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Original Article

Apoptosis status and proliferative activity in mucopolysaccharidosis type I mice tongue mucosa cells

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ABSTRACT

Background: Mucopolysaccharidosis type I (MPS I) is caused by a deficiency of alfa-iduronidase (IDUA), which leads to intralysosomal accumulation of glysosaminoglycans. Evidences point secondary events like oxidative stress on lysosomal storage diseases including MPS I. Patients with MPS I present a wide range of oral clinical manifestations, including tongue hypertrophy, hypertrophyc alveolar process, and carious teeth. However, the mechanisms by which these alterations occur are still not fully understood. The aim of this study was to analyze the proliferative activity as well as apoptosis in tongue mucosa cells from murine model of MPS I.

Materials and Methods: Protein expression of apoptotic markers such as p53, bcl-2 and bax were evaluated in this setting. Ki-67 was used as a proliferative marker. All analyses were made by immunohistochemistry in tongue cells. Statistical analysis was perfomed by Kruskal-Wallis non-parametric test followed by the Dunn's test. $P < 0.05$ was considered for statistic significance.

Results: Histopathological analysis revealed no remarkable differences in tongue mucosa on MPS I mice when compared to control. By contrast, our results demonstrated that bcl-2 immunoexpression was decreased in mice tongue mucosa cells of MPS I mice. p53, bax and ki-67 immunoexpresssion did not show significant differences among controls and MPS I mice.

Conclusion: Taken together, our results suggest that IDUA deficiency, which characterizes MPS I, may induce apoptosis in mice tongue cells as a result of bcl-2 down regulation.

Key Words: Apoptosis, bax, bcl-2, ki-67 mucopolysaccharidosis, p53, tongue mucosa cells

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INTRODUCTION

Mucopolysaccharidosis I (MPS I) is an autosomal recessive disorder caused by deficient or absent activity of the lysosomal hydrolase α-L-iduronidase, which is responsible for the degradation of the glycosaminoglycans (GAGs) heparan sulfate and dermatan sulfate. A deficiency in the enzyme leads to accumulation of these substances in various tissues.[1] MPS I (Hurler, Hurler-Scheie and Scheie syndromes) is one of the most common lysosomal storage disease, with a reported incidence of 1.19 per 100,000 population in Europe.[1]

Patients with MPS I are characterized by skeletal malformations, hernias, cardiac disease and systemic hypertension, hepatosplenomegaly, and flexion contractures.[2] The oral and dental findings of MPS I include tongue hypertrophy, hypertrophyc alveolar process, carious teeth, hyperplastic gingiva, high-arched palate, short mandibular rami with abnormal condyles, spaced hypoplastic peg-shaped teeth with retarded eruption; and localized dentigerous cyst-like radiolucencies.[3] Despite the well documented oral and dental features of MPS I, little evidence on cell signaling has been demonstrated in tongue mucosa cells.

Apoptosis is a tightly regulated process of genetically

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programmed cell death by which senescent, damaged, and superfluous cells are eliminated from the body. Apoptosis is involved in the etiology of many chronic, often degenerative processes, including cardiac remodeling after myocardial stress.[4] Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. The Tp 53 gene encodes a nuclear phosphoprotein, so called the p53 protein.[5] This consists of 393 amino acids and comprises four amino-terminal transactivation domain, a core DNA-binding domain, carboxy terminal tetramerization and regulatory domains, with different functions.[6] The gene regulated by p53 protein is involved with cell cycle inhibition, and apoptosis by extrinsic pathway.[7] The bcl-2 proto-oncogene was originally discovered by analysis of the t (14;18) chromosomal translocation associated with human follicular B-cell lymphoma.[8] The bcl-2 gene encodes a membrane protein localized to the nuclear membrane, the inner surface of mitochondria, and the endoplasmic reticulum.[8] It is the most important gene of the bcl-2 family and has been shown to be an inhibitor of apoptosis.[9] Bax, another member of the bcl-2 family, is considered to be a major effector of apoptosis.[10] Bax forms heterodimers with bcl-2, in which its distribution is inversely related to that of bcl-2.[10] In MPS I mice splenocytes, an increase of apoptotic cell death was observed probably due to the leakage of cysteine proteases from the lysosomes.[11] However, the role of apoptotic proteins in tongue mucosa cells from MPS I has not still been elucidated so far.

As a result of inappropriate *in vivo* evidence, the aim of this study was to analyze apoptosis status in mouse tongue cells from mice model of MPS I. For this purpose, the histopathological analysis of the tongue tissue as well as immunohistochemistry for p53, bcl-2 and bax were performed. To monitor cell proliferation activity, ki-67 immunoexpression was also evaluated. Certainly such data will contribute to better understanding tissue alterations induced by IDUA deficiency that contributes to MPS I phenotype.

MATERIALS AND METHODS

Animals

All animal procedures were conducted according to the "Guidelines for Ethical Care and Use of Experimental Animals" published by the US National Institute of Health and were approved by the Institutional Ethics Committee of Universidade Federal de São Paulo (UNIFESP).

C57BL/6 Idua+/+ and Idua-/- mice were bred by heterozygous mating, which precursors were kindly provided by Dr. Elizabeth Neufeld (UCLA, USA) and Dr. Nance B. Nardi (UFRGS, Brazil). This MPS I mouse model has been briefly described by Ohmi *et al.,*[12] and is similar to that described by Clarke *et al.*[10] Animals were maintained on a 12 hours light/dark cycle with food and water available *ad libitum*. Genotyping was performed by polymerase chain reaction at the 30th day of life and all the experiments were performed with 6-month-old males and females. The Idua -/- mice are a model of MPS I as they lack enzyme activity, and at 6 months of age the pathology is completely installed.[2]

Histopathological analysis

The tongue tissue obtained from all experimental groups were washed immediately with saline and then fixed in 10% buffered neutral formalin solution. After fixation, the tongue tissue was processed embedding in paraffin. Then, the tongue tissue was sectioned and stained with hematoxylin and eosin (H and E). Histopathological evaluation was performed under light microscope. The pathologist was blinded to the animal category (control or MPS I). Analyzes of the tongue sections were performed, as follows: Presence of hyperplasia; dysplasia; hyperkeratosis; inflammatory process; and/or necrosis.

Immunohistochemistry

Serial longitudinal tongue sections of 4μm were deparaffinated in xylene and rehydrated in graded ethanol, then pretreated by microwave (Brastemp, SP, Brazil) with 10mM citric acid buffer (pH = 6) for 3 cycles of 5 minutes each at 850W for antigen retrieval. They were pre-incubated with 0.3% hydrogen peroxide in PBS (phosphate buffer salin) for 5 minutes for inactivation of endogenous peroxidase, and then blocked with 5% normal goat serum in PBS for 10 minutes. The specimens were then incubated with anti-bax antibody (Santa Cruz Biotechnology, USA) at a concentration of 1:200, anti-p53 antibody (Santa Cruz Biotechnology, USA) at a concentration of 1:200, or anti-bcl-2 antibody (Santa Cruz Biotechnology, USA) at a concentration of 1:50 or anti-ki-67 (Novocastrastra, USA) at a concentration of 1:200. Incubation was carried out overnight at 4^{o}C

within the refrigerator. This was followed by two washes in PBS for 10 minutes. The sections were then incubated with biotin-conjugated secondary antibody anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at a concentration of 1:200 in PBS for 1 hour. The sections were washed twice with PBS followed by the application of preformed avidin biotin complex conjugated to peroxidase (Vector Laboratories, Burlingame, CA, USA) for 45 minutes. The bound complexes were visualized by the application of a 0.05% solution of 3-3'-diaminobenzidine, and counterstained with Harris hematoxylin. For control studies of the antibodies, the serial sections were treated with rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at a concentration of 1:200 in place of the primary antibody. Additionally, internal positive controls were performed with each staining batch.

Quantification of immunohistochemistry

Sections stained using immunohistochemistry were analyzed for the percentages of immunopositive cells in control and experimental animals. A total of 1000 epithelial cells were evaluated in 3 to 5 fields at 400 × magnification. These values used as labeling indices.

Statistical methods

Statistical analysis was perfomed by Kruskal-Wallis non-parametric test followed by the Dunn's test using SPSS software pack (version 1.0). "*P*" value < 0.05 was considered for statistic significance.

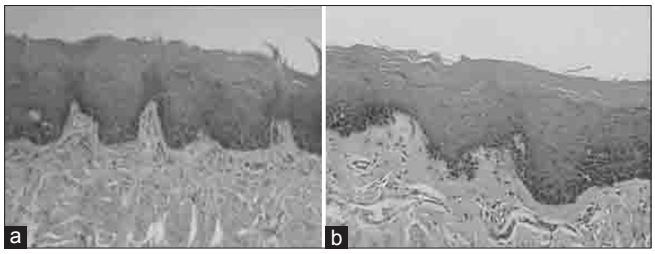

Figure 1: Photomicrographies showing mice with MPS I: (a) Control and (b) MPS I (H and E, ×100)

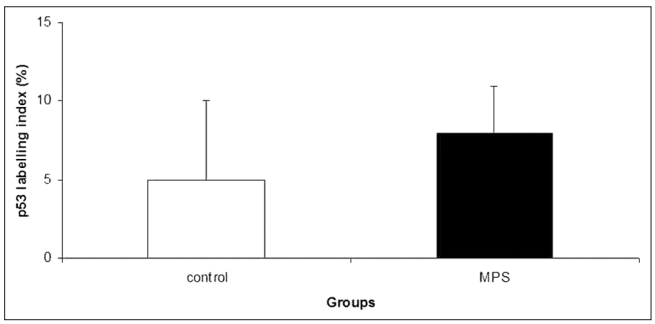

Figure 3: p53 labeling index in the negative control and those suffering MPS I. *P* >0.05

RESULTS

Histopathological findings

Histopathological findings of the control group pointed out tongue tissue presenting ordinary appearance [Figure 1a]. In the same way, MPS I mice did not show remarkable changes [Figure 1b].

Immunohistochemistry

The expression of p53 was detected in the nucleus of the tongue cells. Expression of p53 was weak in all negative controls as well as in the MPS I group [Figures 2a and 2b], respectively. P53 labelling index revealed no significant differences between groups. These numerical data are shown in Figure 3.

Bcl-2 expression was detected either in cytoplasm or in nucleus of the tongue cells. In the normal tongue epithelium, represented by the negative control group, immunostaining with anti-bcl-2 polyclonal antibody was weak and only identified in the basal and suprabasal cell layers [Figure 4a]. By contrast, bcl-2 staining was absent in the group of MPS I mice with remarkable differences when compared to controls [Figure 4b] Such findings are summarized in Figure 5.

Immunoexpression with anti-bax antibody was seen in the prickle and granular layers of the

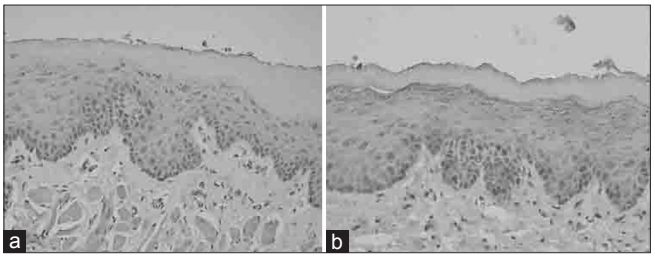

Figure 2: Expression of p53 noticed in the oral mucosa cells (a) Control; (b) MPS I; (×400)

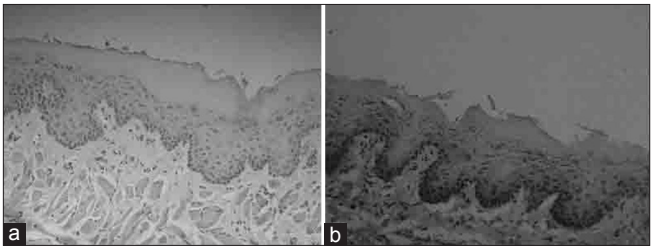

Figure 4: Expression of bcl-2 noticed in the oral mucosa cells (a) Control; (b) MPS I; ×400

epithelium [Figure 6a]. The positivity for bax was homogeneous in MPS I mouse tongue mucosa cells without significant differences between groups [Figure 6b]. Bax labelling index showed no significant statististically differences ($P > 0.05$) between groups [Figure 7].

Finally, Ki-67 positive-nuclei in control group were confined to the basal cell layer of tongue mucosa [Figure 8a]. However, this was observed in the same layer in the MPS I group [Figure 8b] histomorphometric data are showed in Figure 9.

In the negative controls for immunohistochemistry, they confirmed no staining for all antibodies used.

DISCUSSION

The goal of this study was to investigate whether MPS I mice present apoptosis and/or proliferation activity in the tongue tissue. The effects of IDUA deficiency on the histopatological changes and immunohistochemistry for p53, bcl-2, bax and Ki-67 were evaluated. To the best of our knowledge, the approach has not been addressed so far.

Regarding the histopathological examination, MPS I mice revealed no remarkable changes when compared to control group. Therefore, it seems that the enzyme deficiency was not able to induce histopathological changes in tongue tissue of this MPS I mice model. It has been postulated that the storage of GAGs within the oropharynx with associated enlargement of the tonsils and adenoids among MPS I patients can contribute to upper airway complications along with narrowed trachea, thickened vocal cords, redundant tissue in the upper airway and an enlarged tongue.[13] Taken as a whole, our data suggest that enzyme deficiency in MPS I did not induce histopathological changes in tongue cells. Further research will be required to fully clarify this issue.

To further elucidate putative mechanisms of action involving IDUA deficiency on mouse tongue cells, we designed additional experiments by using immunohistochemistry. Regarding p53 immunomarker, our results demonstrated no remarkable differences between groups. However, some studies have argued that engulfment of cells, due to GAGs accumulation and impaired autophagic vacuoles recycling, results in activation of inflammation and eventually in cell death.[14] Therefore, it seems that MPS I did not induce p53 activation in this setting. It is important to emphasize that MPS I in mice did not show signs of tongue involvement, suggesting that substrate accumulation does not occur in this organ. This provides relevant information to determine whether the abnormal pathways observed in MPS I organs only occur in the presence of GAGs accumulation and therefore to assess whether the phenotype observed

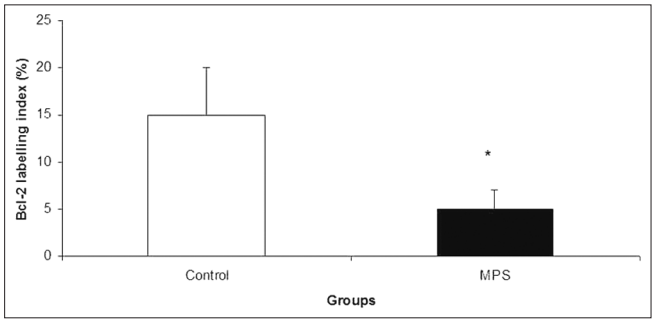

Figure 5: Bcl-2 labeling index in the negative control and those suffering MPS I. $P < 0.05$ when compared to negative control

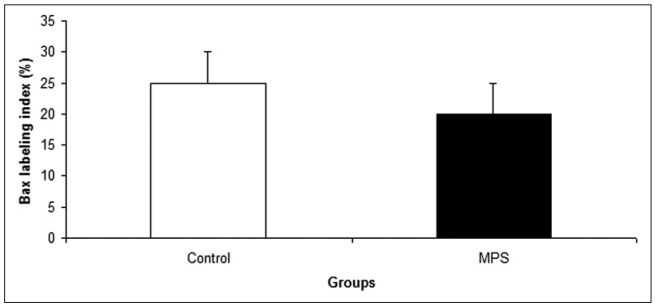

Figure 7: Bax labeling index in the negative control and those suffering MPS I. $P > 0.05$

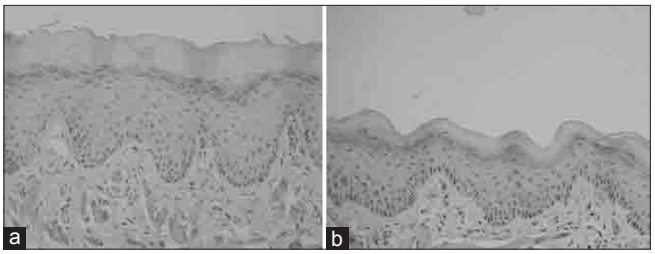

Figure 6: Expression of bax noticed in the oral mucosa cells (a) Control; (b) MPS I; × 400

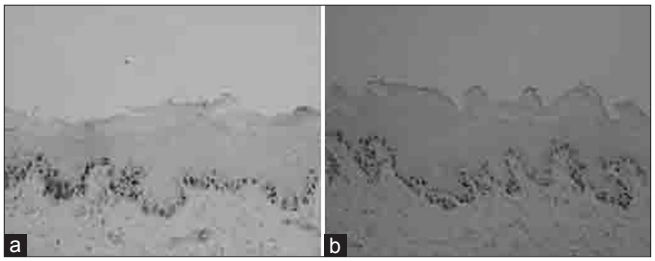

Figure 8: Expression of Ki-67 noticed in the oral mucosa cells (a) Control; (b) MPS I; × 400

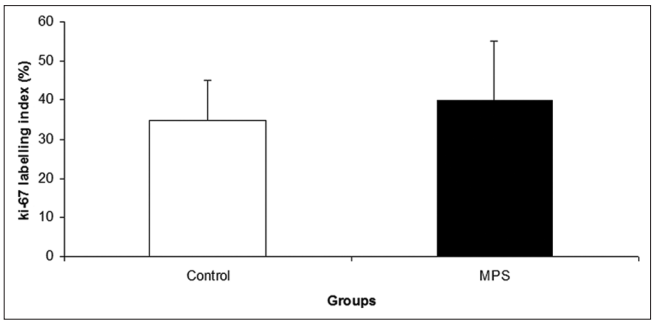

Figure 9: ki-67 labeling index in the negative control and those suffering MPS I. $P > 0.05$

in cells and affected organs is due to lysosomal storage or are consequence of secondary events. It is important to emphasize that MPS I is a progressive disease and, the data we obtained referred to animals 3 months old. This probably explains our negative results. The analysis of older animals could confirm if other modifications in tongue tissues occur or in fact no alterations occurs among these animals.

Taking into consideration intrinsic apoptosis pathway, our results pointed out an effect, since there were differences in bcl-2 expression between groups. Particularly, tongue cells from MPS I mice did not show bcl-2 immunoexpression when compared to control group. The subcellular distribution of bcl-2 is a matter of controversy.[15] For example, Hockenbery reported that bcl-2 is found primarily in the inner mitochondrial membrane.[16] Conversely, others have postulated that bcl-2 resides primarily in the nuclear envelope, endoplasmic reticulum and outer mitochondrial membranes.[17]

Bax did not show remarkable differences between experimental and control groups. By comparison, some authors have revealed apoptotic cell death in central nervous system by means of TUNEL assay.[14] These data clearly indicate that the presence of abnormal degradation pathways, inflammation, and apoptosis is strongly associated with lysosomal storage in MPS tissues. In fact, mitochondria produce metabolic energy and free radicals (that is, reactive oxygen species-ROS), serve as biosensors for oxidative stress, and eventually become effectors of apoptosis.[18] Mice MPS I cells and tissues may suffer increasing oxidative stress resulting in inflammation, which finally triggers cell death responses as observed in different disorders.[19,20] Despite the differences in the type and the amount of metabolites accumulated in lysosomal storage diseases as well as the cells or tissues where storage occurs, the clinical and pathological manifestations are to some extent similar among the diseases, thus suggesting common mechanisms triggered by different genetic defects. Identification of critical cellular mediators within these processes may help develop therapies to target them and biomarkers for follow-up of disease progression and therapeutic intervention. Most importantly, our data support a strong association between lysosomal storage and apoptosis deregulation by bcl-2 down regulation in tongue mucosa cells *in vivo*.

Cell proliferation is regarded as one of the most important biological mechanisms in some degenerative diseases.[21] Our results demonstrated no differences in MPS I model mice. These findings are new being the mechanism unknown yet and so, difficult to explain. Independent of biological mechanism involved to this phenomenon, we assume that MPS I did not interfere with proliferation activity in tongue cells of mice.

CONCLUSION

The results of this study suggest that IDUA deficiency induces bcl-2 down regulation in mice tongue cells, which in turn could impact apoptosis process in these cells. Certainly, this finding offers new insights into the mechanisms underlying the relation between IDUA deficiency and tongue lesions that can occur in MPS I patients.

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