Apoptosis-related Factors (TRAIL, DR4, DR5, DcR1, DcR2, Apoptotic Cells) and Proliferative Activity in Ameloblastomas

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Abstract. Background: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a recently identified death factor that acts as a potent apoptosis inducer in ameloblastomas. Materials and Methods: The expression of TRAIL and its receptors (TRAIL-R), and the location of apoptotic cells were evaluated in 15 cases of ameloblastoma using immunohistochemistry and an in situ DNA nick-end labeling method. The proliferative activity of ameloblastomas was analyzed by determining the Ki-67 labeling index. Results: TRAIL and TRAIL-R were diffusely expressed in ameloblastomas, without clear correlation with the location of apoptotic cells. Apoptosis and proliferation were opposite in the peripheral and central components of the ameloblastomas. In some ameloblastoma variants, apoptosis and proliferation seemed to modify in the same direction. Conclusion: TRAIL and its receptors might be involved in neoplastic transformation of odontogenic epithelium and might suggest some intrinsic regulation of neoplastic cell proliferation and death in ameloblastomas, thus explaining their slow growth and inability to metastasize.

Apoptosis, also known as programmed or physiological cell death, is involved in many important cellular processes, including normal cell turnover and embryonic development, as well as in oncogenesis. It is now believed that clonal expansion and tumor growth result of the deregulation from cell proliferation and death. The survival of aberrant cells resulting from the inhibition of apoptosis is expected to contribute to the development and progression of neoplasms.

Therefore, the evaluation of apoptotic processes and proliferative activity of a neoplastic cell population may be helpful for predicting the biological behavior of the lesion. Death-receptor-induced cell death is one of the main pathways for apoptosis. Tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) is a death factor of the TNF family. TRAIL can interact with two different functional death domain-containing membrane receptors: death receptor 4 (DR4/TRAIL-R1) and DR5/TRAIL-R2. Moreover, there are two additional antagonist decoy receptors for TRAIL: decoy receptor 1 (DcR1/TRAIL-R3) and DcR2/TRAIL-R4, which lack an intracellular signaling domain or have a truncated death domain, and a third soluble receptor, osteoprotegerin (OPG). DcR1, DcR2 and OPG are capable of competitively inhibiting the effect of DR4/DR5 and are unable to activate apoptotic signal transduction upon stimulation (1).

Odontogenic epithelium is responsible for the development of teeth in physiological conditions, but can also give rise to cystic lesions and neoplasms of the jaws. The most frequent tumor arising from odontogenic epithelium is ameloblastoma, characterized by a benign but locally invasive behavior and a high recurrence rate.

Recent studies (2-6) have investigated the expression of apoptosis-related factors and the location of apoptotic cells in tooth germs and ameloblastomas, demonstrating the importance of apoptosis in odontogenesis and tooth eruption, and suggesting a role for apoptosis in the oncogenesis and cytodifferentiation of neoplasms deriving from odontogenic epithelium.

Other studies (2, 5, 7-12) reported differences in cellular proliferative activity, not only between the different components of the same neoplasm, but also between the different variants and histological types of ameloblastoma suggesting that the evaluation of proliferative activity may be useful for explaining the different biological behavior of these lesions.

This study was performed to determine the apoptotic behavior of ameloblastomas by analyzing the role of TRAIL and its receptors (TRAIL-R), and the location of terminally
apoptotic cells using immunohistochemistry and an in situ DNA nick-end labeling method. In addition, the proliferative activity of ameloblastomas was analyzed by determining the Ki-67 labeling index.

Materials and Methods

Tissue sample selection. Ameloblastoma tissue blocks from 15 patients (8 males and 7 females; age range 17-82 years, mean age 40 years), who had been operated on at the hospital of L’Aquila, Italy, were selected. Fourteen ameloblastomas were taken from the mandible and 1 was from the maxilla. The tissue blocks were cut into 3 μm thick sections for histological classification and subsequent immunohistochemical examination. According to the World Health Organization histological typing of odontogenic tumors (13), the tumors comprised 6 solid (1 follicular, 2 plexiform, 2 acanthomatous, 1 granular cell type), 5 multicystic (1 follicular, 3 plexiform, 1 granular cell type), 2 intraluminal unicystic (both plexiform), and 2 peripheral ameloblastomas (1 follicular and 1 plexiform). Four of these ameloblastomas had recurred during follow-up and their recurrences were also analyzed giving a total of 19 cases.

Immunohistochemistry. The tissue sections were deparaffinized, microwaved in 0.01 M citrate buffer (pH 6.0) for 15 min (except for those to be stained with the anti-TRAIL antibody) and immersed in 3% hydrogen peroxide solution for 6 min. After treatment with blocking serum for 30 min, the sections were incubated with primary antibodies at 4°C overnight. The antibodies used were anti-TRAIL (Santa Cruz Biotechnology, CA, USA; diluted 1:100), anti-DR4 (ProSci Incorporated, Poway, CA, USA; diluted 1:200), anti-DR5 (Chemicon International, Temecula, CA, USA; diluted 1:400), anti-TRAIL-R3 (Alexis Biochemicals, San Diego, CA, USA; diluted 1:20) and anti-DcR2 (Chemicon International; diluted 1:300). Staining for Ki-67 was performed using a Vectastain ABC Kit, Burlingame, CA, USA. The chromogenic reactions were visualized with diaminobenzidine (DAB). Finally, the tissue sections were counterstained with hematoxylin.

Terminal deoxynucleotidyl transferase-mediated dUTP-nick end-labeling (TUNEL) assay. The TUNEL assay was performed according to the protocol of an Apop Tag Plus in situ Apoptosis Detection Kit (Intergen, NY, USA). Briefly, the tissue sections were deparaffinized. Proteinase K (20 μg/ml; Serologicals, GA, USA) was applied for 15 min to digest the protein in the tissue sections. Then, the tissue sections were incubated in 3% hydrogen peroxide in PBS for 5 min and immersed in equilibration buffer for 10 sec. Working strength terminal deoxynucleotidyl transferase (TdT) enzyme was applied to the tissue sections for 1 h at 37°C in a humidified chamber. After applying anti-digoxigenin-peroxidase for 30 min in a humidified chamber, tissue sections were immersed in DAB substrate working solution for 6 min. Finally, the tissue sections were counterstained with hematoxylin. Positive and negative controls were provided according to the protocol.

Evaluation and statistical analysis. The expression of TRAIL and its receptors in the lesions were classified as negative, weakly/ focally positive or strongly/diffusely positive. The expression of Ki-67 and TUNEL were analyzed by counting at least 1000 cells, in randomly selected areas, for each tissue section using a x40 lens and an eye-piece graticule. The data were analyzed using Mann-Whitney U-tests and p-values of <0.05 were considered statistically significant. The pattern of expression of TRAIL and TRAIL-R, the percentage of Ki-67 positive cells and the percentage of terminally apoptotic cells were evaluated in relation to the clinico-pathological variants, the WHO histological type and the presence or absence of recurrence.

Results

Immunohistochemical reactivity for TRAIL was detected in the cytoplasm of the neoplastic odontogenic epithelial cells, whereas the expression of TRAIL-R was recognized both in the cell membrane and in the cytoplasm (Figure 1) and both were expressed in most of the samples, with different patterns of reactivity. Only a few cases were negative for TRAIL and DR4, and none for DR5, DcR1 and DcR2. None of the samples showed qualitative or quantitative differences in the expression of TRAIL and its receptors between the central stellate reticulum-like component and the peripheral columnar component of the ameloblastomas. The evaluation of the immunophenotypes of the different histological types of ameloblastoma demonstrated that the expression of TRAIL and TRAIL-R is extremely heterogeneous both in degree and in distribution. The keratinizing cells in the acanthomatous ameloblastomas showed strong and diffuse reactivity for TRAIL (Figure 2A) and TRAIL-R. The granular cells in the granular cell ameloblastomas were strongly positive for TRAIL (Figure 2B) and negative or only faintly positive for TRAIL-R. The reactivity patterns of the different ameloblastoma variants did not show significant differences. The only noteworthy result was that all the cases of unicystic ameloblastoma showed a different degree of reactivity for TRAIL in the cyst lining (positive) and in the intraluminal nodule (weakly and focally positive) (Figure 3).

The evaluation of the expression of TRAIL and TRAIL-R in the recurrent ameloblastomas did not produce any significant results. The ameloblastomas tended to maintain unchanged immunophenotypes in their recurrences; some minor, apparently meaningless variations occurred.

The mean Ki-67 labeling index of the peripheral columnar cells of the ameloblastomas was significantly higher than that of the central stellate reticulum-like cells (p<0.05), with a significant positive correlation (r=0.79). The highest mean proliferative activity were demonstrated in the granular cell ameloblastomas, followed by the plexiform ameloblastomas. The follicular and acanthomatous ameloblastomas exhibited low Ki-67 reactivity. The differences between the labeling indices of the histological types were statistically significant (p<0.05). As regards the mean proliferative activity of the
different variants of ameloblastoma, the peripheral and unicystic ameloblastomas showed the highest values, followed by the multicytic and solid ameloblastomas, the latter characterized by a rather scarce fraction of Ki-67 positive nuclei; the differences were not, however, statistically significant. In the unicystic ameloblastomas, the mean Ki-67 reactivity of the intraluminal nodule was higher than that of the cyst lining, but without statistical significance. The ameloblastomas that recurred exhibited a mean Ki-67 labeling index considerably higher than the ameloblastomas without recurrence, but the difference was not statistically significant.

The TUNEL method revealed a small number of terminally apoptotic cells, located almost exclusively in the central stellate reticulum-like component of the ameloblastomas; only in one sample (a plexiform ameloblastoma occurring in the maxilla) was the presence of apoptotic cells observed in the peripheral columnar component. The apoptotic indices were highest in the granular cell ameloblastomas. The localization of terminally apoptotic cells was wholly seen among the cells of the inner component, mainly in the granular cell clusters (Figure 4). Numerous scattered apoptotic cell fragments were also seen. The plexiform ameloblastomas presented high TUNEL labeling indices in 62.5% and low in 25% of the cases, whereas in 12.5% of the cases, as well as in the follicular and acanthomatous types of ameloblastoma, no terminally apoptotic cells were seen. As regards the apoptotic activity of the different variants of ameloblastoma, the unicystic ameloblastomas showed high indices, with most apoptotic cells located in the intraluminal nodule. In the multicytic/solid type, the apoptotic indices were high in approximately half of the cases and low or zero in the remainder. The peripheral ameloblastomas showed no reactivity by the TUNEL method. The comparison of the apoptotic indices of the ameloblastomas with reference to recurrence did not show significant differences.

Discussion

It has recently been reported that TRAIL is a potent apoptosis inducer in ameloblastomas (14, 15). The expression of TRAIL and its death receptors DR4 and DR5 in ameloblastomas has previously been analyzed only by Kumamoto and Ooya (6). They detected immunohistochemical reactivity for TRAIL especially in the epithelial cells neighboring the basement membrane, whereas in the present study, there were no differences in TRAIL expression between the central stellate reticulum-like component and the peripheral columnar component of the ameloblastomas. Moreover, in the ameloblastoma variants, these authors found a low TRAIL expression in the keratinizing cells of acanthomatous ameloblastomas; in contrast, in the present study the acanthomatous areas were strongly and diffusely positive for TRAIL. They also identified increased TRAIL expression in the granular cells of granular cells ameloblastomas, a finding confirmed by the present study. Kumamoto and Ooya recognized diffuse reactivity for DR4 and DR5 in ameloblastomas and widespread expression of these receptors was also found in most of the present samples. Moreover, the present immunoreactivity for DcR1 and DcR2 showed that the death receptors were also extensively expressed in the ameloblastomas, without differences between the central and the peripheral components. In addition, in all of the present cases of unicystic ameloblastoma, a different degree of reactivity for TRAIL was found in the cyst lining (positive) than in the intraluminal nodule (weakly and focally positive). Furthermore, the pattern of expression of TRAIL and TRAIL-R tended to remain unchanged in the ameloblastomas recurrences.

Kumamoto and Ooya (6) found no clear correlation between the expression of TRAIL and TRAIL-R and the localization of apoptotic cells analyzed in their previous studies. In fact, they detected apoptotic cells detached from the basement membrane in ameloblastomas, and markedly increased apoptotic activity in the keratinizing and granular cells in the ameloblastoma variants (2, 4). The present study confirmed the presence of terminally apoptotic cells almost exclusively in the central stellate reticulum-like component of the ameloblastomas. Moreover, our results were in agreement with the high apoptotic index of the granular cell ameloblastomas but, in contrast to previous studies (2, 16), failed to demonstrate increased apoptotic activity in the acanthomatous ameloblastomas. Among the ameloblastoma variants, the unicystic ameloblastomas had the highest apoptotic index, with most apoptotic cells located in the intraluminal nodule, whereas the peripheral ameloblastomas showed no reactivity by the TUNEL method. The recurrent ameloblastomas did not exhibit significant differences in apoptotic activity.

Several studies have analyzed the cellular proliferative activity of ameloblastomas as assessed by proliferating cell nuclear antigen (PCNA) and Ki-67 labeling indices. Positive nuclei were found predominantly in the basal cell layers of ameloblastomas (2, 5, 7, 9-11). Also in the present study, the mean Ki-67 labeling index of the peripheral cells was statistically significantly higher than that of the central cells. No significant difference between the proliferative activities of the different histological types was found in one study (12), while higher proliferative activity of follicular ameloblastomas than of plexiform ameloblastomas (5, 9-11) and higher labeling indices in the plexiform type (8) have also been reported. The latter study also found that acanthomatous ameloblastomas had the highest percentages of positive nuclei. The present study showed statistically significant differences in labeling indices between the different histological types, granular cell
ameloblastomas exhibiting the highest Ki-67 labeling index, followed by plexiform ameloblastomas, whereas the follicular and acanthomatous ameloblastomas showed low Ki-67 reactivity. Unicystic ameloblastomas have been shown to have a lower proliferative activity than the solid type (5, 8, 10, 11), supporting the contention that the lower recurrence rates of this variant are the result of a lower proliferative capacity. In contrast to all previously reported studies, statistically significant higher PCNA and Ki-67 labeling indices in unicystic ameloblastoma than in the solid and multicystic variants was demonstrated (7, 17), suggesting that the lower recurrence rates of the unicystic variant appeared rather to be a reflection of the different morphology, which provided greater accessibility and ease of surgery. This observation is supported by the present results, which demonstrated high Ki-67 labeling indices in the peripheral and unicystic variants. In the unicystic ameloblastomas, the mean Ki-67 reactivity of the intraluminal nodule was higher than that of the cyst lining, as in the study of Kim and Yook (12). In both studies, however, the differences were not statistically significant. In the present study, the recurrent ameloblastomas exhibited a mean Ki-67 labeling index considerably, although not significantly, higher than the ameloblastomas without recurrence, which was consistent with PCNA results (8, 12) and suggested that proliferative activity could be of practical importance in predicting the biological behavior of ameloblastomas.

Kumamoto (2) provided further evidence that Ki-67 positive nuclei are especially evident in the cells neighboring the basement membrane and showed that Ki-67 reactivity in granular cell ameloblastomas tends to be lower than in plexiform and follicular ameloblastomas. He suggested that apoptosis might be the opposite of cell proliferation in ameloblastomas. This observation was confirmed by Sandra et al. (16), who demonstrated that ameloblastomas have two relatively distinct patterns, an anti-apoptotic proliferating site in the outer layer (periphery) and a pro-apoptotic differentiating site in the inner layer (center). The present results corroborated the low apoptotic and high proliferative activities of the peripheral columnar component, and the opposite behavior of the central stellate reticulum-like component of ameloblastomas. Furthermore, in the present study, the apoptosis and proliferation of at least the granular cell, acanthomatous and unicystic ameloblastomas seemed to modify in the same direction.

In conclusion, TRAIL, its death receptors DR4 and DR5 and its decoy receptors DcR1 and DcR2 are diffusely expressed in ameloblastomas, confirming that TRAIL and its associated molecules might be involved in neoplastic transformation of odontogenic epithelium. Nevertheless no clear correlation between the expression of TRAIL and its receptors and the apoptotic behavior of ameloblastoma cells was found. The evaluation of proliferative activity may be useful to explain the different biological behavior of ameloblastomas. Opposing

Figure 1. Immunohistochemical expression of TRAIL and its receptors in a case of follicular ameloblastoma. DR 4, 5: death receptor 4, 5; DcR1, 2: decoy receptor 1, 2.
apoptosis and proliferation occur in the peripheral and central components of ameloblastomas, and may occur in the same direction in some types of ameloblastoma. This might suggest some intrinsic regulation of neoplastic cell proliferation and death in ameloblastomas, which might control the development and the progression of these lesions, thus explaining their slow growth and inability to metastasize.

References


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