FULL PAPER   *Anatomy*

Distribution of Apoptotic Cells and Apoptosis-Related Molecules in the Developing Murine Palatine Rugae

Hajime AMASAKI[1], Miyuki OGAWA[1], Jun NAGASAO[2], Ken-ichiro MUTOH[2], Nobutsune ICHIHARA[3] and Masao ASARI[3]

[1]*Department of Veterinary Anatomy, Nippon Veterinary and Animal Science University, 1–7–1 Kyonan-cho, Musashino-shi, Tokyo 180–8602, Japan,* [2]*Department of Veterinary Anatomy, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada-shi, Aomori 034–8628 and* [3]*Department of Anatomy and Physiology, School of Veterinary Medicine, Azabu University, Sagamihara-shi 229–8501, Japan*

ABSTRACT. Distribution of apoptotic cells and expression of the apoptosis-related factors p53, bcl-2 and bad during morphogenesis of the murine palatine rugae (PR) were examined histochemically using the terminal deoxynucleotidyl transferase-mediated UTP nick end-labeling (TUNEL) technique and specific antibodies against apoptosis and cell cycle-related molecules. Formation of the PR rudiment was controlled by cell proliferation and apoptosis in the palatal epithelium. TUNEL-positive cells were detected only at the epithelial placode area at 12.5–13.5 days post coitus (dpc), but only a few cells were positive at the protruding PR area at 14.5–16.5 dpc. Bcl-2 protein was expressed mainly in the areas outside of those containing TUNEL-positive cells at 15.5 –6.5 dpc. P53 protein was not detected throughout gestation. Bad was detected in the epithelial layer at 13.5 and 15.5 dpc and overlapping the apoptotic area at 13.5–15.5 dpc. Apoptosis of palatal epithelial cells might therefore involve spatiotemporally regulated expression of bad during murine PR development.
KEY WORDS: apoptosis, bad, bcl2, morphogenesis, palatal ridge.

Palatine rugae (PR) are formed as a corrugated pattern on the hard plate of the murine oral roof [1, 2, 4, 20, 21]. PR patterns form quite regular structures. We have studied PR pattern formation mechanisms [1, 2, 20, 21]. PR develop similarly to other organs in the epithelium, eg., hair follicles [7, 8], and feather buds [7, 9]. Epithelium and subepithelial mesenchyme of the epithelial organs develop under the reciprocal controls of each different tissue. Pattern formation of PR could also involve spatiotemporally regulated epithelial cell proliferation and apoptotic death, as occurs with chick feather [9] and scale [22, 23] development. Our previous study indicated that differential cell apoptosis during placode formation might maintain the epithelial population in the PR rudiment [20]. Apoptosis may regulate cell population and play a role in organogenesis [5, 14, 18]. Apoptosis can be induced by growth factor withdrawal, glucocorticoids [13, 26], fas ligand/fas interaction [12, 19], killer T cells [17], ionising radiation, drugs, oxygen radicals [3], tumour necrosis factor α (TNFα) TNF-receptor 1 interaction [15] etc. However, the signals inducing epithelial cell apoptosis at the PR epithelial placode are not known.

In the present study, we examined the spatiotemporal distribution of apoptotic cells by using the terminal deoxynucleotidyl transferase-mediated UTP nick end-labeling (TUNEL) technique and assessing the presence of p53 (anti-tumor apoptosis enhancement factor) [10], bcl-2 (B cell lymphoma/leukaemia-2) [24], bad (apoptosis enhancement factor) [25] molecules by immunochemistry during PR formation in the mouse.

MATERIALS AND METHODS

Animals: All animals were kept in an air-conditioned room, and fed standard laboratory food pellets and water *ad libitum*. Pro-estrous female mice were mated with male mice overnight. Gestation was confirmed by the presence of a vaginal plug next morning, and this time point was designated as 0.5 days post coitus (dpc). Each animal was sacrificed by cervical dislocation, and fetuses were removed from the uterus on the selected dpc. Dissected fetal heads were fixed in Bouin's solution for microscopic observation. The experimental procedure and care of animals were in accordance with the guideline of the Animal Care and Use Committee of Nippon Veterinary Animal Science University.

Detection of apoptotic cells: Deparaffinized sections were digested with proteinase K (Sigma-Aldrich Co., St. Louis, Missouri, U.S.A.; 20 μg/ml) for 5 min at room temperature. After inactivation of endogenous peroxidase activity with 2% H_2O_2, apoptotic cells were identified by *in situ* apoptosis detection kit (Takara Bio Inc., Outsu, Shiga., Japan) based on the TUNEL technique. All procedures were performed according to the manufacturer's instructions. Negative control staining check was carried out as normal procedure of TUNEL method only except for non-terminal deoxynucleotidyl transferase reaction. Positive control sample was used mouse thymuses at 6 months old. Section was assessed by light microscopy and photographed.

Detection of the cell cycle and apoptosis-related factors p53, bcl-2 and bad: Endogenous peroxidase was inactivated
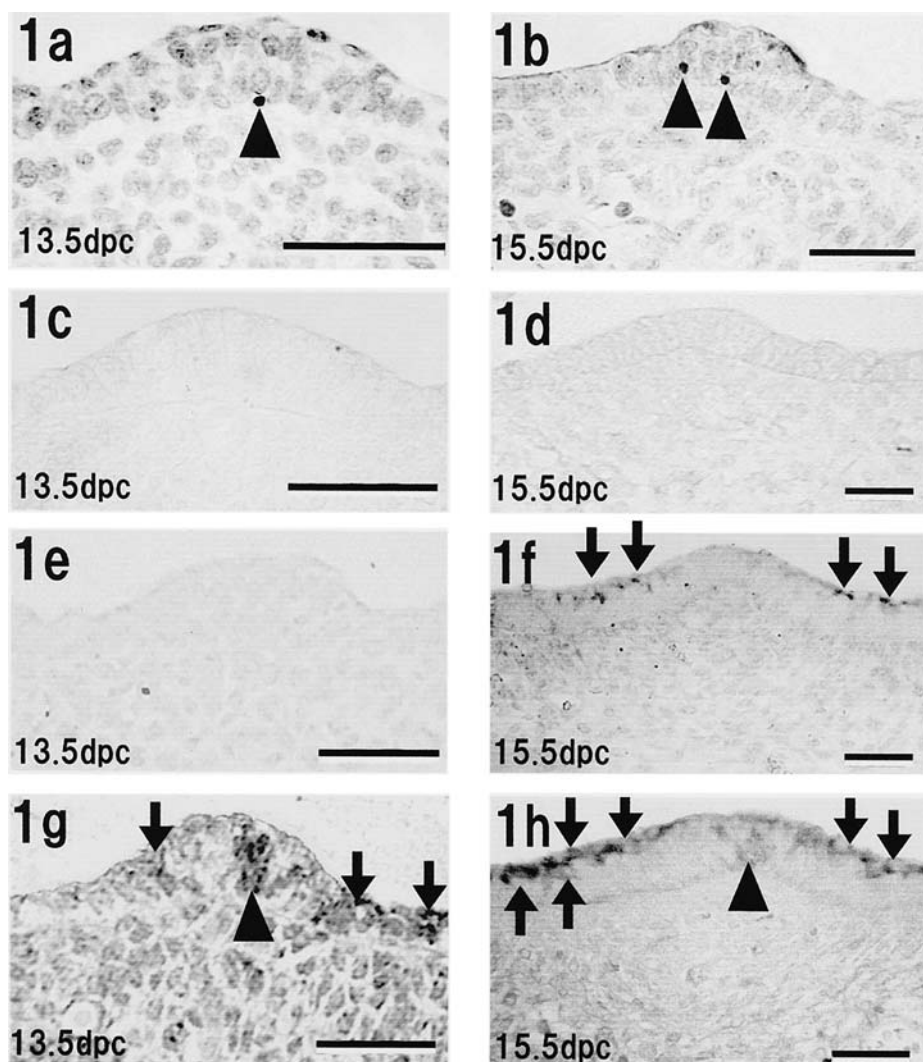
in deparaffinized sections with 2% H_2O_2. Prior to first antibody incubation, each section was treated with the microwave method for the anti-masking signals [6]. The section was then incubated with a 1st antibody specific for p53, bcl-2 or bad (Transduction Labs. Inc., Lexington, KY, U.S.A.; working dilution of all antibodies was used 25 μg/ml). Detection of the first antibody was carried out using the avidin-biotin complex (ABC kit; Nichirei Bio. Sci. Co., Tsukiji, Tokyo, Japan) method and 3, 3'diaminobenzydine (DAB) coloration, and sections were examined and photographed using an optical microscope after application of the nuclear contrast stain, methyl green.

RESULTS

Distribution of TUNEL-positive cells: TUNEL-positive cells were detected during the period 12.5–16.5 dpc. They were identified in the palatal epithelial layer mainly at the placode area from 12.5–13.5 dpc and the protruding ridge area from 14.5–16.5 dpc (Figs. 1a and 1b; arrows).

Expression of p53, bcl-2 and bad: P53 was not detected anywhere in the epithelial layer during the gestation period examined (Figs. 1c and 1d).

Bcl-2 was not detected before 14.5 dpc (Fig. 1e), and was expressed mainly at the upper layer of the epithelium of the

Figs. 1a and 1b. Distribution of TUNEL-positive cells. Arrows indicate the apoptotic bodies. At 13.5 dpc, the apoptotic body was located at the central area of PR epithelial placode in Fig. 1a. At 15.5 dpc, apoptotic bodies were seen at the central area of PR protrudi ng rudiments in Fig. 1b. Bar, 50 μm.
Figs. 1c and 1d. Expression of p53. No p53 positive signals were observed at 13.5 dpc and 15.5 dpc. Bar, 50 μm.
Figs. 1e and 1f. Expression of bcl-2. At 13.5 dpc, no bcl-2 positive signals were observed. Arrows indicated the bcl-2 positive signals. At 15.5 dpc, bcl-2 positive signals were detected at the upper area of epithelium of the inter area of PR protruding rudiments. Bar , 50 μm.
Figs. 1g and 1h. Expression of bad. At 13.5 and 15.5 dpc, arrows indicate the bad positive signals at the periderm area. Arrows indicate bad positive signals at outer area of EP and PR. Arrowheads indicate bad signals at the central area of PR protrusion area. Bar, 50 μm.

Table 1.   Distribution patterns of TUNEL-positive cells and p27, p53, bcl-2 and bad expressions

	12.5 dpc	13.5 dpc	14.5 dpc	15.5 dpc	16.5 dpc
TUNEL	±	+	+	+	±
p27	–	–	–	+	+
p53	–	–	–	–	–
bcl-2	–	–	–	+	+
bad	±	+*	+*	+*	–

+*: Positive signals at the cental area of PR epithelium and the periderm

interprotruding PR areas from 15.5–16.5 dpc (Fig. 1f; arrows; Table 1).

Bad was expressed at the periderm area and also at the epithelial cells at the central area of EP and PR at 13.5–15.5 dpc (Figs. 1g and 1h; arrows; Table 1).

Distribution patterns of TUNEL-positive cells and expression of p53, bcl-2 and bad molecules were indicated in Table 1. The pattern of TUNEL positivity nearly corresponded to the expression of bad at the central area of PR epithelium, but expression of bcl-2 showed a pattern inverse to that of TUNEL staining.

DISCUSSION

Murine palatal rugae (PR) are formed as 9 corrugated patterns on the oral roof. Each edge of PR is orientated aborally, according to our previous scanning electron microscopical and gross anatomical examinations [20]. Murine PR develop through two steps; the first step being the formation of the epithelial placode (EP), and the second protrusion and growth in the aboral direction. The formation of the EP is an important stage influencing the point of formation the PR and other organs in the epithelium. The development and growth of PR are controlled reciprocally by epithelium-mesenchymal interactions. It is known that the expression of certain extracellular matrix (ECM) molecules is spatiotemporally regulated during PR development [1, 2]. Spatial and temporal changes of cell proliferation, especially between the placode and interplacode areas, are closely related to the development of the chick scale [22, 23]. In our previous study based on the distribution of cells incorporating BrdU and on TUNEL analysis, it was suggested that spatial and temporal variations in cell proliferation and/or apoptosis of the epithelium were intimately involved with murine PR formation [20]. Apoptosis plays an important role in controlling cell populations during human embryogenesis [16]. However, the inducing signals for apoptosis in the placode area and the PR protruding area had thus far not been elucidated. The present examination revealed that bcl-2 was expressed mainly at the interprotruding PR areas at 15.5–16.5 dpc. The balance between levels of bcl-2 versus bad influenced apoptosis, with bcl-2 acting as an anti-apoptotic factor. After completion of the construction of the PR protruding structure, epithelial cell apoptosis therein coincided with the expressions of bcl-2. P53

expression was not detected in developing PR in the present study. It is therefore concluded that normal PR organogenesis did not require p53 expression. Bad expression was detected in the epithelial layers at 13.5–15.5 dpc, and it was expressed at the central area of epithelial placode and protruding areas at this time. Expression of strong bad signals coincided with the distribution of apoptotic epithelial cells within the placode area of the PR rudiment. High levels of bad expression relative to bcl-2 levels induced apoptosis. The present results therefore supported the notion that strong bad expression induced apoptosis of PR epithelial cells at the placode area and might control cell populations generating the placode area. Bad is a member of the bcl-2 family, which includes bak (bcl-2 homologous antagonist / killer) [11]. In contrast to bcl-2, these bcl-2 members induced apoptosis. Bad overexpression relative to bcl-2 enhanced apoptosis. The apoptosis of PR epithelial cells observed in the present study might be switched on by spatiotemporally -regulated overexpression of bad. High levels of bad might in turn be induced by decreased availability of some growth factor. Therefore, the apoptosis previously observed at the PR placode during formation of epithelial placode may have been due to growth factor withdrawal.

In conclusion, this study indicated that epithelial cell apoptosis-related molecules may be involved temporally and spatially in regulating the developing PR in mice. The control of cell apoptosis during placode formation may be closely related to the maintenance of the epithelial population in the PR placode and rudiment. Apoptosis present at the placode area might be induced by the lack of some growth factors.

REFERENCES

1. Amasaki, H., Matsumoto, S., Takanosu, M. and Daigo, M. 1991a. Distributions of extracellular matrix and carbonic anhydrase-III during bovine palatine ridge development. *J. Vet. Med. Sci.* **53**: 1031–1036.
2. Amasaki, H., Matsumoto, S. and Daigo, M. 1991b. The distributions of the fibronectine, laminine, type I and IV collagens and the carbonic anhydrase isozyme III during bovine ruminal epithelial development. *Acta Anat.* **140**: 169–174.
3. Amstad, P. A., Liu, H., Ichimiya, M. Berezesky, I. K., Trump, B. F., Buhimschi, I. A. and Gutierrez, P. 2001. BCL-2 is involved in preventing oxidant-induced cell death and in decreasing oxygen radical production. *Redox. Rep.* **6**: 351–362.
4. Babiarz, B. S., Wee, E. L. and Zimmerman, E. F. 1979. Palate morphogenesis. III. Changes in cell shape and orientation during shelf elevation. *Teratology* **20**: 249–278.
5. Cappello, P., Tarozzo, G., Benedetto, A. and Fasolo, A. 1999. Proliferation and apoptosis in the mouse vemeronasal organ during intogeny. *Neurosci. Let.* **266**: 37–40.

6. Cattoretti, G., Becker, M. H., Key, G., Duchrow, M., Schluter, C., Galle, J. and Gerdes, J. 1992. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J. Pathol.* **168**: 357–63.

7. Chuong, C. M., Patel, N., Lin J., Jung, H. S. and Widelitz, R. B. 2000. Sonic hedgehog signaling pathway in vertebrate epithelial appendage morphogenesis: perspectives in development and evolution. *Cell Mol. Life Sci.* **57**: 1672–1681

8. Chuong, C. M. and Noveen, A. 1999. Phenotypic determination of epithelial appendages: genes, developmental pathways, and evolution. *J. Investig. Dermatol. Symp. Proc.* **4**: 307–311.

9. Desbiens, X., Queva, C., Jaffredo, T., Jaffredo, T., Stehelin, D. and Vandenbunder, B. 1991. The relationship between cell proliferation and the transcription of the nuclear oncogenes c-myc, c-myb and c-ets-1 during feather morphogenesis in the chick embryo. *Development* **111**: 699–713.

10. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A. Jr., Butel, J. S. and Bradley A. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature (Lond)* **356**: 215–221.

11. Farrow, S. N., White, J. H., Martinou, I., Raven, T., Pun, K. T., Grinham, C. J., Martinou, J. C. and Brown, R. 1995. Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. *Nature (Lond)* **374**: 731–733.

12. Hatakeyama, S., Tomichi, N., Ohara-Nemoto, Y. and Satoh, M. 2000. The immunohistochemical localization of Fas and Fas ligand in jaw bone and tooth germ of human fetuses. *Calcif. Tissue Int.* **66**: 330–337.

13. Monkawa, T., Hiromura, K., Wolf, G. and Shankland, S. J. 2002. The hypertrophic effect of transforming growth factor-beta is reduced in the absence of cyclin-dependent kinase-Inhibitors p21 and p27. *J. Am. Soc. Nephrol.* **13**: 1172–1178.

14. Pan, H. and Griep, A. E. 1994. Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: implication for tumor suppressor gene function in development. *Genes Develop.* **8**: 1285–1299.

15. Pezzano, M., Samms, M. and Guyden, J. C. L. 2001. TNF and Fas-induced apoptosis during negative selection in thymic nurse cells. *Ethn. Dis.* **11**: 154–156.

16. Polakowska, R. R., Piacentini, M., Barlett, R., Goldsmith, L. A. and Haake, A. 1994. Apoptosis in human skin development: morphogenesisi, periderm, and stem cells. *Develop. Dinamics.* **199**: 176–188.

17. Screpanti, V., Wallin, R. P., Ljunggren, H. G. and Grandien, A. 2001. A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. *J. Immunol.* **167**: 2068–2073.

18. Shigemura, N., Kiyoshima, T., Kobayashi, I., Matsuo, K. and Yamazu, H. 1999. The distribution of BrdU- and TUNEL-positive cells during odontogenesis in mouse lower first molars. *Histochem. J.* **31**: 367–377.

19. Song, J., Sapi, E., Brown, W., Nilsen, J., Tartaro, K., Kacinski, B. M. Craft, J., Naftolin, F. and Mor, G. 2000. Roles of Fas and Fas ligand during mammary gland remodeling. *J. Clin.Invest.* **106**: 1209–1220.

20. Takanosu, M., Amasaki, H., Iwama, Y., Ogawa, M., Hibi, S. and Suzuki, K. 2002. Epithelial cell proliferation and apoptosis in the developing murine palatal rugae. *Anat. Histol. Embryol.* **31**: 9–14.

21. Takanosu, M., Amasaki, H., Matsumoto, S. and Kimata, K. 1996. Distributions of chondroitin sulphate proteoglycans and peanut agglutinin binding molecules during bovine fetal palatine ridge formation. *J. Anat.* **189**: 109–115.

22. Tanaka, S. and Kato, Y. 1983a. Epigenesis in developing avian scales. I. Qualitative and quantitative characterization of fine cell populations. *J. Exp. Zool.* **225**: 257–269.

23. Tanaka, S. and Kato, Y. 1983b. Epigenesis in developing avian scales. II. Cell proliferation in relation to morphogenesisis and differentiation in the epidermis. *J. Exp. Zool.* **225**: 271–283.

24. Tsujimoto, Y., Cossman, J., Jaffe, E. and Croce, C. M. 1985. Involvement of the bcl-2 gene in human follicular lymphoma. *Science* **228**: 1440–1443.

25. Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B. and Korsmeyer, S. J. 1995. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* **80**: 285–291.

26. Zou, M. H., Shi, C. and Cohen, R. A. 2002. High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H(2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells. *Diabetes* **51**: 198–203.