Hormonal Basis for the Gender Difference in Epidermal Barrier Formation in the Fetal Rat

Abstract

Previous studies have shown that ontogeny of the epidermal permeability barrier and lung occur in parallel in the fetal rat, and that pharmacologic agents, such as glucocorticoids and thyroid hormone, accelerate maturation at comparable developmental time points. Gender also influences lung maturation, i.e., males exhibit delayed development. Sex steroid hormones exert opposite effects on lung maturation, with estrogens accelerating and androgens inhibiting. In this study, we demonstrate that cutaneous barrier formation, measured as transepidermal water loss, is delayed in male fetal rats. Administration of estrogen to pregnant mothers accelerates fetal barrier development both morphologically and functionally. Competent barriers also form sooner in skin explants incubated in estrogen-supplemented media in vitro. In contrast, administration of dihydrotestosterone delays barrier formation both in vivo and in vitro. Finally, treatment of pregnant rats with the androgen antagonist flutamide eliminates the gender difference in barrier formation. These studies indicate that (a) estrogen accelerates and testosterone delays cutaneous barrier formation, (b) these hormones exert their effects directly on the skin, and (c) sex differences in rates of barrier development in vivo may be mediated by testosterone. (J. Clin. Invest. 1996. 97:2576–2584.) Key words: fetal skin development • transepidermal water loss • stratum corneum lipids • lamellar bilayers

Introduction

The principal function of the skin is to provide a protective barrier against transcutaneous water loss. The epidermal permeability barrier resides in the outermost layers of the mammalian epidermis, the stratum corneum, and is mediated by hydrophobic lipids organized into multiple lamellar membranes (1–3). Precursor lipids are packaged in lamellar bodies in stratum granulosum cells and delivered to the extracellular spaces of the stratum corneum by exocytosis of lamellar bodies (1–3). The epidermal lamellar body secretory system develops during late gestation to allow for the transition from aqueous to atmospheric environment. Immaturity of the epidermal permeability barrier can have extreme consequences in preterm infants, including fluid and electrolyte imbalance, increased caloric requirements resulting from evaporative heat loss, and predisposition to sepsis (4–7).

The development of an analogous lamellar body secretory system in the type II alveolar cell pneumocyte has been studied widely in humans and in experimental animals (8–11). Although lung surfactant contains predominantly phospholipids, a class of lipids absent from barrier lipids, both secretory systems deliver lipid to their respective extracellular domains (1–3, 12–14). Moreover, the epidermal and lung lamellar secretory systems exhibit striking similarities in their developmental timetables, with both maturing in the third trimester in humans and other animals (4, 8–11, 15). In the fetal rat, lamellar bodies first appear in skin and lung on gestational d 18; secretion begins on d 19 and 20, respectively; and maturation of both tissue systems is complete by d 21 of gestation (normal parturition occurs on d 22) (15, 16). Furthermore, glucocorticoids and thyroid hormone accelerate lung maturation (reviewed in reference 17), and, as we have shown, glucocorticoids accelerate epidermal barrier maturation in utero in the rat (18), and both glucocorticoids and thyroid hormone accelerate fetal rat barrier formation in vitro (19). Other hormones are also recognized to affect pulmonary development; for example, estrogen accelerates, while testosterone inhibits lung maturation (20–26). Additionally, a gender difference in the timing of lung maturational events has been noted, with males demonstrating a delay in lung development during late gestation (27–30). Whether there are similar male–female differences during epidermal development, and whether these differences may contribute to sex differences in survival of preterm infants has not been previously examined. The purpose of the present study was to determine if a sex-specific difference exists during cutaneous barrier development, and whether the sex steroid hormones, estrogen and testosterone, affect epidermal barrier formation.

Methods

Materials. Timed pregnant Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA.). Diethylstilbestrol (DES),1 dihydrotestosterone and estrogen accelerates and androgens inhibiting. In this study, we demonstrate that cutaneous barrier formation, measured as transepidermal water loss, is delayed in male fetal rats. Administration of estrogen to pregnant mothers accelerates fetal barrier development both morphologically and functionally. Competent barriers also form sooner in skin explants incubated in estrogen-supplemented media in vitro. In contrast, administration of dihydrotestosterone delays barrier formation both in vivo and in vitro. Finally, treatment of pregnant rats with the androgen antagonist flutamide eliminates the gender difference in barrier formation. These studies indicate that (a) estrogen accelerates and testosterone delays cutaneous barrier formation, (b) these hormones exert their effects directly on the skin, and (c) sex differences in rates of barrier development in vivo may be mediated by testosterone. (J. Clin. Invest. 1996. 97:2576–2584.) Key words: fetal skin development • transepidermal water loss • stratum corneum lipids • lamellar bilayers

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Methods

Materials. Timed pregnant Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA.). Diethylstilbestrol (DES),1 dihydrotestosterone; EGA, estimated gestational age; TEWL, transepidermal water loss.

1. Abbreviations used in this paper: DES, diethylstilbestrol; DHT, dihydrotestosterone; EGA, estimated gestational age; TEWL, transepidermal water loss.
and lead citrate, and examined with a Zeiss 10A electron microscope for electron microscopy. Sections were stained with uranyl acetate.

Results

icance was determined using a Student's t-test.

Centrations of 0.01–100 nM.

agen membranes, and submerged in 4 ml of hormone- and serum-

from d 17 EGA fetuses. Explants were placed dermis down onto col-

was made in d-20 pups.

Control animals were injected subcutaneously during the same time period. TEWL was measured, and sex determination

ated water loss solely through the epidermis, as described previously (19). Samples were then weighed hourly for 6 h using a Cahn balance (sensitivity 0.001 mg).

Lanthanum penetration was examined with electron microscopy as an independent method of assessment of barrier integrity (32, 33). Explants were minced and incubated first in 4% lanthanum nitrate in modified Karnovsky’s fixative at room temperature for 1 h, then rinsed, incubated, and processed for electron microscopy, as described previously (15).

Light microscopy, histochemistry, and electron microscopy. 10–15 frozen sections of skin samples taken from six to eight different pups were examined. 10-μm sections were stained with hematoxylin. Nile red was applied to 5-μm frozen sections, which were examined using a Leitz fluorescence microscope equipped for epifluorescence (E. Leitz, Inc., Rockleigh, NJ) (15). Parallel samples were minced into 1-mm³ pieces, fixed in modified Karnovsky’s fixative, and processed for electron microscopy. Sections were stained with uranyl acetate and lead citrate, and examined with a Zeiss 10A electron microscope (Carl Zeiss, Inc., Thornwood, NY) (15).

Statistics. Results are presented as mean±SEM. Statistical signif-

icance was determined using a Student’s t test.

Results

Gender differences in rates of barrier development. Our laboratory has shown previously that on d 19 EGA all pups lack a measurable barrier to water loss, whereas on d 21 all exhibit a competent barrier (15). However, on d 20, a wide range in barrier competence is observed. To determine whether this variance could be gender related, we obtained pups from seven different litters (10 males and 10 females) on d 20, and from three different litters (10 males and 10 females) on d 21, and measured TEWL. As shown in Fig. 1, males exhibit significantly poorer barriers in comparison with female littermates on d 20 EGA. All pups on d 21 exhibited competent barriers, and there was no significant difference between males and females. These studies demonstrate gender-related differences in rates of barrier maturation in male vs. female rats.

Estrogens accelerate barrier development in utero. To de-

termine whether estrogen, which accelerates lung development in the rat, also accelerates barrier maturation, we examined the effects of maternal DES treatment on fetal barrier formation. Pregnant rats were injected with 1 mg/kg DES on d 16–18 of gestation, a treatment similar to that which accelerates lung maturation. Pups were then obtained on d 20 and their barrier to water loss measured. As described previously (15), control

Figure 1. The formation of a competent barrier is delayed in males. TEWL was measured in d 20 and d 21 EGA fetal rats with a Meeco electrolytic water analyzer. Fetal sex was assessed by microscopic internal examination. Results are reported as mean ± SEM. n (d 20) = 24 males and 24 females. n (d 21) = 10 males and 10 females. *P < 0.01.

Figure 2. Effect of maternal estrogen treatment on fetal barrier function. Pregnant rats were injected with 1 mg/kg DES or with 0.1% ethanol in peanut oil on d 16–18 of gestation and TEWL was measured in d-20 fetal rats. Results are reported as mean±SEM, n = 16. *P < 0.005.
pups on d 20 EGA again exhibited poor barriers to water loss (5.90 ± 0.92 mg/cm² per h; n = 16). In contrast, d-20 pups of DES-treated mothers all displayed highly competent barriers (Fig. 2) (TEWL: 0.38 ± 0.11 mg/cm² per h; n = 16), with TEWL values similar to those of untreated control d-21 pups (15). Thus, maternal estrogen treatment accelerates barrier maturation in vivo.

To ascertain whether the functional changes observed with estrogen treatment could be attributed to accelerated structural changes, we next examined skin sections from d-20 fetuses after maternal DES vs. vehicle treatment. As seen in Fig. 3, DES treatment (B) results in an increase in the thickness of the stratum corneum over controls (A). We next determined the pattern of lipid deposition in the skin samples using nile red histochemistry. Control d-20 pups showed little membrane pattern staining in the stratum corneum (Fig. 4 A). In contrast, the stratum corneum of DES-treated pups showed intense staining in a multi-layered membrane pattern, indicative of deposition of lipids in the stratum corneum extracellular membranes (Fig. 4 B), comparable to the morphology of epidermis during normal gestation (15). Thus, development of a competent barrier on d 20 in DES-treated fetal rats was accompanied by the accelerated development of a well-defined multilayered stratum corneum with increased deposition of neutral lipid in a membrane pattern.

Ultrastructural studies also demonstrated accelerated maturation in fetal epidermis after maternal DES treatment. Controls showed secreted lamellar material that had not undergone the final processing steps to form mature lamellar unit structures in the extracellular spaces of the lower stratum corneum (Fig. 5 A). Mature bilayers were limited to the interstices of the upper stratum corneum. In contrast, DES treatment resulted in lamellar unit structures throughout the stratum corneum interstices of both the upper and lower stratum corneum (Fig. 5, B and C). Taken together, these studies indicate that the accelerated functional development produced by maternal estrogen treatment can be ascribed to parallel changes in epidermal structure.

Effects of estrogen on barrier development are due to direct effects on the skin. To determine if the acceleration of barrier ontogenesis by estrogen occurs through a direct effect of the hormone on the skin, we next determined whether DES would accelerate barrier development in an in vitro skin explant model. Fetal rat skin, when obtained at d 17 EGA, and incubated in a serum- and hormone-free medium for 4 d, exhibits morphological and functional indications of barrier maturation that precisely parallel the timetable for barrier maturation in utero (19). The effect of DES on barrier formation was dose dependent. A maximal effect was seen with 100 nM DES (data not shown), a similar concentration to that which accelerates lung maturation in vitro. As shown in Fig. 6, epidermal water loss values in explants incubated for 2 d in 100 nM DES decreased significantly as compared to controls (DES 0.063 ± 0.006 vs. control 0.097 ± 0.007 mg/mm² per h, n = 9, P < 0.005).

As an additional index of barrier integrity, penetration of a water-soluble, electron-dense tracer, lanthanum, was examined in explants cultured in the presence or absence of DES for 2 d. We have previously shown that inhibition of lanthanum permeation parallels declining TEWL measurements during barrier maturation (19). Whereas extracellular lanthanum permeated throughout the epidermal interstices in control explants, indicating an incompetent barrier, in DES-treated explants, lanthanum only reached the stratum granulosum-stratum corneum interface and did not permeate the stratum corneum interstices, indicating establishment of a barrier to outward water movement (data not shown). These studies demonstrate that DES accelerates the maturation of barrier competence in vitro using two independent parameters.

We next ascertained the structural basis for the acceleration of barrier competence induced by DES treatment. As we...
have reported previously (19), a well defined stratum corneum again was not present in d 17 EGA fetal rat skin incubated for 2 d in hormone- and serum-free media (Fig. 3C). In contrast, explants grown for 2 d in DES-supplemented media showed a distinct stratum corneum in hematoxylin-stained sections (Fig. 3D). Likewise, in sections stained with nile red, control explants display a predominantly cytosolic pattern of fluorescence, limited to the nucleated layers (Fig. 4C), while DES-treated explants showed intense staining in a membrane pattern throughout the stratum corneum (Fig. 4D). Finally, on electron microscopy, lamellar material was unorganized and mature lamellar unit structures were absent in controls after 2 d of incubation (Fig. 5F), as described previously (19). In contrast, mature lamellar unit structures appeared throughout the stratum corneum interstices in DES-treated explants (Fig. 5, D and E). The light and electron microscopic features in explants treated with DES for 2 d closely resembled the morphologic features of untreated skin after 4 d in culture (19). These results show that the acceleration of barrier function produced by DES in vitro can be explained by parallel changes in epidermal structural maturation. Moreover, these results show that DES accelerates barrier ontogenesis through a direct effect on skin maturation.

**Androgens delay barrier maturation in utero.** Whereas the foregoing studies demonstrate a beneficial effect of estrogens on barrier maturation, they do not entirely account for the observed sex difference in skin maturation, since both male and female fetuses are exposed to maternal estrogens during gestation. Therefore, we next determined whether exogenous androgens, administered during the same time period and in the same concentration as has been shown to delay lung maturation, could have a deleterious effect on barrier development. Pregnant rats were injected with DHT on d 13–18. Pups were delivered from two different mothers on d 20 (n = 8) or d 21 (n = 8) for TEWL measurements. As shown in Fig. 7, maternal treatment with DHT caused a dose-dependent delay in barrier formation. While little difference in morphology was evident between control and DHT-treated fetal skin, ultra-
structural indicators of delayed development were noteworthy. Whereas in the interstices of the upper stratum corneum of control epidermis on d 20 lamellar body contents have been further processed to form mature lamellar unit structures (Fig. 8, A and B), secreted material in the stratum corneum of DHT-treated pups did not undergo complete processing (Fig. 8 C). Failure to process secreted lamellar body contents is also evident during normal gestation at d 19 EGA (19). In contrast,
in d-21 pups, maternal DHT treatment did not effect TEWL (control: 0.29±0.10 mg/cm² per h; treated: 0.31±0.09, respectively). Thus maternal androgen treatment delays but does not prevent barrier maturation.

**Effects of androgens on barrier development are due to direct effects on the skin.** To determine if DHT, like estrogen, glucocorticoids, and thyroid hormone, directly affects the skin, fetal skin explants were incubated for 3 or 4 d in the presence or absence of DHT. The maximal effect was observed with 100 nM DHT. As seen in Fig. 9, epidermal water loss in treated explants was increased by ~25% after 3 or 4 d in culture. Although no effect on the morphology of the explants was seen by light microscopy (data not shown), electron microscopy revealed disorganized, unprocessed lamellar material in multiple layers of the stratum corneum interstices in explants incubated for 4 d with DHT (Fig. 8 E). In contrast, control explants exhibited mature bilayers throughout the extracellular spaces of the stratum corneum (Fig. 8 D), and unprocessed material was limited to the stratum granulosum-stratum corneum interface. These data demonstrate that DHT delays barrier maturation by direct effects on the skin.

**Maternal antiandrogen treatment eliminates the gender difference in fetal barrier function.** Previous studies have shown that maternal treatment with flutamide, an androgen antagonist, eliminates the gender difference in lung maturation in the fetal rat (34). To determine whether the delay in epidermal barrier formation in males can also be attributed to the higher levels of androgens in the male fetuses, we followed a protocol similar to that which eliminated the gender difference in lung development: pregnant rats were injected with either flutamide or vehicle daily between d 13 and d 18. TEWL was measured in the pups of three control litters (n = 12) and three treated litters (n = 12) on d 20. As seen in Fig. 10, treatment with flutamide totally eliminated the gender difference in TEWL in d-20 fetuses by lowering the TEWL level in the treated males to the level of both treated and untreated females. These results imply that androgens are responsible for the delay in barrier maturation in male vs. female pups.

**Discussion**

Gender differences in the mortality rates of premature infants have long been recognized, with males displaying a poorer prognosis (35). Because lung immaturity, until the recent advent of surfactant replacement therapy, has been the major cause of death in these infants, sex-specific differences in lung maturation rates have been sought. Several aspects of lung development, both morphological and functional, have been shown to be delayed in the male (27–30). Furthermore, administration of exogenous androgens inhibits lung maturation in vivo and in vitro in several mammalian species (24–26, 34, 36). Finally, studies with androgen antagonists have provided further evidence that androgenic hormones are responsible for the male delay in lung maturation (34).

While lung surfactant replacement therapy has resulted in significant improvements in mortality rates of prematurity, gender differences in survival persist (37). This recent study of viability of the extremely low birthweight infant in the postsurfactant replacement therapy era again demonstrated a deleterious effect of male gender on morbidity and mortality (37). Indeed, of all variables examined, only male gender was associated both with increased mortality and with poor outcome (severe sequelae). Skin immaturity, with the destabilizing effects of increased transepidermal water loss, the increased caloric requirements imposed by energy loss through evaporation, and the increased susceptibility to systemic bacterial and fungal infections by organisms of the integumental “normal” flora consequent to immaturity of skin barrier function could contribute to the poorer prognosis in male infants.

The fetal rat provides useful in vivo and in vitro models for the study of skin barrier maturation. In this study, we have demonstrated that male rat fetuses exhibit retarded barrier maturation. Moreover, we have shown that, as in lung maturation, estrogens and androgens exert opposite effects on barrier formation.
formation. Estrogen accelerates formation of a competent epidermal barrier to water loss, and accelerates maturation of the morphological correlates of a competent barrier, including: (a) formation of a distinct, multilayered stratum corneum by light microscopy; (b) deposition of neutral lipid in a membrane pattern throughout the stratum corneum, as shown by nile red fluorescence microscopy; and (c) formation of mature lamellar unit structures in the stratum corneum intercellular domains, on electron microscopy. These morphologic features parallel those that are present when a competent barrier is formed dur-
prenatal androgen exposure is associated with a number of malformations and abnormal developmental progression (17, 18). The role of androgen in fetal barrier development in vivo could occur indirectly, by stimulating other factors such as corticosteroids, which are potent stimulators of barrier maturation in fetal rats (17). However, our demonstration that estrogen also accelerates barrier maturation in fetal skin organ cultures maintained in a serum- and otherwise hormone-free medium indicates that estrogen also has a direct effect on skin maturation.

While our studies demonstrate that estrogen promotes barrier formation, an absolute requirement for estrogen is not present, because skin explants from d 17 EGA fetal rats form a competent barrier in hormone-free media, in parallel to the developmental time course in utero. Moreover, studies in mice with estrogen receptor-disrupted genes suggest that estrogen is not crucial for development or survival, as these animals live to adulthood with normal gross external phenotypes (41). Rates of barrier maturation, however, have not been examined in this model; thus, a delay in maturation could be present. Additionally, maximal estrogen stimulation probably does not occur in vivo, since additional estrogen significantly accelerates maturation. The precise role of estrogens in normal barrier maturation remains to be determined.

Androgens are more likely to be the mediators of gender differences in skin maturation. Testosterone levels begin to rise in the male fetal rat on d 16 EGA (39), close to the time of onset of epidermal stratification. In the present study, we have shown that the male fetus displays a less competent barrier than the female on d 20 EGA, but by d 21 both males and females have fully competent barriers. Moreover, maternal administration of DHT caused a significant delay in barrier formation, suggesting that this gender difference is due to inhibitory effects by androgens. Since DHT treatment did not affect barrier function on d 21, androgens retard but do not block barrier formation. Additionally, DHT appears to have a direct effect on the skin since the addition of DHT to fetal skin explants in vitro also delayed barrier maturation. DHT, a very potent androgen, was used because this metabolite of testosterone does not aromatize to estrogen, avoiding any estrogenic effects in response to androgen treatment. In addition, tissues which display 5α-reductase activity, as skin and fetal lung do, are often DHT dependent. Finally, late gestational treatment of pregnant rats with flutamide totally abolished the gender difference in fetal barrier function, further suggesting the primacy of androgenic effects.

In conclusion, our study demonstrates a sex difference in epidermal barrier maturation, with males exhibiting delayed development. While estrogens accelerate barrier maturation, the observed gender difference in skin maturation is probably attributable to an inhibitory androgen effect in males. The continued increased morbidity and mortality of males in the postsurfactant replacement therapy era indicates that gender differences in epidermal barrier maturation contribute to the increased vulnerability of male newborns.
differences in organ maturity other than lung are likely. Dis-proportionate skin immaturity in males is a possible contribu-
tor to this differential prognosis. Further understanding and
treatment of skin barrier immaturity may be of critical impor-
tance to this new frontier.

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