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Dietary protein restriction rapidly reduces transforming growth factor β1 expression in experimental glomerulonephritis

(extracellular matrix/transforming growth factor β/glomerulonephritis)

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ABSTRACT **Dietary protein restriction has been shown to slow the rate of loss of kidney function in humans with progressive glomerulosclerosis due to glomerulonephritis or diabetes mellitus. A central feature of glomerulosclerosis is the pathological accumulation of extracellular matrix within the diseased glomeruli. Transforming growth factor β1 (TGF-β1) is known to have widespread regulatory effects on extracellular matrix and has been implicated as a major cause of increased extracellular matrix synthesis and buildup of pathological matrix within glomeruli in experimental glomerulonephritis. In the present study, it is shown that administration of a low protein diet to rats rapidly reduces the elevated expression of TGF-β1 mRNA and TGF-β1 protein that is known to occur within glomeruli after induction of glomerulonephritis. Compared to a normal protein diet, glomerulonephritic rats receiving the low protein diet did not develop an increase in glomerular extracellular matrix and showed significantly less proteinuria. Glomeruli isolated from glomerulonephritic rats fed the normal protein diet showed a marked increase in proteoglycan synthesis on day 7 of disease and were demonstrated to be secreting increased amounts of TGF-β1 into the medium, whereas glomeruli at the same point in time isolated from rats on a low protein diet showed no increase in proteoglycan production or TGF-β1 secretion. These results suggest that a mechanism of the rapid therapeutic effect of a low protein diet on experimental glomerulonephritis is through suppression of TGF-β1 expression and prevention of the induction of extracellular matrix synthesis within the injured glomeruli.**

Accumulation of extracellular matrix leading to glomerulosclerosis is the central pathological feature of progressive kidney disease due to glomerulonephritis or diabetes mellitus (1). Morphometric analysis of glomeruli from diabetic patients showed that the expanding volume of the glomerular mesangium (matrix and cells) correlated with the clinical onset of proteinuria, hypertension, and a decreased glomerular filtration rate (2). The factor(s) responsible for the buildup of extracellular matrix in human glomerular disease is largely unknown.

Recently, transforming growth factor β1 (TGF-β1) has been shown to have widespread effects on extracellular matrix (3). In various cell lines, TGF-β1 is known to (*i*) increase production of proteoglycans, fibronectin, and collagens (4, 5), (*ii*) decrease secretion of proteases and increase levels of protease inhibitors (6), and (*iii*) stimulate expression of extracellular matrix receptors on cells (7). We have found that TGF-β1 has dramatic effects on the production of extracellular matrix components by cultured rat glomerular mesangial (8) and epithelial cells (9). A principal action of TGF-β1 on both cell types is to regulate the synthesis of two chondroitin/dermatan sulfate proteoglycans, biglycan and decorin, both of which can bind TGF-β1 (23). In an experimental model of glomerulonephritis in the rat, we have found a close association between elevated expression of the TGF-β1 gene and the development of glomerulonephritis (10). Seven days after glomerular injury, at the time of significant extracellular matrix accumulation, the glomeruli showed a 5-fold increase in TGF-β1 mRNA and a nearly 50-fold increase in production of biglycan and decorin. Treatment of glomerulonephritic rats with an antiserum directed at a synthetic peptide from mature human TGF-β1 blocked the induction of proteoglycan synthesis and prevented the histologic accumulation of extracellular matrix in glomeruli (11). Taken together the findings implicate TGF-β1 as playing a central role in the pathogenesis of glomerulonephritis.

In humans with progressive glomerular disease, a reduction in dietary protein intake has been advocated as being effective in slowing the rate of loss of renal function (12). The mechanism of the protein effect in preserving renal function has been suggested as being a reduction in glomerular pressure and/or plasma flow (13, 14). In our model of experimental glomerulonephritis, a low protein diet rapidly suppressed the expected increase in glomerular extracellular matrix. We now provide evidence that a mechanism of the therapeutic effect may be reduction of TGF-β1 gene expression that results in suppression of the TGF-β1-induced increase in matrix synthesis in the injured glomeruli.

METHODOLOGY

Experimental Diets. Two defined formulae protein diets were used (Teklad, Madison, WI). The normal protein diet (22% protein; TD 86550) contained 25% casein and the low protein diet (6% protein; TD 86551) contained 7% casein. Both diets were identical in fat and mineral content. Sucrose and cornstarch provided 91% of the carbohydrate and the diets were made isocaloric (3.5 kcal/g; 1 cal = 4.184 J) by increasing the amount of sucrose in the low protein diet. To equalize caloric intake per body weight among the experimental groups, the amount of normal protein chow fed to the rats was adjusted at 3-day intervals to equal the amount of chow consumed by rats fed the low protein diet. All animals had free access to tap water during the study.

Experimental Model. Glomerulonephritis was induced in Sprague–Dawley rats (4–6 wk old) by intravenous injection of anti-thymocyte antiserum as described (10). Control rats received an equivalent injection of preimmune serum. To determine the effects of dietary protein on development of glomerulonephritis, repeat experiments were conducted by using the following four groups: (*i*) six normal control rats fed

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Abbreviation: TGF-β1, transforming growth factor β1.

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the normal protein diet, (*ii*) six normal control rats fed the low protein diet, (*iii*) six glomerulonephritic rats fed the normal protein diet, and (*iv*) six glomerulonephritic rats fed the low protein diet. Both the normal and low protein diets were started on day 1, at the time of anti-thymocyte or preimmune serum administration, and maintained until the time of sacrifice. Kidneys for immunohistologic examination and isolation of glomeruli were harvested from normal control rats before (day 0) and 7 days after preimmune serum injection and from glomerulonephritic rats on days 1, 4, 7, 14, and 28 after injection of anti-thymocyte antiserum. At each time point the following measurements were also made: serum creatinine, 24-h urinary protein excretion, systolic blood pressure, and body weight as described (10). Kidney tissue was prepared for light and immunofluorescence microscopy by standard published methods (10). Glomerular extracellular matrix was evaluated by an observer unaware of the source of the tissue. Thirty glomeruli were selected at random and the percentage of the glomerular area occupied by extracellular matrix was estimated by using a quantitative scale as described (10, 11).

Bioassay of TGF-β1. The ability of TGF-β1 to induce production of proteoglycans by normal rat mesangial cells in culture was used as a bioassay to detect TGF-β1 in conditioned medium from glomerular cultures as described (10). To include measurement of both mature and precursor TGF-β1 activity in the bioassay, aliquots of the conditioned medium were acidified to pH 3.2 for 1 h by addition of 1 M HCl. The transiently acidified medium was brought to pH 7.4 with 1 M NaOH and dialyzed against serum-free RPMI medium for 24 h at 4°C.

Growth Factors and Antibodies. Porcine platelet TGF-β1 was obtained from R & D Systems (Minneapolis). The polyclonal TGF-β1 neutralizing antibody was prepared as described (10). A second polyclonal antibody (anti-LC) made against a synthetic peptide corresponding to the first 30 amino acids of mature TGF-β1 was kindly provided by K. C. Flanders and M. B. Sporn (National Institutes of Health). Rabbit antibodies produced against synthetic peptides from the core proteins of human biglycan and decorin were a generous gift from L. W. Fisher (National Institutes of Health). Fluorescein isothiocyanate-conjugated antisera to rabbit IgG were obtained from ICN.

Molecular Identification by Immunoprecipitation and Enzyme Digestion. Identification of proteoglycans in conditioned medium was carried out by digestion with enzymes specific for glycosaminoglycan side chains and immunoprecipitation using polyclonal antibodies to synthetic peptides from the proteoglycan core protein as described (8, 10).

Electrophoretic Technique. Samples were analyzed by SDS/PAGE, fluorography, and densitometry as described (8, 10, 11).

Northern Blot Analysis. Total RNA was prepared from control and nephritic glomeruli and RNA blotting was carried out as described (10, 11). A porcine TGF-β1 cDNA probe and a rat glyceraldehyde 3-phosphate cDNA probe were kindly provided by M. B. Sporn.

Statistical Analysis. Differences between groups in proteinuria, creatinine, blood pressure, body weight, and matrix score were analyzed by *t* test as described (10, 11).

RESULTS

Effect of Dietary Protein on Experimental Glomerulonephritis. Injection of anti-thymocyte antiserum into a rat produces acute glomerulonephritis as a result of antibody and complement-mediated selective injury to mesangial cells (15–17). The glomerular lesion is characterized on days 1 and 4 by a reduction in extracellular matrix and cell number due to lysis of a portion of the mesangial cells, followed by a marked increase of the extracellular matrix and cell number on days 7 and 14, and then resolution over a 3-month period. The low protein diet suppressed the expansion of the glomerular extracellular matrix that occurred in glomerulonephritic rats fed the normal protein diet but did not interfere with healing of the areas of mesangial cell lysis, the net effect being that the glomeruli appeared nearly normal on day 7 in glomerulonephritic rats receiving the low protein diet. The therapeutic effect of the low protein diet on glomerular histology is shown in quantitative terms in Fig. 1*A*. The low protein diet also reduced the amount of proteinuria in glomerulonephritic rats (Fig. 1*B*). Control rats did not show any changes in glomerular matrix nor did they manifest proteinuria. Renal function as measured by serum creatinine levels did not change in this model and no differences were noted between control and glomerulonephritic groups (data not shown). Normal control and glomerulonephritic rats receiving the normal protein diet tended to have higher body weights (Fig. 2*A*) and blood pressures (Fig. 2*B*) than did the groups being fed the low protein diets; these differences became significant on days 14 and 28.

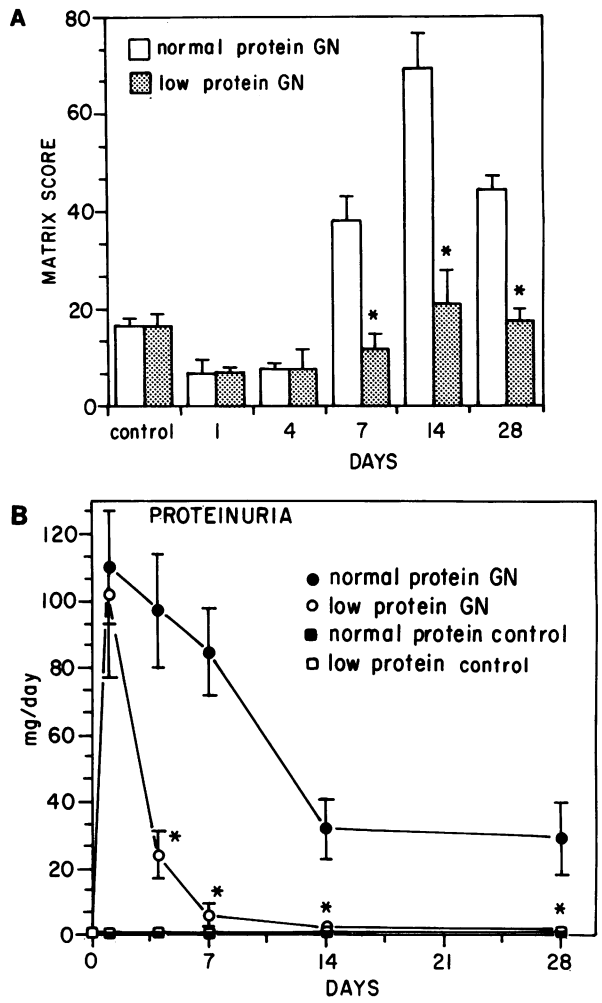

FIG. 1. Effect of dietary protein on extracellular matrix expansion and proteinuria in experimental glomerulonephritis (GN). (*A*) Values are a quantitative score of the percentage of glomerular area occupied by extracellular matrix after induction of glomerulonephritis. The score was determined in 30 glomeruli from each group of six rats at each time point. (*B*) Proteinuria during the course of experimental glomerulonephritis. The 24-h urinary protein excretion was measured in each group of six rats receiving the experimental diets. *, $P < 0.001$ (glomerulonephritic rats receiving normal protein diet compared to low protein diet). Values are means ± SD.

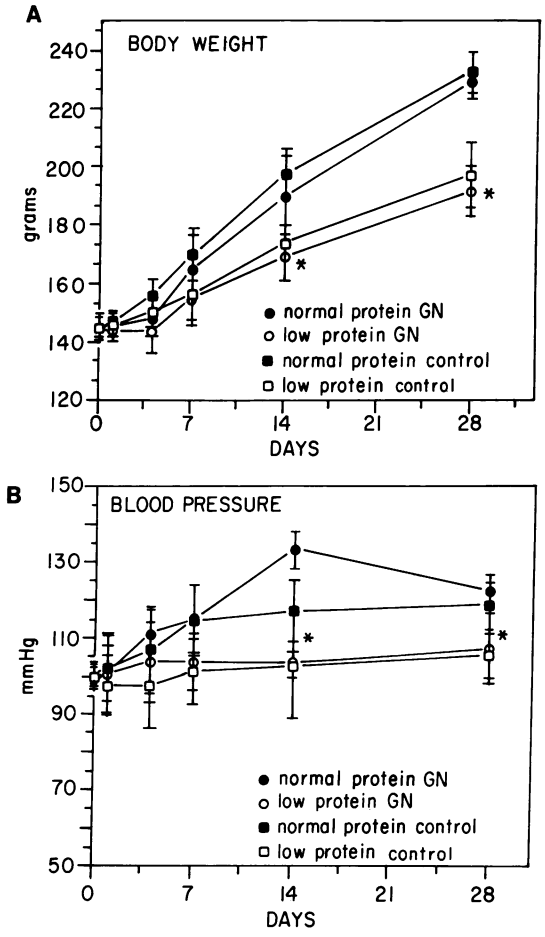

FIG. 2. Body weights and blood pressure during the course of experimental glomerulonephritis (GN). (*A*) Body weights. (*B*) Systolic blood pressures in experimental groups (n = 6 rats per group) fed either a normal or low protein diet. *, $P < 0.01$ (low protein groups compared to normal protein). Values are means ± SD.

Expression of TGF-β1 in Glomeruli. There was a clear increase in TGF-β1 mRNA in glomeruli of glomerulonephritic rats receiving the normal protein diet on day 7 of disease (Fig. 3*A*). Rats fed the low protein diet showed no increase in mRNA and maintained levels that were the same as those in normal control rats. The level of message of the control enzyme, glyceraldehyde-3-phosphate dehydrogenase, remained unchanged in each of the control and glomerulonephritic groups (Fig. 3*B*). The presence of TGF-β1 protein in glomeruli was detected with an antibody (anti-LC) that reacts with cells that are synthesizing TGF-β1. In glomeruli from glomerulonephritic animals fed the normal protein diet, there was an increase in TGF-β1-positive cells on day 7, whereas there was no increase in TGF-β1 protein staining from normal control levels in glomerulonephritic animals fed the low protein diet (Fig. 4).

Proteoglycan Production in Glomeruli. Glomerulonephritic animals being fed both low protein and normal protein diets were sacrificed 1, 4, 7, 14, or 28 days after induction of disease. The glomeruli were isolated, placed in culture, and biosynthetically labeled to identify newly synthesized proteoglycans. In glomerulonephritic rats on the normal protein diet, on day 4 there was a clear increase of two proteoglycan bands centered at 220 and 120 kDa (Fig. 5*A*). These proteoglycans showed a further increase on day 7; whereas, in glomerulonephritic rats fed the low protein diet, no increase in proteoglycan synthesis was observed at any time (Fig. 5*B*).

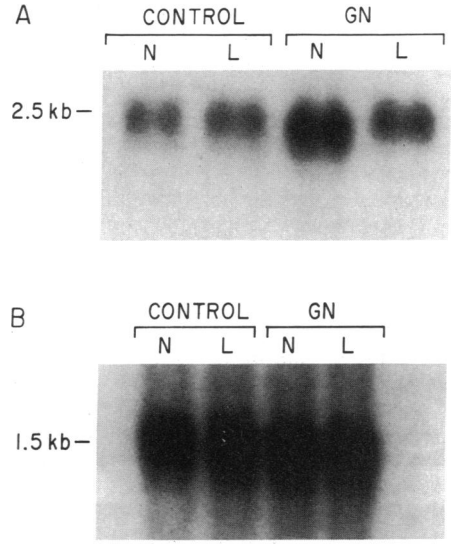

FIG. 3. Northern blot analysis of glomerular TGF-β1 mRNA. Total RNA was isolated on day 7 from kidneys of normal control (control) or glomerulonephritic (GN) rats while receiving either a normal protein (lanes N) or low protein (lanes L) diet. Total RNA from glomeruli was probed for TGF-β1 mRNA (*A*) or mRNA for glyceraldehyde-3-phosphate dehydrogenase (*B*). There is a 3-fold increase in TGF-β1 mRNA in glomeruli from the glomerulonephritic rats fed the normal protein diet compared to the other groups. The levels of mRNA of the control enzyme did not change in any of the groups. The position of each RNA was determined from markers and is shown on the left. kb, Kilobases.

We and others have shown that the ability to stimulate proteoglycan production is a relatively specific property of TGF-β1, compared to other growth factors, when added to normal rat mesangial cells in culture (8, 10). Thus, the response of mesangial cell cultures to conditioned medium from nephritic glomeruli can be considered as a bioassay for TGF-β1. Medium conditioned by glomeruli from both glomerulonephritic and control rats on day 7 receiving both diets were added to normal cultured mesangial cells. There was a striking stimulation of proteoglycan synthesis by the conditioned medium from the glomerular culture established from glomerulonephritic rats fed the normal protein diet, whereas medium conditioned by glomeruli from glomerulonephritic rats fed the low protein diet did not stimulate proteoglycan synthesis as did the conditioned medium from the two groups of normal control rats (Fig. 6). The proteoglycan-stimulating activity in the medium conditioned by the nephritic glomeruli was reduced by 85% by addition of a specific neutralizing antibody raised against a synthetic peptide from TGF-β1 (data not shown). The proteoglycans synthesized by nephritic glomeruli and induced in mesangial cell cultures by conditioned medium from nephritic glomeruli were shown by specific enzyme digestion and immunoprecipitation with anti-synthetic peptide antibodies to be biglycan and decorin (data not shown).

DISCUSSION

Our results show a striking therapeutic effect of protein restriction in preventing the buildup of glomerular extracellular matrix in experimental glomerulonephritis. It is important to emphasize how quickly the low protein diet was effective in this model. All experimental animals were eating normal protein chow until the time of induction of disease when the low protein diet was first administered. A clear protective effect of low protein was noted on day 7 of disease, when marked extracellular matrix accumulation was present

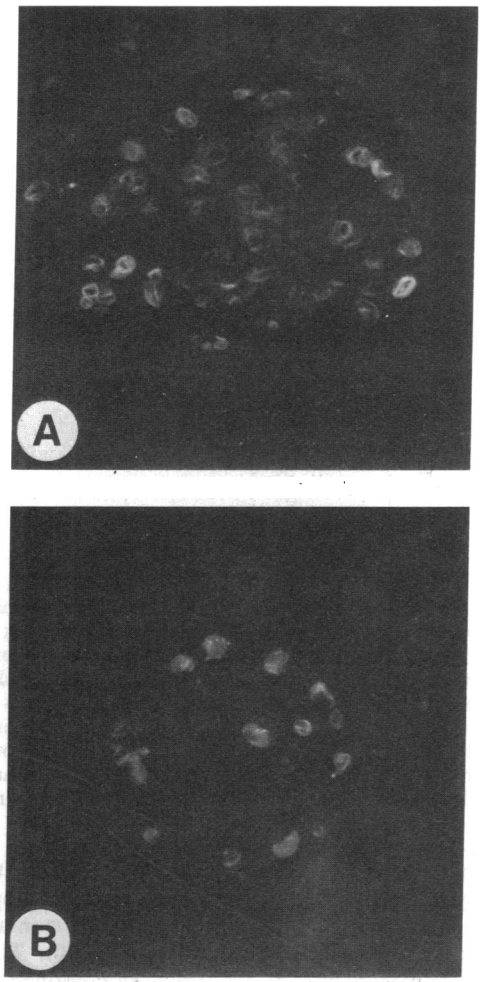

FIG. 4. Immunofluorescence micrographs of glomeruli stained with an antibody to TGF-β1. On day 7 of glomerulonephritis, there are many more TGF-β-positive cells in glomerulus of a rat fed a normal protein diet (*A*) compared to a rat fed a low protein diet (*B*). (×500.)

in glomerulonephritic rats receiving the normal protein diet. Of interest is that low protein gave protection from a pathological increase in extracellular matrix but did permit a normal repair of glomerular matrix to a control level by day 14 after immunologic injury. Suppression of glomerular matrix buildup by a low protein diet was accompanied by a significant parallel reduction in proteinuria, an important clinical indicator of glomerular damage.

We have previously demonstrated a major role for TGF-β1 in modulating the mesangial matrix expansion in this model of glomerulonephritis (10, 11). As demonstrated in the current study, glomerulonephritic rats receiving a normal protein diet showed elevated levels of TGF-β1 mRNA and TGF-β1 protein in glomeruli along with increased production of the proteoglycans biglycan and decorin, known to be induced by TGF-β1 (8). Medium conditioned by the nephritic glomeruli stimulated normal rat mesangial cells to produce proteoglycans; this activity was specifically blocked by addition of anti-TGF-β1. In nephritic glomeruli from rats fed the low protein diet, TGF-β1 mRNA and TGF-β1 protein were not elevated, proteoglycan synthesis was not increased, medium conditioned by the nephritic glomeruli did not induce proteoglycan production by normal mesangial cells, and there was no pathologic increase of glomerular matrix.

The mechanism by which dietary protein reduction affects TGF-β1 expression is unknown. Although we have emphasized TGF-β1, there are two additional isoforms of TGF-β (TGF-β2 and -3) in mammalian cells (3). We cannot exclude an important role for TGF-β2 and/or -3 in experimental glomerulonephritis and the therapeutic effect of protein restriction. The demonstration of the rapidity of the therapeutic action of low protein and the fact that both normal control and

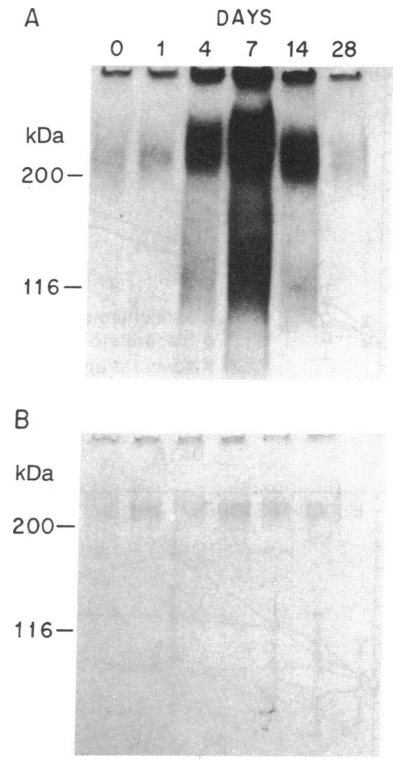

FIG. 5. Proteoglycan production by cultured nephritic glomeruli. Equal numbers of glomeruli were isolated from glomerulonephritic rats receiving a normal protein (*A*) or a low protein (*B*) diet on days 1, 4, 7, 14, and 28 after induction of glomerulonephritis. Day 0 represents normal control glomeruli. There is a 6-fold increase in proteoglycan production on day 4 and a 24-fold increase on day 7 observed in the glomerulonephritic rats receiving a normal protein diet (*A*). Induction of proteoglycan synthesis was suppressed by a low protein diet (*B*). Molecular mass markers are shown on the left.

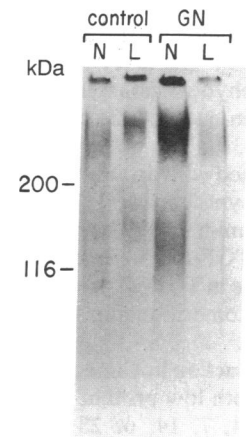

FIG. 6. Bioassay of TGF-β1 using cultured normal rat mesangial cells. Medium conditioned by glomeruli isolated on day 7 from glomerulonephritic (GN) rats induced a clear increase in proteoglycan production compared to similar medium from normal control rats (control) receiving the same diet. The low protein (lanes L) diet prevented the expected stimulation of proteoglycan production by the nephritic glomeruli. Lanes N, normal protein diet. Molecular mass markers are shown on the left.

glomerulonephritic rats receiving the diet continued to grow throughout the experimental period argues against an effect related to general nutritional deficiency or malnutrition. However, caloric intake may have been a factor, since the rats on the low protein diet gained weight more slowly than the ones on the normal diet. The observation that low protein did not alter glomerular mRNA levels of a control constitutive enzyme indicates that the reduction in TGF-β1 expression represents a specific effect. This view is supported by the work of others that showed that expression of renal renin mRNA is reduced by low protein without affecting mRNA levels of renal or liver angiotensinogen (18). Protein restriction in the rat has also been shown to decrease mRNA levels of insulin-like growth factor I in the liver, to have no effect on the amount of growth hormone receptor mRNA, and to increase mRNA levels of insulin-like growth factor binding protein 2 (19). In these studies, the effects of dietary proteins were found to be complex and were shown to influence both transcriptional and posttranscriptional events depending on the protein being studied.

It will be important to elucidate the basis of the dietary effect on TGF-β1 expression because it may lead to additional ways of regulating phenomena in which TGF-β plays a role. These include inflammatory events such as the glomerulonephritis model we have studied and possibly also a role of TGF-β as a tumor promoter (20–22). The rapid response of TGF-β1 expression to the dietary changes in the glomerulonephritis model will make such studies feasible.

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