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Immunohistochemical Localization of Epidermal Growth Factor in the Developing Rat Gonads

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ABSTRACT—Epidermal growth factor (EGF) is known to have various endocrine, paracrine and autocrine roles in adult mammalian tissues. In order to clarify the participation of EGF in rat gonadal differentiation, immunohistochemical localization of EGF as chronologically studied in perinatal rat gonads. Sprague-Dawley rat gonads from gestational day (GD) 13 to postnatal day (PD) 21 were fixed in acetic acid-free Bouin's solution and stained with a polyclonal antibody against mouse EGF by using avidin-biotin complex technique. Immunohistochemical reactivity was positive in almost all cell types in the prenatal male gonads. Male germ and Leydig/interstitial cells showed a positive reactivity from GD 15 to 21. Slight and moderate staining were seen in the Sertoli/supporting cells from GD 13 to 21. After birth, positive expression was not seen in any types of cells in male gonads except for germ cells on PD 21. On the other hand, in prenatal female gonads positive signs were found in the interstitial cells from GD 14 to 21 and in the granulosa cells on GD 21. During the postnatal period from PD 5 to 21, the granulosa and theca cells were slightly positive and the interstitial cells moderately positive. Wolffian ducts in males and Müllerian ducts in females were stained during the prenatal period. These results indicate that EGF shows stage-specific patterns of expression in the developing rat gonadal cells and may be involved in the rat gonadal development and diffferentiation.

INTRODUCTION

Epidermal growth factor (EGF), a single-chain polypeptide of 53 amino acid residues, was first isolated from the submandibular gland of male mice [8]. This peptide is a potent mitogen for a wide variety of cell types *in vivo* and *in vitro* and distributes in various tissues and fluids in mammals [7]. EGF is mainly synthesized in the submandibular gland of mice [5] and rats [22], and its synthesis is under the control of androgens [6] and thyroid hormones [9].

EGF is involved in many physiological functions in the adult gonads [24]. EGF reduced follicle-stimulating hormone (FSH)-stimulated testosterone production in rat Leydig cells *in vitro* [15], which was due to a regulation in 17β-hydroxylase activity [25]. EGF also attenuated FSH-stimulated aromatase activity in the Sertoli cells [2] and the granulosa cells [15], but stimulated Sertoli cell proliferation [16]. FSH-stimulated inhibin production in the Sertoli cells [20] and plasminogen activator production in the granulosa cells were increased by EGF [10]. Although basic properties and functions of EGF have been reported in adults, little is known about its contribution to the fetal gonadal development and diffferentiation.

In order to clarify the participation of EGF in developing rat gonads, immunohistochemical expression of EGF was chronologically studied in the fetal and prepubertal rat gonads from gestational day (GD) 13 to postnatal day (PD) 21.

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MATERIALS AND METHODS

Aminals

Crj: CD (Sprague-Dawley) rats from 13 to 20 weeks of age were housed in constant temperature (23±1°C), relative humidity (55±10%) and light-dark cycle (light on 7:00–19:00). Animals were given a commercial diet (CRF-1, Charles River Japan Co.) and tap water *ad libitum*. Cohabitation was done in the evening of vaginal proestrus in the 1:1 basis of male: female. In the next morning, copulation was checked by the presence of sperm in the vaginal smear. The day when sperm-positive smear was found was designated as GD 0, and the day when litter was found was designated as PD 0.

Preparation of tissues for immunostaining

Dams were sacrificed from GD 13 to 21 and neonates on PDs 5, 11 and 21 by diethyl ether anesthesia. The gonads and genital ducts dissected from more than three fetuses and pups in different litters were fixed in acetic acid-free Bouin's solution for a few hours at 4°C. The sexes of fetuses were determined as described by Agelopoulou et al. [1]. Then the tissues were dehydrated through a series of graded concentrations of ethanol and xylene, embedded in paraffin and sectioned in 5 μm thickness. The male rat submandibular glands on PD 35 were also dissected and fixed in the same fixative, for checking the specificity of the antibody used.

Immunohistochemistry

Sections were deparaffinized with xylene and hydrated in decreasing concentrations of ethanol. The sections were incubated with 0.5% periodic acid (Sigma Chemical Co.) in 50 mM Tris-buffered saline (TBS, pH 7.6, Wako Pure Chemical Industries, Ltd.) at room temperature for 30 min to block endogenous peroxidase. Sections were subsequently rinsed with 50 mM TBS for 20 min, blocked non-specific staining with 1.5% normal goat serum in 50 mM

TBS for 20 min. Then, sections incubated overnight at 4°C with the polyclonal antibody against mouse EGF raised in the rabbit (Collaborative Research Inc.) at 0.02 mg/ml in 10 mM PBS. Dose response study indicated that this concentration of the antibody gave optimal labelling results. Follwing this incubation the sections were rinsed with TBS and then treated with 0.5% biotinylated goat anti-rabbit secondary antibody (Vector Lab, Inc. ABC kit Elite) diluted in 50 mM TBS for 30 min at room temperature. Sections were again washed in TBS and subsequently incubated with 2% avidin-biotin complex (Vector Lab, Inc, ABC kit Elite) in 50 mM TBS for 40 min at room temperature. Avidin and biotin were prepared at least 30 min before applied to the sections to allow the complex to form. The sections were again washed in TBS, and the bound antibody was visualized with 0.05% 3,3′-diaminobenzidine tetrachloride (Dojindo Laboratories) in 50 mM TBS (pH 7.2) and 0.02% hydrogen peroxide for 5 min. These sections were counterstained with hematoxylin.

Controls included (a) replacing the primary antibody with normal rabbit serum, (b) using the primary antibody that had been pre-incubated overnight at 4°C with 0.04 mg/ml mouse EGF (Chemicon International, Inc.) before this mixture was applied to the section to check specificity of the primary antibody, and (c) omitting the primary antibody to check the specificity of the secondary antibody.

RESULTS

Specificity of antibody

Preparations which were stained with the antibody to EGF, and with the immunoneutralized antibody by pre-incubated with the antigen were shown in Fig. 1. EGF antibody stained the granular convoluted tubule cells of submandibular gland in male rats on PD 35, but the neutralized antibody did not stain any cells. Therefore, these results showed that this polyclonal antibody specifically stained EGF-containing cells, because the rat submandibular gland was confirmed to contain EGF-positive cells [22].

Immunohistochemical localization

Immunohistochemical expression of EGF in the developing rat gonads was summarized in Table 1. In male gonads, many germ cells were moderately stained from GD 15 to 21 and PD 21 (Fig. 2.A-C). A few Sertoli/supporting cells and almost all Leydig/interstitial cells showed positive reactivity; the staining intensity was moderate or slight in Sertoli/supporting cells from GD 13, the time when the supporting cells were firstly recognized by histological examination, to GD 21 and marked in Leydig/interstitial cells from GD 15 to 21 (Fig. 2.A-C). On the other hand, in female gonads, a few interstitial cells expressed slight reactivity from GD 14 to 21 and moderate from GD 21 to PD 21 (Fig. 2.E-G). A few theca cells slightly stained from PD 5 to 21 and a few granulosa cells slightly stained from GD 21 to PD 21 (Fig. 2.E-G). But the germ cells in females were not shown any positive reactivity during the experimental period (Fig. 2.E-G).

The epithelial cells of the genital ducts were stained in sex-specific manner during the prenatal period: the Wolffian ducts in males were moderately or markedly stained from GD 16 to 21 (Fig. 2.D) and the Müllelrian ducts in females were slightly or moderately stained from GD 16 to PD 21 (Fig. 2.H).

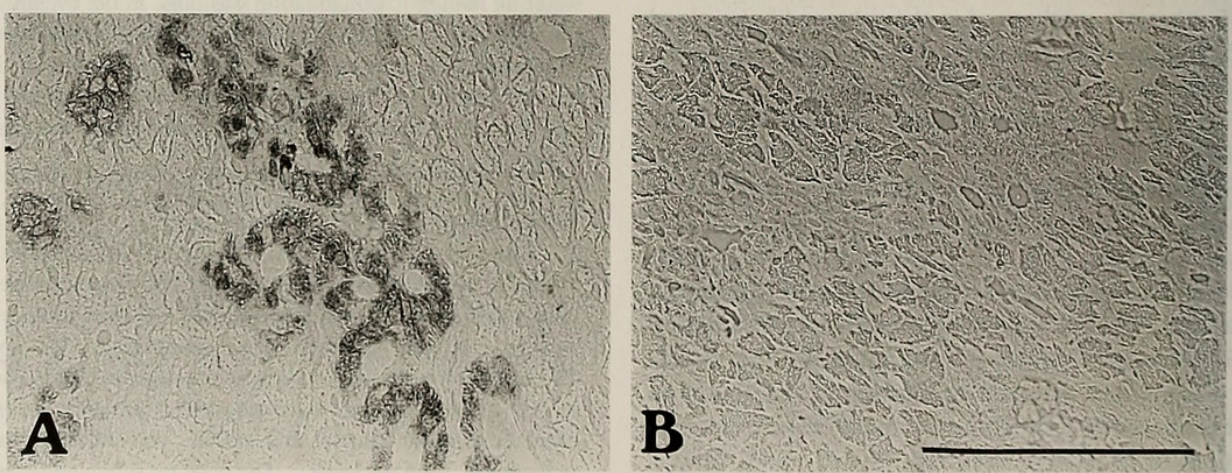

FIG. 1. Demonstration of staining specificity. Male rat submandibular glands on PD 35 incubated with the EGF antibody (A), or with the neutralized antibody (B). Staining is seen in the granular convoluted tubule cells of A, but those of B. Bar: 50 μm.

FIG. 2. Immunohistochemical localization of EGF in the gonad of perinatal rats. (A) Male gonad on GD 14. The Sertoli/supporting cells (arrow) show slight staining. (B) Male gonad on GD 17. In the Leydig/interstitial cells (arrow head), and the moderate stainings in the germ (thick arrow) and the Sertoli/supporting cells (arrow) express marked staining. (C) Male gonad on PD 5. Gonadal cells do not express any positive sign. (D) Male Wolffian duct and on GD 17. The epithelial cells (arrow) show marked staining. (E) and (F) Female gonads on GDs 14 and 17. The interstitial cells (arrow) express slight staining. (G) Female gonad on PD 5. The interstitial cells (arrow head) are stained moderately and the granulosa (arrow) and theca (thich arrow) cells slightly. (H) Female Müllerian duct on GD 17. The epithelial cells (arrow) are moderately stained. All sections are shown in the same magnification. Bar: 50 μm.

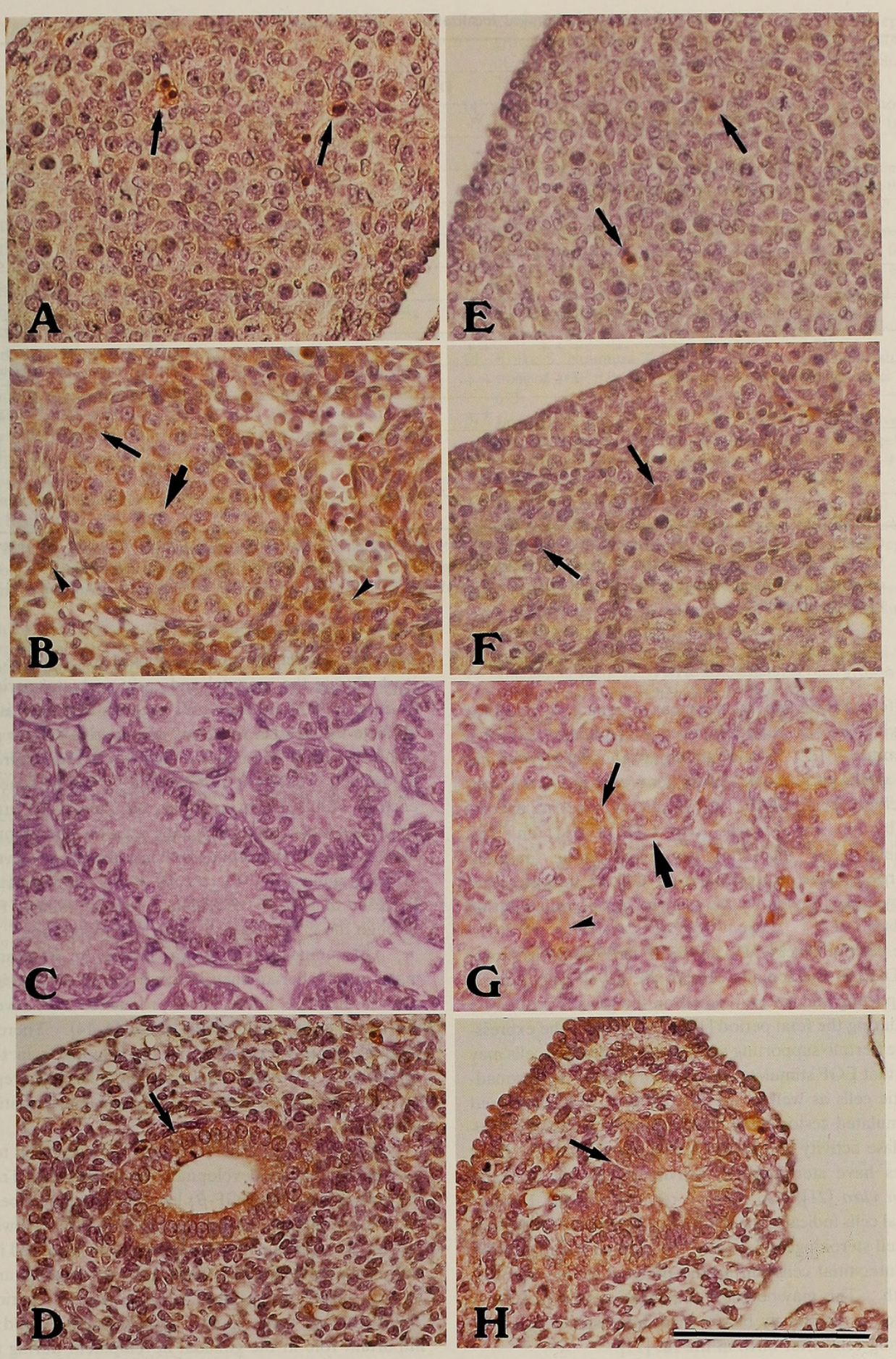
A
E
B
F
C
G
D
H

TABLE 1. Immunohistochemical localization of EGF in the developing rat gonad

		Male						Female					
		Gonad				Duct		Gonad				Duct	
		G	S	P	L	W	M	G	Gr	T	I	W	M
GD	13	−	+/−	*	*	−	*	−	*	*	*	−	*
	14	−	+/−	*	*	−	−	−	−	*	+/−	−	−
	15	++	+/−	*	+++	−	−	−	−	*	+/−	−	−
	16	++	+/−	*	+++	++	−	−	−	*	+	−	++
	17	++	++/−	*	+++	+++	*	−	−	*	+	−	++
	18	++	++/−	−	+++	++	*	−	−	*	+	−	+
	19	++	++/−	−	+++	++	*	−	−	*	+	*	+
	20	++	++/−	−	+++	++	*	−	−	*	+	*	+
	21	++	+/−	−	+++	++	*	−	+/−	*	++	*	++
PD	5	−	−	−	−	−	*	−	+/−	+/−	++/−	*	++
	11	−	−	−	−	−	*	−	+/−	+/−	++	*	+
	21	++	+/−	−	−	−	*	−	+/−	+/−	++	*	+

GD: Gestational day, PD: Postnatal day, G: Germ cell, S: Sertoli/Supporting cell, P: Peritubular cell, L: Leydig cell, W: Wolffian duct, M: Müllerian duct, Gr: Granulosa/Supporting cell, T: Theca cell, I: Interstitial/Stromal cell, Grade, −: Not detectable, +: Slight but above background levels, ++: Moderate, +++: Marked staining, *: The cells or tissues were not found in that day.

DISCUSSION

Differentiation and development of gonads are complex events which involve various cell-cell interactions. EGF is a potent mitogen [12, 13] and affects to gonadal steroid and peptide syntheses [15, 26]. Male germ cells showed a positive reactivity from GD 15. Germ cells in males are encompassed by the somatic cells from GD 14 ([11] and this experiment) and thereafter undergo mitotic division. Therefore, positive expression from GD 15 indicates that EGF may have a function in the germ cell proliferation during the fetal period.

The positive reactivity to anti-EGF antiserum was shown from GD 13 in the somatic cells of the developing gonads. The supporting and interstitial cells in the gonads proliferate rapidly during the fetal period [19]. Therefore, the expression in the Sertoli/supporting and Leydig/interstitial cells may indicate that EGF stimulates the proliferation of these gonadal somatic cells as well as the germ cells. EGF decreased FSH-stimulated testosterone production by regulating 17β-hydroxylase activity in Leydig cells *in vitro* [15, 25]. Fetal rat testes have steroidogenic activity during the prenatal period *in vitro* [21]. So positive reactivity in the Leydig/interstitial cells indicates that EGF may contribute to the fetal Leydig cell steroidogenesis. But the marked staining of the Leydig/interstitial cells during the prenatal period was lost after birth. This may be due to a regression of fetal Leydig cells which begin shortly before birth and replace to adult-type Leydig cells after birth in the rat [11].

The interstitial cells in female gonads have not been known to participate in physiological roles on the ovary, at least, during the prenatal and early postnatal period. The positive staining in these cells suggests that EGF is likely to mediate the ovarian functions in autocrine and/or paracrine fashions. EGF stimulates or inhibits the immature rat granulosa cell proliferation depending on the presence or absence of FSH [3]. In addition, the theca cells produce an EGF-like substance, which may regulate the granulosa cell functions [23]. Together with these results, positive staining in the granulosa and theca cells during the perinatal period suggests that EGF contributes to the follicle formation through the granulosa and theca cell poliferation.

The immuno-reactivity was seen in the Wolffian ducts in males and Müllerian ducts in females. EGF plays important roles in the male and female reproductive-tract development during the perinatal or critical period [4, 14]. Therefore, the immuno-positive expression in the gonoducts in this stage supports the previous findings that EGF has been reported to be concerning with the development and differentiation in the gonoducts.

Other growth factors have also been reported to participate in the gonadal development in rat fetuses. Transforming growth factor-β (TGF-β) localized in many types of cells in developing fetal gonads [18], like EGF. However, inhibin-α, a member of TGF-β superfamily, localized in Sertoli cells at the time of seminiferous tubule formation and in the Leydig cells during the late gestational period [17]. Together with these results, growth factors could play an important role in the gonadal development during the perinatal period. The mechanisms and roles of these growth

factors on gonadal development are under investigation.

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