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DIFFERENTIAL EXPRESSION OF TRANSFORMING GROWTH FACTOR-α AND EPIDERMAL GROWTH FACTOR DURING POSTNATAL DEVELOPMENT OF RAT SUBMANDIBULAR GLAND

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SUMMARY: The concentration and the localization of transforming growth factor (TGF)-α and epidermal growth factor (EGF) in the submandibular glands (SMGs) of male Wister rats of different ages (postnatal 0 to 10 weeks of age) were examined. Highest levels of TGF-α were seen early, at postnatal day 0; the levels dropped thereafter in an age-dependent manner, while EGF was not detectable before the third postnatal week. Immunoreactive localization of EGF was restricted to the granules of the granular convoluted tubule (GCT) cells in the mature SMGs, whereas TGF-α was observed throughout postnatal development over the entire duct system. TGF-α was demonstrated in the cytoplasm at early stages when the GCT granules were not observed and was also located on the granules at the late stage, as was the case for EGF, indicating that TGF-α is colocated with EGF in the mature SMG. These results demonstrate the differences between the expression of TGF-α and that of EGF in the developing rat SMG.

Transforming growth factor-α (TGF-α) is a single polypeptide sharing structural and functional homology with epidermal growth factor (EGF) and is a member of the EGF family of structurally related mitogenic polypeptides (1,2). TGF-α and EGF are derived from membrane-anchored precursors, are biologically active via their ability to interact with the EGF receptor, and exert a variety of similar biological activities both *in vivo* and *in vitro* (3). They are known to effect epithelial and

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Abbreviations used: EGF, epidermal growth factor; EIA, enzyme immunoassay; GCT, granular convoluted tubule; PMSF, phenylmethylsulfonylfluoride; MoAb, monoclonal antibody; PoAb, polyclonal antibody; SMG, submandibular gland; TGF-α, transforming growth factor-α.

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mesenchymal cell proliferation, migration, and differentiation. There is no evidence for a distinct TGF-α receptor. EGF was isolated from the submandibular glands (SMGs) of adult male mice in 1962 (4), and the primary sites of synthesis of EGF were demonstrated to be the salivary glands and kidneys in humans, rats and mice (5). Up until 1990 only EGF (and not TGF-α) had been reported to be expressed in the SMG (6,7). Recent studies demonstrate that salivary glands tissue does express TGF-α (8,9). Although TGF-α synthesis has usually been associated with transformed and tumor cells (10), expression of TGF-α mRNA has also been noted in normal tissues (11). TGF-α and/or EGF also exhibits neurotrophic effects upon certain populations of neurons in culture and in tissues (12,13), therefore, both are now accepted as integral regulators of growth in normal tissues (14,15). Differential expression of TGF-α during prenatal development of the mouse embryo was reported earlier (16). Recently, we developed a simple and reliable two-site sandwich enzyme immunoassay (EIA) for rat TGF-α employing monoclonal and polyclonal antibodies with defined epitopes (17,18), and, using it, demonstrated TGF-α to be present in both human and rat SMGs (18). Based on these observations, we decided to determine the content and the localization of TGF-α and EGF peptides in developing rat SMGs (postnatal day 0 to week 10), by EIA and immunohistochemical staining.

MATERIALS AND METHODS

Morphological development of the rat SMG is complete between 7 and 10 weeks of age (19). Therefore, five groups of male Wister rats of different ages were used: postnatal day 0 (within 24 hr after birth) and 1 week, 3 weeks, 5 weeks, and 10 weeks of age. Each group contained 4 rats. The animal protocol was reviewed and approved by the institutional panel. Paired SMGs were carefully dissected out under anesthesia with sodium pentobarbital. Sublingual glands and connective tissue capsule were removed under the microscope. The right SMG was stored at - 80°C for the EIA determination and Western blot analysis. The left one was minced and immersed in the Zamboni's fixative at 4°C (20), and then embedded in Epon 812 plastic resin. One-μm-thick sections were first treated with KOH-saturated ethanol to remove the plastic resin and then were immunostained with anti-TGF-α IgG or anti-rat EGF serum by the peroxidase-anti peroxidase method of Sternberg (21). Anti-rat EGF serum was kindly donated by Dr. M. Kashimata (Meikai University, Japan). Rat recombinant TGF-α was a gift of Hoechst Japan Limited. Phenylmethylsulfonylfluoride (PMSF), porcine pancreatic elastase (Boehringer Mannheim, Germany), and other reagents used were all commercially provided. The polyclonal antibody (PoAb) against human TGF-α (hTGF-α [1-50]) was produced in rabbits (17). Anti-hTGF-α monoclonal antibody (MoAb) was prepared according to a previously published procedure (17). The immunoreagents and sandwich EIA were essentially similar to those described previously (18,22), consisting of solid phase-immobilized anti-hTGF-α IgG (PoAb) and IgG_1 (MoAb) labeled with β-D-galactosidase. EGF was measured by a two-site sandwich ELISA utilizing two kinds of MoAbs, as described previously (23). Protein content was determined by protein dye binding, with bovine serum albumin employed as a standard, by use of a Bio-Rad protein-assay kit (24). For the EIA, tissue samples were homogenized with buffer containing 50 mM sodium phosphate buffer, pH 7.2/0.25 M sucrose/protease inhibitors (leupeptin, pepstatin, and antipain, each 5 μg/ml; PMSF 0.1 mM), then centrifuged at 100,000*g* for 30 min, as previously reported (25). Western blot analysis was performed by use of 14 % gels and PVDF membranes (22). Detection of TGF-α on PVDF membranes was with an Immunblot kit (Bio-Rad) using PoAb against TGF-α.

RESULTS AND DISCUSSION

The calculated levels of immunoreactive TGF-α and EGF in the rat samples determined by EIA are shown in Fig.1. We found the highest content of TGF-α in the rat SMG, *i.e.*, 377.8 ± 48.5 (S.E.M.) pg/mg protein, at the earliest stage examined (postnatal day 0). By postnatal week 5 the level had declined to 72.8 ± 3.5 pg/mg and remained low, being 65.0 ± 3.0 pg/mg at the tenth postnatal week. In contrast, no EGF was detected in the SMG from postnatal week 0 to 3 (immature SMGs). At postnatal week 5, the samples registered a low content of EGF (82.5 ± 55.5 pg/mg), and then showed a drastic elevation in content at 10 weeks (611.3 ± 137.0 pg/mg). The level of immunoreactive TGF-α in the SMG at week 10 (mature SMG) was less than 11 % of the level of EGF. When we subjected extracts of rat SMGs (postnatal 0 day to 10 weeks) to SDS-PAGE and Western blot analysis using anti-TGF-α IgG (PoAb), all samples gave high-density 15~20-kDa bands, which did not correspond to the relative molecular mass of authentic rat TGF-α (5.6 kDa), as indicated in Fig.2A. If the 15~20-kDa species were indeed TGF-α precursors, it might be expected that treatment with elastase would yield a product with the size and properties of mature TGF-α (26). Following such treatment the 15~20-kDa species yielded a product of 6 kDa that reacted with anti-TGF-α IgG (Fig.2B). These data indicate that TGF-α is present as a precursor form (pro-TGF-α) in the rat SMG. The density of the TGF-α bands decreased gradually in an age-dependent manner, which well correlated with the EIA data (Fig.1).

The immunohistochemical localization of TGF-α in SMGs was determined, and the results were compared to those for EGF (Fig 3). Positive immunostaining of EGF was demonstrated only on the granules of the GCT cells of the SMG; it was not apparent in sections prepared from neonates to 3 weeks old, was very rare in 5-week

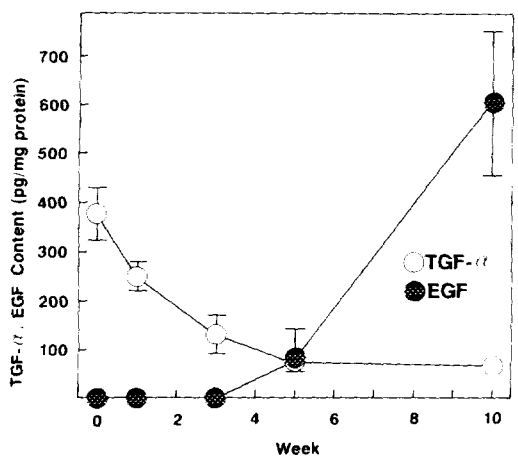

Fig.1. TGF-α and EGF Content in Rat Submandibular Gland. The levels of the two growth factors were determined by EIA for samples from rats of various postnatal ages.

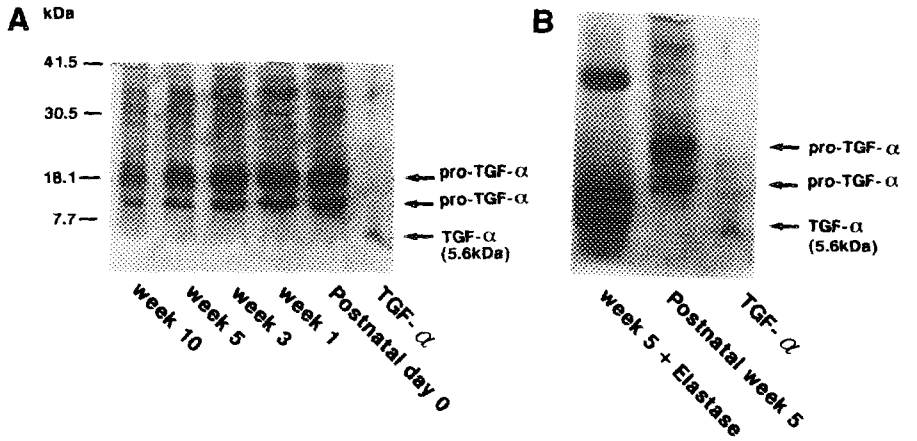

Fig.2. TGF-α in Rat Submandibular Glands by Western Blot Analysis.
(A) Extracts of SMG from rats of different ages were subjected to SDS-PAGE (14 % gel) and then immunostained with anti-TGF-α IgG.
(B) The extract containing 15~20-kDa TGF-α-related species were lyophilized and incubated in 50mM glycylglycine, pH 8.8, containing porcine pancreatic elastase (5 mg/ml) for 1 hr at 25 °C. The samples were then subjected to electrophoresis and Western blot analysis.

specimens (Fig.3a), but was remarkable in 10-week ones (Fig.3c). This result confirmed that of a previous report (6). TGF-α, on the other hand, was detected in the SMGs of both immature and mature rats; TGF-α in the SMGs of 5-week-old rats was localized in the basolateral cytoplasm of cells of the entire duct system, including the intercalated duct, the GCT, and the striated duct (Fig.3b). The localization pattern of immunoreactive TGF-α changed in the mature SMG. Positive staining for TGF-α was remarkably intense on the granules located in the luminal region of GCT cells, as was the case for EGF, demonstrating that TGF-α and EGF are co-present in the same granules of GCT cells in the mature SMG. In addition, TGF-α at the basolateral region was still observed in the other duct cells as it was in immature glands (Fig.3d).

Our present studies using EIA and immunohistochemical staining clearly show the differential expression of TGF-α and EGF in the SMG of male rats during postnatal development. This EIA is applicable to determine the concentration of rat TGF-α as a practical procedure that was heretofore unavailable. The epitope recognized by anti-TGF-α IgG, is identical between human and rat TGF-α's (17). Therefore, we could utilize this antibody for Western blot analysis and immunohistochemical staining. The most striking finding we made is that TGF-α itself is present in the rat SMG from an early stage (postnatal day 0), whereas EGF is not present at this time. In several *in vitro* systems, murine and human TGF-α's were functionally interchangeable with murine and human EGF's (27,28). Murine EGF is known to cause precocious opening of the eyelids in newborn mice (4). TGF-α is similarly as active as EGF in this function (29). TGF-α is also reported to accelerate incisor eruption in neonatal mice, as is EGF (30). These findings combined with the present data suggest that TGF-α in the immature rat SMG plays an important role in morphogenesis.

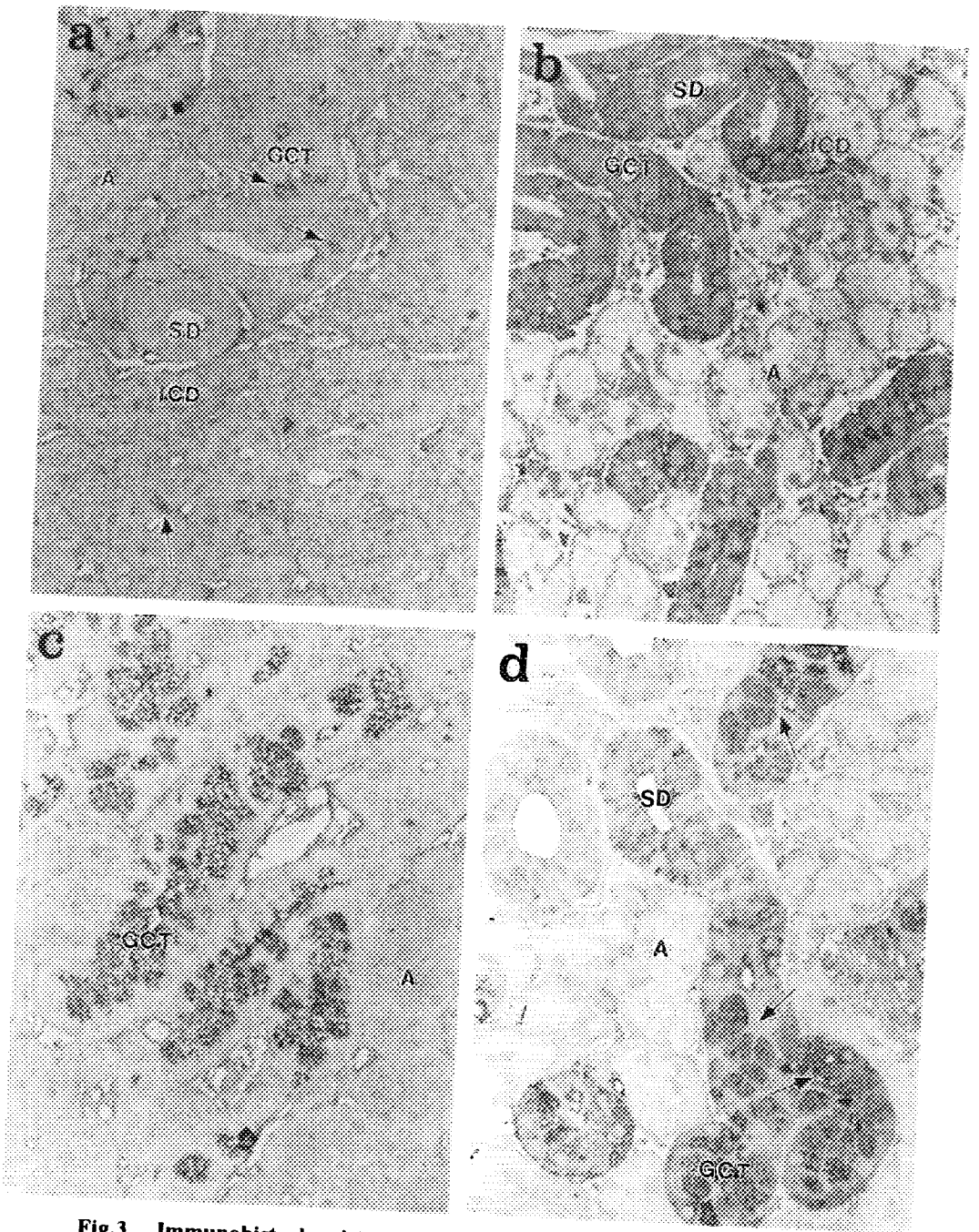

Fig.3. Immunohistochemistry of TGF-α and EGF in rat SMGs.
(a) SMG of 5-week-old rat immunostained for EGF, x375. Only a few GCT cells have granules immunoreactive for EGF (arrows); and cells in other components such as acini (A), intercalated duct (ICD), and striated duct (SD) are negative.
(b) SMG of 5-week-old rat immunostained for TGF-α, x375. Intense immunostaining is observed over the duct system including ICD, GCT, and SD. Acini (A) are not stained.
(c) SMG of 10-week-old rat immunostained for EGF, x375. Immunoreactivity is restricted to the granules of GCT cells.
(d) SMG of 10-week-old rat immunostained for TGF-α, x375. Immunoreactivity is most intense in the granules of GCT cells (arrow), and the basal cytoplasm of other duct cells is also positive.

Our immunohistochemical results showed a difference in localization pattern for TGF-α between immature and mature rats. In the immature SMG, TGF-α was localized throughout the cytoplasm of cells of the entire duct system; whereas in the mature gland the immunostain was most intense in the GCT granules. Moreover, Western blot analysis demonstrated a common immunoreactive band at the molecular weight region around 15~20-kDa in tissues prepared from rat SMG's (Fig.2). Although molecular mass of TGF-α is 5.6kDa, its precursors can range from 11 to 40 kDa, and have similar biological activity (7,26,31). Immunohistochemical localization of TGF-α was examined earlier by Wu *et al.* (8), and they indicated that TGF-α was present in the entire duct system in the rat SMG except on the granules of the GCT cells, which finding disagrees with our observations. However, in line with the present results, Humphreys-Beher *et al.* showed specific immunostaining of TGF-α in the GCT region in SMGs; and they also detected TGF-α in saliva, which suggests salivary glands to be an exocrine source for a second member of the EGF-like growth factor family in the oral cavity (9). Our finding in mature rats that TGF-α immunostain could be demonstrated on the granules of GCT cells supports their suggestion. Furthermore, our immunohistochemical demonstration of TGF-α in the duct cells at the early postnatal stage suggests the possibility that TGF-α may contribute to the growth and differentiation of the duct system prior to the onset of EGF expression. In fact, the duct system of rats or mice is undifferentiated at the perinatal stage and differentiates quickly postnatally, indicating the action of some inducer for normal morphogenesis. From these considerations, it is likely that the function of TGF-α found in the rat SMG may change during the development of the gland from one of inducer of morphogenesis of the ductal system in the early stage to one of growth factor for the oral cavity after the maturation of the gland.

The removal of the SMG's abolishes almost completely the serum EGF and reduces serum TGF-α to half its normal content in rats (32). This observation indicates that the SMG is the exclusive source of EGF and that some additional source of TGF-α must exist. Given that TGF-α and EGF bind to the same receptor and have structural similarity and biological function similarity, it is tempting to speculate that TGF-α is the endogenous ligand for the EGF receptor in the SMG especially in the early postnatal stages (postnatal 0 day to 3 weeks).

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REFERENCES

1. Derynck, R. (1988) Cell **54**, 593-595.
2. Massague, J. (1990) J. Biol. Chem. **265**, 21393-21396.
3. Brachmann, R., Lindquist, P.B., Nagashima, M., Kohr, W., Lipari, T., Napiel, M., and Derynck, R. (1989) Cell. **56**, 691-700.

4. Cohen, S. (1962) J. Biol. Chem. **237**, 1555-1562.
5. Sporn, M. B., and Roberts, A. B. (1985) Nature, **313**, 745-747.
6. Gresik, E., and Barka, T. (1977) J. Histochem. Cytochem. **25**, 1027-1035.
7. Salomon, D.S., Kim N., Saeki T., and Ciardiello F. (1990) Cancer Cells **2**, 389-397.
8. Wu, H.H., Kawamata, H., Wang, D.D., and Oyasu, R. (1993) Histochem J. **25**, 613-618.
9. Humphreys-Behar, M.G., Macauley, S.P., Chegini, N., Setten, G., Purushotham, K., Stewart, C., Wheeler, T.T., and Schultz, G.S. (1994) Endocrinology **134**, 963-970.
10. Todaro, G.J., Fryling, C., and DeLarco, J.E. (1980) Proc. Natl. Acad. Sci. USA **77**, 5258-5262.
11. Lee, D.C., Rose, T.M., Webb, N.R., and Todaro, G.J. (1985) Nature **313**, 489-491.
12. Morrison, R.S., Kornblum, H.I., Leslie, F.M., and Bradshaw, R.A. (1987) Science **238**, 72-75.
13. Mogi, M., Harada, M., Kondo, T., Riederer, P., Inagaki, H., Minami, M., and Nagatsu, T. (1994) Neurosci. Lett. **180**, 147-150.
14. Skinner, M.K., and Coffey, R.J.Jr (1988) Endocrinology **123**, 2632-2638.
15. Coffey, J.R. Jr, Sipes, N.J., Bascom, C.C., Graves-Deal, R., Pennington, C.Y., Weissman, B.E., and Moses, H.L. (1988) Cancer Res. **48**, 1596-1602.
16. Twardzik, D.R. (1985) Cancer Res. **45**, 5413-5416.
17. Inagaki, H., Katoh, M., Kurosawa, O.K., and Tanaka, S. (1990) J. Immunol. Meth. **128**, 27-37.
18. Mogi, M., Inagaki, H, Kojima, K, Minami, M., and Harada, M. (1995) J. Immunoassay in press.
19. Fukuda, M. (1967) Histochemie **8**, 342-354.
20. Stefanini, M., DeMartio, C., and Zamboni, C. (1967) Nature **216**, 173-174.
21. Sternberger, L.A., Hardy, P.H. Jr, Cuculis, J.J., and Meyer, H.G. (1970) J. Histochem. Cytochem. **18**, 315-333.
22. Mogi, M., Kojima, K., and Nagatsu, T. (1984) Anal. Biochem. **138**, 125-132.
23. Katsuura, M., and Tanaka, S. (1989) J. Biochem. (Tokyo) **106**, 87-92.
24. Bradford, M.M. (1976) Anal. Biochem. **72**, 248-254.
25. Mogi, M., and Guroff, G. (1991) Biochem Biophys Res. Commun. **177**, 624-629.
26. Ignotz, R.A., Kelly, B., Davis, R.J., and Massague, J. (1986) Proc. Natl. Acad. Sci. USA **83**, 6307-6311.
27. Twardzik, D.R., Todaro, GJ., Marquardt ,H., Reynolds, F.H. Jr., and Stephensonb, J.R. (1982) Science **216**, 894-897.
28. Derynck, R., Roberts, A.B., Winkler, M.E., Chen, E.Y., and Goeddel, D.V. (1984) Cell **38**, 287-297.
29. Smith, J.M., Sporn, M.B., Roberts, A.B., Derynck, R., Winkler, M.E., and Gregory, H. (1985) Nature **315**, 515-516.
30. Tam, J.P. (1985) Science **229**, 673-675.
31. Luetteke, N.C., Michalopoulos, G.K., Teixido, J., Gilmore, R., Massague, J., and Lee, D.C. (1988) Biochemistry **27**, 6487-6494.
32. Kurachi, H., Morishige, K., Amemiya, K., Adachi, H., Hirota, K., Miyake, A., and Tanizawa, O. (1991) Cancer Res. **51**, 5956-5959.