AN ONTOGENETIC STUDY ON ENDOTHELIN-PRODUCING CELLS IN THE RAT LUNG AND SUBMANDIBULAR GLAND¹

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We ontogenetically examined endothelin (ET) in the lungs and submandibular glands of Wistar rats at various stages, from fetuses to the aged, using specific polyclonal ET-antibody. In the lung, strong ET-immunopositive cells first appeared at 13 days of gestation in the bronchial epithelia, and the strongest immunoreactivity at 17 days; thereafter, the reactivity gradually decreased, disappearing after birth. However, the gene expression of ET-1, ET-3, the receptor A (ET-AR), and B (ET-BR) was observed continuously throughout all the stages. In the submandibular glands, ET-immunopositive cells were first observed in the duct cells at 18 days of gestation; these cells gradually increased in number after birth. In the adult rat, the immunoreactivity was confined predominantly to the granular convoluted tubule. This reactivity was found in every age group, including the aged (3-year-old) rats. All the gene expression of ET-1, ET-3, ET-AR, and ET-BR was observed in the submandibular glands of postnatal and adult rats. We speculate that the ET in these tissues may possibly act as a growth factor.

Endothelin (ET), a potent vasoconstrictor peptide, is known to have three chemical forms, i.e., ET-1, ET-2, and ET-3 (31). Although no specific role of ET in individual organs has been elucidated, ET-3 is thought to be the main component of the endothelin isopeptides that are found in many organs in the rat (30). Several studies of the biological significance of ETs in mammals have been reported (8, 13-15, 18-20, 25-27) to date; however, few have focused on the immunohistochemical localization of ET-producing cells (except in the case of vascular endothelial cells) or their ontogenesis. Thus, the particular ET isotypes produced by various cell types and the occasions on which the isotypes are produced are not clear. Both ontogenetic and histochemical approaches may be useful for clarifying these points.

We produced an ET-antibody specific for the C-terminal active portion of ET, and we used it in a histochemical investigation to detect ET. We also designed oligonucleotide primers for PCR to detect the gene expressions of ETs and their receptors, according to the findings in previous reports (18, 25, 26, 30).

We conducted a study using immunohistochemistry and PCR techniques, and report herein the existence and distribution of ET and their receptors in the rat lungs and submandibular glands at various stages in the growth process, from fetal to aged.

MATERIALS AND METHODS

Antibody Preparation

The C-terminal (9 amino acids) peptide of ET-1 which is the active region and well-preserved in the ET family, was prepared by a peptide synthesizer. This synthetic peptide was coupled to bovine thyroglobulin with *n*-maleimide caproic acid *N*-hydroxysuccinimide ester (Sigma Chemical Co; St. Louis, USA), and used as the immunogen. Male Japanese white rabbits were sensitized with this immunogen, emulsified in Freund's complete adjuvant. After sensitization, uncoupled ET-1 was emulsified in incomplete adjuvant and injected into the rabbits as booster every 10 days. The specificity of the antibody (ET-antibody) purified from serum of immunized rabbits was evaluated by enzyme-linked immunosorbent assay (ELISA), and by immunohistochemical absorption tests.

Immunohistochemical Study

Male Wistar rats (fetal, neonatal, and adult) were used. Neonatal and adult rats were anesthetized with sodium pentobarbital and perfused with a fixative con-

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taining 4% formaldehyde, 0.2% picric acid, and 0.1 M sodium phosphate buffer (pH 7.2) through the right (for lungs) or left (for submandibular glands) ventricle. The lungs and submandibular glands were removed and immersed in the same fixative for 24 hr. Fetal rats were quickly removed from a pregnant mother and immersed in the same fixative. The sections (20 μ m-thick) were cut in a cryostat at -20°C and placed on gelatin-coated glass slides. The immunostaining was performed with the ABC method described in our previous report (29). The sections were pretreated with 1% H_2O_2 in phosphate-buffered saline (PBS) and incubated in a moist chamber for 48 hr with primary antibody against endothelin (1/3000). After being rinsed in PBS, the sections were incubated in a moist chamber at room temperature for 3 hr with goat anti-rabbit γ -globulin diluted at 1/250 in PBS. After the sections were treated with avidin-biotin complex (Vectastain kit, Vector Lab; Burlingame, USA) solution diluted to 1/250 in PBS in a moist chamber for 80 min at room temperature, they were reacted with 3,3'-diaminobenzidine in Tris-HCl buffer (pH 7.6) containing H_2O_2 for 15 min. They were then stained with hematoxylin, dehydrated, mounted with Entellan, and examined with a light microscope.

Polymerase Chain Reaction (PCR) Study

The lungs of fetal (19 and 21 days), neonatal and adult (3 months and 3 years) rats and the submandibular glands of 3-week-, 3-month- and 3-year-old rats were used in this study. These organs of rats in stages earlier than those could not be examined because they were too small to be removed. A First-Track mRNA Isolation Kit (Invitrogen Co., San Diego, USA) was used to obtain total mRNAs from 1 g of sample; that is, the total mRNAs were separated from the excised tissues by affinity chromatography on oligo (dT) cellulose. The first strand cDNA was generated by reverse transcription of mRNA, using a cDNA Cycle Kit (Invitrogen Co.), which contains oligo (dT) primers, four dNTPs, and reverse transcriptase. The synthesized cDNA was concentrated in

sterile water and was subjected to amplification of the PCR. The mixture for PCR consisted of firststrand cDNA, four dNTPs, the specific primers, 2.5 units/100 μ l of *Taq* DNA polymerase (*AmpliTaq* DNA Polymerase, Perkin-Elmer Cetus Co., Norwalk, USA) and PCR buffer (containing 1.5 mM Mg^{++}). Using a Zymoreactor AB-1800 (Atto Co., Japan), the amplification reactions were performed for 35 cycles, which consisted of a melting step at 95°C for 1 min and a combined annealing and extension step at 60°C for 1 min. The PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE) with ethidium bromide staining. Specific oligonucleotide primers (Fig. 1) for PCR were designed according to the rat DNA sequences (18, 25, 26, 30) by analyzing the data of the GenBank.

RESULTS

ET-immunoreactivity (ET-IR) in the lung was first detected in the bronchial cuboid epithelia in a 13-day fetus (Fig. 2A); the reactive intensity remained constant up to 17 days of gestation. The ET-IR was always found diffusely in almost all the peripheral bronchial epithelial cells. The epithelium in the stem of the bronchial tree was immunonegative (Fig. 2B). Thereafter, ET-IR decreased gradually according to the differentiation of the lung, finally disappearing after birth (Fig. 2C).

In the submandibular gland, the ET-IR was first observed in undifferentiated duct cells of an 18-day fetus (Fig. 3A), and gradually increased after birth (Fig. 3B). At this stage, the ET-IR was found in only a few parts of the duct. About 12 weeks after birth, when the granular convoluted tubule (GCT) cells had matured, the ET-IR was observed predominantly in this portion of the duct system (Fig. 3C). No ET-IR was found in any acinar cells. In adult rats, the ET-IR was diffusely distributed in the cytoplasm of almost all the GCT cells. ET-IR in the GCT cells was strongest in the adults (3 months) and remained strong, even in the aged (3-years-old) rats (Fig. 3D), in-

ET-1	primer 1	5'-GAGCTCCAGAAACAGCTGTCTT-3'
	primer 2	5'-CAGCTGCTGATAGATACACTTC-3'
ET-3	primer 1	5'-GCTGCACGTGCTTCACTTAT-3'
	primer 2	5'-AACTCACTCAGGAGTGTGA-3'
ET-AR	primer 1	5'-AGCAATGGCTCAATGCATGGCT-3'
	primer 2	5'-ATGACGAAGCCGATTGCTTCTG-3'
ET-BR	primer 1	5'-GCAGATTGCCTTGAATGACCAC-3'
	primer 2	5'-TCAGAAGTTCACACCTCTGAGG-3'

FIG. 1. Specific oligonucleotide primers for PCR.

FIGS. 2A-C. Photomicrographs shows rat lung immunostained with ET-antibody.

- A. ET-IR in the bronchial epithelia of a 13-day fetus. $\times 200$
 B. A 17-day fetus. The peripheral epithelia of the bronchial tree are immunopositive (arrow), while the epithelium in the center of the tree is negative (arrowheads). $\times 200$ C. A 3-month-old rat. No ET-IR was observed. $\times 200$

dependent of the aging process.

The ET-IR in the GCT-cells of the adult submandibular gland was absorbed by adding 1 mg of ET-1 (Fig. 4), ET-2, or ET-3 to 10 ml of diluted (1/3000) primary antibody solution, while the ET-IR was not affected by the addition of a large amount (100 mg) of big ET-1 to the 10 ml of the working solution of the primary antibody.

The DNA fragments amplified by the PCR procedure were observed as 434 base pairs (ET-1), 86 base pairs (ET-3), 497 base pairs (ET-AR), and 164 base pairs (ET-BR) on the 6% polyacrylamide gel (Fig. 5). These findings, i.e., the gene expressions of ET-1, ET-3, ET-AR, and ET-BR, were obtained in both the lungs and the submandibular glands of all examined stages. The results of the present study using immunohistochemistry and PCR technique are summarized in Table 1.

DISCUSSION

ETs are now considered to have not only vasoconstrictive but also cell-growth stimulatory activity (3, 12). ETs and their receptors have been demonstrated to exist in many organs of the rat, using immunohistochemistry (8), radioimmunoassay (13, 14, 20), *in situ* hybridization (19), Northern blotting analysis (18, 25, 26), and autoradiography (15, 23,

27). However, *in vivo* studies of ET-producing cells, except for vascular endothelial cells (31), have lagged behind the *in vitro* studies. Since the ET content in various organs and tissues is very low, staining with ET antibodies is difficult to accomplish. In addition, it is possible that ET is detected at only some stages of development. For this reason, we carried out ontogenetic investigations of ET in the rat lung and submandibular gland. Prior to this investigation, we prepared a polyclonal ET-antibody specific to the C-terminus of ET-1, 2, and 3. The result of the histochemical absorption test indicates that this antibody caps the C-terminus of the ETs and neutralizes ET-activities by antigen-antibody reaction.

ETs and their receptors in the rat lung have already been studied by various approaches (14, 15, 18-20, 23, 25-27, 30, 32); however, there are very few ontogenic studies on the distribution of ET-producing cells, i.e., investigations of the stages at which ET appears and on the cells which contain it. Until now, only a paucity of information is available on the ET in the rat lung, such as: the expression of ET-3 mRNA in the 19-day fetus and in adult rats by an *in situ* hybridization technique (19), high concentrations of ETs by radioimmunoassay (32), and enzyme immunoassay (20). The fetal lung develops in 3 stages, glandular, canalicular, and alveolar (16). In the present study, ET-IR was first detected in the bronchial

FIGS. 3A-D. Photomicrographs show the ET-containing cells in the rat submandibular gland.
 A. An 18-day rat fetus. ET-IR first appeared. $\times 200$
 B. A neonatal rat (1 week after birth). ET-IR is gradually increased in number and density. $\times 200$
 C. A 3-month-old rat. ET-IR is localized in GCT-cells. $\times 200$
 D. A 3-year-old rat. $\times 200$

epithelium at 13 days, when the lung tissue appeared. The ET-IR was observed successively up to the 17th day, the end of the glandular stage. The epithelium at this stage is composed of cuboidal cells and is rich in glycogen (1). We found that the ET-IR in the epithelial cells was gradually reduced during the canalicular and alveolar stages in a manner similar to that of glycogen granules (1). Our findings indicate that the level of ET is highest from the 13th to the 17th day of gestation in the fetus, after which its decrease until birth. After birth, the ET-IR became negative, although gene expression was detectable. The PCR method also indicated the presence of gene expression of ET-1, ET-3, ET-AR, and ET-BR mRNA in the lung on and after the 18th day of gestation, which was the stage at which we were able to excise the lung tissue from the living fetus. Because of the lower ET

FIG. 4. Photomicrograph shows no immunoreactivity in the adult rat submandibular gland, using the primary ET-antibody preabsorbed by adding 1 mg of ET-1 in the 10 ml of working antibody solution. $\times 100$

FIG. 5. This photograph shows polyacrylamide 6% gel electrophoresis of PCR products from the sample of a 3-month-old rat. M: ϕ X174/HaeIII digest
 1: ET-1 (Salivary gland) 2: ET-1 (Lung) 3: ET-3 (Salivary gland) 4: ET-3 (Lung)
 5: ET-AR (Salivary gland) 6: ET-AR (Lung) 7: ET-BR (Salivary gland) 8: ET-BR (Lung)

TABLE 1. A summary of the appearance of ETs detected by an immunohistochemical (IHC) technique and a polymerase chain reaction method (PCR).

	Fetus	13	15	17	19	21day	Neonate	Adult(3month)	Aged(3years)
Salivary gland	—	—	—	±	±	±	±*	+	+
Lung	—	+	+	+	±*	—*	—*	—*	—*

(+:Positive, ±:Indeterminate, -:Negative by IHC)
 (*:Positive by PCR)

content in the adult rat lung, ETs are not immunohistochemically stained. Considering that the fetal lung does not respire, it is possible that the ET in the lung may have a function other than being involved in respiration or regulation of bronchial constriction.

With regard to the submandibular gland, only the gene expressions of ET-1 and ET-BR mRNA in the adult rat have been studied by Northern blotting hybridization (25, 30). However, the ontogenesis of ET-containing cells in this gland had been unknown, until we found that ET-IRs were localized predominantly in the GCT. The GCT located between the intercalated duct and the striated duct of the submandibular gland (24) is differentiated from the distal portion of the striated duct, and is considered to be the secretory portion of the ductal system. The secretory granules of GCT cells, which are more prominent in male rodents than in females, have been shown to contain nerve growth factor (2, 4, 22), epidermal growth factor (2, 5, 22), renin (2, 9), and esteropeptidases (2, 7). Epidermal growth factor has also been reported to exist in the normal ductal system and ductal origin tumor cells of humans (28), in addition to atrial natriuretic polypeptide in the submandibular glands of rats (21). ET, which may have cell-proliferation and -differentiation activity, is thought to co-exist in these cells with the above-mentioned bioac-

tive substances and to be co-secreted from GCT cells into saliva. In the rat, GCT first appears at 20 days after birth; it differentiates gradually and is fully mature at 12 weeks of age (17). Epidermal growth factor is first detectable by immunohistochemistry in the male mouse GCT cells at 20 days of age (11). However, ET appeared in the undifferentiated duct cells of 18-day fetuses where the secretory granules first appeared (6). Since the GCT cells had not yet differentiated in the fetus, it is possible that these cells may begin to differentiate in parallel with the appearance of ET. After the differentiation of GCT cells, the ET-IR was found predominantly in these cells. Our PCR method also indicated the presence of the gene expression of ET-1, ET-3, ET-AR, and ET-BR mRNA in the postnatal and adult submandibular gland. Since the fetal gland was too small to remove from the fetus, we were unable to carry out the procedure for the detection of gene expression in this tissue.

The finding that immunopositive cells were first detected at the time of lung maturation indicates that ET may play an important role in maturation and differentiation as a growth factor. Moreover, the coexistence of ET-AR and ET-BR in the same organ indicates that ET may also act as a local humoral agent. On the other hand, ET in the submandibular gland is co-secreted with growth factors and other bioactive

peptides into exocrine saliva. However, the specific function of ET in each organ still remains unknown. In addition to ontogenetic studies combining immunohistochemistry and PCR, such as this one, further studies using electron microscopes or *in situ* hybridization should be performed in other organs and/or other species to elucidate this matter.

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