Acta clin Croat 2004; 43:211-215

THE EXPRESSION OF PROLIFERATING CELL NUCLEAR ANTIGEN AND RETINOBLASTO MA PROTEIN IN TRANSPLANTED FETAL RAT LACRIMAL GLAND

Ana Katušić[1], Ljiljana Šerman[1], Floriana Bulić-Jakuš[1], Gordana Jurić-Lekić[2], Maja Vlahović[1] and Klaus Kratochwil[3]

[1]Department of Biology, [2]Department of Histology and Embryology, School of Medicine, University of Zagreb, Zagreb, Croatia; [3]Institute of Molecular Biology, Salzburg, Austria

SUMMARY – A model of ectopic organogenesis of the rat fetal lacrimal gland was developed to study lacrimal gland organogenesis. It was done by its transplantation under the renal capsule. In transplants, expression of the tumor suppressor retinoblastoma gene (Rb) and proliferation cell nuclear antigen (PCNA) was assessed at the protein level. Eyes of 17- and 20-day-old rat fetuses were isolated under a dissecting microscope. Lacrimal glands were found and transplanted under the renal capsule of an adult male. After 14 days, transplants were routinely prepared for immunohistochemistry. DAKO Animal Research kit (Peroxidase) was used for detection of monoclonal mouse anti-PCNA and monoclonal mouse anti-human retinoblastoma gene product. In transplants, teratoma-like structures developed that contained lacrimal gland epithelial cells and ducts as well as epidermis. PCNA signal was detected in the nuclei of excretory duct cells and in epidermal basal layer cells. Some transplant cells were found to have the ability of proliferation preserved even after a 14-day period. PCNA signal was absent in well differentiated epithelial cells of lacrimal gland. Intranuclear Rb protein expression was only detected in several scattered cells, indicating the proliferating compartment to be usually larger than the differentiating one in fetal tissues. Assessment of the PCNA and Rb gene expression could prove important for elucidation of pathologic processes of the lacrimal gland, such as tumors or dry eye syndrome.

Key words: *Lacrimal apparatus – embryology; Organ culture; Retinoblastoma protein – analysis; Animal*

Introduction

Development of the lacrimal gland is an example of epithelial-mesenchymal interaction. In mouse, a single budlike invagination of the conjunctival epithelium at the temporal extremity of the eye is the initial sign of lacrimal gland formation[1]. The mesenchymal cells that surround the point of epithelial budding are periocular cells of neural crest origin[2]. Tubular invagination of the lacrimal gland extends and branches multiple times to give the lobular structure of the mature gland[1].

The type of morphogenesis[3] that accompanies development of the lacrimal gland has been studied in detail in several other organ systems including the limb[4], the lung[5-7], and teeth[8]. As the result of these analyses, a selection of soluble signaling molecules has been implicated in generating morphology of this type. These include sonic hedgehog[9,10] and members of the bone morphogenetic protein (BMP)[11] and fibroblast growth factor (FGF) families[12]. In particular, FGF10, also known as keratinocyte growth factor-2 (KGF2), has been implicated in budding outgrowth of epithelia[13-15].

The proliferating cell nuclear antigen (PCNA) is a well-known endogenous proliferation marker and its detection is often used to establish the proliferation status of cells in animal and human tissues[16,17]. On the other hand, tumor suppressor genes are considered to be negative growth

Correspondence to: *Floriana Bulić-Jakuš, Ph.D.,* Department of Biology, School of Medicine, University of Zagreb, Šalata 3, HR-10000 Zagreb, Croatia
E-mail: floriana@mef.hr

Received May 7, 2004, accepted May 14, 2004

regulators in development and differentiation[18,19]. Rb gene is a prototype of a tumor suppressor gene, and while inactivation of both Rb alleles seems to cause the tumor retinoblastoma, absent or altered Rb protein expression was also detected in other tumor types[20]. Rb protein is an important negative regulator of the cell cycle progression but its phosphorylation during G1 phase by Cdk-cyclin complex causes progression to S-phase[21]. Rb has been shown to promote terminal differentiation and to prevent cell cycle re-entry[22].

The purpose of this study was to investigate the ability of cell proliferation in conjunction with expression of the tumor suppressor Rb gene and PCNA in rat fetal lacrimal gland in order to get better insight into the lacrimal gland organogenesis.

Material and Methods

Transplantation and in vivo organ culture

Fisher rats were mated overnight and sacrificed in ether after 17 and 20 days of pregnancy. Eyes were microsurgically isolated from the embryos under a dissecting microscope. Fetal lacrimal glands were found behind neural retina and transplanted to the ectopic site under the renal capsule of an adult male. Rats were anesthetized and paravertebral incision was made through the skin and muscle to approach the kidney. An incision was made on one renal capsule of each rat and a small pocket was formed under the capsule to place the transplant. Transplants were grown *in vivo* for 14 days.

Immunohistochemistry

After 14 days, the transplants were routinely fixed in St. Marie fixative (90% ethanol, 1% glacial acetic acetate) and embedded in paraffin. Serial sections (5 mm) were put on silanized slides (S 3003; DAKO, Glostrup, Denmark) and air-dried for 24 hours at room temperature. Sections were routinely deparaffinized and placed in a jar filled with PBS (pH=7.4). Prior to application to the specimens, primary antibodies were diluted with 0.05 mol dm^{-3} Tris-HCl buffer, pH=7.6, containing 1% bovine serum albumin and labeled by mixing for 15 minutes in a solution with the biotinylation reagent, a modified biotinylated anti-mouse immunoglobulin. The blocking reagent (normal mouse serum in Tris-HCl buffer containing carrier protein and 15 mmol dm^{-3} sodium azide) was then added for 5 minutes to the mixture to inhibit the appearance of endogenous signal. Monoclonal Mouse Anti-PCNA, Clone PC10 (M 0879, DAKO) was diluted to 1:25; Monoclonal Mouse Anti-Human Retinoblastoma Gene Product, Clone Rb1 (M 7131, DAKO) was diluted to 1:25. Negative control was performed by avoiding the step of incubation with primary antibodies. DAKO Animal Research kit (Peroxidase) was used for primary antibody visualization according to the manufacturer's instructions. The specimens were incubated with Peroxidase Block for 5 minutes. Biotinylated primary antibody and streptavidin-peroxidase complex were applied step by step for 15 minutes each. After each incubation step, the slides were washed in the buffer twice for 3 minutes. Prior to staining, the DAB + Substrate-Chromogen complex was made by mixing the Buffered Substrate solution (pH=7.5; containing hydrogen peroxide and a preservative) with the Liquid DAB (3,3'-diaminobenzidine) + Chromogen solution. The complex was immediately applied on the specimens for 5 minutes and washed with distilled water. Hematoxylin was used for counterstaining. The slides were covered with 50% glycerol in PBS.

Results

Development of teratoma-like structures at the ectopic site

After 14 days of *in vivo* cultivation of rat fetal lacrimal gland at the ectopic site under the renal capsule, the appearance of teratoma-like structures was observed, containing not only lacrimal gland but also hair-like structures. The success of transplantation presented by the development of teratoma-like structures was 85.71% in 17-day-old rat fetuses and 50% in 20-day-old rat fetuses (Table 1).

PCNA expression in transplants of rat fetal lacrimal gland

PCNA was detected in tissues of lacrimal gland transplants of 17- and 20-day-old rat fetuses. The intranuclear PCNA signal was intensive within particular excretory duct

Table 1. Transplantation of fetal lacrimal gland under renal capsule of an adult male

Days of pregnancy	No. of isolated fetuses	No. of lacrimal glands transplanted	Teratoma-like structures observed
17	7	7	6
20	6	6	3

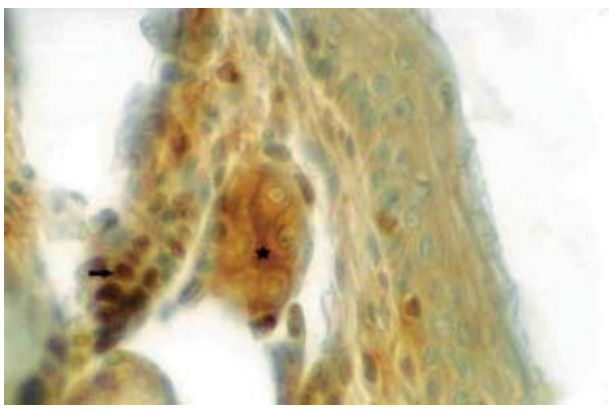

Fig. 1. Proliferating cell nuclear antigen (PCNA) expression in the rat fetal lacrimal gland transplant under renal capsule. Intranuclear PCNA signal is visible in excretory duct cells of lacrimal gland (arrow). Note the absence of signal in lacrimal gland (asterisk). PCNA signal is also visible in basal layer epidermal cells. (IHC staining, DAB, hematoxylin counterstaining, X40).

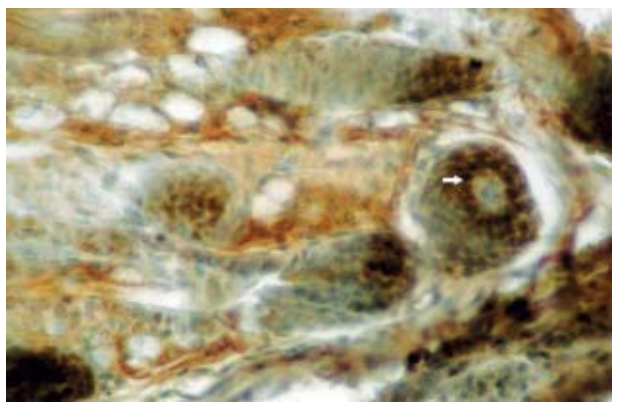

Fig. 2. Proliferating cell nuclear antigen (PCNA) expression in the rat fetal lacrimal gland transplant under renal capsule. PCNA signal is detectable in the nuclei of epidermal cells (arrow), positive control. (IHC staining, DAB, hematoxylin counterstaining, X20).

cells of lacrimal gland. Lacrimal gland cells were negative for PCNA (Fig. 1). In epidermis, the signal was found mainly in the cells of the basal layer (Figs. 1 and 2).

Rb protein expression in transplants of rat fetal lacrimal gland

Rb protein expression was also detected in rat fetal lacrimal gland transplants. It was found as an intranuclear signal restricted to several cells scattered through the transplant. It seems that lacrimal gland ducts did not contain Rb protein (Figs. 3 and 4).

Discussion

The expression of PCNA and Rb protein was detected in rat fetal lacrimal gland grown in a newly established model of lacrimal gland organogenesis. Moreover, analyzing protein expression at a single cell level it was possible to detect differences within the tissues constituting the transplant, probably as a result of differential expression of genes which encode for those proteins, similarly as for Rb protein during mouse development[23].

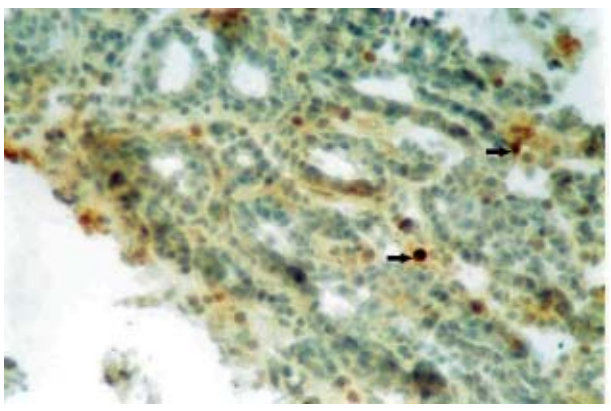

Fig. 3. Retinoblastoma protein (Rb) expression in the rat fetal lacrimal gland transplant under renal capsule. Intranuclear Rb signal is visible (arrow). (IHC staining, DAB, hematoxylin counterstaining, X20).

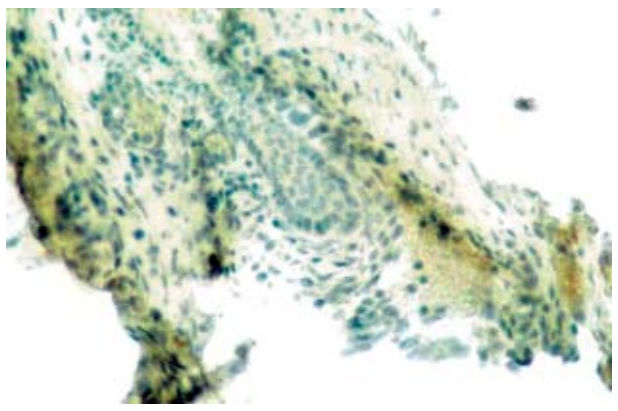

Fig. 4. Transplant of the rat fetal lacrimal gland under renal capsule, negative control. (IHC staining, DAB, hematoxylin counterstaining, X20).

PCNA signal was detected in the nuclei of excretory duct cells and in the epidermal basal layer cells. This indicated that some cells had preserved the ability of proliferation even after the 14-day period. PCNA signal was absent from the well differentiated epithelial cells of lacrimal gland that had obviously exited the cycling compartment. PCNA signal has been correlated with proliferation[24], and is especially abundant in fetal tissues[25]. It can be assumed that cells with PCNA expression have not yet proceeded to the process of terminal differentiation[26].

In the present study, intranuclear Rb protein expression was detected in a number of cells scattered across the transplant. These cells could be assumed to have passed through the process of differentiation at the time of detection. The small number of Rb positive cells indicates the proliferating compartment to be larger than the differentiating one in these fetal tissues, similarly to the observation in fetal testis[25].

Studies of an endogenous cell proliferation marker such as PCNA and a tumor suppressor gene such as Rb could prove relevant for the clarification of the pathologic processes in lacrimal gland, e.g., tumors[27,28] or dry eye syndrome[29,30].

Acknowledgments

The authors thank Ms. Đurđica Cesar and Ms. Jasna Ljubek for their technical assistance. The study was supported by a grant No. 108049 (Experimental approach to mammalian reproductive biology) from the Ministry of Science and Technology of the Republic of Croatia.

References

1. KAMMANDEL B, CHOWDHURY K, STOYKOVA A, APARICIO S, BRENNER S, GRUSS P. Distinct cis-essential modules direct the time-space pattern of the Pax6 gene activity. Dev Biol 1999;205:79-97.
2. JOHNSTON MC, NODEN DM, HAZELTON RD, COULOMBRE JL, COULOMBRE AJ. Origins of avian ocular and periocular tissues. Exp Eye Res 1979;29:27-43.
3. HOGAN BL. Morphogenesis. Cell 1999;96:225-33.
4. MARTIN GR. The roles of FGFs in the early development of vertebrate limbs. Genes Dev 1998;12:1571-86.
5. PETERS K, WERNER S, LIAO X, WERT S, WHITSETT J, WILLIAMS L. Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. EMBO J 1994;13:3296-301.
6. HOGAN BL, YINGLING JM. Epithelial/mesenchymal interactions and branching morphogenesis of the lung. Curr Opin Genet Dev 1998;8:481-6.
7. WEAVER M, YINGLING JM, DUNN NR, BELLUSCI S, HOGAN BL. Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. Development 1999;126:4005-15.
8. PETERS H, BALLING R. Teeth. Where and how to make them. Trends Genet 1999;15:59-65.
9. BELLUSCI S, FURUTA Y, RUSH MG, HENDERSON R, WINNIER G, HOGAN BL. Involvement of sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. Development 1997a;124:53-63.
10. PEPICELLI CV, LEWIS PM, McMAHON AP. Sonic hedgehog regulates branching morphogenesis in the mammalian lung. Curr Biol 1998;8:1083-6.
11. GRAFF JM. Embryonic patterning: to BMP or not to BMP, that is the question. Cell 1997;89:171-4.
12. MASON IJ, FULLER-PACE F, SMITH R, DICKSON C. FGF-7 (keratinocyte growth factor) expression during mouse development suggests roles in myogenesis, forebrain regionalization and epithelial-mesenchymal interactions. Mech Dev 1994;45:15-30.
13. BELLUSCI S, GRINDLEY J, EMOTO H, ITOH N, HOGAN BL. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development 1997b;24:4867-78.
14. OHUCHI H, NAKAGAWA T, YAMAMOTO A, ARAGA A, OHATA T, ISHIMARU Y, YOSHIOKA H, KUWANA T, NOHNO T, YAMASAKI M *et al.* The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. Development 1997;124:2235-44.
15. SEKINE K, OHUCHI H, FUJIWARA M, YAMASAKI M, YOSHIZAWA T, SATO T, YAGISHITA N, MATSUI D, KOGA Y, ITOH N *et al.* FGF10 is essential for limb and lung formation. Nat Genet 1999;21:138-41.
16. SANDERS EJ, FRENCH AS. Cell proliferation in the gastrulating chick embryo. Development 1993;118:389-99.
17. MOKRY J, NEMEČEK S. Immunohistochemical detection of proliferative cells. Sbor ved Praci LF UK Hradec Kralove 1995;38:107-13.
18. GUTMANN DH. Tumor suppressor genes as negative growth regulators in development and differentiation. Int J Dev Biol 1995;39:895-907.
19. NIKITIN AYu, SHAN B, FLESKEN-NIKITIN A, CHANG KH, LEE WH. The retinoblastoma gene regulates somatic growth during mouse development. Cancer Res 2001;61:3110-8.
20. KORNBLAU SM, CHEN N, Del GIGLIO A, O'BRIEN S, DEISSEROTH AB. Retinoblastoma protein expression is frequently altered in chronic lymphocytic leukaemia. Cancer Res 1994;54:242-6.
21. HARBOUR JW, LUO RX, Dei SANTI A, POSTIGO AA, DEAN DG. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell 1999;98:859-69.
22. LEE Z. The retinoblastoma gene: a prototypic and multifunctional tumor suppressor. Exp Cell Res 2001;264:2-18.

23. SZEKELY L, JIANG WQ, BULIC-JAKUS F, ROSEN A, RINGERTZ N, KLEIN G, WIMAN KG. Cell type and differentiation dependent heterogeneity in retinoblastoma protein expression in SCID mouse fetuses. Cell Growth Differen 1992;3:149-56.
24. SANDERS EJ, VAREDI M, FRENCH AS. Cell proliferation in the gastrulating chick embryo: a study using BrdU incorporation and PCNA localization. Development 1993;118:389-99.
25. MARIC S, BULIC-JAKUS F, JEZEK D, JURIC-LEKIC G, KOS M, VLAHOVIC M. Expression of the proliferating cell nuclear antigen and protein products of tumour suppressor genes in the human foetal testis. Andrologia 2004;36:24-30.
26. ALBERTS B, BRAY D, LEWIS J, RAFF M, ROBERTS K, WATSON JD. Molecular biology of the cell. 3rd ed. New York: Garland Publishing Inc., 1994.
27. DEVOTO MH, CROXATTO JO. Primary cystadenocarcinoma of the lacrimal gland. Ophthalmology 2003;110:2006-10.
28. FRIEDRICH RE, BLECKMANN V. Adenoid cystic carcinoma of salivary and lacrimal gland origin: localization, classification, clinical pathological correlation, treatment results and long-term follow-up control in 84 patients. Anticancer Res 2003;23(2A):931-40.
29. TSUBOTA K, TAKEUCHI T. Quantitative analysis of lacrimal gland function in dry eye patients. Exp Eye Res 2003;76:233-40.
30. UBELS JL, AUPPERLEE MD. Down-regulation of androgen receptor expression and inhibition of lacrimal gland cell proliferation by retinoic acid. Exp Eye Res 2002;75:561-71.

Sažetak

EKSPRESIJA NUKLEARNOG ANTIGENA STANIČNE PROLIFERACIJE I PROTEINA RETINOBLASTOMA U PRESAĐENOJ FETALNOJ SUZNOJ ŽLIJEZDI ŠTAKORA

A. Katušić, Lj. Šerman, F. Bulić-Jakuš, G. Jurić-Lekić, M. Vlahović i K. Kratochwil

Razvijen je model ektopične organogeneze fetalne suzne žlijezde štakora za ispitivanje organogeneze suzne žlijezde. To je postignuto presađivanjem suzne žlijezde ispod bubrežne kapsule. U presatcima je procjenjivana ekspresija gena tumorske supresije retinoblastoma (Rb) i jezgrenog antigena proliferirajućih stanica (PCNA) na razini bjelančevina. Oči fetusa štakora starih 17 i 20 dana izolirane su pod disekcijskim mikroskopom. Suzne žlijezde su pronađene i presađene pod bubrežnu kapsulu odraslog mužjaka. Nakon 14 dana su presatci rutinski pripravljeni za imunohistokemijsku analizu. Za otkrivanje monoklonskog mišjeg anti-PCNA i monoklonskog mišjeg anti-humanog proizvoda gena retinoblastoma uporabljen je DAKO Animal Research test (peroksidaza). U presatcima su se razvile teratomu slične strukture koje su sadržavale epitelne stanice suzne žlijezde i kanale, te epidermis. Signal PCNA otkriven je u jezgrama stanica odvodnog kanala, te u stanicama epidermnog bazalnog sloja. Time je pokazano kako su neke stanice presatka zadržale sposobnost proliferacije i nakon 14-dnevnog razdoblja. Signal PCNA bio je odsutan u dobro diferenciranim epitelnim stanicama suzne žlijezde. Unutarstanična ekspresija proteina Rb otkrivena je samo u nekoliko razasutih stanica, što pokazuje da je proliferirajući odjeljak u fetalnim tkivima obično veći od diferencirajućeg odjeljka. Procjena ekspresije PCNA i gena Rb mogla bi biti važna za pojašnjenje patoloških procesa u suznoj žlijezdi, kao što su tumori ili sindrom suho g oka.

Ključne riječi: *Suzni aparat – embriologija; Kultura organa; Protein retinoblastoma – analiza; Životinja*