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Bcl-2 expression is essential for development and normal physiological properties of tooth hard tissue and saliva production

Mohammad Ali Saghiri[a,*], Armen Asatourian[b], Zafer Gurel[a], Christine M. Sorenson[c], Nader Sheibani[a,d,e]

[a] Department of Ophthalmology and Visual Sciences, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States
[b] Angiogenesis and Regenerative Medicine Sector, Dr. H. Afsar Lajevardi Research Cluster, Shiraz, Iran
[c] Department of Pediatircs, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States
[d] Department of Biomedical Engineering, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States
[e] Department of Cell and Regenerative Biology, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States

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ABSTRACT

Background: Apoptosis plays a fundamental role in appropriate tissue development and function. Although expression of Bcl-2 has been reported during tooth and submandibular gland (SMG) development, the physiological role Bcl-2 plays during these processes has not been addressed. This study was performed to evaluate the impact of Bcl-2 expression on the formation and properties of tooth hard tissue, and saliva production.

Methods: Twenty-four mice (12 males and 12 females) were divided into three groups of eight (n=8): group A (Bcl-2 +/+), group B (Bcl-2 +/-), and group C (Bcl-2 -/-) and subjected to micro-CT analyses. The mineral content of first molars was analyzed by X-Ray diffraction (XRD) and scanning electron microscopy (SEM) color dot map. The surface microhardness was determined by Vickers test on labial surfaces of incisors. Saliva was collected from different groups of mice after subcutaneous injection of pilocarpine.

Results: Samples from Bcl-2 -/- mice showed significantly smaller micro-CT values, lower and poor crystallinity of hydroxyapatite (HA), and lowest surface micro hardness. SMG from Bcl-2 -/- mice showed remarkable reduction in size, consistent with reduced saliva accumulation.

Conclusions: The absence of Bcl-2 expression in SMG did not affect the expression of other Bcl-2 family members. Thus, Bcl-2 expression influence on the formation and properties of tooth hard tissue, and saliva accumulation.

Significance: Bcl-2 expression has a significant impact on the mineralogical content of enamel crystals of tooth structure. Lack of Bcl-2 expression led to impaired production of enamel ACP crystals.

1. Introduction

Programmed cell death (PCD) or apoptosis is a physiological process by which a cell terminates its life [1,2]. The apoptotic process, in addition to determining cell fate, also plays an important role in various cellular and tissue developmental stages including the formation of tooth structure [3]. The pro-apoptotic Bcl-2 family members maintain the integrity of mitochondria and regulate apoptosis. Bcl-2 family members include anti-apoptotic members Bcl-2, Bcl-w, Bcl-XL, A1 and Mcl-1, as well as pro-apoptotic members Bak, Bax, Bad, Bid, Bok, BNIP3, Blk, Bik and Bim proteins [4]. Among these proteins, Bcl-2 and Bax are prominent molecules with antagonistic effects on survival. The Bcl-2/Bax ratio has a determining effect on cell survival such that the overexpression of Bcl-2 leads to increased cell survival, while Bax overexpression causes apoptosis [5].

In the developing tooth, Bcl-2 is detected in the enamel organ, ameloblasts, odontoblasts, and subodontoblastic layers [6,7]. During the enamel formation and maturation about 50% of the ameloblast cells undergo apoptosis [8]. Irregular patterns of apoptosis can result in dysmorphogenesis and dental diseases including hypodontia, oligodontia, agenesis, and even periodontal disease [9]. In contrast to studies that mainly focused on the role of Bcl-2 expression after

Abbreviation: SMG, Submandibular gland; QPCR, Quantitative Polymerase Chain Reaction Analysis; SEM, Scanning Electron Microscope; HA, Hydroxyapatite; XRD, X-Ray diffraction; MHV, Microhardness Value

* Correspondence to: University of Wisconsin School of Medicine and Public Health, 1111 Highland Avenue, 9418 WIMR, Madison, WI 53705, United States.
E-mail address: saghiri@wisc.edu (M.A. Saghiri).

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development, LeBrun *et al.* demonstrated that Bcl-2 is expressed in stem cell populations, and tissues derived from endocrine and neural cells [10]. Thus, Bcl-2 may be involved in tissue morphogenesis contributing to formation of cell condensations, which are committed to create more differentiated structures such as salivary glands [10]. After maturation of tooth structure and salivary gland the expression of Bcl-2 family members declines such that limited expression of Bcl-2 is observed following birth [11], but it has been shown to play a critical role in dental tissue regeneration [12]. Although, Bcl-2 expression in salivary gland cells with basal cell differentiation is shown to be effective in formation of pleomorphic adenomas [13], the role Bcl-2 expression plays in the development and function of salivary glands remains unknown.

Appropriate development of salivary glands and production of saliva play crucial roles in tooth health and function. Salivary glands are one of the most important exocrine glands with critical roles in tooth homeostasis by secreting saliva [14]. Saliva has multiple functions inside the oral cavity including lubrication, digestion, taste, pH buffering, vocalization, debridement/lavage, antibacterial, antifungal, anti-viral, and immunological activity [15]. It also maintains the teeth post-eruptive maturation of enamel, and remineralization of tooth structures after demineralization by acid diffusion [16]. Unfortunately, the impact of Bcl-2 expression on these activities remains largely unknown. Here we investigated the impact of Bcl-2 expression in tooth morphogenesis and physical properties, as well as in salivary gland function. The effect lack of Bcl-2 has on the formation and properties of tooth hard tissue and saliva production were evaluated.

2. Materials and methods

2.1. Tooth analysis and sample preparations

All animal care and procedures were in agreement with the Principles of Laboratory Animal Care and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health. Twenty-four mice (twelve males and twelve females) were arranged in three groups (n=8, 4 males and 4 females) including: 1) Eight wild types that were used as the control group (Bcl-2 +/+); 2) Eight heterozygous mice (Bcl-2 +/-); and 3) eight homozygous mice with germline deletion of Bcl-2 (Bcl-2 -/-). The generation and screening of the Bcl-2 transgenic mice have been previously described [17]. We maintained a breeding colony of Bcl-2 +/- mice, and the progeny with the desired genotype used for this study. The evaluation of samples was performed on postnatal day 21.

2.2. The micro-CT analysis

The micro-CT analysis of experimental groups was performed by anatomic micro-CT scanning. Animals were sacrificed, the heads were wrapped in dry gauze, and scanned on the micro-CT unit using 3D micro-CT (Inveon, Siemens, Malvern, PA) with a 20 μm voxel size. All scans were visually evaluated by a dental clinician in a masked manner.

2.2.1. The mineralogical evaluations

According to Kim et al. [18] the components of five biological calcium phosphates including hydroxyapatite (HA), β-tricalcium phosphate (TCP), octacalcium phosphate (OCP), amorphous calcium phosphate (ACP), and dicalcium phosphate dehydrate (DCPD) were evaluated in the first molar of each mouse. Two left quadrants of mandible from each group of mice were selected randomly, most of the soft tissue was removed physically, and the mandibular bone was vertically grooved on buccal and lingual side, surfaced with diamond disk (KG Sorensen, 7015; Barueri -SP, Brasil) and a low-speed hand piece without entering the periradicular space, and then spilt longitudinally with a chisel to gently isolate the molar teeth. Samples were washed with ethanol and phosphate buffer saline (PBS; pH 7.4). The roots of the first molars were then sectioned 3 mm below the cemento-enamel junction and fixed on a metal slab, placed in 10% formalin and subjected to X-Ray Diffraction (XRD). The XRD analysis was performed as previously described by us [19] using a X-Ray diffractometer with Cu Ka radiation (40 kV, 30 mA). The operation was carried out in a step-scanning mode over a theta-2 range of 5–80 degrees with a step width of 0.01 and a count time of 10 s per step. In addition, samples in each group of mandible were randomly selected, and prepared for electron microscopy. This part of the study was similar to that previously reported by us [20]. Dot map was employed to determine the inorganic trace element distributions as previously described [20].

Table 1
List of primers used in this study.

Primer	Sequence (5'–3')
Bad-forward	GGAGCAACATTCATCAGCAG
Bad-reverse	TACGAACTGTGGCGACTCC
Bak1-forward	CCACATCTGGAGCAGAGTCA
Bak1-reverse	TGTCCAGATGCCATTTTTCA
Bax-forward	CCAAGAAGCTGAGCGAGTGTCT
Bax-reverse	AGCTCCATATTGCTGTCCAGTTC
Bcl2-forward	GGAGAGCGTCAACAGGGAGA
Bcl2-reverse	CAGCCAGGAGAAATCAAACAGAG
Bcl-XL-forward	CCTTGGATCCAGGAGAACG
Bcl-XL-reverse	CAGGAACCAGCGGTTGAA
Bim-EL-forward	AGTGTGACAGAGAAGGTGGACAATT
Bim-EL-reverse	GGGATTACCTTGCGGTTCTGT
Mcl1-forward	TTCCTTTACTGTTGGCGTGTT
Mcl1-reverse	AAAATGGCCAGTGAAGAGCA
RpL13A-forward	TCTCAAGGTTGTTCGGCTGAA
RpL13A-reverse	GCCAGACGCCCCAGGTA

2.3. The surface microhardness evaluations

The surface microhardness was evaluated as previously described by us [21]. Briefly, eight incisors from each group of mice were isolated and, after debridement of soft tissues by root scaling instruments, were stored in PBS at room temperature for less than 72 h. Samples were individually embedded in acrylic resin and the enamel of labial surfaces was prepared for microhardness measurements. The superficial enamel surface of teeth was flattened and polished with water cooled carborundum discs 1200 grit silicon carbide paper in order to remove about 200 μm enamel. The surface to be tested was fitted with a Vicker's diamond microhardness tester (Buehler Ltd, Lake Bluff, IL) and a 200 N load was used to make indentations in the enamel surface. The indentations were separated by 100 μm from each other on the labial surface. The loaded diamond was allowed to rest on the surface for 15 s. The indentations were carefully evaluated by an optical microscope and the average lengths of two diagonals were used to measure the microhardness value (MHV).

2.4. Salivary gland analysis and saliva collection

Saliva collection was performed according to methods presented by Marmary *et al.* [22]. Briefly, mice were anesthetized and placed in a holder for easier handling, and then were given pilocarpine 80 mg/kg in 0.1 mL subcutaneously. The saliva was collected in two 5-min intervals using a simple suction device. The animal's nose remained unobstructed for unhindered respiration. According to Lin *et al.* [23] tubing was used to collect the saliva from the oral cavity, while the nose was allowed to remain free for unhindered respiration. Saliva collection was performed every 15–30 s for 10 min and accumulated on ice. The investigator was masked to the mice genotype for saliva collections.