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THE ANALYSIS of APOPTOSIS in AMELOBLASTOMA: EVALUATION of Bcl-2, Bcl-X, Bax, Bak

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Abstract

The apoptotic behavior of ameloblastomas was studied using antibodies against the apoptosis related proteins, Bcl-2, Bcl-X, Bax, and Bak. Most of the outer layer cells were predominantly stained by the anti-Bcl-2 antibody, while most of the inner layer cells were stained by antibodies against the apoptosis modulating proteins, Bax and Bak. Among the Bcl-2 family, Bcl-2 was the most ubiquitously expressed protein in ameloblastomas, while Bcl-X was expressed in the greatest concentrations. The acanthomatous areas overexpressed the apoptosis modulating proteins, especially Bak. The outer layer, whose cells had higher apoptotic inhibitor activity than inner layer cells, is suggested to be an active part.

Introduction

An ameloblastoma is a slow growing, non-metastatic, but highly recurrent tumor. Ameloblastoma cells have various proliferating activities depending on the histological type and cytological pattern.[1,2] Both apoptosis and the proliferating activity of the cell are implicated in the development of ameloblastoma.[3] In the previous investigation, we examined the expression of Bcl-2.[4] However, since other Bcl-2 family proteins are expressed in different patterns and regulate cell life at different stages of cell differentiation,[5] it seems important to analyze the expression pattern of these proteins in ameloblastoma. Accordingly, in the present study, we analyzed the apoptotic behavior of ameloblastoma using antibodies against Bcl-2, Bcl-X, Bax and Bak proteins in relation to the histological type and cytological pattern.

Materials and Methods

Tissue Sample Selection

We selected ameloblastoma tissue blocks from 32 patients, 20 males and 12 females, who had been operated on at our hospital. When the tumors were classified according to the World Health Organization International, Histological Typing of Odontogenic Tumors, 2nd Ed., 9 were follicular, 9 were plexiform, 5 were unicystic, 3 were basal cell, 3 were acanthomatous and 3 were desmoplastic ameloblastomas.

Immunohistochemistry

For immunohistochemistry, an antigen retrieval method was employed. The first antibodies were diluted as follows: mouse monoclonal antibody to Bcl-2 (100/D5, novocastra, Newcastle, UK) was diluted 50 times, while rabbit polyclonal antibody to Bcl-X (Dako, Carpiteria, CA, USA), rabbit polyclonal antibody to Bax (Dako, Carpiteria, CA, USA) and goat polyclonal antibody to Bak (N-20, Santa Cruz Biotechnology, USA) were diluted 100 times.

Normal oral mucosae were stained as positive controls. Negative controls were provided by substituting PBS for the first antibodies. The tissue sections were labeled with a streptavidin-biotin method using DAKO-LSAB Kit (LSAB2 Kit Peroxidase, Dako, Carpiteria, CA, USA) and 1:200 diluted biotin-conjugated affinity-purified secondary antibody (Chemicon, Temecula, CA, USA). The tissue sections were visualized with diaminobenzidine (DAB, Histofine, Nichirei, Tokyo, Japan).

Evaluation and Statistical Analysis

At least 1200 cells were counted for each tissue section using a ×40 objective and an eye-piece graticule. Index was expressed as the percentage of positive cells/whole counted cells. The results were evaluated in relation to WHO histological classification and cytological pattern.

Results

Immunohistochemistry

Almost all tissue sections were stained by anti-Bcl-2 antibody. Bcl-2 was detected main-ly in the outer layers of epithelial tissues in ameloblastomas, and only a few cells were positively stained in the inner layers. Anti-apoptotic index of the outer layer cells was significantly higher than that of the inner layer cells ($P<0.05$). Bcl-X and Bax were dispersed rather equally in the outer and inner layers. Bak was more highly expressed in the inner layer cells and the apoptotic index of inner layers was significantly higher than that of the outer layers ($P<0.05$). Acanthomatous areas were densely stained by anti-Bak antibody. There was no significant relation between the apoptotic index and the each type of WHO histological classification. The ratios of anti-apoptotic to apoptotic index showed that the apoptosis was inhibited in the outer layer, but dominant in the inner layer.

Discussion

The family of Bcl-2 related proteins constitutes one of the biologically most relevant classes of apoptosis-regulatory gene products, acting at the effector stage of apoptosis.[6] Immunoreactivity for the Bcl-2 product is present mainly in cell populations, which are long lived, and/or with high proliferation ability[7]. In the present study, Bcl-2 was seen mainly in the outer layers of ameloblastoma. Among the Bcl-2 family proteins tested, Bcl-2 was most prevalently

seen in the ameloblastoma tissues studied (only one case was negative), suggesting that most of the ameloblastoma tissues contained Bcl-2 protein.

Bcl-2 and Bcl-X differ in their ability to associate with at least one proapoptotic family member,[8] and are likely to have other alterations in cell cycle control pathways. In the present study, Bcl-X was stained more strongly than Bcl-2 and its average anti-apoptotic index was higher than that of Bcl-2. It suggested that Bcl-X played a dominant role in the apoptosis of ameloblastoma.

Bax shows extensive amino acid homology with Bcl-2 and forms homodimers and heterodimers with Bcl-2 in vivo. When Bax predominates, programmed cell death is accelerated and the death repressor activity of Bcl-2 is countered.[9] In the present study, the ratio of Bcl-2 to Bax and the ratio of Bcl-X to Bax were significantly higher for the outer layer cells than for the inner layer cells, suggesting that the outer layer cells have the characteristic of apoptotic inhibition, while the inner layer cells were undergoing apoptosis.

In the present study, the ratio of Bcl-2 to Bak and that of Bcl-X to Bak were much higher in the outer layers than in the inner layers, indicating that Bak did not counter the Bcl-2 and Bcl-X in the outer layers. Expression of Bak was much higher in the inner layers, especially, in the acanthomatous ameloblastoma.

For conclusion, ameloblastoma has much apoptosis inhibiting protein than the apoptosis modulating protein, considering that Bcl-2 was found in almost all samples and Bcl-X had the highest labelling index. Acanthomatous areas which were stained strongly by Bak and numerous terminally apoptotic cells were located, were suggested as matured and differentiated areas. This study will be useful for understanding the apoptotic behavior of ameloblastoma.

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fracture healing, a number of growth factors and cytokines such as BMP-2, TGF-β, PDGF-B and Osteopontin are present at the elevated level in and around the fracture site. It suggests those factors play an active role in promoting fracture healing but the regulation of this phenomenon in a mandibular fracture has not been clearly understood. Taking this into consideration, we investigated the control mechanism that coordinates recruitment, localization, and function of different cell populations at the mandibular fracture.

Material and Methods

48 male Wistar rats, 12-week, weight 320-350 gram, that have had their right ramus mandible fractured with a bending clamp were used in this study. On days 3, 7, 14, 21 after the operation, the animals were sacrificed with perfusion technique. The fractured mandibles were excised, fixed with 4% PFA, decalcified with 20% EDTA, pH 7.4 at 4°C and embedded with OCT compound to prepare 5μm section. Immunohistochemical staining using 3% H_2O_2 for 20 minutes to block the endogenous activity. To evaluate the presence and localization of those growth factors and cytokines during fracture healing, we used goat polyclonal antibody against rat BMP-2, rabbit polyclonal antibody against rat TGF-β and PDGF-B, and mouse monoclonal antibody against rat Osteopontin as primary antibody and incubating for over night at 4°C. Final development of the sections were carried out with 3,3-diaminobenzidine-tetrahydrochloride and observed under the microscope. Counter staining was made 10% Hematoxylin.

Result

Immunohistochemical studies show that on day 3 after fracture BMP-2, PDGF-B and Osteopontin were detected in undifferentiated mesenchymal cells at the thickened periosteum near fracture site while TGF-β was slighily detected. On day 7 after fracture, staining for BMP-2, PDGF-B and Osteopontin were strong in proliferating fibroblasts-like spindle cells and proliferating osteoblasts in the intramembranous ossification and in various types of chondrocytes. TGF-β was detected in proliferating osteoblasts and proliferating chondrocytes. On day 14 after fracture, BMP-2, TGF-β, PDGF-B and Osteopontin were continuously staining in the osteoblastic cells in the newly formed periosteal trabecular bone, but decreasing in the hypertrophic chondrocytes. Immunostaining for TGF-β, PDGF-B and Osteopontin was observed in multinucleated osteoclasts-like cells on the newly formed trabecular bone. On day 21 after fracture, immunostaining for BMP-2 was decreasing in osteoblasts. TGF-β, PDGF-B and Osteopontin were slightly observed on both osteoblats and osteoclasts.

Discussion

There is no doubt to expect that several cytokines and growth factors are present and participate at elevated levels at fracture site during healing. Our results suggest that BMP-2 has the ability to stimulate progenitor cells, moderately stimulate the activity of osteoblasts but has a small effect on mature osteoblasts and chondroblasts. High expression of TGF-β in proliferating chondrocytes suggests that TGF-β may stimulate proliferation of initial callus formations. Also, chondrocytes may synthesize high level of TGF-β. Staining of TGF-β in the hypertrophic chondrocytes cells may show a role of these cells in regulating cartilage calcification and in endochondral ossification to induce the maturation osteoblasts, chondrocytes and also osteoclasts lineage. The expression of PDGF-B in intramembranous ossification was shown to contribute in part to the promotion of osteogenesis by mesenchymal cells from the early to the midphase of fracture healing. We suggest that PDGF-B acts as an autocrine and paracrine factor. Osteopontin was expressed in the proliferating osteoblasts and

in the matrix of new bone during intramembranous and endochondral ossification which may be correlated with mineralization. Osteoclasts are stained by Osteopontin during fracture healing suggest that Osteopontin may facilitate the adhesion of the osteoclasts to the bone surface to initiate bone remodeling.

Conclusion

This study shows that many cytokines and growth factors are expressed in different cellular events according to their response and function to fracture healing. Presumably, those factors are the key regulators of cellular proliferation, differentiation, and synthesis of matrix during fracture healing.

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