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Cellular localization of immunoreactive epidermal growth factor during Wolffian duct differentiation of the fetal mouse

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Abstract Previous studies from this laboratory indicated a role for epidermal growth factor (EGF) in androgen-dependent male reproductive tract differentiation of the fetal mouse. Expression of an EGF-like protein during Wolffian duct differentiation was indicated from the determinations by radioimmunoassay (RIA) and radioreceptor assay. To further characterize the protein and to assess its role in male sexual differentiation, expression of the protein has been analyzed by Western blot assay and its tissue-specific cellular expression has been determined by immunocytochemical assay in the present study. Western blot analysis of the 18-day fetal male reproductive tract detected an immunoreactive band of the predicted 6-kDa size. Immunocytochemical analysis also detected EGF-specific immunostaining in the Wolffian duct derivatives. At day 18 of gestation, the staining was localized predominantly in the epithelial nuclei of the Wolffian duct derivatives whereas at days 14 and 16 of gestation, the staining was equally distributed in the mesenchymal and epithelial sites of the Wolffian duct derivative. The intensity of the staining increased with progression of differentiation during the 14th–18th days of gestation. Prenatal exposure to the antiandrogen flutamide significantly reduced the immunostaining of the duct. Thus, a role for EGF in Wolffian duct differentiation is indicated.

Key words Epidermal growth factor · Androgen · Sexual differentiation · Fetal reproductive tract differentiation

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Introduction

Differentiation of the male reproductive tract is dependent on secretion of testicular hormones, androgen and müllerian inhibiting hormone [14, 15]. Androgen stabilizes the Wolffian duct structure and causes differentiation of the Wolffian duct (progenitor of the male ductal reproductive organs) and urogenital sinus into other male reproductive structures [1, 10, 15, 20, 21]. Although androgens are essential for male reproductive tract differentiation, it is becoming apparent that they are not sufficient to induce normal growth and function. A series of recent observations suggests that growth factors, in particular EGF, may play an important role in the male reproductive system [3, 5, 7, 9, 11–13]. We demonstrated that anti-EGF antibody, added in the organ culture of Wolffian duct differentiation, prevented the Wolffian duct morphogenesis and that this was reversed when the medium was supplemented with exogenous EGF [13]. The presence of EGF receptor in the developing Wolffian duct was demonstrated both by us [9, 11] and other workers [3, 26]. Expression of EGF-mRNA has been demonstrated during the male reproductive tract differentiation [12] and expression of an EGF-like protein was detected during this process by radioimmunoassay (RIA) and radioreceptor assay [13].

The molecular form of the EGF-like protein, identified in a previous study [13], was not determined. Additionally, cellular localization of expression of such protein, correlating with Wolffian duct differentiation, was not assessed. Using Western blot analysis and cytochemical analysis we demonstrated localized expression of EGF at the site of Wolffian duct morphogenesis.

Materials and methods

Animal

CD-1 females from the Charles River breeding laboratory (Wilmington, MA) were bred in our mouse colony. The day of breeding

was counted as day zero of gestation. Pregnant mice were injected s.c. with flutamide (100 mg kg^{-1} day^{-1}) or vehicle from days 13–17 of gestation, and fetuses were removed on day 18 of gestation after cesarean section. The reproductive tract containing Wolffian duct and the adjoining urogenital sinus was isolated from the male fetuses by microdissection. In some experiments, different regions of the reproductive tract were separated further by microdissection and used in the analysis. All animal procedures were carried out under the guidelines of the NIH.

Electrophoresis and Western blot analysis

Ten milligrams of each tissue isolated from the male and female fetuses were homogenized in 100 µl 50 m*M* TRIS, 5 m*M* ethylenediaminetetraceta (EDTA), pH 8.0, containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF) by sonication at 4°C. The protein concentration in the homogenate was determined and an aliquot (approximately 300 µg) was used for Western blot analysis. The sample was heated with electrophoresis-sample buffer [50 m*M* TRIS containing 1 m*M* EDTA, 0.1 *M* sucrose, 3% sodium dodecyl sulfate (SDS), pH 6.8] at 95°C and electrophoresed as described elsewhere [18] using 16 × 14 cm polyacrylamide-SDS gel (5% stacking gel and 12% separating gel). A low molecular standard containing myoglobin and its fragments I, II and III, the molecular weight ranging from 16 kDa to 2.5 kDa (Sigma, St. Louis, MO) was used with each gel run. After electrophoresis, the proteins were transferred for only 30 min electrophoretically to a nitrocellulose membrane using Hoffer's semidry trans blot apparatus according to the method described elsewhere [23]. Our initial transfer for a longer period (more than 1 h) resulted in a complete loss of EGF protein due to transfer of the protein through the nitrocellulose membrane and therefore the time was adjusted to avoid the loss. The transfer of the proteins was quickly checked by staining the nitrocellulose paper with Ponceau S (Sigma, St. Louis, MO) stain followed by destaining with water if this blot was planned to be used for immunodetection. For detecting proteins only, the blots were stained for 5 min with Coomassie blue followed by destaining with 40% methanol for 10 min.

Western blot analysis was carried out using specific anti-EGF serum (Collaborative Research, Bedford, MA). The antibody is specific for EGF and has no cross-reactivity to TGFα. The nitrocellulose membrane, after the protein transfer, described above, was washed 3 times with TBS buffer (100 m*M* TRIS-saline buffer, pH 8.0) containing 0.2% Tween-20 over a 15-min period. The blot was then incubated with the blocking buffer [1.5% goat serum, 3% milk powder and 1% bovine serum albumin (BSA) in TBS] for 2 h. The blot was washed and then treated with the anti-EGF antibody (1:500 dilution of the supplied serum) in blocking buffer for 16 h at 4°C and subsequently washed. This was next incubated with a biotinylated anti-rabbit antibody (Vector Laboratory, Burlingame, CA) for 1 h at room temperature according to the manufacturer's instructions and washed extensively. The nitrocellulose paper was then incubated in the ABC-alkaline phosphatase reagent (Vector Laboratory) for 1 h at room temperature using a reaction kit. The reactive bands were visualized with an ABC-alkaline phosphatase substrate kit (Vector Laboratory) according to the manufacturer's instructions. The immunoreactive bands were estimated using densitometric analysis.

Immunocytochemical analysis

Immunocytochemical analysis of EGF was carried out as described elsewhere [8]. In brief, the tissue was fixed in 4% buffered formalin for 3–4 h. The tissue was then washed with PBS and equilibrated in 20% sucrose-PBS solution for 16 h. The tissue was then mounted in Tissue Tek-OCT compound (Miles Labs., Elkhart, IN) and frozen. Serial transverse sections (10 mµ) were cut from each tissue using a microtome, and alternate sections were collected in two slides, one of which was used for immunostaining and the other for control staining. The sections providing maximum information on the intracellular structures of a specimen (usually mid-sections) were chosen for analysis. The two best slides thus selected were treated with 0.06% pronase solution for 8–10 min at room temperature and the reaction was stopped by washing the slides with PBS. The slides were then treated with a blocking solution containing 1.5% goat serum in PBS for 1 h in a humid atmosphere. One of the slides was treated with 1:200 dilution of EGF antibody (Collaborative Research anti-EGF serum) and the other was treated with 1:200 anti-EGF serum pretreated with excess of EGF (10 µg/ml for 1 h). The immune reaction was detected with an immunoperoxidase staining kit (Vector Laboratory) according to the manufacturer's instructions. For each tissue, a control reaction was carried out in each experiment and the analysis was repeated at least 3 times for each tissue.

Results

Identification of EGF in the developing male reproductive tract

Western blot analysis using anti-EGF antibody revealed a reactive band, at the expected size of 6 kDa (Fig. 1, lane –) in the 18-day male reproductive tract containing Wolffian duct derivative and urogenital sinus. The staining of the reactive band was reduced by pretreatment of the antibody with authentic EGF (2 µg/ml; Fig. 1, lane +), suggesting EGF specificity of the 6-kDa band. No immunoreactive band was detected using the urological sinus alone (data not shown). Thus, the immunoreactive band detected in the 18-day reproductive tract containing Wolffian duct derivative and urogenital sinus was associated with EGF expression at the Wolffian duct tissue only. The 6-kDa band was found also at day 14 and day 16 but the intensity of staining

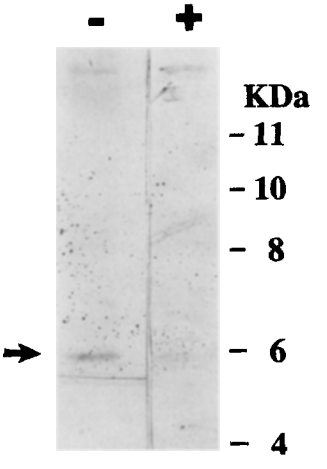

Fig. 1 Western blot analysis of expression of EGF in the 18-day fetal male reproductive tract. Lane – represents the reaction with a protein around 6 kDa size using untreated antibody (1:500 dilution) and lane + represents the reaction using antibody pre-adsorbed with EGF (2 µg/ml). Total protein 300 µg was applied in each lane. The molecular weight standard contained myoglobin fractions of different sizes ranging from 2.5 to 11.2 KDa

was significantly less at these ages and could not be determined accurately.

Cytochemical localization of EGF expression during Wolffian duct differentiation

The role of EGF in Wolffian duct differentiation was further assessed by examining EGF immunoreactivity at the site of Wolffian duct differentiation. As shown in Figs. 2 and 3, EGF-specific staining was noted in the epithelial nuclei of the Wolffian duct and its derivative, epididymis and efferent ductules. A lower level of staining was also noted in the mesenchymal nuclei of these tissues (Figs. 2, 3). The intensity of the staining decreased gradually along the efferent duct, epididymis head and vas deferens, correlating with testosterone transport from the testis through these segments of the Wolffian duct. The immunostaining disappeared following exposure to the antibody pretreated with EGF (Figs. 2C, 3C). Flutamide, an inhibitor of androgen binding to its receptor, almost completely abolished the immunostaining of the Wolffian duct (Fig. 2D), suggesting a role for androgen receptor in EGF expression in the developing duct.

Expression of EGF at different stages of Wolffian duct differentiation

Cytochemical expression of EGF was determined at days 14, 16 and 18 of gestation, the period of Wolffian duct differentiation. A low level of EGF expression was found at the onset of Wolffian duct differentiation at day 14 (Fig. 4). The expression increased with progression of differentiation. The expression at day 18 was significantly higher than that at days 14 and 16 of gestation. No significant difference in the expression of EGF was noticed in the mesenchyme vs epithelium at days 14 and 16, but at day 18 the expression was significantly higher in the epithelium (the site of ductal morphogenesis) than in the mesenchyme.

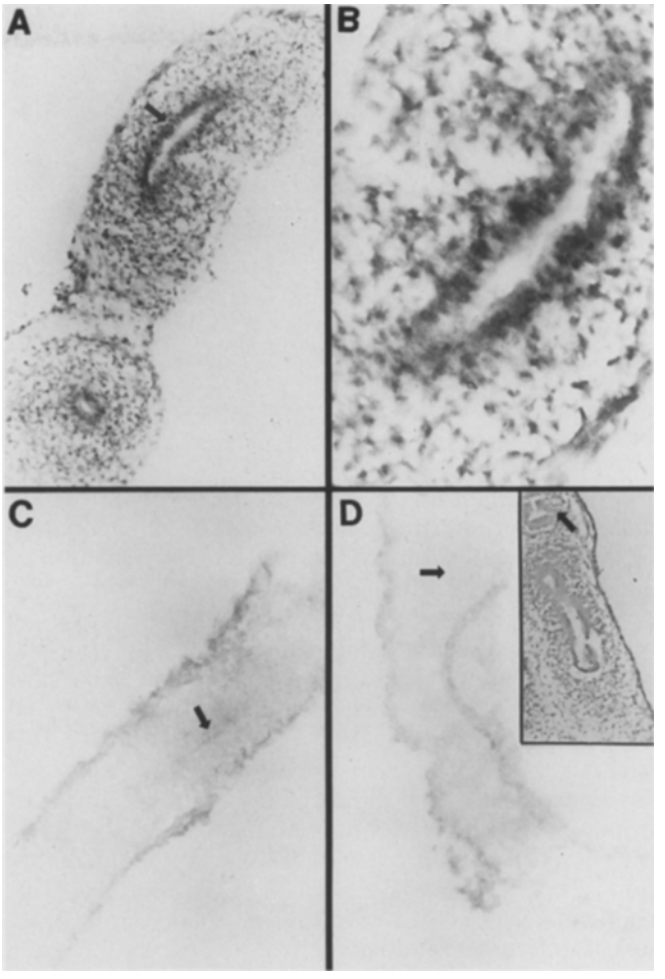

Fig. 2A–D Immunohistochemical analysis of EGF expression in the 18-day fetal ductus deferens. **A**, **B** represent the reaction using anti-EGF serum (1:200 dilution). **A** ×250; **B** ×600. **C** represents the reaction using anti-EGF serum, pre-treated with EGF (10 μg/ml). **D** represents the reaction with flutamide-exposed male reproductive duct. Note the strong immunostaining in the epithelial nuclei and lower staining in the mesenchymal nuclei in **A** and **B**. The stain was absent when examined with the flutamide-exposed duct in **D**. *Arrow* in **A** indicates the ductal region that has been magnified in **B** and *arrow* in **C** and **D** indicates the position of the ductal lumen. *Insert in* **D** is a hemotoxylin-stained section, indicating the ductal configuration in that specimen

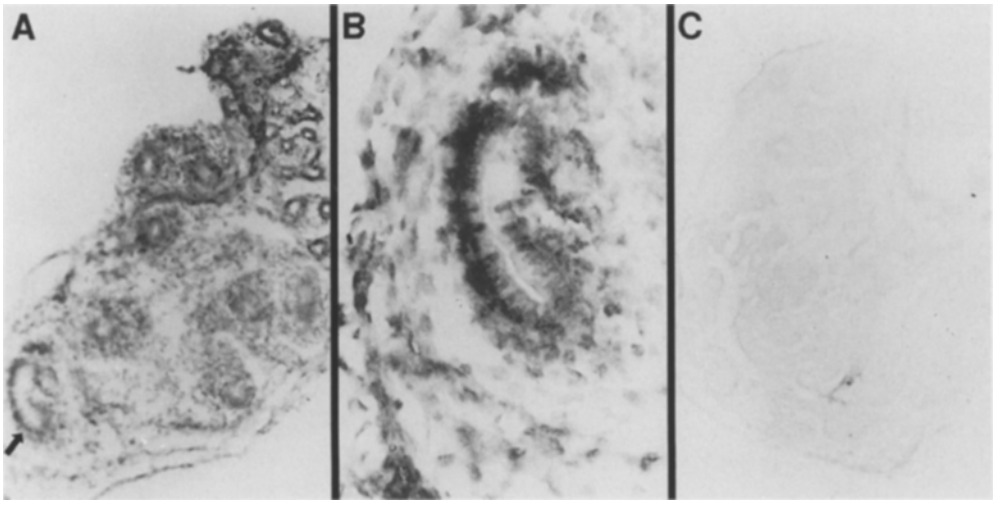

Fig. 3A–C Immunohistochemical analysis of EGF expression in the epididymis. **A**, **B** represent the reaction using immune serum and **C** represents the reaction using EGF-adsorbed immune serum. **A** ×250; **B** ×600; and **C** ×250. Immunostaining was found in both mesenchyme and epithelium, but the epithelium showed higher expression than the mesenchyme. *Arrow* in **A** indicates the structure that has been magnified in **B**

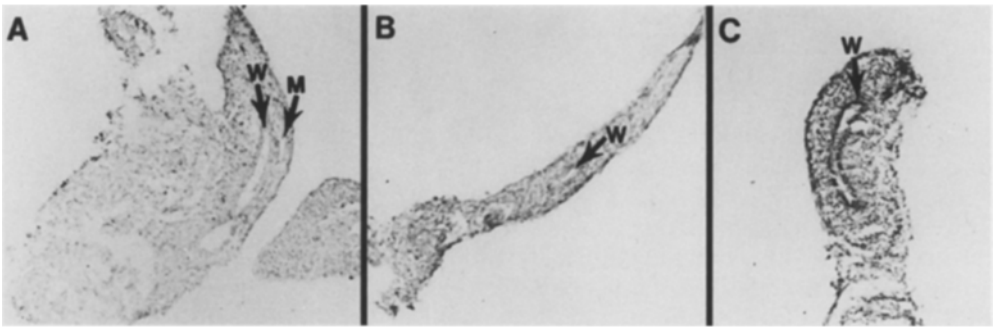

Fig. 4A–C Immunohistochemical analysis of EGF expression at day 14 (**A**), day 16 (**B**) and day 18 (**C**) of gestation, ×200

Discussion

Although significant progress has been made toward understanding the effect of androgen on differentiation of the reproductive organs, its molecular mechanism of action remains poorly understood. Recent observations by different laboratories indicated a role for EGF in male reproductive tract differentiation [9, 11–15]. It was shown that anti-EGF antibody inhibited masculine differentiation in the presence of testosterone and that EGF replacement reversed the inhibition [13]. This suggests that EGF is required in addition to androgen for normal masculine differentiation. The role of EGF in masculine differentiation was further established by the present observation that Wolffian duct differentiation accompanies expression of EGF protein. This cooperative effect of androgen and EGF during male sexual differentiation is similar to the observation made during androgen-induced prostatic cell differentiation and proliferation [19]. It was shown that androgen was not able to induce prostatic cell differentiation unless it was provided with other hormones and growth factors [19].

It has been shown that androgen is able to increase the levels of EGF [4, 24] and its receptor in various tissues [16, 22]. Neonatal androgen was also shown to imprint the EGF receptor expression in response to androgen during the pubertal and adult period [16]. The present finding that flutamide inhibits EGF expression in the male reproductive tract suggests a role of androgen and its receptor in mediating the induction of EGF during Wolffian duct differentiation. This suggestion is further supported by the observations that EGF expression increased in the fetal male duct during the period the testosterone level was reported to increase [15]. Additionally, expression of EGF was found to correlate with the level of testosterone secretion at the different parts of the Wolffian duct, the highest being in the efferent duct and the lowest being in the vas deferens site of the 18-day Wolffian duct. Thus, a role of testosterone in EGF synthesis during Wolffian duct differentiation is indicated.

Immunocytochemical analysis in this study indicated predominant expression of EGF at the nuclear site of the 18-day Wolffian duct whereas at days 14 and 16 the expression of EGF was found equally distributed in the mesenchyme and epithelium. A similar trend was noticed when expression of AR was investigated. Initially, expression of AR was found in the mesenchyme and as the fetus grew older the expression of AR was localized in the epithelial site of the Wolffian duct [2]. This may imply that expression of androgen receptor and EGF in the fetal Wolffian duct is interdependent. The finding that flutamide, blocking androgen action at the level of androgen receptor binding, inhibits EGF expression in the 18-day Wolffian duct supports this speculation.

The biochemical mechanism of EGF modulation of sexual differentiation is not clear at present. EGF has been shown to modulate a variety of biological effects by virtue of its ability to induce phosphorylation and dephosphorylation of cellular proteins through EGF receptor binding [6, 25]. A similar mechanism may be involved in EGF activation of the androgen action during male sexual differentiation. Phosphorylation of androgen receptor is a critical phenomenon in different androgen receptor-mediated action [17] and EGF may play a role in that process. Alternatively, EGF may act in an indirect manner by inducing phosphorylation of a protein and this, in turn, acts in concert with androgen receptor and thereby activates the androgen-responsive system. Studies are in progress to determine the mechanism of EGF modulation of sexual differentiation.

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