SHORT COMMUNICATION

BCL-2 expression in epithelial rest of Malassez, both *in vivo* and *in vitro*

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Introduction

The bcl-2 gene was originally discovered for its involvement in the t(14 ; 18) chromosomal translocation in B-cell lymphomas. The bcl-2 gene product, BCL-2, has been reported to block apoptosis and is involved in tissue development and tumorigenicity^{1,2)}.

The expression of BCL-2 has been demonstrated in the epithelial part of the human tooth germ except for the enamel-forming ameloblasts at their fully differentiated stage³⁾. We showed the localization of BCL-2 in ameloblastoma, suggesting that BCL-2 contributed to the development of ameloblastoma by blocking apoptosis⁴⁾. A part of these odontogenic epithelial cells termed the epithelial rest of Malassez⁵⁾, maintained a long life span in the periodontal ligament even after tooth development was completed. We hypothesized that the epithelial rests expressed the BCL-2 maintained their existence and escaped from apoptosis. Therefore, the present study examined whether the epithelial rests of Malassez expressed BCL-2 in both *in vivo* and *in vitro* conditions.

Materials and Methods

Tissue sections and immunohistochemistry

The premolars and their surrounding tissues from a six month-old pig were fixed in 4% paraformaldehyde in a 0.1 M phosphate buffer. The tissues were sliced at 5 mm thickness and decalcified in 5% trichloroacetic acid solution for 5 days. Sections were dehydrated in a series of graded alcohols, embedded in paraffin and

sectioned at 5 μ m thickness. After treating with 0.3% H₂O₂ in methanol to block endogenous peroxidase, the sections were soaked in a 0.1 M sodium citrate buffer, placed in the 500 w microwave oven and heated intermittently for 10 min. Sections were washed well and then immersed in 10% normal mouse serum to block non-specific binding. After washing with PBS, the samples were incubated with antihuman BCL-2 monoclonal antibody (DAKO, Denmark) (dilution 1/20) at 4°C overnight. The samples were rinsed with PBS and the streptavidin-biotin peroxidase complex method was carried out using a Histofine kit according to the manufacture's instructions (Histofine kit (M) Nichirei, Tokyo). The reactions were visualized by DAB (0.05% diaminobenzidine and 0.005% H₂O₂ in 0.05 M Tris-buffer, pH 7.6) and counterstained with hematoxyline. The primary antiserum was omitted or preimmune serum was substituted for the control. The immunocytochemical staining was replicated 3 times.

Cell isolation and cell culture

Epithelial cells from porcine epithelial rests of Malassez were isolated from the periodontal ligaments and cultured as previously reported⁶⁾. In brief, the explants of periodontal ligaments obtained from an extracted pig premolar were cultured in Dulbecco modified Eagle medium (DMEM) (Nissui, Tokyo, Japan) containing 15% fetal bovine serum (FBS) (Cancera, Canada) and antibiotics [penicillin G potassium 100 μ g/ml (Meijiseika, Tokyo, Japan), kanamycin sulfate 60 μ g/ml (Meijiseika, Japan), amphotericin B 30 μ g/ml (FUNGIZONE®, Bristol-Myers Squibb K. K. Japan) at 37°C in a humidified

atmosphere of 95% air and 5% CO₂. An outgrowth comprised of both epithelial-like and fibroblast-like cells was observed after 14 to 20 days of incubation. The epithelial cells were separated from the two cell types to be more resistant to detachment by 10% dispase (Goudousyusei Inc. Japan). These cells were removed from the rest of the cell population and discarded. Attached cells were cultured and then added to DMEM containing 15% FBS. The separation procedure was repeated 2-3 times and the fibroblasts were disposed. For immunocytochemical staining, the cells were plated on coverslips, and the streptavidin-biotin peroxidase complex method was employed, using the same antibodies as the paraffin sections, omitting the blocking of endogenous peroxidase and counter stained with hematoxyline.

Western blot

Cells were washed twice with ice-cold PBS. Lysate cells were generated with ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 250 mM NaCl, 1%NP-40, 50 mM NaF) containing protease inhibitors (1 mM PMSF, 5 µg/ml aprotinin, and 5 µg/ml leupeptin). Insoluble material was removed by centrifugation at 15,000 rpm for 20 min. The samples were electrophoresed on 15% SDS-polyacrylamide gels. After electrophoresis, gels were either stained with Coomassie blue or transferred to nitrocellulose (Amersham, USA) using a semidryblotting apparatus (Bio-rad, USA). After blocking with 5% BSA in TBS, a membrane was incubated in anti-human BCL-2 monoclonal antibody (Oncogene Science, USA). The expression of BCL-2 was detected by the enhanced chemiluminescence system (Amersham, USA) according to the manufacture's instructions.

Results and Discussion

A number of clusters of the epithelial rests of Malassez were observed in the cementum side of the periodontal ligament from a 6 month-old pig. Immunohistochemical staining of BCL-2 showed that positive reactions were observed in all of the epithelial rests of Malassez (Fig. 1). The localization seemed to be limited to the cytoplasm. The isolated epithelial

rests of Malassez *in vitro* were found to be BCL-2 positive. Most of the cells exhibited the localization of BCL-2 in the cytoplasm (Fig. 2). The localization may correspond to previous reports which demonstrated by immunoelectron microscopy that BCL-2 localized at the nuclear membrane and mitochondria^{7,8}). A few rounded cells expressed the BCL-2 not only in cytoplasm but also in nuclei (Fig. 2). The rounded epithelial cells on the culture dish usually indicated cell death or mitosis. Willingham and Bhalla (1994)⁹ demonstrated the BCL-2 localization in the nuclei of the early prophase or late G₂ stage. They implied that BCL-2 might inhibit endonucleosomal DNA cleavage and apoptosis in normal mitosis. The rounded cell found to be BCL-2 positive in the nuclei may represent early prophase or late G₂ stage at the beginning of mitosis. Because the normal keratinocyte-including cells these epithelial cells did not proliferate well under normal culture conditions but proliferated well under low calcium conditions¹⁰, the rounded cells showing BCL-2 were few. The restricted localization in the cytoplasm *in vivo* may be reflected in the resting of the epithelial cells. By the Western blot analysis of the cell extracts, the BCL-2 immunoreacted with a fine single band (MW : 25 kD) (Fig. 3). The results confirmed biochemically, the BCL-2 expression in the epithelial rests of Malassez.

Although the BCL-2 was originally discovered in B-cell lymphoma, the BCL-2 have been localized in nonlymphoid tissues¹¹). The tissues expressing BCL-2 were grouped as (a) glandular epithelia in which hormones or growth factors regulate hyperplasia and involution, (b) cornified epithelial differentiation such as skin and intestine characterized by stem cells, and (c) long-lived postmitotic cells such as neurons. These tissues seemed to contain long-lived cells, but not all long-lived cell types displayed BCL-2 expression, e. g., muscle and other mesodermal tissues. BCL-2 appeared to be restricted to tissues in which apoptosis molds developed structures or accounted for cell turnover¹¹). Although the epithelial rests of Malassez do not turnover but rest in the periodontal ligament physiologically, an irritation, such as inflammation, derived from infection of the root canal, stimulates the epithelial cells to proliferate leading to cell turn-

Fig. 1 Immunohistochemical staining of BCL-2 showed that positive reactions were observed solely at the epithelial rests of Malassez (arrows).

Fig. 2 The epithelial cells isolated from the rests of Malassez also showed BCL-2 positive.

Although most of the epithelial cells exhibited the localization of BCL-2 in the cytoplasm, some rounded cells expressed the BCL-2 not only in cytoplasm but also in nuclei (arrows).

Fig. 3 Western blot demonstrated BCL-2 immunoreaction of the cell extracts at 25 kD.

over. Therefore, the BCL-2 expression in the epithelial rest of Malassez may be consistent with previous reports.

A number of roles for the epithelial rest of Malassez have been suggested, beside the formation of epithelial lining cysts associated with tooth. One of the roles was a consistently protective role for the ankylosis of periodontal ligaments between bone and cementum^{12,13}. Inoue *et al.* (1995)¹² reported that the ankylosis occurred unless the epithelial rests of Malassez exit during the regeneration of periodontal ligaments. The epithelial cells must be long-lived cells to keep the periodontal ligament from ankylosis under physiological conditions. BCL-2 is well known to block apoptosis. The expression of BCL-2 may maintain the long life of the epithelial rests, avoiding apoptosis.

Slootweg and de Weger (1994)³ showed the localization of BCL-2 in all parts of the enamel organ except the ameloblasts, indicating that these epithelial components contained stem cells and thus could bring about neoplastic formation. The present study demonstrated the localization of BCL-2 in the epithelial rests of Malassez, suggesting that certain odontogenic tumors may be originated from the epithelial rests of Malassez.

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