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Treatment of the Neonatal Rat with Epidermal Growth Factor: Differences in Time and Organ Response

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ABSTRACT. The ability of exogenous mouse epidermal growth factor (EGF) (500 ng/g body weight/day) to retard somatic growth and to alter the timing of integumental maturation was investigated in the newborn rat. The immediate postnatal period (days 0–3) was identified as the critical time for elicitation of EGF effects. Somatic growth retardation produced by EGF persisted through weaning (day 20) and was asymmetric with maximal organ growth retardation present in liver and kidney and with relative sparing of heart and brain. Treatment of newborn rats with EGF on postnatal days 0–3 advanced the mean time of eyelid opening by 146 *versus* 31 h for tooth eruption. In contrast, EGF delayed opening of the external ear canal by approximately 48 h. EGF-treated pups thus exhibited a rearrangement in the normal sequence of craniofacial development. In summary, these data provide new information on 1) the critical time of EGF response in the rat, 2) the existence of an asymmetric pattern of organ growth retardation following perinatal EGF exposure, and 3) the ability of EGF to retard morphogenesis of the external ear. (*Pediatr Res* 20:468–472, 1986)

Abbreviations

EGF, epidermal growth factor
NGF, nerve growth factor

In the early 1960's, while studying the source of NGF, Cohen (1) noted that extracts of adult mouse submandibular gland injected daily into newborn mice and rats resulted in several gross biological effects. These included precocious eruption of the incisors, early opening of the eyelids, and a stunting of body growth and hair formation. Isolation of this "tooth-lid factor" revealed a heat-stable, single-chained protein with a molecular weight of approximately 6000 (1, 2). Over the past decade, spectacular advances have been made in understanding the mechanism of action of EGF and its membrane receptor at the molecular and cellular levels (3, 4). Despite this progress, the physiological role of EGF is still unclear.

In the traditional bioassay, a fixed dose of EGF per gram body weight is injected daily into newborn mice or rats beginning within 12 h of birth (1). Injections are usually continued at 24-h intervals until the time of eyelid opening. The eyelid response is measured as the postnatal day on which eyelid unfusion occurs and is linearly related to the logarithm of the EGF dosage (5). Other parameters of EGF action such as growth retardation, incisor eruption, and the appearance of a fatty liver secondary to excessive triglyceride accumulation (6) have been less well quantified. Moreover, none of the above actions of EGF can be elicited in adults. Thus, the bioassay for EGF is strictly a developmental determination.

Recently we conducted a series of experimental bioassays as part of a program to characterize purified mouse EGF. Herein we report several new observations on EGF effects in the neonatal rat relating to: 1) the critical time of EGF action; 2) the sensitivity of selected organs to EGF induced growth retardation; and 3) an unexpected effect of EGF to delay external ear morphogenesis.

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MATERIALS AND METHODS

Purification of mouse EGF. EGF was routinely purified from the submandibular glands of adult male Swiss Webster mice by a modification of the method of Savage and Cohen (2). Briefly, the crude gland extract was chromatographed at low pH on Bio-Gel P-10, the immunoreactive material was concentrated by ultrafiltration and purified by gradient reverse-phase high performance liquid chromatography on a 30 cm × 4.6 mm id C_{18} column using acetonitrile as secondary solvent essentially as described by Smith *et al.* (7). Using this technique α and β EGF peaks were easily discriminated. Only α EGF was used for *in vivo* injections. The final material was pure as judged on 1) 2D gels following isoelectric focusing in one dimension and polyacrylamide gel electrophoresis in the other as described by O'-Farrell (8) and modified by Anderson *et al.* (9); 2) eyelid opening scores in the EGF bioassay (1); and 3) radioimmunoassay criteria with a specific antiserum (10).

Animal preparations. Pregnant Sprague-Dawley rats (Charles-River) were obtained on approximately the 17th day of gestation and housed singly in our vivarium. Following birth, individual litters were subdivided according to experimental design into appropriate treatment groups distinguished arbitrarily by small, coded, low dorsal, India ink tattoos. Allotted groups contained equal numbers of individual pups, aggregate birth weights, and sex ratios. Total litter size varied from eight to 12. EGF injections were begun within 12 h of birth and were administered subcutaneously between the shoulder blades using a 10 μl Hamilton syringe. α EGF was prepared for injection as a 500 ng/ul solution in normal saline. Treated animals received 500 ng/g body weight/day; control animals received an equal volume of saline using a separate syringe. Treatment periods consisted of EGF administration on postnatal days 0–3, 4–7, or 11–14. Animals were sacrificed by decapitation. Organ weights were measured immediately using a Mettler PE360 top-loading balance (accuracy ± 1 mg). Brain weights refer to the aggregate of all central nervous system tissue above the foramen magnum.

Photomicroscopy. Photomicrographs of 3-day-old rats were obtained using an automated Olympus model PM-10AD Photomicrograph System on a Wild MSA Dissecting Microscope. Pups were lightly anesthetized with ether prior to photography.

Scoring of integumental events. Proper positioning and scoring of incisor eruption was performed as described previously (11). Judgment of external auditory canal opening requires a practiced eye. Examination in this study was performed on animals under light ether anesthesia using a model 41RT American Optical Stereoscopic microscope (magnification 15×). Ear canal opening was judged as non-apposition of epithelial surfaces from the external auditory meatus to the tympanic membrane on at least one side. Examination of the canal is facilitated by gentle traction of the pinna with blunt forceps in a posterior-superior direction. Eyelid opening was scored as present when breakdown of the line of fusion had occurred sufficient to render the underlying cornea visible on at least one side. Generally, eyelid opening proceeds from the center of the line of fusion outward to the canthi.

Statistical analyses. Analysis of variance was used to test for differences in mean body weights and timing of integumental development among control, early (days 0–3), and late (days 4–7) EGF treatment groups. A randomized complete block design (12) was used to eliminate variation due to litter effects. Experiments using only control and one EGF treatment regimen were analyzed by Student's two-tailed t test for unpaired observations unless otherwise noted. A p value < 0.05 was considered significant.

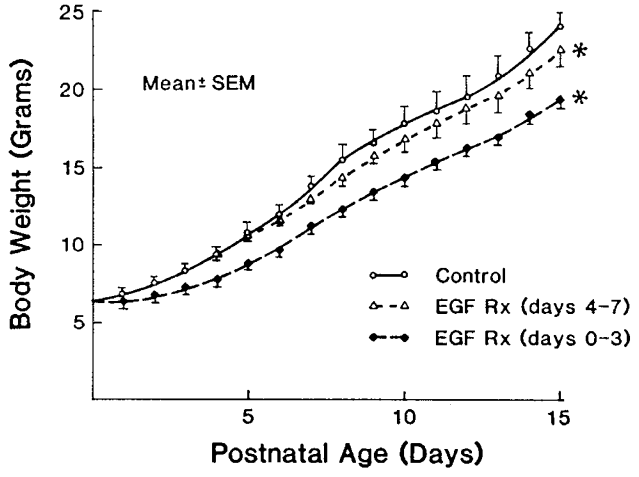

Fig. 1. Postnatal weight gain in littermate rats treated with EGF (500 ng/g body weight/day) during the 1st wk of life. Data represent a total of 72 animals divided equally into control, early, and late treatment regimens. *Asterisks* denote values statistically different ($p < 0.01$) from both control and the other treatment modality on postnatal day 15. All values are presented as mean ± SEM.

RESULTS

Postnatal gain in mean body weight was reduced in littermate rats treated with exogenous EGF (Fig. 1). In the first experiment, six litters were examined containing 12 pups each. Both early (days 0–3) and intermediate (days 4–7) EGF administration produced an apparent trend toward reduction in weight gain by 48 h after initiation of treatment. Statistical significance was achieved by postnatal day 15 in both treatment groups ($p < 0.01$). A similar experiment was conducted following EGF administration on postnatal days 11–14 (500 ng/g body weight/day). A total of five litters of 10 pups each were used with division of littermates into equal numbers of control and treated pups. At the time of sacrifice on day 15, the following data were obtained (controls: body weight 24.7 ± 0.6 g, liver 0.843 ± 0.02 g, brain 1.135 ± 0.02 g; EGF Rx: body weight 24.2 ± 0.7 g, liver 0.892 ± 0.02 g, brain 1.095 ± 0.02 g). No significant differences were ascertained using the 11- to 14-day regimen. Thus, in all, three EGF treatment groups were examined: early (days 0–3), intermediate (days 4–7), and late (days 11–14). The data show that during the first 2 wk of life in the rat, there is a progressive loss of the ability of weight-equivalent doses of EGF to produce somatic growth retardation.

Evaluation of integumental morphogenesis was performed using the same experimental groupings listed above. Initial examination of EGF effects on ear morphogenesis was triggered by the observation that treatment of newborn rats with EGF resulted

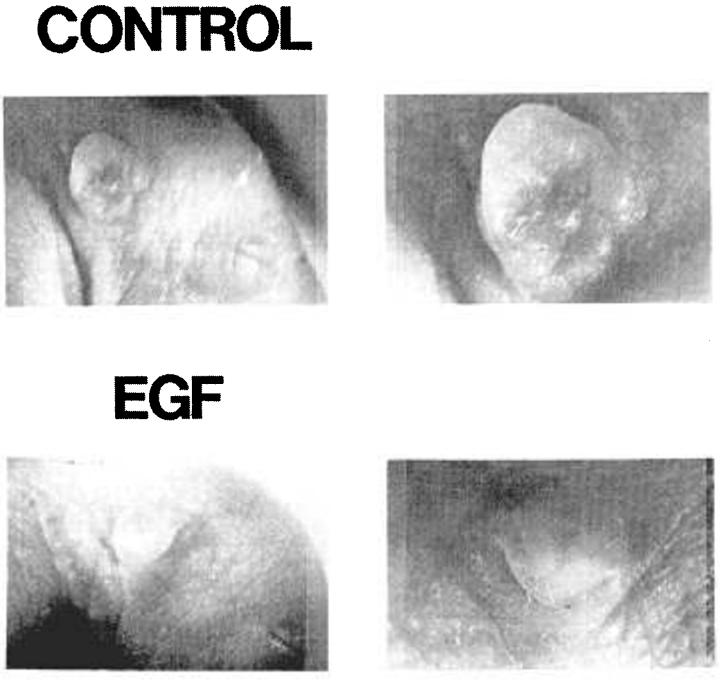

Fig. 2. Early pinna development in control and EGF treated 3-day-old rats. The control pinna has released and has assumed an upright position. EGF treatment (500 ng/g body weight/day) has delayed detachment of the epithelial edge and the pinna remains folded inferiorly against the head. Magnification is 15× (*left panel*) and 30× (*right panel*).

in delayed release and erection of the pinnae (Fig. 2). Normal epithelial detachment of the pinnae occurred in controls ca. 48-72 h after birth followed over the next day by gradual attainment of upright pinnal carriage. EGF-treated pups showed retarded release of the epithelial edge and a 24- to 48-h delay in unfolding and erection of the pinnae. By postnatal day 8, sculpting of the external ear was noticeably poorer in animals treated with EGF on days 0–3 compared to either the 4–7 day regimen or controls. In addition, other distinguishing features were present such as accelerated formation of the oral vestibule, malformation of the vibrissae, and impending eruption of the lower incisors. On the other hand, the late treatment regimen (days 4–7) resulted in a blunted response and pups which were relatively similar in appearance to controls (Fig. 3).

Subsequent observation revealed that in animals treated with EGF on postnatal days 0–3 the mean time of opening of the external auditory canal was delayed by 48 h (Table 1). In contrast, mean eyelid opening and incisor eruption were accelerated by 146 and 31 h, respectively. These results may be compared to previous reports of eyelid opening in neonatal rats treated with equivalent doses of mouse (5) and rat (14) EGF. The data suggest that the full biopotency of EGF to evoke early eyelid opening is confined to the first 3 days of life; *i.e.* later doses produce no greater effect. Treatment regimens, *e.g.* days 4–7 which omit this critical early period, evoke responses which are similar in direction but diminished in magnitude (Table 1). EGF treatment on days 11–14 (*vide supra*) had no effect on time of eyelid opening (controls 13.8 ± 0.2 days *versus* EGF 13.9 ± 0.2 days).

Given the apparent efficacy of early EGF treatment (days 0–3) to reduce somatic weight gain (Fig. 1) and to maximally alter craniofacial development (Table 1), all subsequent studies on organ growth retardation were performed using this early treatment model. Table 2 and Figure 4 present data obtained following sacrifice on postnatal days 15 and 20, respectively. These timepoints were chosen because they coincide with a period of rapid organogenesis in the rat, particularly of the liver (13). EGF treatment produced significant reduction at both ages in body weight, snout-anus length, and liver/brain ratios ($p < 0.05$). Mean body weights and lengths (+ SE) in the 20-day-old animals were as follows: control, 37.9 ± 1.4 g and 11.1 ± 0.1 cm; EGF Rx, 32.8 ± 1.2 g and 10.2 ± 0.2 cm. Additional data obtained in the 20-day-old animals showed significant growth retardation of the kidney with relative sparing of the heart (Fig. 4).

DISCUSSION

Classically, endocrine studies have relied on ablation experiments in order to examine the biological effects of deficiency and replacement of particular hormones (15). With most growth factors, deficiency states (a possibly lethal condition) have not been described other than the immunosympathectomy in developing mammals following administration of antiserum to NGF (16). Other tissue growth factors; *e.g.* EGF, the somatomedins and platelet-derived growth factor appear to have a much broader range of target cells than NGF judging from the tissue distribution of their receptors and the wide range of responsive cell types *in vitro* (17). Moreover, many tissues may synthesize these growth factors making depletion experiments difficult (18). Consequently, studies directed at models of growth factor excess such as the EGF bioassay system may prove exceedingly useful in the dissection of regulatory mechanisms. As Needham (19) pointed out many years ago, the experimental dissociation of major ontogenetic processes such as growth and differentiation is of fundamental importance in the attempt to understand the means by which the developing organism is integrated.

The present results demonstrate that the immediate perinatal period constitutes a critical time for the elicitation of both the growth retarding and the differentiative effects of EGF. Thus, a lower total dose of EGF is more effective in reducing body weight and eliciting early eyelid unfusion than a larger dose given after the first 3 days of life. In the rat and other mammals, birth entails a period of profound metabolic and nutritional changes (20). The rapid loss of sensitivity to exogenous EGF in the newborn rat pup coincides with this period of postnatal adaptation.

Among the "paradoxical" effects of neonatal administration of EGF is the production by a "growth" factor of a prolonged stunting of somatic weight and length (Table 2, Fig. 4). The mechanism for this effect is unknown but the pattern of organ growth retardation which follows early neonatal EGF exposure is similar to that produced by a variety of growth retardation models in the perinatal rat: 1) late gestational ligation of the uterine artery (21); 2) maternal malnutrition (22); 3) overcrowding of litters (23); 4) partial placental destruction by electrolysis (24); and 5) chronic hypoxia (25). In this pattern the rate of growth of susceptible organs; *e.g.* the liver, is markedly reduced compared to the rate of growth of less vulnerable organs, *e.g.* the brain. Thus, these animal models approximate the type of retarded growth seen in humans during the 3rd trimester of pregnancy (26).

In addition to effects on somatic and organ growth, EGF has profound influences on integumental maturation. In the rat, normal ontogenetic development consists of a series of precisely

Table 1. *Effect of EGF treatment* on the timing† of integumental events during rat craniofacial development (postnatal day)*

	Incisor eruption	Ear canal opening	Eyelid unfusion
Control	10.3 ± 0.1	13.3 ± 0.1	14.0 ± 0.2
EGF (days 4–7)	9.5 ± 0.1‡	14.3 ± 0.1‡	11.0 ± 0.1‡
EGF (days 0–3)	9.0 ± 0.2‡	15.3 ± 0.1‡	7.9 ± 0.1‡

* EGF treatment was 500 ng/g body weight/day on the indicated postnatal days.

† Each value is the mean (± SE) postnatal day for 24 animals.

‡ $p < 0.01$ *versus* control and other treatment modality.

Fig. 3. Physical appearance of 8-day-old rats treated with EGF (500 ng/g body weight/day) on postnatal days 0–3 (*left*) *versus* days 4–7 (*middle*). A littermate control is also shown (*right*). Early treatment results in early formation of the oral vestibule and malformation of the vibrissae; the lower incisors have almost erupted through the overlying gingiva. Later treatment results in animals similar in appearance to controls.

Table 2. *Effect of postnatal EGF treatment* on somatic and organ growth in 15-day-old suckling rats*

	Body wt (g)	Body length (cm)	Liver (mg)	Brain (mg)	Liver/brain (mg/mg)
Control ($n = 20$)	26.4 ± 1.0	9.3 ± 0.1	782 ± 22	1171 ± 14	0.67 ± 0.02
EGF* ($n = 20$)	21.5 ± 1.2†	8.4 ± 0.2†	622 ± 29†	1084 ± 23†	0.57 ± 0.02†

* EGF treatment was 500 ng/g body weight/day on postnatal days 0, 1, 2, and 3.
† $p < 0.01$ *versus* controls, all values mean ± SE.

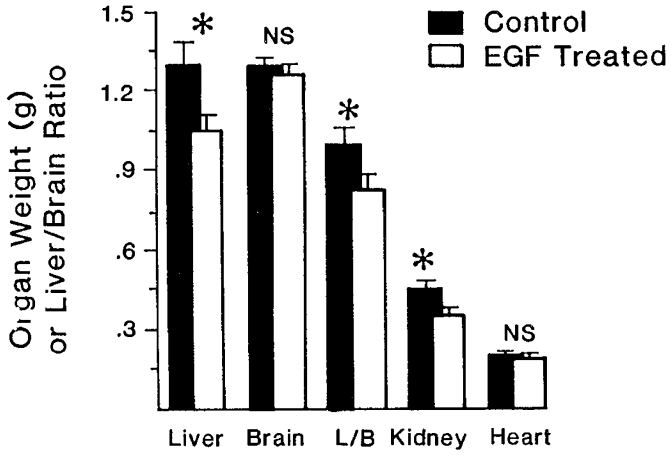

Fig. 4. Effect of EGF (500 ng/g body weight/day) treatment on postnatal days 0–3 to reduce liver and kidney weights and liver/brain ratios in 20-day-old weanling rats (*asterisks* indicate $p < 0.05$). Heart and brain weights were not significantly different from controls (NS). Body weights and snout-anus lengths were also significantly reduced (see "Results").

timed topological discontinuities or "breaks" in a postnatally continuous epidermal sheet. Each of these discontinuities is closely coupled to the organization of higher functions. Thus, in the rat, the normal sequence of topological events and their associated functions are: 1) incisor eruption, 10th postnatal day (mastication); 2) ear canal opening, 12–13th day (hearing); 3) eyelid unfusion, 13–14th day (vision); 4) vaginal opening, 34–36th day (reproduction).

In slow growing rats from large litters, the timing of incisor eruption and eyelid unfusion are relatively unaffected even in the face of extreme growth retardation. These observations led early investigators to suggest that events such as incisor eruption and eyelid unfusion were under "chronological" or "genetic" control (23). During development, the endocrine hormones constitute one of the major mechanisms regulating gene expression. In this regard, the thyroid hormones and, to a lesser extent, the glucocorticoids, have been reported to promote early eyelid opening in rodents (27, 28). Recent evidence suggests that these hormones may be exerting their effects on the integument via mechanisms involving endogenous EGF (10, 11).

Although not widely appreciated, hormones may retard as well as accelerate development. For example, insulin delays maturation of surfactant synthesis in perinatal mammalian lung explants (29). During the course of these experiments it was noted that EGF-treated rat pups exhibited a delayed release of the pinnae. Closer examination revealed an effect of EGF to delay opening of the external auditory canal, resulting in an altered sequence of craniofacial development (Table 1). This finding was unexpected and indicated that EGF could function simultaneously to retard some developmental events while accelerating others in close physical proximity.

Embryologically, mammalian tooth bud, eye, and external ear all form as prenatal epithelial invaginations which fuse superficially (30, 31). Subsequent integumental morphogenesis occurs as a sequence of epithelial "breaks," the order of which is highly species dependent. In humans, for example, eyelid opening is a prenatal event occurring approximately 15 wk before birth while tooth eruption is delayed until the 6th postnatal month. In rodents, the present findings demonstrate that EGF treatment markedly accelerates the time of eyelid opening, has a minimal effect to accelerate incisor eruption and has an opposing action to retard opening of the external ear canal. This aspect of the EGF bioassay is unexplored and offers an experimental insight into the mechanism whereby a specific ontogenetic sequence can be rearranged in response to a molecular signal received at a critical time.

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