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Apolipoprotein E knockout mice have accentuated malnutrition with mucosal disruption and blunted insulin-like growth factor I responses to refeeding*

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Abstract

Apolipoprotein E (apoE) is synthesized mainly in the liver and in the brain and is critical for cholesterol metabolism and recovery from brain injury. However, although apoE mRNA increases at birth, during suckling, and after fasting in rat liver, little is known about its role in early postnatal development. Using an established postnatal malnutrition model and apoE knock-out (ko) mice, we examined the role of apoE in intestinal adaptation responses to early postnatal malnutrition. Wild-type and apoE-ko mice were separated from their lactating dams for defined periods each day (4 hours on day 1, 8 hours on day 2, and 12 hours thereafter). We found significant growth deficits, as measured by weight gain or tail length, in the apoE-ko mice submitted to a malnutrition challenge, as compared with malnourished wild type, especially during the second week of postnatal development ($P < .05$). In addition, apoE-ko animals failed to show growth catch-up after refeeding, compared with wild-type malnourished controls. Furthermore, we found shorter crypts and reduced villus height and area in the apoE-ko malnourished mice, compared with controls, after refeeding. Insulinlike growth factor 1 expression was also blunted in the ileum in apoE-ko mice after refeeding, compared with wild-type controls, which exhibited full insulinlike growth factor 1 expression along the intestinal crypts, villi, and in the muscular layer.

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Taken together, these findings suggest the importance of apoE in coping with a malnutrition challenge and during the intestinal adaptation after refeeding.

Keywords

ApoE; Malnutrition; IGF-1; Small intestine; Growing mice

1. Introduction

Apolipoprotein E (apoE), a 35-kd plasma protein synthesized mainly in the liver and in the brain, is critically involved in cholesterol transport and metabolism. Early studies identified apoE as a key component of plasma cholesterol homeostatic mechanisms [1,2]. ApoE binds with high affinity to lipoprotein particles in the plasma compartment and acts as a ligand for receptor-mediated endocytosis via multiple members of the low-density lipoprotein receptor family [1,3–5].

Several studies have highlighted the critical role of apoE in brain plasticity after injury (in vivo and in vitro models) [6–10]. On the other hand, to date, few studies have addressed the importance of apoE during early postnatal development, although apoE mRNA has been shown to increase at birth, during suckling, and after fasting in the rat liver [11]. In this study, we have investigated whether apoE null mice exhibit developmental deficits, focusing on intestinal healing, after a malnutrition challenge and refeeding, compared with wild-type controls. We have focused on the intestinal maturation during weaning, a transitional time when profound adaptations in small bowel morphology take place at the time of an introduction to a new diet regimen and when the small bowel structure is particularly prone to disruption [12,13].

Malnutrition is known to cause progressive changes in the intestinal permeability [14] and mucosa integrity, ultimately leading to hypoplastic villi and crypts and disruption of its biomechanical properties [15]. Most of these changes have been shown to be reversible after refeeding in a variety of refeeding protocols [16].

To investigate intestinal recovery from a malnutrition challenge, we have examined the intestinal catch-up after re-feeding by monitoring morphological changes in villus height, area, and crypt length and insulin-like growth factorI (IGF-1) expression in the ileal mucosa, which has been successfully used as a model of intestinal adaptation in rodents [17,18] and because distal parts of the small bowel are particularly vulnerable to a single period of food restriction [19].

Our findings suggest that apoE plays a critical role in intestinal healing, possibly by regulating IGF-1 actions in the ileum mucosa, raising the hypothesis that apoE might synergistically act with hormones and growth factors during the healing process in the intestinal mucosa. This animal model might provide further insights into understanding the relationship between apoE and nutritional interventions to improve the devastating effects of enteric infections and undernutrition in developing children in poor areas.

2. Methods and materials

2.1. Undernutrition model

ApoE knock-out (ko) mice were purchased from Jackson laboratories after being generated from B6.apoE^{-/-} at the N10 backcross and constructed from B6.129 apoE^{-/-} mice [20]. C57BL/6J wild types were purchased from Charles River Laboratories (Wilmington, MA). Either purchased pregnant mice or breeding pairs were used to obtain the study pups. Detectable pregnant mice (~12 days pregnant) were then caged individually, with free access to standard rodent chow and water, and were monitored daily for delivery (termed *day 0*). Newborn litters were adjusted to 7 to 8 pups. Undernutrition was induced by separating half the pups in each litter from their lactating dams for defined periods each day (4 hours on day 1, 8 hours on day 2, and 12 hours thereafter), according to protocol adapted from Calikoglu et al [21]. Both apoE-ko and wild-type mice underwent the same protocol for induction of undernutrition. This method has the advantage of providing littermate-control, well-nourished pups to compare with undernourished ones. At day 21, the study pups were removed from lactating dams and housed in a new cage, at which time they were fully weaned to a regular chow diet and water until the end point at day 27, *ad libitum*. Pups were euthanized after being refed with free access to chow diet (irradiated Harlan Teklad LM-485 for mice; Harlan Teklad, Madison, WI) for 7 days after weaning. Euthanasia was done by cervical dislocation after anesthesia with sodium pentobarbital (3–4 mg/100 g IP). Weight and tail length were recorded daily until euthanasia. A thermal pad was used to warm the pups during daily measurements (28°C ± 2°C). Protocols from this study were previously approved by the Institutional Animal Care and Use Committee at the University of Virginia.

2.2. Physical growth

Experimental mice were monitored carefully by daily inspection of weight gain and skeleton growth during the suckling time at days 1 to 20 (nourished and malnourished wild type [*n* = 11] and malnourished and nourished apoE-ko [*n* = 6]) and after refeeding (nourished and malnourished apoE-ko [*n* = 5] and nourished and malnourished wild type [*n* = 8]); at days 21 to 27, the skeletal growth was evaluated by assessing tail length by means of measuring gently the animal tail from the basis to the tip, using a digital caliber and a card board (to the nearest 0.1 mm). All measurements were conducted before starting the procedures of daily mice separation (8 to 10 AM). Care was taken to keep the same degree of handling during this process for both apoE-ko and wild-type pups.

2.3. Intestinal morphometry

Villus height and crypt depths were measured from slides stained with hematoxylin and eosin on a light microscope (BH-2, Olympus, Tokyo, Japan), *n* = 4 for each group, equipped with a high-resolution digital camera that was connected to a computer with an image capture program. Villus height was measured from the baseline to the villus tip. The crypt depth was measured from the baseline to the crypt bottom. The villous surface area was estimated by creating an apex-basis conical diagram on digital images at low magnification, and values were averaged and converted to a percentage of the phosphate-buffered saline control group, as described previously by Carneiro-Filho et al [22]. At least 10 clear

longitudinal sections of villi and crypts were selected and counted for each sample (4 samples for each group). All morphometric measurements were done blindly with NIH Image J 1.34 S (National Institutes of Health, Bethesda, MD) analysis software.

2.4. Mitotic index

In order to evaluate the role of apoE in healing the injured small intestinal mucosa, we studied the mitotic index in the ileum of study mice ($n = 4$ for each group) at day 27, with or without a previous malnutrition challenge, after 7 days of refeeding, by blindly counting well-defined mitotic figures at crypt bases. The mitotic figures per crypt were scored in 20 longitudinal crypt sections stained with hematoxylin and eosin. Measurements were done under light microscopy at high magnification ($400\times$). The absolute values were averaged to produce the mitotic index of each group.

2.5. Western blots

In brief, ileal segments (0.5 cm to the ileocecal valve) were harvested and immediately frozen in liquid nitrogen. Thawed specimens were pulverized in glass homogenizers, containing lysis buffer and then transferred to test tubes with protease inhibitor and centrifuged at 14000 rpm. Supernatants were assayed using the bicinchoninic acid method, BCA Protein Assay Kit (Pierce, Rockford, IL) to standardize 200 μg of protein product. Samples were loaded into 15% denaturing polyacryamide gels (Amersham Biosciences, UK), and gels were transferred overnight and then blotted onto nitrocellulose membranes. Membranes were incubated with rabbit antihuman IGF-1 antibody (at dilution of 1:500) for 1 hour and then rinsed 3 times in rinsing buffer then incubated in a biotinylated secondary antibody and rinsed as described above. Each membrane was washed and exposed to Kodak X-Omat AR film (Kodak, Rochester, NY).

2.6. Immunohistochemistry

Briefly, immunohistochemistry was performed on fixed, paraffin-embedded samples sectioned at 5 μm ($n = 4$ for each group). Serial sections incubated with antibodies specific for IGF-1, directed to an epitope corresponding to amino acids 49 to 118 representing mature IGF-1 of human origin and to IGF-1 α receptor (rabbit polyclonal IgG, 200 $\mu\text{g}/\text{mL}$), were used at a dilution of 1:100, providing a useful tool to examine sites of increased IGF-1 expression and its receptor on the ileal tissue. Both antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Binding of the primary antibodies was detected by the avidin-biotinylated peroxidase method (VectaStain Kit, Vector Laboratories, Burlingame, CA). Negative controls, which consisted of omission of the primary antiserum, were uniformly negative. Positive controls were used to confirm immunostaining.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) with post hoc Bonferroni multiple comparison test was used to assess mean differences between groups [23]. $P < .05$ was considered statistically significant. Data are represented as mean $\pm$ SEM. Statistical analyses were performed using SPSS-PC version 13.0 (SPSS Inc, Chicago, IL).

3. Results

In this model, as expected, we have found significant growth deficit and wasting during the suckling time, as measured by daily tail length and weight gain, in the study mice submitted to a malnutrition challenge, as compared with their respective nourished controls (Fig. 1A and B). The apoE-ko malnourished mice have slower tail length gain, as compared with the malnourished wild type already at day 6. In addition, the malnourished apoE-ko animals showed a further significantly slower pace of weight gain throughout the first and second week of postnatal development, as compared with the nourished controls and the malnourished wild type, as early as day 2 (Fig. 1A) ($P < .001$). Surprisingly, the malnourished wild type reached the same relative gain of tail length as the nourished apoE-ko (Fig. 1B). In addition, a subset of apoE-ko animals failed to show weight catch-up after refeeding with chow diet, as compared with the other groups since day 22, 2 days after starting the full refeeding protocol (Fig. 1C) ($P < .05$). No statistical difference was seen during the burst of skeleton gain after refeeding between both malnourished groups (Fig. 1D). Nevertheless, only the challenged malnourished wild types reached the highest burst of growth, even higher than the nourished apoE-ko and wild-type controls, as an expected response to reaching growth catch-up ($P < .01$).

In addition, we found shorter crypts and villi in the malnourished apoE-ko mice, as compared with wild-type controls and greatly reduced absorptive area, after 1 week of full refeeding with standard chow diet, at day 27 (Fig. 2). Crypts were remarkably compromised by the malnutrition challenge in the apoE-ko mice, exhibiting blunted mitotic activity. Western blotting and immunohistochemistry techniques showed that IGF-1 expression was also blunted in the ileum from apoE-ko mice after refeeding (Fig. 3). On the other hand, a burst of epithelial proliferation was seen within the crypts from the wild-type refeed controls challenged by malnutrition (Fig. 3). Representative histology of study ileal sections are shown in Fig. 4.

Paralleling the higher mitotic index, a full IGF-1 expression was seen along the intestinal crypts and villi and within the muscular layer from these refeed mice (Fig. 5). No significant differences were observed in the IGF-1 α receptor in the ileum from the wild-type and apoE-ko malnourished mice along the mucosa, although a slightly higher expression was observed conspicuously along the muscular layer. In addition, the expression of the IGF-1 α was higher in the nourished wild type when compared with the apoE-ko mice (Fig. 6). The IGF-1 receptor immunostaining was seen scattered along the lamina propria, marking stromal cells, preferentially distributed within the villi and the periglandular connective tissue from both wild-type and apoE-ko well-nourished mice (Fig. 6). Taken together, these findings suggest the importance of apoE during the intestinal adaptation after malnutrition and refeeding.

4. Discussion

Apolipoprotein E, a 35-kd plasma protein synthesized mainly in the liver, is involved in cholesterol transport and metabolism. ApoE is also expressed in other tissues, notably the brain, which is a prolific tissue in terms of apoE production [8,24]. As a key determinant of plasma cholesterol homeostasis, apoE, a 299-amino acid secretory protein, binds with high

affinity to lipoprotein particles in the plasma compartment and acts as a ligand for receptor-mediated endocytosis via multiple members of the low-density lipoprotein receptor family [1,25].

ApoE is the principal apolipoprotein in the brain and cerebrospinal fluid [26]. Several observations have implicated a role for apoE in the injured nervous system [27,28]. In addition, apoE null mice have been used as a model to study Alzheimer's disease-like effects and impaired cognitive function [29]. Expression of apoE mRNA by astrocytes in the hippocampus increases after experimental damage to the entorhinal cortex [30]. Oligodendrocytes and macrophages increase expression of apoE after optic and sciatic nerve injury; apoE protein accumulates to 5% of total extracellular protein after peripheral nervous system injury; and apoE binds to and potentiates the biologic activity of neurotrophic factors [31,32]. However, to date, few studies have highlighted the apoE role in intestinal tissue remodeling during early development and adaptation against a malnutrition injury.

The intestinal maturation is dependent on a rapid surge of corticosteroids during the weaning time, mainly via IGF-1 expression in the small bowel [33,34], modulating the intestinal adaptation to fit a new diet regimen of solid food, at the same time when glutamine and arginine metabolism is critical to enterocytes [35,36] in the small intestine is the highest during early development [37–39]. Among extrahepatic tissues, the adrenal gland has one of the highest concentrations of the apoE mRNA and the highest rate of apoE synthesis [40]. In this regard, it is known that apoE-ko mice exhibit dysfunction of the hypothalamic-pituitary-adrenal axis, leading to increased basal adrenal corticosterone levels in these mice with age [41].

Interestingly, the apoE mRNA in the rat liver abruptly increases at birth and rises again during the suckling period, suggesting a critical role during early postnatal development. Moreover, liver apoE mRNA rises in 10-hour-fasted suckling rats as compared with controls, suggesting that the apoE gene expression in the rat liver cells changes during development in relation to insulin and glucagon levels [11]. The importance of cholesterol and phospholipid contents to the integrity of the small intestine microvillus membrane during the weaning transitional changes [42,43], which might be influenced by apoE, has been suggested. Furthermore, increased apolipoprotein gene expression accompanies the differentiation in intestinal cell lines in vitro [44]. Recently, apoE was found to play a role in establishing the integrity of tight junctions in intestinal cell lines [45]. This is reinforced by the evidence of blood brain barrier disruption in apoE-null mice, especially after injury [26,46]. The blood brain barrier is mainly composed of tight junctions between endothelial cells, similar to the intestinal barrier along the enterocytes; therefore, we speculate that growing apoE null mice exhibit impaired intestinal barrier function, which might further explain the sustained mucosa atrophy and failure to thrive seen in these malnourished animals after refeeding.

The apoE differential expression along the small intestine may influence cholesterol availability to enterocytes, which is concentrated in the microvillus membrane during intestinal development and strongly regulated primarily by changes in the diet regimen [47,48]. The role of the apoE cholesterol complex in intestinal adaptation is reinforced by

the evidence that apoE is secreted by enterocytes (in a polarization-dependent manner) due to 25-OH-cholesterol induction in differentiated Caco-2 cells, and this secretion is reduced by lipopolysaccharide [49]. Our findings suggest a synergistic effect between IGF-1 system and apoE during the intestinal mucosa healing after the malnutrition challenge.

Early weaning, altered nutrient status, and changing of diets can severely affect patterns of early growth and related hormone secretion, and refeeding may effectively restore normal physiologic and physical development [50,51]. Circulating growth factors, such as IGF-1, have been implicated in the control of the intestinal epithelial proliferation and influencing cell migration and differentiation on in vitro and in vivo models of intestinal healing [33], effects that may be stimulated and regulated by critical nutrients [52] upon refeeding.

The responsiveness to the rigorous malnutrition protocol imposed by this study was strongly influenced by the apoE genetic background because the malnourished wild-type animals tolerated the malnutrition schedule, responded much better after refeeding, and adapted in a faster pace to the weaning. In addition, these mice recovered promptly from the malnutrition stress by rapidly gaining weight and skeletal growth and by healing the intestinal mucosal lesions, including adaptive enhancement of the intestinal absorptive area, as measured by villus area and height, as compared with the apoE-ko mice. These findings suggest that apoE has a critical role in intestinal maturation and adaptation to undernutrition, supporting our initial findings that apoE polymorphisms have cognitive developmental consequences after early childhood diarrhea in Brazilian shantytown children [53], and that this model may be helpful in elucidating potential mechanisms and approaches to improving the devastating long-term effects of undernutrition in developing children. The mechanisms and the role of apoE in enterocytes' adaptive responses during intestinal maturation and adaptation remain to be explored.

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Fig. 1.

Relative weight (A) and tail length (B) gain from the experimental mice during the malnutrition schedule and after weaning and the refeeding protocol (nourished and malnourished wild type [$n = 11$] and for malnourished and nourished wild type [$n = 6$]). The catch-up of growth is shown by linear changes in weight and tail length, following refeeding, as a percentage of initial weight (C) and tail length (D) at day 21 (nourished and malnourished apoE-ko mice [$n = 5$] and nourished and malnourished wild-type mice [$n = 8$]). Statistical analyses were done from raw data using 1-way ANOVA corrected by Bonferroni test. The significant level was set at $P < .05$. The results are shown as mean $\pm$ SEM.

Fig. 2.

Morphometric analyses of villus height (A), crypt depth (B), and villus area (C) from experimental mice ($n = 4$ for each group). The pixel values were calibrated and averaged to micrometers. Villus area was calculated as a percentage of the nourished wild-type group. Statistical analyses were done from raw data using 1-way ANOVA and corrected by Bonferroni test. The significance level was set at $P < .05$.

Fig. 3.

Mitotic Index obtained from ileal crypts from the experimental groups at day 27 after weaning and refeeding ($n = 4$ for each group), when compensatory proliferation is required to repopulate the surface epithelium. The bars represent the mean mitotic index of 40 well-defined longitudinal crypts per group chosen at random. Results are shown as mean $\pm$ SEM.

Fig. 4. Representative hematoxylin and eosin histology (original magnification $\times 100$) of distal ileal sections from the experimental mice at day 27 after weaning and refeeding.

Fig. 5. Blunted expression of IGF-1 in the ileum mucosa from malnourished apoE null mice at day 27 (B and D), after the refeeding protocol (day 21–27), in comparison with the rehabilitated malnourished wild-type controls (A and C), using immunoblotting and immunohistochemistry, respectively. Distal ileum sections were used at 200× magnification for panels C and D.

Fig. 6.

Insulinlike growth factor α receptor expression from distal ileal sections (original magnification $\times 100$). Note stronger and scattered immunostaining marking mucosa stromal cells and the muscular layer in the nourished wild-type ileum and poor expression of the IGF-1 receptor in the malnourished ileum, mostly restricted to the muscular smooth cells, even after refeeding. Burst of mitotic activity is seen along the crypts of the rehabilitated wild-type mice challenged previously by malnutrition. Arrowheads indicate high proliferative crypts in the refeed wild-type ileum.