Effects of Dietary Protein and Tumor Necrosis Factor on Components of the Insulin-Like Growth Factor-I Pathway in the Colon and Small Intestine in Protein-Depleted Rats

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Intestinal cell growth is markedly affected by nutrient intake and the presence of cytokines. Since insulin-like growth factor-I (IGF-I) is an important hormone regulator of intestinal proliferation, this study examined the effects of dietary protein content and tumor necrosis factor (TNF) on mRNA levels of IGF-I, IGF-I receptor, IGF binding protein-3 (IGFBP-3), and IGFBP-4 and on the histology of the colon, jejunum, and ileum in protein-malnourished rats. After 2 weeks of feeding a 2% casein diet, rats continued on the 2% casein diet or were refed with a 20% casein diet and received daily intraperitoneal injections of either TNF (50 μg/kg) or saline for 4 days. The abundance of mRNA in the intestine was determined by RNA dot-blot analysis, and morphology measurements were performed by light microscopy. Simultaneous refeeding with the 20% casein diet and administration of TNF led to a modest increase in IGF-I and IGFBP-4 mRNA abundance in the colon. However, in the jejunum and ileum, refeeding had no effect but TNF caused a decrease in IGF-I and IGFBP-3 mRNA levels in malnourished rats. Refeeding with the 20% casein diet resulted in relatively modest histologic changes, which were greater in the colon versus the small intestine. The decreased magnitude of histologic changes in the order of the colon, ileum, and jejunum may reflect a response to a gradient of amino acid availability from intraluminal nutrients. These data demonstrate that TNF has distinct effects on colon and small intestine mRNA, but these mild changes had only a slight impact in the colon and did not translate into identifiable histologic changes in the small intestine. Combined protein restriction and TNF administration had only a modest effect on intestinal mRNA levels and mucosal histology.

INSULIN-LIKE GROWTH FACTOR-I (IGF-I) is a polypeptide growth factor that promotes cell proliferation in the intestine, as well as many other tissues.[1,2] IGF-I regulates cell growth by binding to specific IGF-I receptors, a process modulated by IGF binding proteins (IGFBPs).[3-5] The functions of IGFBPs are complex, depending on the type of IGFBP, the cell system, and the experimental conditions.[6,7] Among the proposed functions of IGFBPs are the prolongation of plasma IGF-I half-life, control of IGF transport from the vascular compartment, and regulation of the interaction between IGF-I and its receptors on the cell surface.[8] IGF-I may act in an endocrine, paracrine, or autocrine manner to regulate cell growth.[2,9,10]

Nutritional status has marked effects on intestinal cell growth[11] and also is known to regulate the IGF-I pathway.[12] Protein restriction significantly decreases plasma IGF-I and IGFBP-3 by decreasing their mRNA abundance in the liver.[13-15] However, little is known about the role of dietary protein in the regulation of the IGF-I pathway in the intestine.[16] Tumor necrosis factor (TNF) is a cytokine with important effects on intestinal cell growth and metabolism.[17-19] It has been shown to increase intestinal protein synthesis in experimental animals[20] and intestinal epithelial cell lines[21] and is also known to increase hepatic IGF-I mRNA abundance,[15] but the effects of TNF on components of the IGF pathway in the intestine have not been investigated. Similarly, the potential interactive effects of dietary protein and TNF in the intestine have not been examined.

We have previously reported the effects of nutritional factors and TNF administration on hepatic IGF-I and albumin mRNA and plasma IGF-I and albumin levels.[15] The results showed that refeeding protein-restricted rats a 20% casein diet significantly increased the body weight, liver weight, liver protein content, and plasma IGF-I concentration in comparison to the 2% casein diet. TNF administration did not have additional effects on body weight, liver weight, liver protein content, or plasma IGF-I in rats maintained on the 2% casein diet or refed the 20% casein diet. In the present study using the same animals from this malnutrition model of dietary protein restriction and refeeding, we examined the effects of dietary protein content and TNF on the mRNA for IGF-I, IGF-I receptors, IGFBP-3 and -4, and histologic parameters in the colon and small intestine.

MATERIALS AND METHODS

Animal Procedures and Experimental Design

Male Sprague-Dawley rats (N = 24; Taconic Farms, Germantown, NY) weighing 180 to 200 g were acclimated for 5 days in a controlled environment (12-hour light/dark cycle and 24°C). During this period, the rats were housed individually in wire-bottomed cages and given free access to water and a standard laboratory diet (Prolab; Agway Country Foods, Syracuse, NY). They were then switched to the AIN 76 diet with 2% casein ad libitum for 14 days. On the afternoon of day 15 (2 PM), the rats were started on one of two diets of different protein content (Table 1) and were administered daily intraperitoneal injections of saline or TNF (50 μg/kg/d; Genentech, San Francisco, CA) until day 19. The dose of TNF was based on our previous experience.[22] A total of four experimental groups were studied: two continued on the 2% casein diet and received saline (2% casein + saline, n = 6) or TNF (2% Casein + TNF, n = 6) injections, respectively; the remaining two groups were repleted with the AIN 76 diet modified to contain 20% casein and received saline (20% casein + saline, n = 6) or TNF (20%

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Table 1. Diet Composition (g/kg)

Ingredient	2% Casein	20% Casein
Casein	20	200
DL-Methionine	0.3	3
Cornstarch	192.2	150
Sucrose	640.5	500
Cellulose	50	50
Corn oil	50	50
Salt Mix #200000	35	35
Vitamin Mix #300050	10	10
Choline bitartrate	2	2
Energy (J/g)	16.16	16.16

NOTE. Diets were purchased from Dyets (Bethlehem, PA). Salt Mix #200000 includes the following (mg/kg diet): calcium 5,200, phosphorus 4,000, potassium 3,600, sodium 1,020, chloride 1,560, sulfur 337, magnesium 507, iron 35, copper 6.0, manganese 54.0, zinc 30.0, chromium 2.0, iodine 0.2, and selenium 0.1. Vitamin Mix #300050 includes the following (mg/kg diet): thiamine 6, riboflavin 6, pyridoxine HCl 7, niacin 30, calcium pantothenate 16, folic acid 2, biotin 0.2, cyanocobalamin 10, and menadione sodium bisulfite 0.8, plus vitamin A 4,000 IU, vitamin E 175 IU, and vitamin D_3 1,000 IU.

casein + TNF, n = 6) injections. All animals were pair-fed to the 2% casein + TNF group, since these animals consumed the least amount of food. Body weight was recorded on days 15 and 19.

On day 19, the rats were killed by decapitation at 10 AM. Blood was collected into chilled sodium EDTA tubes and centrifuged, and the plasma was stored at −20°C for analysis of IGF-I. The small intestine was removed from the ligament of Treitz to the ileocecal junction. The entire colon from the cecum to the anus was removed. Segments (2-cm) were obtained from the first 10 cm of proximal jejunum, the last 10 cm of distal ileum, and the last 5 cm of distal colon. The bowel segments were immediately opened by longitudinal incision, rinsed with phosphate-buffered saline, marked on the serosa with India ink, pinned to a paraffin block, and immersed in Formalin for histology. The remaining intestinal samples were quickly flushed with ice-cold saline until free of contents, blotted dry, frozen in liquid nitrogen, and stored at −80°C for subsequent RNA isolation and analysis.

RNA Extraction and Dot-Blotting

Total colon and small intestine RNA was extracted by the TRI Reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Aliquots of total RNA were denatured, and a 10-μg sample was applied to the nylon membranes (Gene Screen Plus; Dupont NEN Products, Boston, MA) using a dot-blot vacuum manifold apparatus (Schleicher and Schuell, Keene, NH). After RNA immobilization by UV cross-linking, the blots were hybridized with ^{32}P-labeled rat cDNA probes for IGF-I (from Peter Rotwein, Washington University, St Louis, MO), IGF-I receptor (isolated in our laboratory),[23] and IGFBP-3 and -4 (from Dr S. Shimisaki, Whittier Institute, La Jolla, CA). A murine 18S ribosomal cDNA probe was used as a control for total RNA loading. Blots were hybridized overnight at 42°C and washed according to methods described by the manufacturer of the nylon membranes. Optimal hybridization and washing conditions for each respective cDNA probe were previously established by Northern blot analysis.[23] Blots were exposed in a Phosphorimager cassette, and the relative intensities were quantified by a Phosphorimager system (Molecular Dynamics, Sunnyvale, CA). The concentrations of mRNA for IGF-I, IGF-I receptor, and IGFBP-3 and -4 are expressed as arbitrary units relative to the 20% casein + saline group after correcting for 18S ribosomal RNA content.

Histologic Analysis

Morphologic analysis of the colon and small intestine was performed as described previously.[24] In brief, histologic sections of colon, jejunum, and ileum stained with hematoxylin and eosin were examined in double-blind fashion by light microscopy (magnification ×40). Crypt-villus units were identified that included, in continuity, the bottom lumen of the crypt, a continuous crypt-villus junction, and the tip of the villus. Crypt depth, crypt cell number, and villus height were measured using a calibrated eyepiece micrometer. The mean crypt cell width was determined by dividing the crypt depth by the cell number on one side of the crypt. At least five villi and crypts were measured in each specimen, and mean values were determined for each sample.

Statistical Analysis

Data are presented as the mean ± SEM. Group means were compared by two-way ANOVA (20% *v* 2% casein and saline *v* TNF), and correlations were also examined using the SYSTAT statistical software package (SYSTAT, Evanston, IL). Significance was defined as a P value less than .05. Comparisons among groups were determined by Fisher's least-significant difference (LSD) (SYSTAT) when ANOVA was found to be significant at the 95% confidence level.

RESULTS

Analyses of IGF-I, IGF-I Receptor, and IGFBP-3 and -4 mRNA in the Colon and Small Intestine

Four days of 20% casein diet repletion modestly increased the colonic IGF-I mRNA content by 10% compared with the 2% casein diet ($P < .05$; Table 2 and Fig 1). Colonic IGF-I mRNA levels were also increased 25% and 21% after TNF injection in animals refed the 20% casein diet and maintained on low protein, respectively. There was a significant 27% decrease in colonic IGF-I receptor mRNA after dietary protein repletion ($P < .001$). Consistent with the decreased IGF-I downregulation of IGF-I receptor mRNA content in protein-restricted animals, colonic IGF-I receptor mRNA was inversely correlated with the plasma IGF-I level ($r = .6$, $P < .001$). In the jejunum, both IGF-I and IGFBP-3 mRNA levels were decreased by TNF in rats fed the 2% casein diet ($P < .05$ and .01, respectively; Table 3). By contrast, IGF-I receptor and IGFBP-4 mRNA were increased by TNF injection ($P < .001$ and .005, respectively), reaching statistical significance only in animals consuming the

Table 2. Colonic Content of IGF-I, IGF-I Receptor, IGFBP-3, and IGFBP-4 mRNA by Dot-Blot Analysis

Group	No.	IGF-I*	IGF-IR†	IGFBP-3	IGFBP-4‡
20% Casein + saline	6	1.00±0.04	1.00±0.07¶	1.00±0.06	1.00±0.06
20% Casein + TNF	6	1.25±0.05§	1.03±0.06¶	1.02±0.09	1.14±0.08#
2% Casein + saline	6	0.9±0.04‖	1.27±0.09	0.94±0.10	0.83±0.05
2% Casein + TNF	6	1.09±0.06	1.27±0.04	1.01±0.09	1.01±0.08

NOTE. Values are the mean ± SEM normalized to the value in the 20% casein + saline group.

*$P < .05$, 20% casein *v* 2% casein, *$P < .001$, saline *v* TNF, †$P \leq .001$, 20% casein *v* 2% casein, ‡$P < .05$, 20% casein *v* 2% casein and saline *v* TNF (all by 2-way ANOVA).

§$P < .05$ *v* all, ‖$P < .05$ *v* 2% casein + TNF, ¶$P < .05$ *v* 2% casein, #$P < .01$ *v* 2% casein + saline by Fisher's LSD.

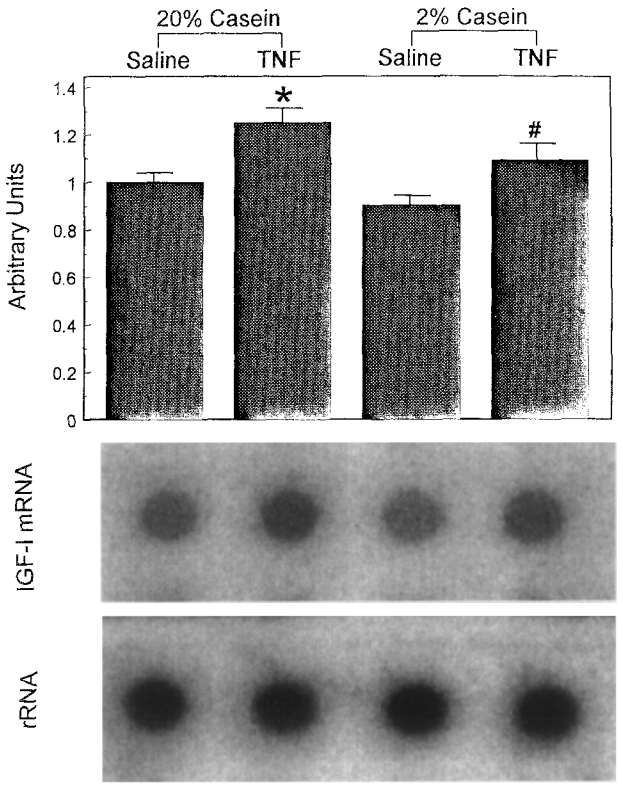

Fig 1. Effects of dietary protein content and TNF on IGF-I mRNA abundance in colon. IGF-I mRNA concentration is expressed as arbitrary units relative to the 20% casein + saline group after normalizing to ribosomal mRNA. Data are expressed as the mean ±SEM. $P < .05$, 20% casein *v* 2% casein, and $P < .001$, saline *v* TNF, by 2-way ANOVA. *$P < .05$ *v* all and #$P < .05$ *v* 2% casein + saline by Fisher's LSD.

2% casein diet. In the ileum, TNF increased IGF-I mRNA in the 20% casein group ($P < .001$) and decreased IGF-I and IGFBP-3 mRNA in the 2% casein group ($P < .01$ by LSD; Table 4). Local production of IGF-I mRNA in the ileum was significantly correlated with IGFBP-3 mRNA ($r = .68$, $P < .001$), and IGF-I receptor mRNA with IGFBP-4 mRNA ($r = .71$, $P < .001$), respectively.

Table 3. Jejunal Content of IGF-I, IGF-I Receptor, and IGFBP-3 and IGFBP-4 mRNA by Dot-Blot Analysis

Group	No.	IGF-I*	IGF-IR†	IGFBP-3‡	IGFBP-4§
20% Casein + saline	6	1.00±0.02	1.00±0.03	1.00±0.04	1.00±0.02
20% Casein + TNF	6	1.03±0.02	1.10±0.03	1.06±0.03	1.12±0.02
2% Casein + saline	6	0.97±0.03	1.02±0.03	0.99±0.04	1.09±0.05
2% Casein + TNF	6	0.86±0.02‖	1.25±0.05‖	0.85±0.02‖	1.33±0.08‖

NOTE. Values are the mean ± SEM normalized to the value in the 20% casein + saline group.

*$P < .001$, 20% casein *v* 2% casein, $P < .05$ for interaction between diet and treatment.

†$P < .05$, 20% casein *v* 2% casein, $P < .001$, saline *v* TNF.

‡$P < .005$, 20% casein *v* 2% casein, $P < .01$ for interaction.

§$P = .005$, 20% casein *v* 2% casein, $P < .005$, saline *v* TNF (all by 2-way ANOVA).

‖$P < .01$ *v* by Fisher's LSD.

Table 4. Ileal Content of IGF-I, IGF-I Receptor, and IGFBP-3 and IGFBP-4 mRNA by Dot-Blot Analysis

Group	No.	IGF-I*	IGF-IR	IGFBP-3†	IGFBP-4
20% Casein + saline	6	1.00±0.03	1.00±0.04	1.00±0.04	1.00±0.05
20% Casein + TNF	6	1.11±0.03§	1.15±0.06	1.02±0.03	1.21±0.08
2% Casein + saline	6	0.99±0.02	1.14±0.06	0.87±0.05	1.13±0.09
2% Casein + TNF	6	0.85±0.03‡	1.09±0.08	0.81±0.06‖	1.15±0.08

NOTE. Values are the mean ± SEM normalized to the value in the 20% casein + saline group.

*$P < .001$, 20% casein *v* 2% casein, $P < .001$ for interaction with TNF.

†$P = .001$, 20% casein *v* 2% casein by 2-way ANOVA.

‡$P < .01$ *v* all.

§$P < .05$ *v* 20% casein + saline and 2% casein + saline.

‖$P < .01$ *v* 20% casein + saline and 20% casein + TNF by Fisher's LSD.

Histology of the Colon and Small Intestine

Refeeding protein-restricted rats a 20% casein diet significantly increased colonic crypt depth, which was associated with increased crypt cell width ($r = .80$, $P < .001$; Table 5). The cell number in the bottom third of the colonic crypt was increased by TNF in 20% casein diet–repleted rats ($P < .05$ for interaction). The 20% casein diet repletion increased the mean villus height in the jejunum as compared with the 2% casein protein restriction, with a suggestive further increase with TNF (Table 6). However, in the jejunum, dietary protein content had no effect on crypt depth, crypt cell number, crypt cell width, or villus surface area. Similar to the jejunum, dietary protein repletion increased the mean villus height in the ileum, although this did not reach statistical significance (Table 7). However, ileal crypt cell width increased significantly with 20% casein diet refeeding compared with the 2% casein diet ($P = .01$). Dietary protein content had no effect on villus height, crypt depth, crypt cell number, or villus surface area in the ileum.

Table 5. Crypt Depth, Cell Number in the Entire Crypt and the Bottom Third of the Crypt, and Crypt Cell Width in the Colon

Group	No.	Crypt Depth (μm)*	Crypt Cell No.	Cell No. in Bottom Third of Crypt†	Crypt Cell Width (μm)*
20% Casein + saline	6	178.1 ± 8.9‡	46.2 ± 2.3	36.9 ± 1.0	7.8 ± 0.6§
20% Casein + TNF	6	186.8 ± 8.2§	51.0 ± 1.7	42.2 ± 0.7‖	7.5 ± 0.2‡
2% Casein + saline	6	142.2 ± 5.8	46.0 ± 1.2	39.8 ± 0.7	6.1 ± 0.1
2% Casein + TNF	6	140.8 ± 5.7	46.2 ± 1.6	36.4 ± 0.7	6.1 ± 0.3

NOTE. Values are the mean ± SEM.

*$P < .001$, 20% casein *v* 2% casein, †$P < .05$ for interaction, by 2-way ANOVA.

‡$P < .05$ *v* 2% casein + saline and 2% casein + TNF.

§$P < .005$ *v* 2% casein + saline and 2% casein + TNF.

‖$P < .05$ *v* 20% casein + saline and 2% casein + TNF by Fisher's LSD.

Table 6. Villus Height, Crypt Cell Number, Crypt Cell Width, and Villus Surface Area in the Jejunum

Group	No.	Villus Height (μm)*	Crypt Depth (μm)	Crypt Cell No.	Crypt Cell Width (μm)	Villus Surface Area (μm²)
20% Casein + saline	6	571.0 ± 17.1	122.4 ± 3.1	41.4 ± 0.3	5.9 ± 0.1	216.6 ± 12.0
20% Casein + TNF	6	618.9 ± 26.2†	115.3 ± 5.0	41.2 ± 0.4	5.6 ± 0.2	280.6 ± 27
2% Casein + saline	6	528.0 ± 20.8	113.4 ± 8.3	40.2 ± 0.8	4.8 ± 0.7	211.5 ± 18.6
2% Casein + TNF	6	522.2 ± 26.5	107.8 ± 3.7	39.6 ± 0.7	5.5 ± 0.1	217.0 ± 28.6

NOTE. Values are the mean ± SEM.

*$P < .01$, 20% casein *v* 2% casein by 2-way ANOVA.

†$P < .05$ *v* 2% casein + saline and 2% casein + TNF by Fisher's LSD.

TNF did not affect these histologic indices in the jejunum or ileum.

DISCUSSION

It has been demonstrated that long-term dietary protein restriction results in a decrease in plasma IGF-I and liver IGF-I mRNA.[13,25-27] We previously reported a significant increase (28%) in plasma IGF-I following refeeding with adequate dietary protein compared with an equal-energy but low-protein diet in the animals used in the present study.[15] Although the current study demonstrates an increase in colonic IGF-I and IGFBP-4 mRNA as a consequence of dietary protein refeeding, these changes are much smaller than the increases in the plasma IGF-I level and hepatic IGF-I mRNA. Refeeding with a 20% casein diet resulted in modest but significant downregulation of IGF-I receptor mRNA in the colon, which appears to be the converse of the previously documented upregulation of IGF-I receptor mRNA with fasting.[28] The inverse correlation of the colonic IGF-I receptor mRNA with the plasma IGF-I level is consistent with the possibility that during times of nutrient restriction, when the circulating (endocrine) source of IGF-I is diminished, increased IGF-I receptor expression in the colon may function as part of a compensatory response to preserve the colonic mucosa.

TNF injection in normal rats produces different metabolic changes depending on the organ involved. For example, TNF has been shown to increase liver protein content but decrease muscle protein content.[29] In the present study, TNF significantly increased colonic IGF-I mRNA in dietary protein–repleted animals, consistent with previously reported findings in the liver of the same animals.[15] This is also consistent with the reported increase in intestinal protein synthesis in response to TNF,[20] an anabolic effect of TNF that is presumably operative only when sufficient amino acids are available.[15] TNF did not affect IGF-I mRNA in the jejunum of dietary protein–repleted animals, although there was a modest TNF effect in the ileum; however, there was a further decrease in IGF-I mRNA abundance in both the jejunum and ileum in rats with continued malnutrition. The different changes in IGF-I mRNA induced by TNF in the colon and small intestine support the concept of tissue-specific IGF-I regulation.[30] One possibility is that there is a functionally important gradient of malnutrition from the jejunum to ileum to colon related to the distance from the limited dietary protein. With dietary protein repletion, there might be a greater response in the most distal (ie, most malnourished) colon. The mechanism for the decrease in IGF-I mRNA in the small intestine induced by TNF in malnourished conditions is not known. There is evidence that infusion of TNF significantly reduces plasma growth hormone (GH) levels by 67% to 81% in rats.[31] Since GH is a major regulator of IGF-I mRNA in nonhepatic tissues,[32-34] the decrease in IGF-I mRNA in the small intestine in response to TNF may result from decreased GH secretion or TNF-induced GH resistance under conditions of dietary protein restriction.

IGFBPs in serum or expressed locally in different tissues have major roles in regulating the IGF-I system.[9,35] By binding circulating IGF-I, IGFBP-3 may prolong the plasma half-life of IGF-I and reduce the amount of free IGF-I available for receptor binding.[36] During long-term dietary protein restriction in rats, serum IGFBP-3 declines and a similar change in the liver IGFBP-3 mRNA level is also observed.[37] There are no previous reports on IGFBP mRNAs in the intestine under protein-restricted conditions. Dietary protein refeeding had parallel but minor effects to increase jejunal IGF-I and IGFBP-3 mRNAs and decrease IGF-I receptor and IGFBP-4 mRNAs, respectively. The increase in IGFBP-4 mRNA in the jejunum by the combination of dietary protein restriction and TNF in this study occurred in association with decreased plasma IGF-I. Determining whether IGF-I regulates intestinal IGFBP-4 gene expression, and the role of IGFBP-4 in promoting or inhibiting IGF-I actions in the intestine, will require further investigation.

The histologic findings of the present study confirm our previous finding that dietary protein restriction diminishes

Table 7. Villus Height, Crypt Depth, Crypt Cell Number, Crypt Cell Width, and Villus Surface Area in the Ileum

Group	No.	Villus Height (μm)	Crypt Depth (μm)	Crypt Cell No.	Crypt Cell Width (μm)*	Villus Surface Area (μm²)
20% Casein + saline	6	343.2 ± 12.7	135.3 ± 12	43.8 ± 1.2	6.1 ± 0.2	114.2 ± 9.2
20% Casein + TNF	6	378.7 ± 36.5	132.2 ± 11.7	41.6 ± 0.4	6.3 ± 0.5	121.7 ± 16.6
2% Casein + saline	6	324.9 ± 20.4	132.2 ± 10.1	48.8 ± 1.6	5.5 ± 0.3	104.4 ± 11.5
2% Casein + TNF	6	318.8 ± 14.4	108.3 ± 7.1	43.4 ± 0.8	5.0 ± 0.2†	94.7 ± 5.7

NOTE. Values are the mean ± SEM.

*$P = .01$, 20% casein *v* 2% casein by 2-way ANOVA.

†$P < .05$ *v* 20% casein + saline and 20% casein + TNF by Fisher's LSD.

epithelial growth in the distal part of the bowel.[24] The decrease in crypt cell size as measured by cell width in the colon with continued dietary protein restriction is presumably due to decreased cellular protein content, because there was no difference in the water content of the intestine among the rats (data not shown). The histologic effect of combined TNF and protein repletion was only evident in the cell number in the bottom third of the crypt, where cells are rapidly proliferating. The more modest changes in histology in the jejunum and ileum may be a consequence of the optimal exposure of this portion of the bowel to the limited supply of nutrients.

The present study compared the different responses of colon and small intestine histology and mRNAs for IGF-I, IGF-I receptor, and IGFBP-3 and -4 to changes in dietary protein content and administration of TNF. Although dietary protein has similar effects on IGF-I, IGF-I receptor, and IGFBP-3 mRNAs in the colon and small intestine, the magnitude is minor compared with the change in plasma IGF-I, and the histologic changes are also modest. Although TNF exerted different effects on colon and small intestine mRNA—anabolic to colon under refeeding and catabolic to small intestine under prolonged malnutrition—the relatively small local changes in IGF-I, IGF-I receptor, and IGFBPs by the interaction of dietary protein and TNF were not associated with major histologic changes in the small intestine. The decreased magnitude of histologic changes in the order of the colon, ileum, and jejunum may reflect the more distal location from amino acids available from the diet and intestinal secretions.

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