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ESTABLISHMENT AND CHARACTERIZATION OF 12S ADENOVIRAL E1A IMMORTALIZED RAT SUBMANDIBULAR GLAND EPITHELIAL CELLS

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Rat submandibular gland (RSMG) cells have been immortalized with a retrovirus vector which encodes the adenovirus 12S E1A gene product. The immortalized cells were epithelial-like in nature and displayed a ß-adrenergic coupled rise in intracellular cAMP upon exposure to norepinephrine. Western- and lectin-blotting experiments of cell lysates demonstrated the presence of blood group A-reactive oligosaccharides. Such oligosaccharides are characteristic of RSMG mucin-glycoproteins. These cells appear to be suitable for use in analysis of cell-specific factors which regulate RSMG glycosylation.

Salivary mucins play a central role in protecting the hard and soft tissues of the mouth (1). Despite their import, little is known about the mechanisms which regulate the biosynthesis of these molecules. In part, this is due to lack of suitably characterized cell lines which synthesize these glycoconjugates. Unfortunately, salivary gland epithelium has proven difficult to maintain in long-term culture (for review, see 2-4 and references cited therein). Moreover, only a few salivary cell lines have been established (5-8), and these have proven to be ductal in origin.

Human adenoviruses transform some epithelium, albeit at low efficiency (9). In part, this relates to variation in adenovirus infectivity of different cell types. To circumvent this, Cone and colleagues have constructed a retrovirus vector expressing the 12S early-region 1A (E1A) gene product (rv12S) of adenovirus. This construct has

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been used to immortalize epithelial cells from several non-salivary tissues (9). The studies reported here indicate that the 12S adenovirus E1A gene product can also be used to immortalize cells from rat submandibular gland (RSMG). Based upon multiple criteria, these cells are epithelial-like in nature and are responsive to ß-adrenergic stimulation. Moreover, they appear capable of synthesizing blood group-A reactive oligosaccharides, which typify the carbohydrate structures of the RSMG mucin-glycoprotein (10,11).

MATERIALS AND METHODS

Primary cell cultures - Primary cultures of RSMG cells were prepared from either 2- or 7-d Long Evans strain rats; and 2- or 28-d Wistar strain rats (Charles River Laboratories, Kingston). Minced tissue was treated with collagenase/dispase (Boehringer) (2.5 mg each/ml phosphate buffered saline, pH 7.0) for 30 min at 37°C with constant shaking. Cell aggregates were harvested by centrifugation (100 x g for 3 min) and the resultant pellet was resuspended in Dulbecco modified Eagle medium (DME) (GIBCO) with 5% fetal calf serum (FCS) and 100 U of penicillin and 100 mg of streptomycin/ml. Cells remaining in suspension after 2 min were plated at a density of 5×10^5 cells/60 mm dish in DME - 5% FCS, and were maintained at 37°C in a humidified atmosphere of 10% CO_2 in air.

Infection, selection, and cloning of cells - Primary RSMG, grown to about 70% confluency in 2d, were infected with 1ml of virus stock (derived from ψ2 cells, kindly supplied by Dr. R.D. Cone, which harbor a retrovirus vector encoding both the 12S adenovirus E1A and neomycin phosphotransferase gene products) in the presence of 8 µg of polybrene (Aldrich Chemical Co.) for 2.5 h at 37°C with occasional shaking. Following infection, RSMG cells were maintained in DME - 10% FCS for 4d and then subjected to G418 (GIBCO) selection (medium containing G418 at 0.5 mg/ml, changed twice weekly for 2 weeks). G418-resistant cells were either trypsinized and replated on 100 mm dishes for expansion in DME - 10% FCS, or cloned using cloning cylinders.

Cell growth and morphology - To visualize cell colonies, cells were fixed with methanol and stained with Giemsa stain. Growth curves were constructed and doubling times were calculated from the logarithmic phase. Growth in soft agar was attempted in DME - 5% FCE made 0.5% with respect to agar (9). For electron microscopy, cells were processed as described previously (11).

Western- and lectin-blot analysis - Confluent cells grown on 100 mm dishes were collected by either trypsinization or exposure to 4mM N,N,N',N' - tetracetic acid (EGTA) in a Hank's balanced salt solution buffered with 10 mM N-2-hydroxyethyl-piperazine-N'-2-thane sulfonic acid (HEPES), pH 7.4. Cells were lysed by brief sonication in Laemmli sample buffer (12), containing a protease cocktail inhibitor, including 10 mM EDTA, 5 mM benzamidine HCL, 1 mM soybean trypsin inhibitor, 5 mM pepstatin, 1 mM phenylmethylsulfonyl fluoride [added fresh]). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide (10%) electrophoresis (12), and the separated proteins were analyzed by Western blot analysis as described previously (10). Primary antibodies included: (i) monoclonal antibody (Mab) M73 (a gift of Dr. E. Harlow) specific for the E1A gene product (13); (ii) Mab LE41 (a gift of Dr. E.B. Lane) specific for cytokeratin 8(14); and (iii) Mab 1F9, specific for the type 3 blood group A-reactive oligosaccharides of the RSMG mucin glycoprotein (10). In addition, nitrocellulose blots were probed with either Phaleolus lunatus lectin, specific for GalNAc α 1,3 (Fuc α 1,2) Gal ß 1,3, GalNAc, or Vicia villosa lectin, specific GalNAc α 1,3 Gal. Control incubations were carried out in the presence of 100 mM N-Acetyl Galactosamine. Each lectin was conjugated to alkaline phosphatase (E.Y. Laboratories),

and reactive materials were localized by exposure to nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toludine salt (BRL-GIBCO). All secondary antibodies were pre-sorbed with cell lysate (24h, 4°C) to eliminate non-specific interactions.

Intracellular cAMP measurements - Cells, grown to confluence in 6-well plates (GIBCO) were extracted as described previously (15). cAMP concentrations were detected by radioimmune assay (Amersham).

RESULTS

Proliferation and immortalization of RSMG epithelial cells by the 12S E1A adenovirus gene product - Infection of adult (60d) RSMG primary cultures with the retroviral vector rv12S failed to yield proliferating cells. In contrast, when primary cells derived from either 2- or 7-d old animals were used, a rapid proliferation of epithelial-like cells, which formed discrete islands of tightly packed polygonal cells, was observed (Fig. 1A). Following G418 selection (Fig. 1B), the surrounding spindle-like fibroblasts died away and epithelial-like cell islands emerged, which were indistinguishable from one another (Fig. 1C). Several colonies were dissociated by trypsin treatment and replated (RSMG 2 - derived from 7d Long Evans RSMG; RSMG 3 - derived from 2d Long Evans RSMG and RSMG 4 - derived from 2d Wistar RSMG). Several clonal lines were established from the replated cells and were designated as RSMG 2-A3, 2-C2, 4-4B, 4-B4, and 4-C3. Each clonal line displayed similar growth rates within 2 - 3 passages of subculture. The population doubling time of clone 2-C2 was 21 h. This line has been cultured continuously for approximately 1 year without any signs of crisis. However, all attempts to grow 2-C2 cells in soft agar failed.

Western blot analysis of these cells indicates that they express E1A cross-reactive protein. Further, all cells were shown to express cytokeratin cross-reactive proteins (data not shown). Electron micrographs of clone 2-C2 revealed prominent intercellular junctions between adjacent cells (Fig. 2), moderate amounts of rough endoplasmic reticulum, and some cells exhibited well-developed Golgi apparatus. However, no evidence of secretory granules was observed.

Characterization of RSMG-like properties of immortalized cells - Protein secretion of RSMG is under ß-adrenergic control (16). We therefore evaluated clone 2-C2 for ß-adrenergic responsiveness. The cAMP content of 2-C2 cells increased in a dose-

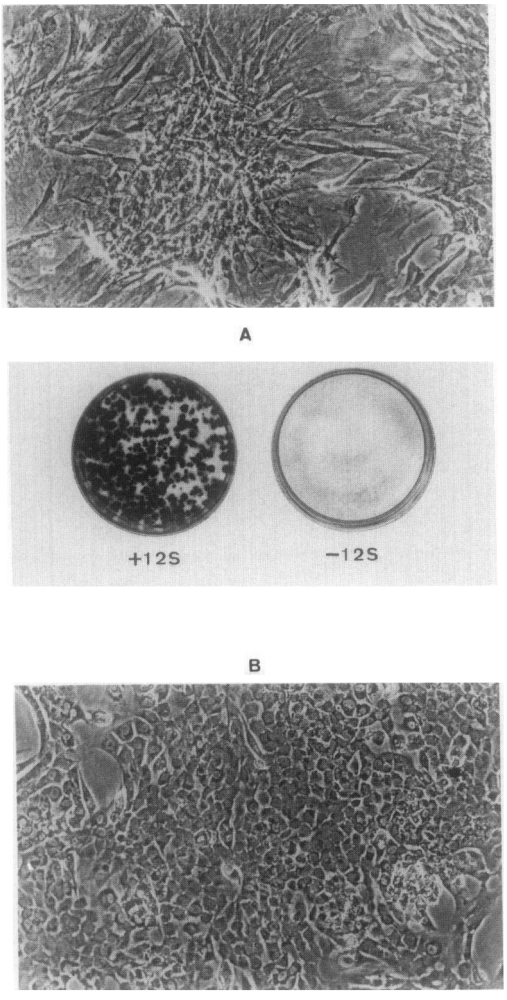

Fig. 1. (A) Phase contrast photomicrograph (x100) of rv12S treated RSMG-2 (derived from 7d Long Evans rats). (B) Colony formation in rv12S treated cells following G418 selection. Cells were visualized with Giemsa stain. (C) Phase contrast photomicrograph (x100) of rv12S treated RSMG-2 following G418 selection.

dependent manner to norepinephrine (Fig. 3A). The resting concentration of cAMP in these cells was 9.5 ± 0.6 pmole/well/10 min, and the maximum increase in cAMP content was over 100-fold relative to the resting level (≈ 100 μm norepinephrine). Accompanying this increase in intracellular cAMP level was a pronounced morphological shrinkage (data not shown). cAMP stimulation was completely reversed by the ß-adrenergic antagonist propranolol (10 μM) (Fig. 3B); in addition, propranolol completely inhibited the norepinephrine-induced change in 2-C2 morphology.

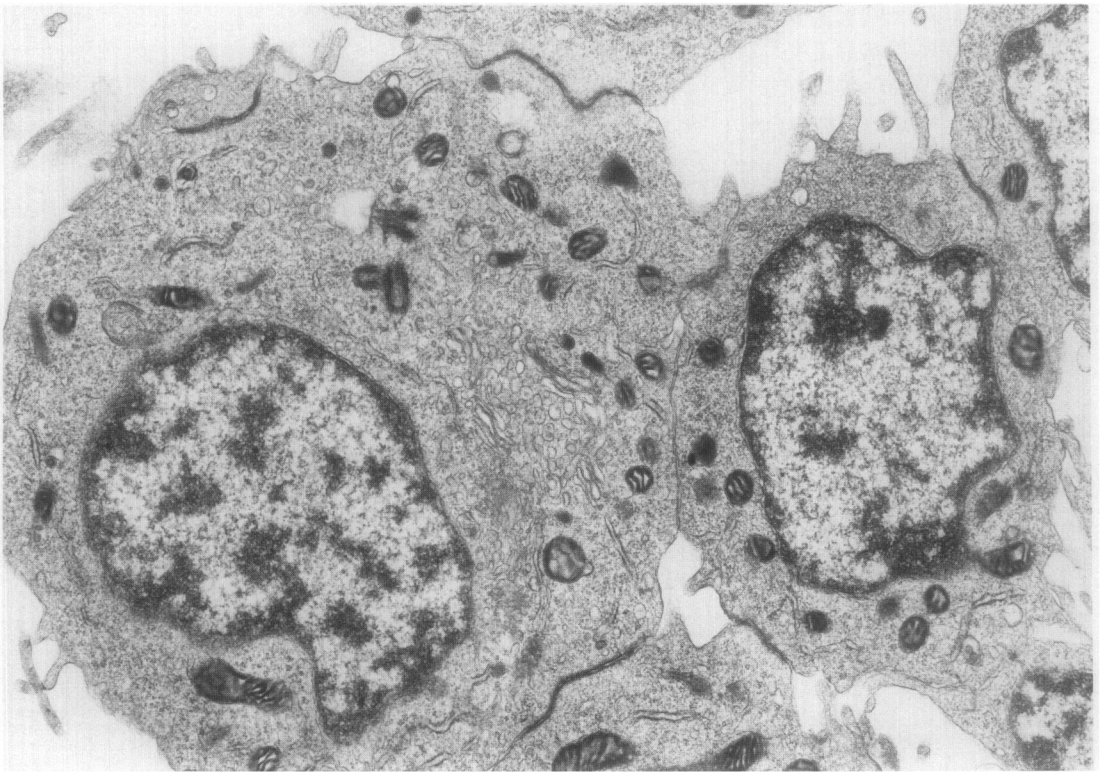

Fig. 2. Electron micrograph (x17,000) of cell line 2-C2.

RSMG synthesize and secrete a highly glycosylated blood group A-reactive mucin glycoprotein (10, 17). Using Mab 1F9 (specific for the type 3 blood group-A RSMG mucin oligosaccharide) and two lectins with blood group-A specificity, we demonstrated that cell lines 2C-2 and 4B-2 (not shown) each synthesize a series of blood group-A

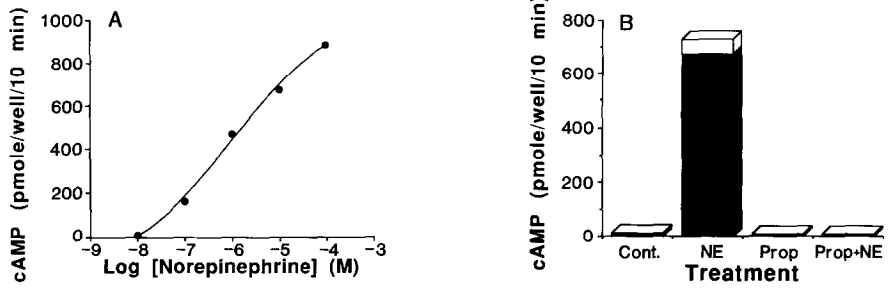

Fig. 3. (A) Dose dependent ß-adrenergic stimulation of intracellular cAMP content of 2-C2 cells. (B) Effect of propranololol on norepinephrine stimulation of intracellular cAMP content of 2-C2 cells. Data in both experiments are expressed as means +/- standard error of the mean from 2 independent experiments. Each point was assigned in triplicate.

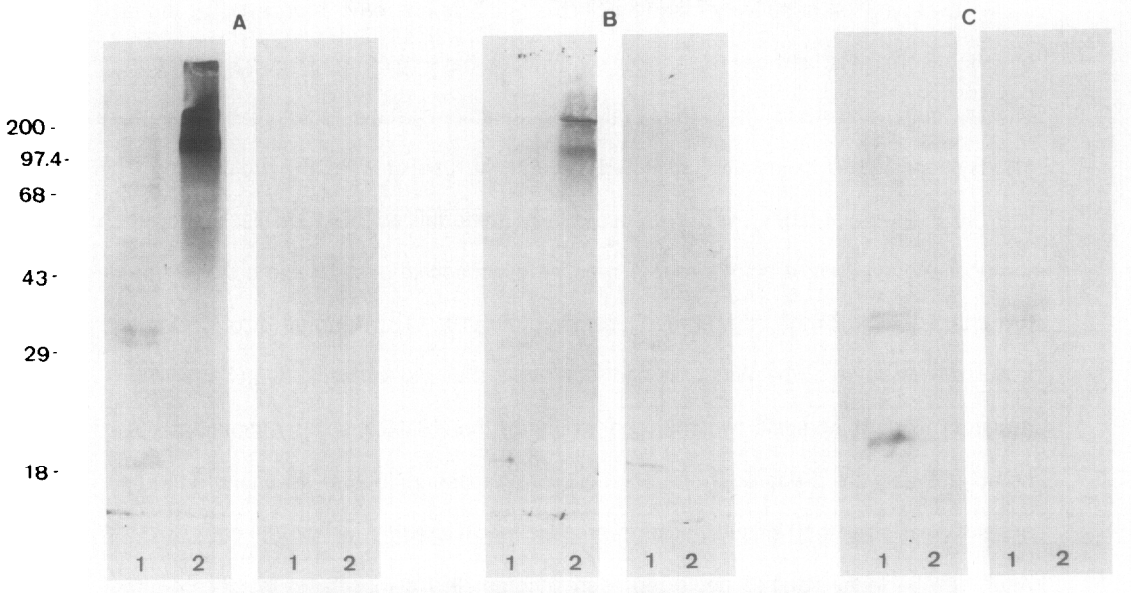

Fig. 4. Western and lectin-blot analysis of RSMG (control) extract (lane 1) and 2-C2 cell lysates (lane2). Panel A: left, probed with Mab1F9; right, probed with secondary antibody alone. Panel B: left, probed with V. villosa lectin; right, probed with V. villosa lectin in presence of 0.1M GalNAc. Panel C: left; probed with P. lunatus lectin; right, probed with P. lunatus lectin in presence of 0.1M GalNAc.

reactive species (Fig. 4). Significantly, cells in culture for approximately 1 year retain the ability to synthesize these glycoconjugates.

DISCUSSION

RSMG cells have been immortalized with the retrovirus vector which encodes the adenovirus 12S E1A gene product. The cells obtained are rapidly proliferating, yet do not appear to be transformed since they fail to grow in soft agar. In common with observations made with thyroid gland (9), incompletely developed RSMG (derived from 2d and 7d old rats) proved to be a more suitable target for immortalization than fully developed adult RSMG. All immortalized cells appeared epithelial-like in nature. In particular, the cloned cell line 2C-2 was observed to have prominent intercellular junctions between cells, suggesting the presence of epithelial "tight-junctions." Additionally, this cell line proves responsive to ß-adrenergic stimuli, with the intracellular levels of cAMP being increased over 100 times greater than control values.

Examination of salivary gland mucin biosynthesis has been hampered by the lack of suitably characterized cell lines. While advances have been made using short-term primary culture systems (16), the analysis of cellular-specific factors which regulate glycosylation would be greatly facilitated through the use of a rapidly proliferating cell line. In the present study, we have successfully immortalized RSMG cells, which retain the ability to synthesize blood group-A reactive oligosaccharides. These oligosaccharides are characteristic of the RSMG mucin-glycoprotein (10, 11). A range of glycosylated species was detected by Western- and lectin-blot analysis, including material with an apparent relative molecular weight equal to that of the RSMG mucin. Although cellular homogenates were prepared in the presence of protease inhibitors, we cannot yet rule out the possibility that these species represent mucin break-down products. Alternatively, these species could represent incompletely glycosylated mucins or new species. Since E1A gene products are known to induce transcriptional activation (17, and references cited therein), it is plausible that the expression of new glycosylatable substrates and/or the activity of glycosyltransferases involved has been altered. Currently, we are examining the nature of the glycosylated products from these cells to distinguish among these possibilities.

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REFERENCES

1. Tabak, L.A., Levine, M.J., Mandel, I.D., and Ellison, S.A. (1982) J. Oral Biol. 11, 1-17.

2. Redman, R.S., Quissell, D.O., and Barzen, K.A. (1988) In Vitro Cell. and Dev. Biol., 24, 734-742.

3. Brown, A.M., Rusnock, E.J., Sciubba, J.J., and Baum, B.J. (1989) J. Oral Pathol. Med. 18, 206-213.

4. Durban, E.M. (1989) In Vitro Cell. Dev. Biol. 26, 33-43.

5. Hayashi, Y., Nishida, T., Yoshida, H., Yanagawa, T., Yura, Y., and Sato, M. (1987) Cancer, 60, 962-968.

6. Sato, M., Axuma, M., Hayashi, Y., Yoshida, H., Yanagawa, T., and Yura, Y. (1987) Cancer Res. 47, 4453-4459.

7. Hayashi, Y., Yoshida, H, Nagamine, S., Yanagawa, T., Yura, Y., Azuma, M., and Sato, M. (1987) Cancer 60, 1000-1008.

8. Giard, D.J., Aronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H., and Parks, W.P. (1973) J. Natl. Cancer Inst. 51, 1417-1421.

9. Cone, R.D., Grodzicker, T., and Jaramillo, M. (1987) Mol. and Cell. Biol. 8, 1036-1044.

10. Tabak, L.A., Mirels, M., Monte, L.D., Ridall, A.L., Levine, M.J., Loomis, R.E., Lindauer, F., Reddy, M.S., and Baum, B.J. (1985) Arch. Biochem. Biophys. 242, 383-392.

11. Moreira, J.E., Tabak, L.A. , Bedi, G.S., Culp, D.J., and Hand, A.R. (1989) J. Histochem. and Cytochem. 37, 515-528.

12. Laemmli, U. (1970) Nature, 227, 680-685.

13. Harlow, E., Franza, B.R., and Schley, C. (1985) J. Virol. 55, 533-546.

14. Lane, E.B. (1982) J. Cell. Biol. 92, 665-673.

15. He, X., Ship, J., Wu, X., Brown, A.M., and Wellner, R.B. (1989) J. Cell. Physiol. 138, 527-535.

16. Quissell, D.O. and Tabak, L.A. (1989) In Handbook of Physiology-The Gastrointestinal System III (J.G. Forte, Ed.), pp. 79-91, Amer. Physiol. Soc., Bethesda, MD.

17. Jones, N.C., Rigby, P.W.J., and Ziff, E.B. (1988) Genes & Dev. 2, 267-281.