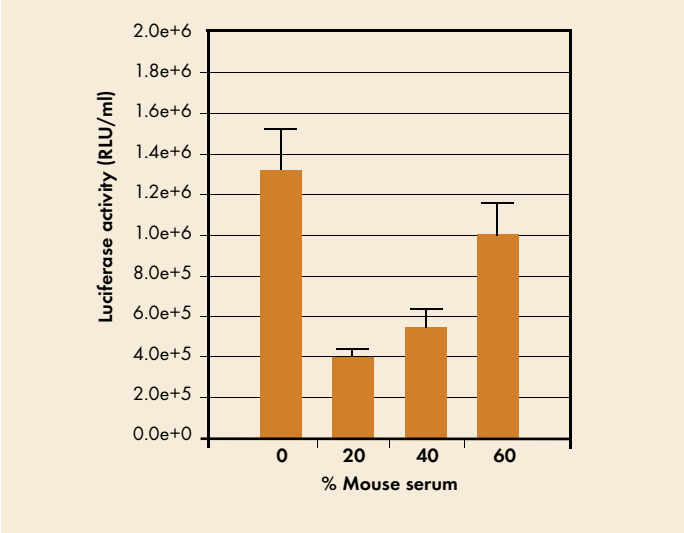

Figure 2. Luciferase expression in SCCVII cells transfected with Metafectene-pCMV.Luc complexes in the presence of varying concentrations of mouse serum. Data represent the mean ± SD obtained from triplicate wells.

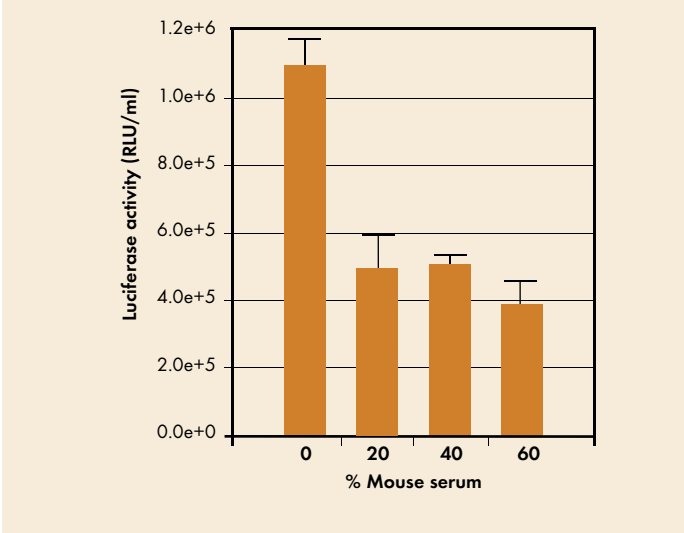

Figure 3. Luciferase expression in SCCVII cells transfected with GeneJammer-pCMV.Luc complexes in the presence of varying concentrations of mouse serum. Data represent the mean ± SD obtained from triplicate wells.

become available recently, the polycationic liposome, Metafectene, and the polyamine reagent, GeneJammer were examined. Metafectene has been used for gene delivery to a variety of cells in culture, including prostate cancer cells, human cervical carcinoma cells, and mouse fibroblasts.[23-25] Significantly, Metafectene has been employed in delivering the gene encoding bone morphogenetic protein, BMP-2, to tissues surrounding implants.[26] GeneJammer has been used for transfection of zebrafish embryos.[27]

GeneJammer- and Metafectene-plasmid complexes were shown to transfect murine SCCVII cells efficiently both in the absence and presence of mouse serum. These cells were chosen since they are employed in the generation of SCC tumors in an oral cancer model in C3H/HeJ mice.[4] Using the adenovirus HSV-tk vector for intratumoral injection and systemic GCV administration, Sewell et al. have demonstrated tumor regression and improved animal survival in this model.[4,28] The results also demonstrated that the delivery of the HSV-tk gene by Metafectene, followed by GCV treatment, causes extensive cytotoxicity even in the presence of 60 percent mouse serum. Thus, it is likely that Metafectene will be useful for the delivery of genes in biological milieu, either for intravenous injection in the treatment of disseminated cancers or for intratumoral injection in the therapy of oral SCC. Although GeneJammer facilitated serum-resistant gene delivery to SCCVII cells, the presence of ethanol in the formulation of this reagent may preclude its use in vivo. Current studies in the authors' laboratory are directed toward cancer cell-specific delivery and expression of reporter (Luciferase and ß-galactosidase) and suicide (HSV-tk) genes. Cancer cell-specific cytotoxicity is likely to leave neighboring normal cells intact, and to enhance the eradication of tumors, especially when combined with immunotherapy. This approach to the treatment of head and neck cancers is expected to have minimal side-effects, in contrast to current therapies. CDA

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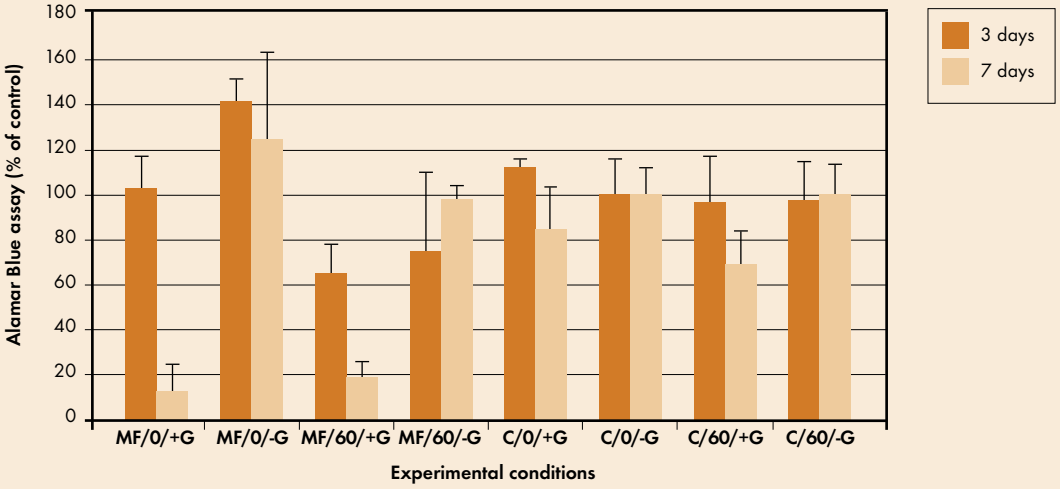

Figure 4. Cytotoxicity of HSV-tk + ganciclovir in SCCVII cells in the absence (MF/0) or presence (MF/60) of 60 percent mouse serum. The cells were transfected with the pCMV.HSV-tk plasmid using Metafectene. Cell viability was measured by the Alamar Blue assay on Days 3 and 7 post-transfection. Results are expressed as a percentage of mock-transfected controls not treated with GCV (C/0/-G and C/60/-G), respectively. Data represent the mean ± S.D. obtained from triplicate wells.

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Cariogenic Virulence Characteristics of Mutans Streptococci Isolated From Caries-active and Caries-free Adults

Gloria Khoo; Ling Zhan; Charles Hoover, PhD; and John D.B. Featherstone, MSc, PhD

ABSTRACT

The objective of the study was to compare aciduricity (ability to live in acid), acidogenicity (ability to produce acid), and intracellular polysaccharide production of mutans streptococci (MS) strains isolated from caries-active (CA, with one or more cavitated lesions) and caries-free (CF, with no clinically observable new caries in the last five years) adults. Forty-three MS strains from 17 of 17 CA adults, and 14 strains from eight of 12 CF adults were investigated. MS isolates' growth, survival, and pH reduction in pH 3.5-7.0 broths were evaluated to compare their acidogenicity and aciduricity. Extracellular water-soluble polysaccharide (WSP) and water-insoluble polysaccharide (WISP) was extracted from MS culture in BHI broth with 5 percent sucrose and assessed by a colorimetric anthrone-sulfuric acid microassay. No significant differences in mean aciduricity were found between CA and CF MS isolates ($P>0.05$, t test). However, significantly more CA subjects (29 percent) were colonized by MS strains with aciduricity above the average than CF subjects (13 percent, Fisher's exact test, $P<0.05$). Furthermore, CA MS strains produced significantly more acid at pH<5 (Mann-Whitney, $P<0.05$) and significantly more CA subjects were colonized with more acidogenic MS at pH<4.5 (Fisher's exact test, $P<0.01$). Similarly, CA MS isolates produced significantly more WISP than CF (Mann-Whitney test, $P<0.01$) while no statistical difference was found in WSP between the two groups. More CA subjects were colonized by multiple strains with aciduricity, acidogenicity, and polysaccharide synthesis ability above average. The study indicated that differences in acidogenicity, aciduricity, and polysaccharide synthesis in strains of MS may partially contribute to increased caries activity.

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Dental caries is one of the most common infectious diseases found in all human populations. The mutans streptococci (MS) group of oral bacteria (particularly, *Streptococcus mutans* and *Streptococci sobrinus*) is one of the major causative bacterial groups in human dental decay. Previous studies have reported positive relationships between salivary levels of MS and caries prevalence or future caries increment and salivary MS level have been identified as an important part of caries risk assessment.[1-10] Although salivary MS were proven to be part of reliable caries risk assessment criteria in those studies, there were some subjects with high MS infection who did not develop caries lesions. In these studies, there was no consideration of possible virulence variations in different MS strains. A study by Bowden also showed that high numbers of *S. mutans* associated with the development of a lesion at one site may not result in caries at a second susceptible site with high levels of the same organism in the same subject.[11] This indicated that there might be factors other than MS quantity alone contributing to caries risk.

Recent studies on bacterial genetic polymorphism have found that MS strains are characterized by a high degree of genetic diversity.[12-18] MS infection, however, is usually transmitted amongst family members, most commonly mother and child, who therefore share common MS strains.[19-37] Caries-active subjects tend to have multiple strain MS infections compared to the caries-free subjects.[14,18,21] Three major virulence characteristics of these bacteria are likely to be important, namely acidogenicity (ability to produce acid), aciduricity (ability to live in acid) and glucan synthesis (ability to form extracellular polysaccharides when feeding on carbohydrates such as sucrose). In vitro, differences in cariogenic potential (aciduricity, acidogenicity, and glucan synthesis) of different MS strains have been observed.[38-42]

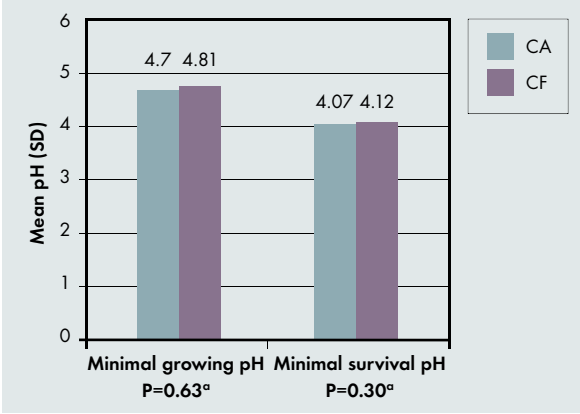

Figure 1. The bar graph presents the mean and SD of the minimal growing pH and minimal survival pH of mutans streptococcus strains isolated from caries-active (CA) subjects vs. caries-free (CF) subjects.

**Student t test*

Only a few previous studies have compared the cariogenic potential of MS isolates from caries-active and caries-free subjects. MS isolates from caries-active subjects have been reported to produce more extracellular water insoluble glucan than MS isolates from caries-free subjects.[43-46] Conflicting results have been reported on aciduricity, acidogenicity, and biofilm formation of MS and subjects' caries status.[40,44,46,47] Although these results suggest that some MS strains may exhibit strong virulence characteristics that enhance their ability to colonize, survive, and induce caries formation, further studies are needed to investigate the relationship between MS virulence factors and caries status.

The aim of the present study was to compare cariogenic virulence factors, namely aciduricity, acidogenicity, and extracellular polysaccharide synthesis ability of MS strains isolated from caries-active and caries-free adults.

Methodology

Subjects and Strains of Mutans Streptococci

After approval by the Committee on Human Subject Research of the University of California, San Francisco, two adult populations from an ongoing NIH-funded study "Caries Management by Risk Assessment" were utilized in this study. Seventeen caries-active subjects with 1-7 frank cavities and more than five DMFT, and 12 caries-free subjects with no new cavities or restorations due to caries during the last five years were recruited. The authors chose these criteria to eliminate clinically observable caries activity for at least five years prior to the present study. MS were enumerated from stimulated whole saliva cultured on Mitis Salivarius Sucrose Bacitricin agar, MSSB. Five randomly selected typical MS colonies were isolated from each MS-positive subject and confirmed as MS by a sugar fermentation test. These strains were stored in TSB (tryticase soy broth) with 20 percent glycerol at -80ºC until further study of MS aciduricity, acidogenicity, extracellular polysaccharide synthesis, and genetic diversity. *S. mutans* 25175 and *S. sobrinus* 6715 were used as reference strains in all assays.

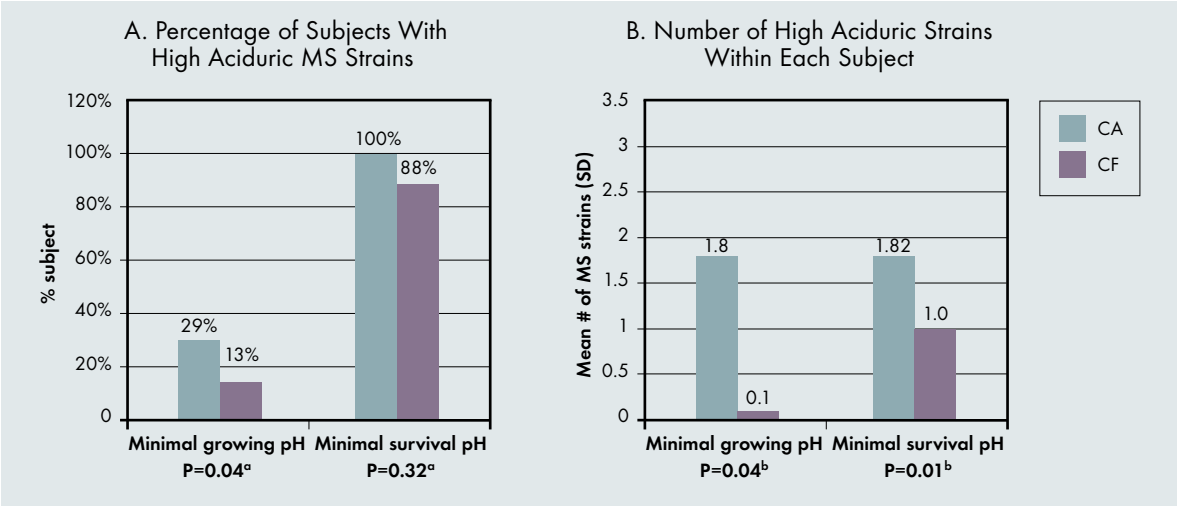

Figure 2. Panel A presents percentage of subjects who had MS strains with the minimal growing pH and the minimal survival pH below average. Panel B presents the mean and SD of number of MS strains, which had the minimal growing pH and the minimal survival pH below average, within each subject.

**Statistically significant; [a]Fisher exact test; [b]Mann-Whitney test*

Mutans Streptococci Aciduricity and Acidogenicity Assays

Mutans streptococci aciduricity was evaluated by two parameters, namely the bacterial minimal growth pH and the minimal bacterial survival pH. The MS strains were recovered on MSSB plates and then grown overnight in pH 7 TPY (tryptone peptone yeast) broth. The overnight cultures were then inoculated into TPY broths at pH 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 for 48 hours. Aciduricity of each bacterial strain was assessed by visible bacterial growth in TPY broth after 48 hours. Broth with no visible bacterial growth was plated on BHI to check for the bacterial survival. The lowest pH with visible bacterial growth in broth was defined as the MS minimal growth pH and the lowest pH with MS growth on BHI (brain heart infusion) plates was defined as the MS minimal survival pH.

To evaluate acidogenicity, the initial and final pH of each MS strain was measured with an Accumet pH meter 900 (Fisher Scientific, USA) before and after 48-hour incubation in BHI broth with 5 percent glucose under anaerobic conditions. The pH drop between initial and final reading was defined as its acidogenicity measurement.

Extracellular Polysaccharide Synthesis Assays

For polysaccharide extraction, MS strains were inoculated into BHI broth with 5 percent sucrose and incubated at 37ºC for 48 hours. The cultures were centrifuged (10,000g) for 30 minutes at 4ºC and the supernatant was collected for water soluble polysaccharide (WSP) extraction. The pellet was washed two times with PBS (phosphate buffered saline) and supernatants were combined for WSP extraction. The pellets were dissolved in 0.5N NaOH for 30 minutes in a 60ºC water bath and used for water insoluble polysaccharide (WISP). The NaOH incubation was repeated twice and the supernatants were collected by centrifugation and combined with the previous portions for WISP extraction. Three volumes of ethanol was added to culture supernatants or NaOH extracts for WSP or WISP and they were stored in the cold overnight to precipitate the polysaccharides. The precipitate was collected by centrifugation and dissolved in 5 ml deionized water for WSP and 0.5 N NaOH for WISP.[48,49] An Anthrone-sulfuric acid colorimetric microassay was used to quantify the polysaccharide in the extracts. For the anthrone-sulfuric acid colorimetric assay, 200 µl of extracted WPS or WISP was mixed with 5 ml of anthrone solution. The samples were heated in a boiling water bath for 12 minutes, and then cooled to room temperature. From each sample, 200 µl of was transferred into a 96-well microtiter plate and the absorbance was measured at 620 nm using a UV/visible Mircroplate Spectrophotometer.[50,51] Polysaccharide concentrations were measured against glucose standard solutions.

Statistical Analysis

The statistical analysis tests were performed at the strain level (between all strains from CA vs. CF) and at the subject level (subjects with strains with

virulence characteristics above average between the CA and CF groups). To compare the aciduricity, acidogenicity, and extracellular polysaccharide synthesis between CA and CF MS strains, the data distribution was analyzed first for continuous data. If the data showed a normal distribution, the student t test was used. Otherwise, the Mann-Whitney nonparametric test was used. To compare the number of subjects with more virulent strains above average between the CA and CF group, Fisher's exact test was used. The average is defined as the overall mean of each virulence factor for all the strains. The Mann-Whitney test was used to compare the number of strains that were more virulent than average within each subject.

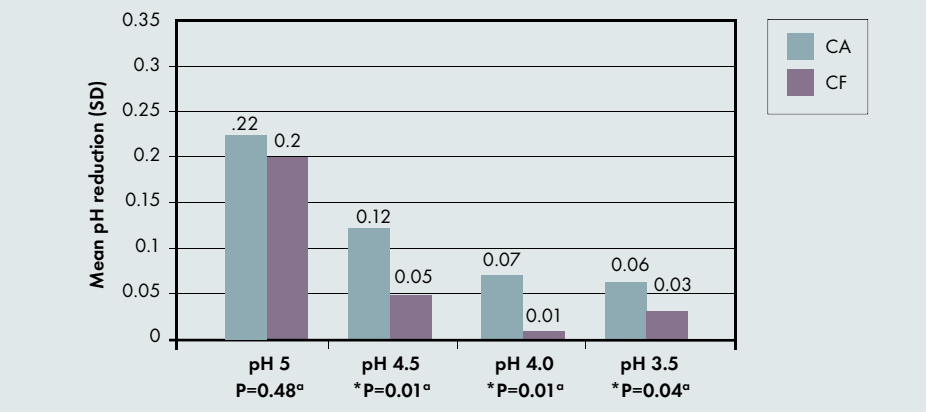

Figure 3. The bar graph presents the mean and SD pH reduction of mutans streptococcus strains isolated from caries-active (CA) and caries-free (CF) subjects.
**Statistically significant; [a]Mann-Whitney test*

Results

Mutans colonization levels and MS isolates from CA and CF subjects

All CA subjects had high MS levels over 10,000 CFU/ml of saliva, while only eight of 12 CF subjects had MS infection with only two CF subjects having MS over 10,000 CFU/ml (detailed results not shown). As previously reported, 43 different MS genotypes were identified from the 17 CA adults by AP-PCR (arbitrarily primed polymerase chain reaction) 37 genotypes were *S. mutans* and six were *S. sobrinus*.[18] Only 14 MS genotypes were identified from the CF adults and they were all *S. mutans*.

Mutans Streptococci Aciduricity and Acidogenicity

For aciduricity measurements, the mean (SD) minimal growth pH of MS strains from CA and CF groups was 4.70 (0.36) and 4.81 (0.15), respectively, with a range of 4.0-5.5. The mean (SD) survival pH of CA and CF MS strains were 4.06 (0.29) and 4.11 (0.36), respectively, with a range of 3.5-5.0 (**Figure 1**). There were no statistically significant differences in the mean aciduricity between CA and CF MS strains at strain level (t test, P>0.05). As shown in **Figure 2**, at subject level, significant more CA subjects (29 percent) had MS strains with minimal growth pH below average than CF subjects (13 percent, Fisher's exact test, P<0.05). All CA subjects had MS strains with minimal survival pH below average compared with 88 percent in the CF group (Fisher exact test, P>0.05). The mean (SD) of MS strains with minimal growth or survival pH below average in CA subjects was 1.80 (1.13) and 1.82 (0.64), while it was 0.13 (0.35) and 1.00 (0.53) in CF subjects, respectively, which were statistically significant (Mann-Whitney test, P<0.05).

For acidogenicity, there was no statistically significant difference between CA and CF strains on acid production in broth with pH ≥ 5.0 (**Figure 3**). However, MS strains from CA subjects produced significantly more acid at pH<5 (Mann-Whitney, P<0.05). No CF subjects were found to have MS strains with acid production ability at pH below 4.5 while 59 percent and 53 percent of the CA subjects still had one or more MS strains capable of generating acid at pH 4.0 and 3.5 respectively (Fisher's exact test, P<0.01) (**Figure 4**).

Mutans Streptococci Extracellular Polysaccharide Synthesis

MS isolates from CA subjects produced significantly more WISP with mean (SD) as 0.47 (0.58, CA) and 0.23 (0.13, CF) (Mann-Whitney test, P<0.01) while no statistically significant difference was found in WSP between the two groups with mean (SD) as 3.85 (2.93, CA) and 3.76 (2.38, CF), respectively (**Figure 5**).

More CA subjects had MS strains with WSP and WISP synthesis ability above average than CF subjects but there was no statistically significant difference between the two groups (**Figure 6**). Similarly, CA subjects also tended to have more MS strains with WSP and WISP synthesis ability above average but there was no statistically significant difference between the two groups.

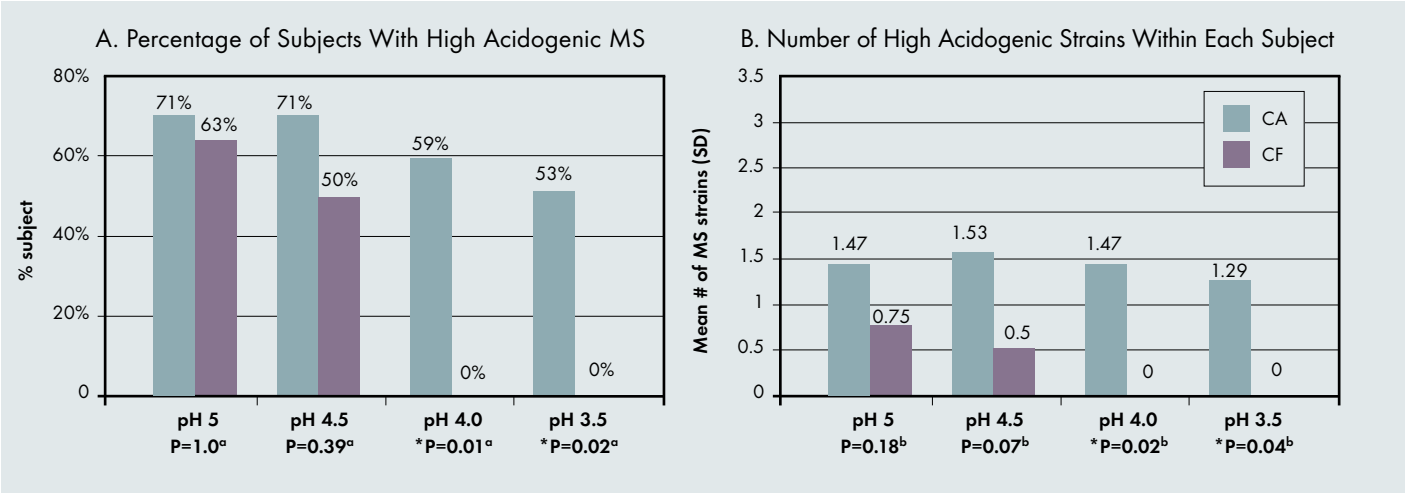

Figure 4. Panel A presents the percent of subjects with acid production ability at pH below 4.5 in caries-active (CA) and caries-free (CF) groups. Panel B presents the number of MS with acid production ability at pH below 4.5 within each subject.

**Statistically significant; [a]Fisher exact test; [b]Mann-Whitney test*

Discussion

Acidogenicity is one of major cariogenic virulence factors of the mutans streptococcus group. Mutans streptococci can metabolize a wide variety of carbohydrates and generate acid as byproducts. Early studies showed that acid production by *S. mutans* and *S. sobrinus* was significantly higher than other oral streptococci at neutral pH.[39-41] Moreover, rapid acid production by some MS strains persisted at lower than neutral pH.[38,39,41] The major acid product of MS when metabolizing sucrose and glucose is lactic acid.[47] Rapid acid production by MS enabled them to decrease plaque pH on the tooth surface dramatically and to initiate demineralization of tooth enamel or dentin. Although previous studies suggested variability in acidogenicity and aciduricity among different MS strains, no correlation between acid production of MS and caries status was found by Kohler and Napimoga, who compared MS acid production in broth at pH 5.5 and neutral pH, respectively.[38-41,46,47] In the authors' study, no significant difference was found between CA and CF group either at the overall strain level or subject level at pH ≥ 5.0. This agreed with the results of Kohler and Napimoga. However, MS isolates from CA subjects produced significantly more acid in TPY broth at pH ≤ 4.5 than those from CF subjects. When the acidogenicity was analyzed at the subject level, none of the CF subjects had MS strains producing acid at pH ≤ 4.0, whereas more than half of the CA subjects were infected with multiple strains being able to continue acid production below pH ≤ 4.0. Very importantly in this study, the authors found that MS in the CA group were able to produce acid at pH values well below 5.5 where enamel and dentin can dissolve rapidly.

Aciduricity is another cariogenic virulence factor of the MS group. Previous studies showed that *S. mutans* and *S. sobrinus* also had greater acid tolerance than other oral bacteria.[38,39] The high aciduricity of MS species enables them to survive and flourish during an acidic environmental challenge generated by them or by other species. It is the acidification of the local environment by the acid end-products of mutans streptococci that inhibits the survival of other competing bacterial species, enabling mutans streptococci to maintain their niche in the oral flora, incidentally causing dental caries in its host.[52] To the best of the authors' knowledge, no data on the relationship of aciduricity of MS clinical isolates and caries status has been published. The authors' results show that MS clinical isolates from the adults in this study were very aciduric. Most MS isolates were still able to grow at pH≤ 5.0 and survive at pH ≤4.5 while *S. sanguinis* 10556 and *S. gordonii* 10558 stopped growing at pH 5.5, and were killed at pH 5.5 or 5.0 (unpublished data). Although no significant difference in mean aciduricity was found amongst MS strains from the CA and CF groups, the CA

group (29 percent) had significantly more subjects infected by MS strains with minimal growth pH ≤ 4.5, and all CA subjects had MS strains that survived at pH ≤ 4.0. Moreover, CA subjects were also infected with multiple high aciduric strains compared to CF subjects. These results indicate that cariogenic potential of MS strains might vary between subjects and that the presence of multiple strains in CA patients may contribute to their increased caries risk.

MS clinical isolates in the present study were able to synthesize extracellular water soluble polysaccharide and extracellular water insoluble polysaccharide using sucrose as the substrate. The WSP serves as an energy store to extend acid production when the carbohydrate source is limited. The WISP is the major matrix component that promotes dental plaque formation, which in turn enhances MS accumulation on tooth surfaces. However, no statistically significant differences in WSP between CA and CF groups at neither strain nor subject level were found in the present study. This finding suggests that WSP synthesis does not play a critical role in caries risk. However, CA MS isolates produced significantly more WISP than CF isolates, which is consistent with other studies.[43-46] The finding further supports the importance of WISP formation by MS in cariogenicity.

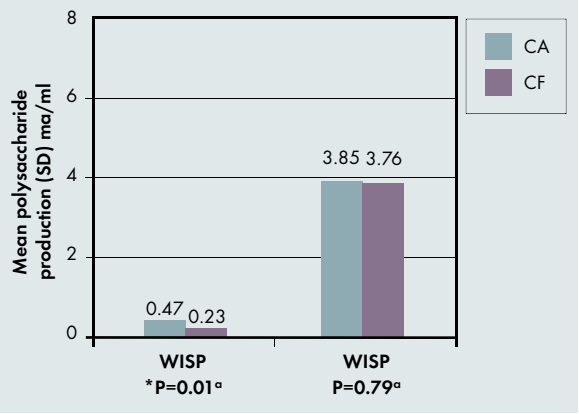

Figure 5. The bar graph presents the mean and SD of extrracellular water soluble polysaccharide (ESP) and water insoluble polysaccharide (WISP) of mutans streptococcus strains isolated from caries-active (CA) and caries-free (CF) subjects.

**Statistically significant; [a]Mann-Whitney test*

Conclusions and Clinical Relevance

Overall, the authors' results showed that MS strains from CA were more virulent in aciduricity, acidogenicity, and polysaccharide synthesis than those in the CF group. Furthermore, more CA subjects were infected by multiple strains with aciduricity, acidogenicity, and polysaccharide synthesis ability above average. The results indicate that differences in acidogenicity, aciduricity, and polysaccharide synthesis in strains of MS at least partially account for caries activity. The results emphasize that dental caries is a bacterial disease and that there are strains of bacteria that are more virulent than others, some highly virulent. These highly virulent strains were transmitted to the child initially, most likely from the parent or caregiver. Currently it is not possible to identify these strains in the clinical setting. However, in order to deal with children with high caries activity, it is necessary to control the bacterial activity and the transmission of the multiple virulent strains. When a high caries challenge is identified by ongoing caries activity antibacterial treatments, such as chlorhexidine rinses, for the older child and for parents or caregivers of younger children are likely to help. In the future, it is likely that better antibacterial treatments will be available for elimination of cariogenic bacteria in children as well as adults, and potentially that chairside identification of these strains will be possible as part of routine dental practice. CDA

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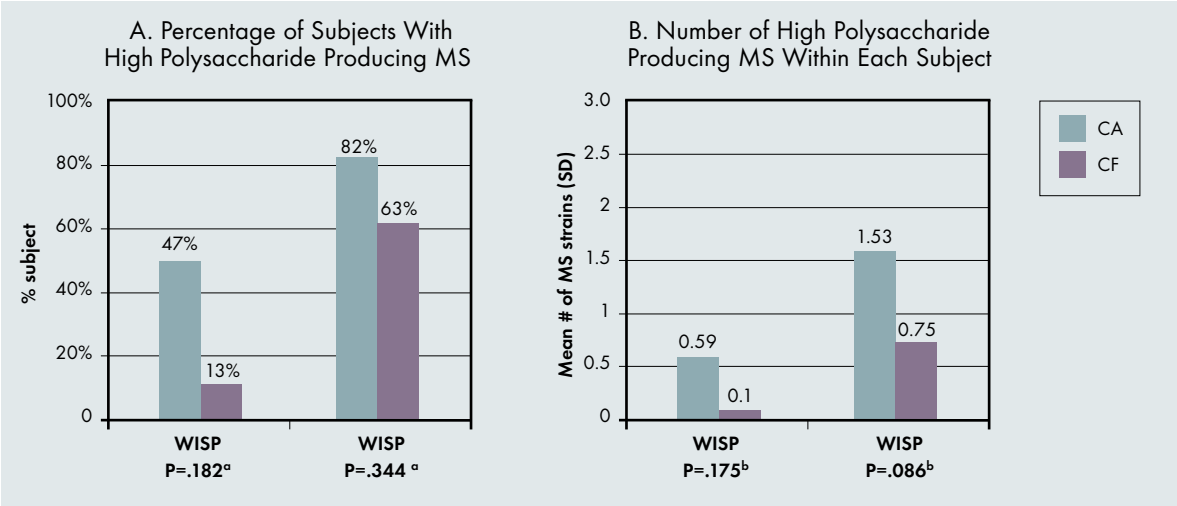

Figure 6. Panel A presents the percentage of subjects with polysaccharide production ability above average in caries-active (CA) and caries-free (CF) groups. Panel B presents the number of MS with polysaccharide production ability above average.

**Statistically significant; [a]Fisher exact test; [b]Mann-Whitney test*

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Robert E. Horseman, DDS

The Divine Ms. O

Back in the summer of 1939, you might have encountered at your corner newsstand the huddled figure of a 19-year-old youth whose only claim to adulthood beyond his size 12 feet, was the fact he owned a can of Burma Shave used but twice in the past month. Clad in the dirty corduroys and black Keds of the day, he thumbed hurriedly through the pages of the current *Photoplay* magazine before the proprietor of the newsstand sent him packing.

The boy was searching for pictures of Olivia de Havilland, a comely confection with whom he had become besotted since she was first spotted four years previously as Hermia in "A Midsummer Night's Dream." She was four years his senior, an unobtainable goddess, especially for a youth whose salary had peaked at the $24 per month he received from his paper route. The bulk of that went for the replacement of trousers whose right legs were forever being devoured by his insatiable bicycle chain.

It took me nearly 50 years to finally forgive Ms. de Havilland for succumbing to the lecherous wiles of Errol Flynn.

It took me nearly 50 years to finally forgive Ms. de Havilland for succumbing to the lecherous wiles of Errol Flynn, chewing the scenery as Robin Hood in the 1938 production of "The Adventures of Robin Hood and His Merry Hoodlums." Even now, in moments of nostalgia laced with masochism, I continue to look for evidence that the lovely Olivia is alive and well, still radiant at 89.

Unfortunately, I shall never find her regal likeness again in *Photoplay*, if, indeed, it still exists. That venerable publication has given way to a new generation of tabloid-inspired magazines such as *US, Life & Styles* and *In Touch*. Within their pages each month, if you have the buck ninety-nine to invest and are a member in good standing of the tattooed, body-pierced, under-20 crowd, you can be treated to the latest hyperbole from the entertainment world.

Served up weekly are the latest offerings of the paparazzi and cinematic dirt-diggers. Jimmy Fiddler, Louella Parsons and Hedda Hopper, the high priests of gossip monger-

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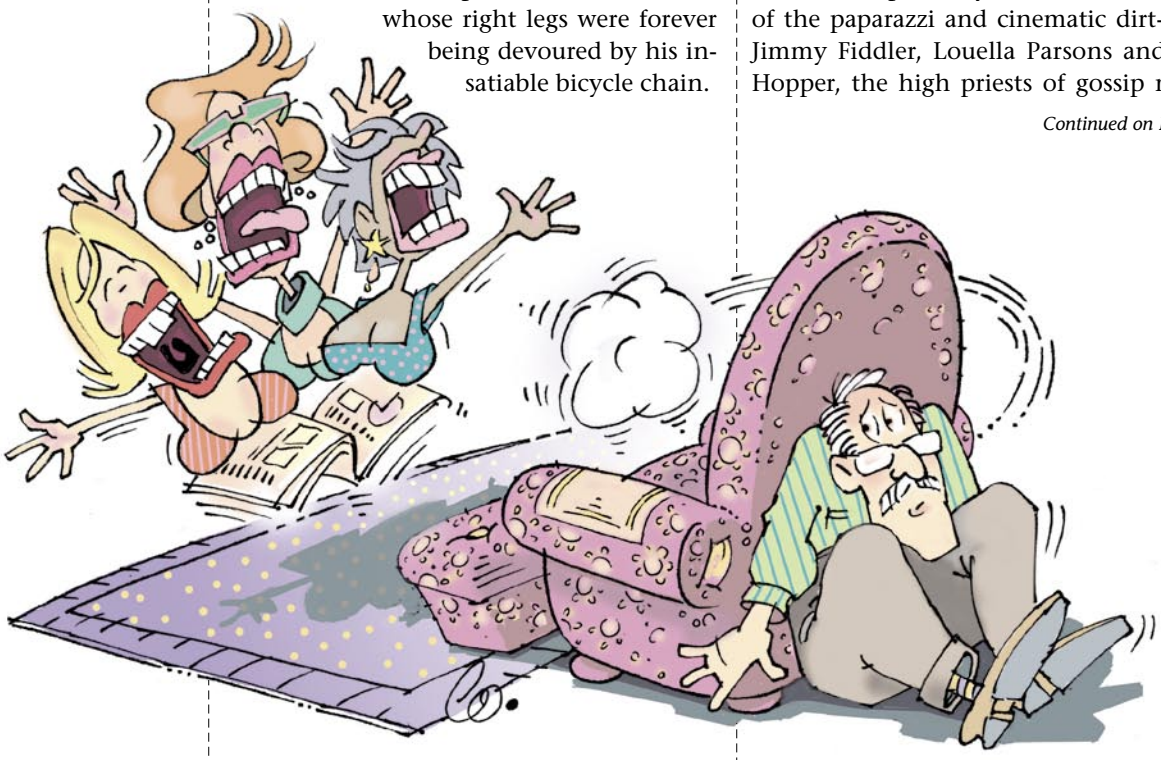

Bowser's mouth was large enough to accommodate a medium-sized watermelon. Darwin would have been enthralled.

Continued from Page 1018

ing in the '30s and '40s would never recognize the present-day genre. For example, we read in a piece holding the rapt attention of today's adolescent readers, "She (Sienna Miller) cries and cries — but look, no baggy eyes! As her world crumbles and her relationship with Jude (Law) hangs in the balance, how does Sienna stay so stunning?" It turns out she uses a cooling mask of cucumber and aloe vera amalgamated with a hydrating gel of wheat proteins. Curling her lashes has also helped assuage her grief in discovering her Significant Other has been smooching his child's babysitter on the side.

The point is — if there is one — the pop culture magazines appear to have unintentionally illuminated a medical-genetic phenomenon that has escaped the scrutiny of the entire scientific community. My own research has determined an actor-comedian by the name of Joe E. Brown, born in 1892, first gave hint that something was afoot. Mr. Brown's popularity and main claim to fame blossomed in the 1920s when it was noted he had a mouth approximately four times the size of a normal oral cavity. Not long afterwards, a comedienne-singer named Martha Raye (born 1916) assumed the distaff role with a mouth that was arguably even larger. It was rumored that if the two of them were to be in the same room and inhaled simultaneously, the windows and doors would implode.

Dentists of the era, if they noticed at all, failed to recognize this as anything beyond a one-time physiological anomaly instead of holding the potential of being the greatest thing for the profession since the introduction of the air-driven handpiece. To her credit, Ms. Raye did her best for dentistry during her stint as spokeswoman for a denture adhesive that she allegedly used herself.

A few years later, a quaint musical ensemble who couldn't get no satisfaction materialized, featuring an artist with lips seldom seen outside the pages of medical texts on food allergies. Singer Carly Simon arrived to challenge the oral dimensions of Martha Raye and a person called Bowser fronted with a troupe of other mutants calling themselves Sha Na Na. Bowser's mouth was large enough to accommodate a medium-sized watermelon. Darwin would have been enthralled.

Both the evolutionists and the Intelligent Design camps would have remained blissfully unaware of the part show business was playing in this outsize mouth phenomenon if it weren't for the magazines now featuring on every page the mouths of Julia Roberts, Jessica Simpson, Britney Spears, and a host of other female stars and wannabes. The depicted mouths are always open to the fullest extent their TMJs will allow, giving us a perfect view without retraction of the facial surfaces of the upper second molars, including an occasional glimpse of the uvula and pharyngeal tonsils. In short, a dentist's fondest dream come true. This windfall for dentists, however, is not necessarily shared by startled readers. Confronted with full-color prints of gaping mouths and unnaturally blinding white teeth, the effect is similar to what one would experience suddenly facing down a pack of insanely hungry wolves.

What is important now to both dentists and genetic engineers is to sort out whether the mega-mouth syndrome will continue to be featured primarily by females as a result of a yet unidentified rogue gene or is simply another well-kept plastic surgery secret like universal nose jobs and silicone/saline implants.

In any event, in my dotage, I still cherish the diminutive mouth of Ms. de Havilland, however unobtainable. Bless you, Olivia, wherever you are! CDA