ORIGINAL ARTICLE

α-fetoprotein involvement during glucocorticoid-induced precocious maturation in rat colon

Min Chen, Peng Sun, Xiao-Yan Liu, Dan Dong, Jun Du, Luo Gu, Ying-Bin Ge

Min Chen, Lian Yun Gang Higher Vocational Technical College of Traditional Chinese Medicine, Lianyungang 222006, Jiangsu Province, China

Peng Sun, Dan Dong, Jun Du, Luo Gu, Ying-Bin Ge, Department of Physiology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Xiao-Yan Liu, The Laboratory Center for Basic Medical Sciences, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Author contributions: Chen M and Sun P performed the animal experiment, RT-PCR and immunofluorescent staining and wrote the paper; Liu XY performed part of RT-PCR and immunofluorescent staining; Dong D and Du J carried out Western blotting and statistical analysis; Ge YB contributed to the the paper writing and experimental design; Gu L and Ge YB contributed equally to this work.

Correspondence to: Ying-Bin Ge, MD, PhD, Department of Physiology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. ybge@njmu.edu.cn

Telephone: +86-25-86862022 Fax: +86-25-86862022

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Abstract

AIM: To investigate the role of α-fetoprotein (AFP), a cancer-associated fetal glycoprotein, in glucocorticoid-induced precocious maturation in rat colon.

METHODS: Colons from suckling Sprague-Dawley rats were used in this study. Corticosterone acetate at a dose of 100 μg/g body weight was given to normal pups on days 7, 9 and 11 after birth to induce hypercorticoidism. Control animals were injected with identical volumes of normal saline. Some rats receiving corticosterone 7 d after birth were also treated with mifepristone (RU38486), a glucocorticoid cytoplasm receptor antagonist to investigate the effects of glucocorticoids (GCs). The morphological changes of the crypt depth and villous height of the villous zone in colon were observed as indices of colon maturation. Expression levels of AFP in colons were detected by reverse transcriptase polymerase chain reaction and Western blotting. To identify the cellular localization of AFP in developing rat colons, double-immunofluorescent staining was performed using antibodies to specific mesenchymal cell marker and AFP.

RESULTS: Corticosterone increased the crypt depth and villous height in the colon of 8- and 10-d-old rats with hypercorticoidism compared with that in the control animals (120% in 8-d-old rats and 118% in 10-d-old rats in villous height, $P = 0.021$; 145% in 8-d-old rats and 124% in 10-d-old rats in crypt depth, $P = 0.017$). These increases were accompanied by an increase of AFP expression in both mRNA and protein (2.5-folds in 8-d-old and 2.5-folds in 10-d-old rats higher than in control animals, $P = 0.035$; 1.8-folds in 8-d-old and 1.3-folds in 10-d-old rats higher than in control animals, $P = 0.023$). Increased crypt depth and villous height and increased expression of AFP in the colon of rats with hypercorticoidism were blocked by mifepristone. Both had positive staining for AFP or vimentin, and overlapped in mesenchymal cells at each tested colon.

CONCLUSION: GCs promote the development of rat colon. AFP appears to be involved, in part, in mediating the effects of GCs in the developmental colon.

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INTRODUCTION

Glucocorticoids (GCs) have been routinely used in clinical practice to prevent diseases of prematurity such as respiratory distress syndrome. GCs treatment may reduce the incidence of necrotizing enterocolitis[1-3], in which a typical immaturity of the intestine[4-6] indicated that GCs may enhance intestinal maturation. Although this regulation seems to be mediated by the GCs receptor pathway, the precise regulatory mechanisms have not yet been documented.

The effect of GCs may require interactions with another tissue or cell type, such as mesenchyme[7]. During late embryogenesis, the mouse colon develops from a pseudostratified, undifferentiated endoderm to a single-layered columnar epithelium accompanying with mesenchymal maturation. Mammalian α-fetoprotein (AFP) is a single-chain glycoprotein with a molecular mass ranging from 66 to 72 kDa and 3%-5% carbohydrate (glycan) content. Recent reports showed that the AFP was expressed and produced in mesenchymal cells and was considered as an important growth factor with a specific function in gastrointestinal development, including pancreas and colon[8-10]. In contrast, AFP enhancer segment contains a sequence resembling the steroid hormone response element and the enhancer activity was mediated by dexamethasone in a dose-dependent manner[11]. Thus, AFP seems to play a role in GCs-induced precocious gastrointestinal maturation.

Numerous investigators have reported the ability of GCs to stimulate intestinal maturation in postnatal rodents[12-14]. However, little is known about the involvement of GCs in rat colon during development.

MATERIALS AND METHODS

Chemicals

Corticosterone acetate (CA), gel mount aqueous mounting medium (G0918), and mifepristone were purchased from Sigma Chemical (St. Louis, USA). TRIZOL reagent was purchased from Invitrogen Life Technologies (Burlington, Ontario, Canada). Takara RNA polymerase chain reaction (PCR) 3.0 Kit was from Takara (Dalian, China). Rabbit anti-goat IgG conjugates and goat polyclonal anti-AFP were from Santa Cruz Biotech (USA). Monoclonal antibody of anti-vimentin was from Chemicon International (Temecula, CA, USA). Protease inhibitor cocktail was from Roche (Mannheim, Germany). Nitrocellulose membranes were from Bio-Rad (Hercules, CA, USA), BCA Protein Assay Kit and enhanced chemiluminescence reagents were from Pierce (Rockford, IL, USA) and CBL202 from Chemicon International, Inc. Temecula (CA, USA).

Animals

Pregnant female Sprague-Dawley rats in late gestation were purchased from Nanjing Medical University Animal Centre. After arrival, cages were checked twice daily for pups. The day of birth was designated as day 0, and experiments were conducted at various ages thereafter. Litter size was restricted to 12 pups per dam that was performed at day 2 postpartum. All animals were kept in a temperature-controlled room (22 ± 1℃) and maintained on a 12-h light-dark cycle. To prevent the stress of removing one animal from a cage on sequential days, all the animals in a cage were killed on a single day. Young rats were never separated from their mothers except during the period of fasting. During this period, the pups were placed in cages artificially warmed by electric light bulbs. Before studies, all rats were fasted for various times. To prevent mortality from prolonged fasting, the 8- and 10-d-old rats were fasted for 18 h, and 14-d-old rats were fasted for 24 h. Experiments were designed using littermate controls disregarding the sex of the pups. All animals were killed between 8 and 10 am[15]. CA was given at a dose of 100 μg/g body weight by intraperitoneal injection to normal pups on days 7, 9, and 11 to induce hypercorticoidism. Control animals were given identical volumes of normal saline[16]. To observe the effect of GCs on suckling rats, another experiment was designed as follows: 100 μg/g body weight of CA was given once to pairs of 7-d-old pups (HC group) by intraperitoneal injection. Control pups (C group) were injected with identical volumes of normal saline at the same day. Pups were treated with 50 μg/g body weight mifepristone (17β-hydroxy-11β-4-dimethyl-aminophenyl-17α-propynylestra-4,9-diene-3-one, RU38486), a glucocorticoid cytoplasm receptor antagonist alone (M group), or 100 μg/g hydrocortisone plus 50 μg/g mifepristone (H+M group)[17].

Colons from control and hypercorticoidism rats at 8, 10 and 14 d of age were used in this study and five rats were used in each age stage. Samples were fixed in 4% paraformaldehyde overnight at 4℃ followed by a standard protocol of dehydration and paraffin embedding. Five-micrometer sections were prepared for morphological and fluorescence immunohistochemical studies. Total RNA and lysate were extracted from tissues at each time point for the reverse transcriptase PCR (RT-PCR) and Western blotting analysis. The study protocol was approved by the Nanjing Medical University Animal Care and Use Committee.

Morphology

Routine hematoxylin and eosin-stained sections for light microscopic (LM) evaluation were used to study the hormonal effects in the morphological development of colon. Slides were viewed under an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan). Morphologic measurement made by the same technician was blinded to the different treatment groups. Under LM, the crypt depth and villous height of the villous zone were mea-

sured using an image analysis system (NYD100). The group means were obtained based on 10 villi and 20 crypts per slide, with a minimum of five animals in each group.

Serial levels of AFP

Blood samples of rats were centrifuged at 2000 × *g* for 20 min, and sera were stored at -20℃ until analyzed. AFP levels in the rat serum were measured by the routine standard radioactive method used in the Nanjing Clinical Nuclear Medicine Center (Nanjing, China).

RNA expression of AFP

Total RNA was isolated from tissues by Trizol according to the protocol supplied by the manufacturers. cDNA was synthesized using Takara RNA PCR 3.0 Kit in a total volume of 10 μL, containing 0.5 μL avian myeloblastosis virus RT, 0.5 μL random 9 primer, 2 μL 25 mmol/L $MgCL_2$, 1 μL 10 × RT buffer, 1 μL dNTP mixture (each 10 mmol/L), 0.25 μL RNase inhibitor, 1 μL RNA, and 3.75 μL dH_2O. Conditions for RT were: 30℃ for 10 min, 42℃ for 25 min, 99℃ for 5 min, and 5℃ for 5 min. PCR was performed in 50 μL reactions containing 2.5 ng cDNA, 1 μL each primer pair, and 25 μL Premix *Taq* in the Takara RNA PCR kit. PCR was carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Quebec, Canada) to determine the annealing temperature for each paired primers. The following AFP primer pairs were used: 5'-GCTGAACCCAGAG-TACTGCAC-3' (forward), and 5'-GACACGTCGTAG-ATGAACGTG-3' (reverse). Amplification reactions were carried out for 30 cycles at 94℃ for 30 s, 58.4℃ for 30 s, and at 72℃ for 1 min.

The amplified products were 443 bp and analyzed on 1% agarose gels and visualized by ethidium bromide staining. Omitting RT, cDNA or DNA polymerase were adopted in the controls, and showed no reaction bands. The data were normalized by actin.

Protein expression of AFP

The tissues were homogenized in a sample buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail. An equal amount of protein samples were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After transferred to nitrocellulose membranes and blocked with 5% fat-free milk in Tris-buffered saline plus 0.05% Tween 20 overnight at 4℃, polyclonal antibody for AFP and the corresponding secondary antibody were applied. Blots were visualized with enhanced chemiluminescence reagents and exposed to X-Omat BT film. Signal intensity was quantified using a Bio-Rad image analysis system and the results were normalized to band intensities at e18.5. The β-actin was used as an internal control and the primary antibody was omitted for negative controls.

Regional and cellular localization of AFP

Double-immunofluorescent staining of AFP and vimentin, a specific marker of mesenchymal cell, were used to determine the regional and cellular localization of AFP in rat colons. Staining was performed according to the standard procedures. Briefly, the sections were deparaffinized in xylene, cleared with graded ethanol in phosphate buffered saline (PBS), and then placed in 10 mmol/L citrate buffer (pH 6.0) for 15 min at 100℃ for antigen retrieval. The sections were applied to goat anti-AFP polyclonal antibody overnight at 4℃ and then linked with FITC-labeled rabbit anti goat-IgG. After washing by Tris buffered saline (TBS), mouse anti-vimentin monoclonal antibody and rhodamine-labeled anti-mouse IgG were applied. Sections were placed in Gel Mount aqueous mounting medium with a cover glass and were examined under an Olympus BX51 microscope. Controls were treated by omitting the primary or secondary antibodies. No staining was observed under the negative control conditions. Images were taken at a magnification of × 200. An image analysis system (NYD100) was used for quantitative analysis of cell density (cell number/view field) of the AFP-positive cells in the rat colon. Four sections from four rats in each group were used. AFP-positive cells were counted in five randomly selected view fields per section at a magnification of × 400. At least 20 fields in each group were analyzed.

Statistical analysis

All experiments were done in triplicate. The experimental data was analyzed using PDQuest 7.0 software (Bio-Rad Laboratories, Hercules, CA, USA) and one-way analysis of variance and paired *t* test were used. Data were presented as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Morphology

Injecting corticosterone into the suckling animals for 1, 2, and 3 d showed no effect on the animal's intestinal and body weights (data not shown). The crypt depth and villous height after corticosterone treatment were higher in the colon of 8- and 10-d-old rats with hypercorticoidism than in the control animals and did not influence those in the 14-d-old rats with hypercorticoidism (Table 1).
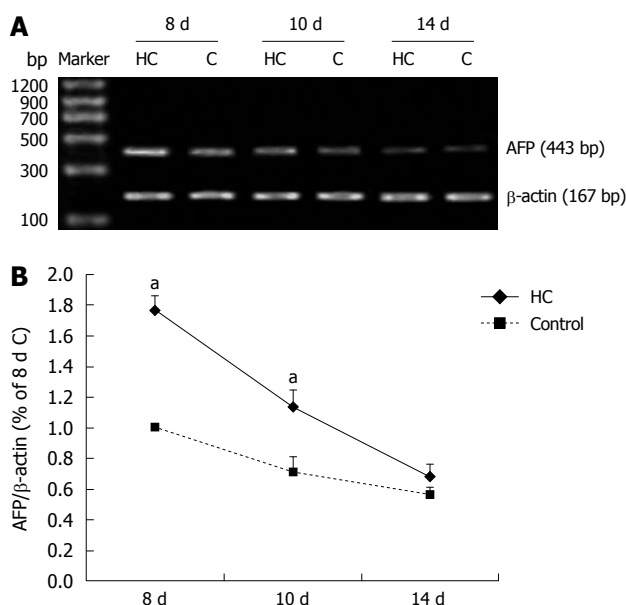
Expression of AFP

The serum AFP levels in the rats were near 18 μmol/L in all measured rats and had no difference between hypercorticoidism and control animals (data not shown). In this study, we analyzed the effect of corticosterone on the level of AFP mRNA by RT-PCR. The AFP mRNA concentration increased significantly after corticosterone treatment, by 2.5-folds in the 8-d-old and 2.5-folds in the 10-d-old rats compared with those in the control animals (Figure 1). The transcriptional regulation of AFP expres-

Table 1 Morphological changes in suckling rat colon treated with corticosterone acetate

	Villous height (μm)			Crypt depth (μm)		
	8 d	10 d	14 d	8 d	10 d	14 d
Controls	124.6 ± 41.4	165.9 ± 34.4	254.3 ± 57.7	26.9 ± 10.3	40.4 ± 14.7	53.0 ± 23.5
CA	151.1 ± 35.1[a]	196.9 ± 29.6[a]	248.5 ± 42.3	39.1 ± 5.7[a]	50.3 ± 13.6[a]	60.5 ± 15.6

[a]$P < 0.05$ *vs* control. CA: Corticosterone acetate.

Table 2 Changes in number of α-fetoprotein-positive cells in suckling rat colon treated with corticosterone acetate (numbers/view field, mean ± SD, $n = 20$)

	8 d	10 d	14 d
Controls	21.6 ± 4.7	14.1 ± 4.3	12.3 ± 5.2
CA	38.2 ± 5.2[a]	23.8 ± 2.5[a]	11.5 ± 2.3

[a]$P < 0.05$ *vs* control. CA: Corticosterone acetate.

Figure 1 Expression of α-fetoprotein mRNA by reverse transcriptase polymerase chain reaction analysis in colons of suckling rats treated with or without hydrocortisone. Molecular weight markers are indicated on the left. A: The α-fetoprotein (AFP) mRNA levels in colons of hydrocortisone-treated animals were higher than in controls of 8- and 10-d-old rats; B: Results are indicated in percentage above the β-actin value and are representative of three independent experiments. [a]$P < 0.05$ *vs* controls; C: Pups received saline; HC: Pups received hydrocortisone.

sion of corticosterone was confirmed by the Western blotting analysis (Figure 2).

Regional and cellular localization of AFP

The AFP positive cells were scattered on the epithelium in either corticosterone treated or untreated rats. The cell density of AFP-positive cells was higher in 8- and 10-d-old corticosterone-treated rats than in control animals (Table 2). Double-immunofluorescent staining for the vimentin and AFP showed that there was complete overlap between the AFP-positive cells and the antibody staining for vimentin in all the tested animals (Figure 3).
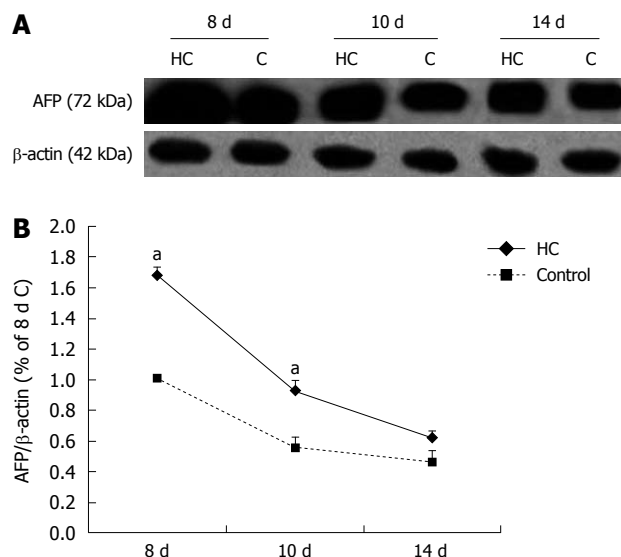
Figure 2 Expression of α-fetoprotein protein by Western blotting analysis in colons of suckling rats treated with or without hydrocortisone as indicated in lanes, respectively. Western blotting analysis using α-fetoprotein (AFP) (C-19), revealed a 72 kDa isoform. A: AFP levels in colons of hydrocortisone-treated animals were higher than in controls of 8- and 10-d-old rats; B: Results are indicated in percentage above the β-actin value and are representative of three independent experiments. [a]$P < 0.05$ *vs* controls; C: Pups received saline; HC: Pups received hydrocortisone.

Effect of mifepristone

The effect of increased crypt depth and villous height in the colon of 8-d-old rats with hypercorticoidism was blocked by mifepristone (Figure 4). The increased expression of AFP was also inhibited in the rats treated with mifepristone (Figure 5).

DISCUSSION

Although rodent colon may differ from human colon in the breadth of its responses to GCs, it is nevertheless a useful model to address issues related to morphological maturation, since the development of gastrointestinal functions is similar in all mammals. The aim of this study was to assess the role of exogenous GCs in the maturation of the colon. Accordingly, we used the rats in the first postnatal week whose circulating concentrations of natural GCs were very low (< 0.5 μg/mL)[18].

Exogenous administration of GCs causes precocious maturation of the intestine. In this study, GCs did result in an increase in villous height and crypt depth of the colon (Table 1) in 8- and 10-d-old rats compared with the age-matched controls. This effect was completely blocked
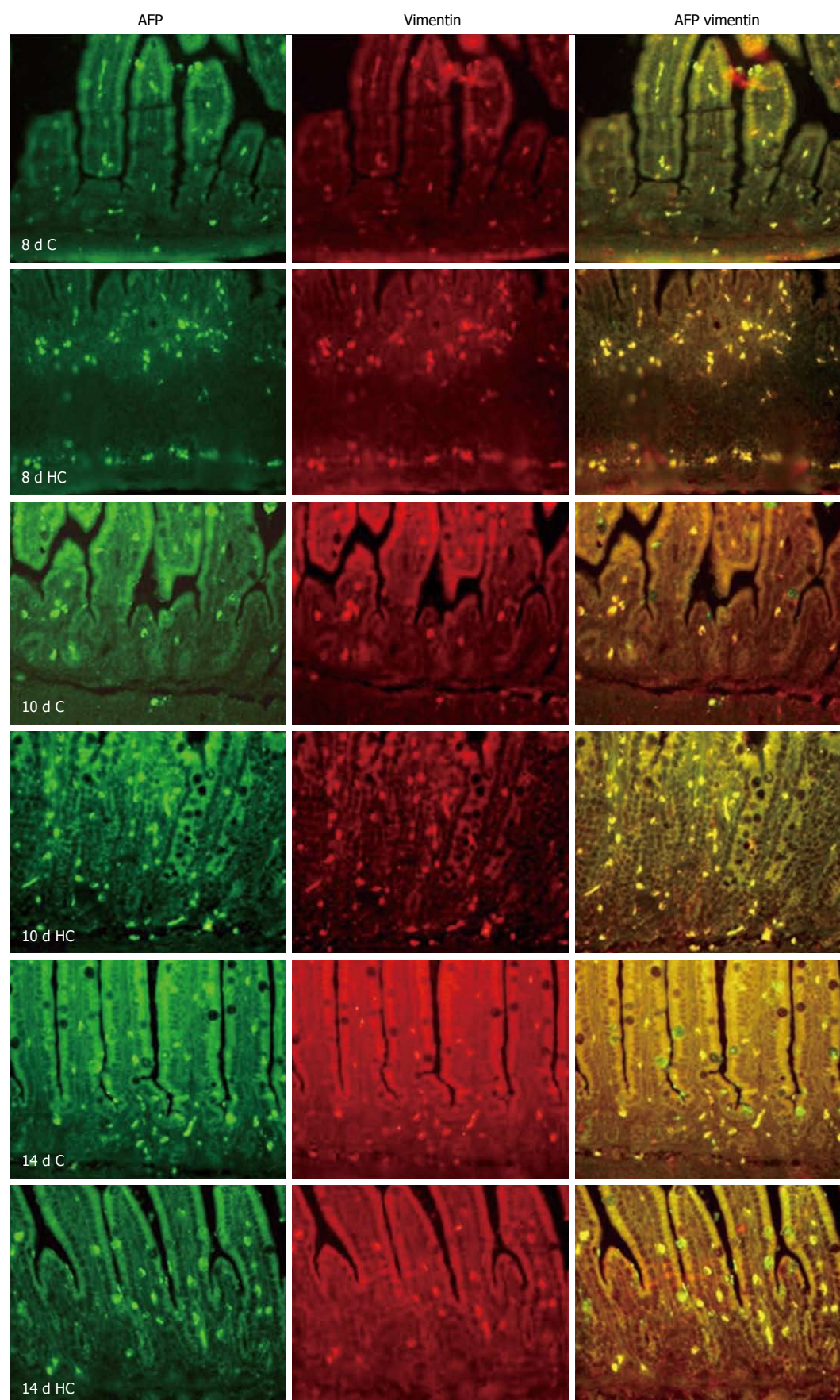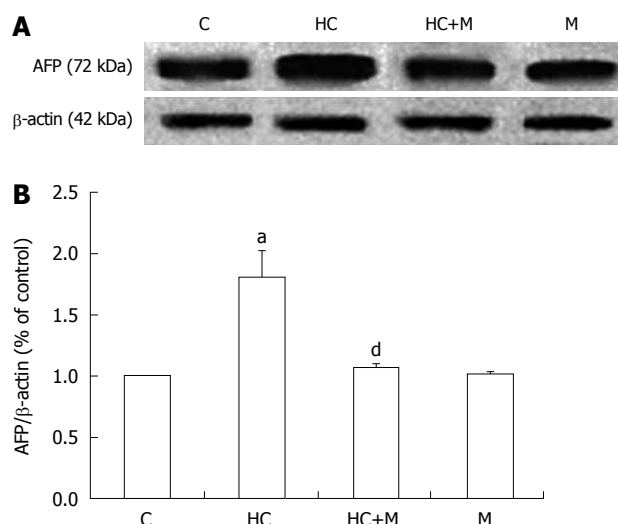
Figure 3 Immunofluorescence localization of α-fetoprotein and vimentin in the suckling rats treated with or without hydrocortisone. Labeling by the α-fetoprotein (AFP) antibody was detected with a fluorescein isothiocyanate (green) labeled secondary antibody. Labeling of vimentin was detected with a rhodamine-(red)-labeled secondary antibody on the same section. The overlap of AFP (green) and vimentin (red) labeling displayed orange color. Double-staining revealed complete colocalization of AFP and vimentin in the same cells of rat colons treated with or without hydrocortisone. All of the primary magnifications are × 200.
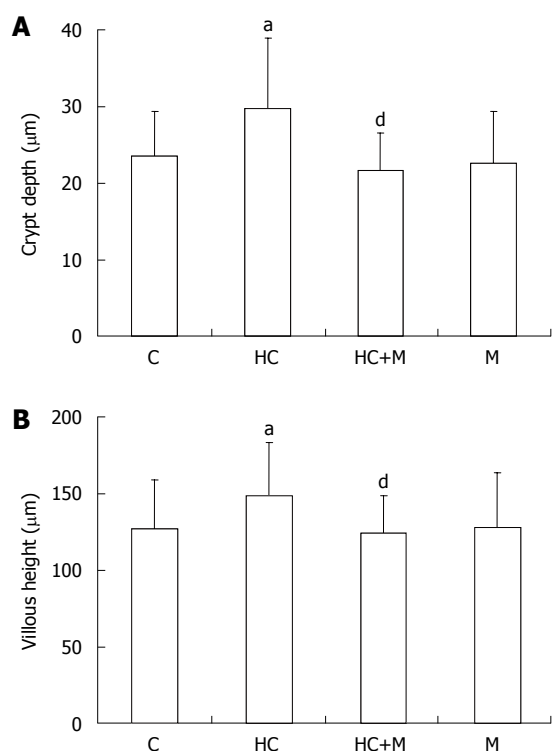
Figure 4 Morphological changes in colons of suckling rats treated with or without hydrocortisone, hydrocortisone + mifepristone and mifepristone alone. Villous height (A) and crypt depth (B) were determined. Each data point (± SD; bars) is the mean of four independent trials. C: Pups received saline; HC: Pups received hydrocortisone; HC+M: Pups treated with both hydrocortisone and mifepristone; M: Pups treated with mifepristone only. [a]$P < 0.05$ *vs* control animals; [d]$P < 0.01$ *vs* hydrocortisone-treated animals.

Figure 5 α-fetoprotein expressions in 8-d-old rat colons treated with or without hydrocortisone, hydrocortisone + mifepristone and mifepristone alone. α-fetoprotein (AFP) expression was determined by the Western blotting using AFP polyclonal antibody (A), and results indicated are in percentage above the β-actin value and are representative of three independent experiments (B). Each data point (± SD; bars) is the mean of four independent trials. C: Pups received saline; HC: Pups received hydrocortisone; HC+M: Pups treated with both hydrocortisone and mifepristone; M: Pups treated with mifepristone only. [a]$P < 0.05$ *vs* control animals; [d]$P < 0.01$ *vs* hydrocortisone-treated animals.

by the mifepristone, a glucocorticoid cytoplasm receptor antagonist (Figure 4). It is possible that GCs had a precocious effect on the development of rat colon in the first postnatal week, and in the rat small intestine as well[19,20].

In contrast, the GCs had no effect on the colon in 14-d-old rats. It has been suggested that immature gastrointestinal tract seems to have more sensitive responsiveness to the regulation of GCs[21]. It is possible that prepartum cellular differentiation progresses until day 10 in rats after birth[22]. On the other hand, cytoplasm receptors of GCs are activated after the binding of GCs that allows their translocation to the nucleus. Of note is the pattern of ontogeny changes in the concentration of GCs receptors in the small intestine as cytoplasm GCs receptors are present in the intestine at all ages, but at higher concentrations during the two postnatal weeks than in older rats[23]. Moreover, GCs receptors themselves have been shown to down-regulate by GCs through GCs enhanced expression of a factor (repressor), which binds the regulatory sequence in the GCs receptor gene promoter[24]. This might serve as a negative feedback control in the GCs response systems under developmental conditions. In 14-d-old rats, the plasma corticosterone of GCs was raised to 5 μg/mL[18], and a loss of responsiveness to GCs for colon development might occur in these rats. No effect of exogenous GCs administration could be also explained by a fall in GCs re-

ceptors in 14-d-old rats with a natural rise in corticosterone level, although this was not tested in this study.

AFP is known to be associated with the successful completion of term pregnancies in mammals and even minute amounts of AFP may still be necessary during human pregnancy[25]. The capability of both up and down modulation of growth and differentiation as a dose-dependent function of AFP has been demonstrated in a multitude of cell types, including placental, ovarian, uterine, and lymphoid, epidermal, endothelial, testicular, breast, and liver[26-30]. In our previous studies, we indicated that AFP was expressed and produced in mesenchymal cells, which might act as a potent paracrine regulator of colonic cell proliferation and organ maturation[10]. In this study, exogenous administration of GCs demonstrated an increased expression of AFP, this effect was also inhibited by mifepristone (Figures 2 and 3). Double-immunofluorescent staining for the vimentin, a mesenchymal cell marker, and AFP showed that the AFP was localized in the mesenchymal cells both in GCs-treated and control colons, similar to our previous study[10].

In present study, the AFP levels in the rat serum were near 18 μmol/L in all measured rats and had no difference between hypercorticoidism and control animals (data not shown). High cortisone concentration in serum prompts the acceleration of colonic ontogenesis, and is not accompanied by an increased level of serial AFP. In the current study, we did observe an increased expression of AFP in both RNA and protein levels in the GCs-treated premature colon of rats. Therefore, mesenchymal cell-derived AFP acted as a potent paracrine regulator in rat developmental colon.

The effect of GCs may require interactions with another tissue or cell type, such as mesenchyme[7]. The epithelial-mesenchymal interactions play an essential role in the control of gastrointestinal epithelial growth and differentiation not only in fetal stages, but also in adults[31,32]. There is no report that GCs could regulate the growth colonic mucosa directly. Our *in vitro* study also showed a negative result for GCs stimulating the proliferation of colonic epidermis (data not shown). The exogenous GCs were blocked, and the effect of accelerating maturation and the increased expression of AFP were also inhibited. Therefore, mesenchymal cell-derived AFP appears to be responsible, in part, in mediating the effects of GCs on developmental colon. Our present study may help us discern whether the epithelial-mesenchymal interactions and the effect of GCs enhance intestinal maturation in the gastrointestinal tract.

The involvement of GCs in AFP expression in the gut still remains an open question. Studies are now in progress in our laboratory using animal and tissue culture models to test it.

In summary, our present study for the first time demonstrated that GCs promote the development of rat colon. AFP appears to be responsible, in part, in mediating the effects of GCs on developmental colon. The exact function of AFP in rat colon development remains to be determined.

COMMENTS

Background
Glucocorticoids (GCs) are considered to play an important role in the maturation of the gastrointestinal (GI) tract. However, the mechanism of GCs on GI system development has not been fully elucidated.

Research frontiers
Although rodent colon may differ from human colon in the breadth of its responses to GCs, it is nevertheless a useful model to address issues related to morphological maturation, since the development of gastrointestinal functions is similar in all mammals. This study assessed the role of exogenous GCs in the maturation of the colon in rats.

Innovations and breakthroughs
This study for the first time demonstrated that GCs promote the development of rat colon. α-fetoprotein (AFP) appears to be responsible, in part, in mediating the effects of GCs on developmental colon. The exact function of AFP in rat colon development remains to be determined.

Applications
This animal model is a useful tool for the study of mechanism of gastrointestinal mucosal proliferation and differentiation *in vivo*.

Terminology
Corticosterone acetate is a chemical compound of GCs. Mammalian AFP is a single-chain glycoprotein with a molecular mass ranging from 66 to 72 kDa and 3%-5% carbohydrate (glycan) content.

Peer review
This is an excellent study.

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