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中山, 勝

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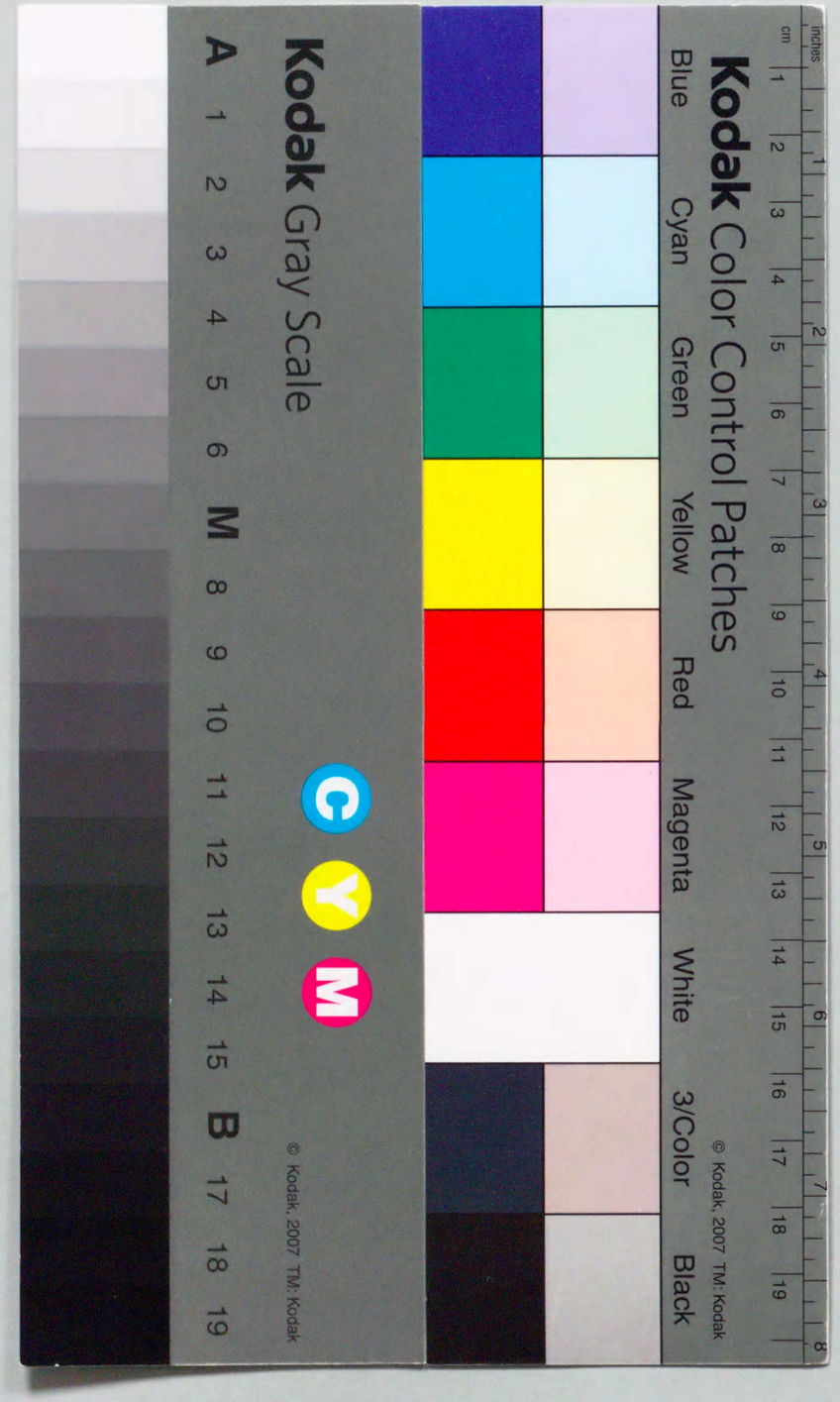

Short- or long-term effects of a low-protein diet on fibronectin and transforming growth factor–β synthesis in Adriamycin-induced nephropathy

MASARU NAKAYAMA, SEIYA OKUDA, KIYOSHI TAMAKI, and MASATOSHI FUJISHIMA

FUKUOKA, JAPAN

Increased synthesis and gene expression of fibronectin or transforming growth factor–β (TGF-β) have been reported to be involved in the progressive process of doxorubicin hydrochloride (Adriamycin)-induced nephropathy. In the present study, the effects of dietary protein restriction on the synthesis and gene expression of fibronectin or TGF-β were investigated by immunoprecipitation, Northern blotting, and TGF-β bioassay in this model after subjects were given either short- or long-term low-protein diets. In the long-term diet experiment, either a normal protein diet (NPD, 20%) or low-protein diet (LPD, 5%) was fed to the Adriamycin rats for 8 weeks after the injection of Adriamycin. An 8-week LPD significantly ameliorated kidney destruction and remarkably reduced the fibronectin synthesis. Furthermore, the significant decreases of the latent TGF-β secretion and the expression of TGF-β1 mRNA were observed in the Adriamycin rats fed an 8-week LPD. In the short-term diet experiment, an NPD or LPD was fed to the Adriamycin rats for 2 weeks at weeks 4, 8, or 16 after the injection of Adriamycin. A 2-week LPD did not ameliorate kidney damage. Although fibronectin synthesis by the renal cortex in the Adriamycin rats was remarkably reduced by a 2-week LPD, there was no significant decrease in the latent TGF-β secretion in the Adriamycin rats. The mRNA expressions of fibronectin or TGF-β1 were not affected by a 2-week LPD in the Adriamycin rats at any stage. In conclusion, decreased fibronectin and TGF-β synthesis may be one of the mechanisms by which the long-term dietary protein restriction ameliorates kidney damage. On the other hand, a 2-week LPD affected the only fibronectin synthesis, which thus suggested that an LPD might exert a quicker influence on the protein synthesis of fibronectin than on the transcriptional events of fibronectin. (J Lab Clin Med 1996;127:29-39)

Abbreviations: DMEM = Dulbecco's modified Eagle medium; ECM = extracellular matrix; FGS = focal glomerular sclerosis; IGF = insulin-like growth factor; LPD = low protein diet; NPD = normal protein diet; SDS = sodium dodecylsulfate; SSC = standard sodium citrate solution; TGF-β = transforming growth factor-β

From the Second Department of Internal Medicine, Faculty of Medicine, Kyushu University.

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Reprint requests: Masaru Nakayama, MD, Second Department of Internal Medicine, Faculty of Medicine, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan.

Doxorubicin hydrochloride (Adriamycin)-induced nephropathy is a model of chronic progressive FGS and interstitial fibrosis in rats.[1] Although the mechanism by which Adriamycin-induced nephropathy progresses to irreversible kidney destruction is not known, the ECM accumulation in the glomeruli or interstitium is a major

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histologic feature that leads to progressive kidney failure. The ECM accumulation may be regulated by growth factors. TGF-β in particular has been shown to have a widespread effect on ECM synthesis by cultured glomerular cells or on mesangial matrix accumulation in experimental glomerulonephritis.[2,3] In our previous study, the cortical synthesis or mRNA expression of fibronectin, one of the major ECM proteins, increased progressively in a pattern similar to the histologic changes in the kidney caused by Adriamycin-induced nephropathy, and TGF-β1 seemed to play an important role in the process of kidney fibrosis and sclerosis by stimulating fibronectin synthesis.[4]

The ameliorative effect of dietary protein restriction on progressive kidney deterioration has been reported in the state of reduced kidney mass in experimentally-induced kidney diseases[5,6] or in humans.[7,8] It has been proposed that the effect of protein restriction is mainly mediated through the prevention of glomerular hyperfiltration.[9,10] Dietary protein restriction also influences immune mechanisms and the production of vasoactive substances,[11-16] which may contribute to kidney injury. However, recent interest has focused on the influence of dietary protein on growth factors in the evolution of kidney disease. El-Nahas et al.[17] reported that the abrogation of compensatory kidney growth by an LPD is accompanied by a reduction in kidney IGF-1 levels. In our previous study, the ameliorative effects of an LPD also correlated with the suppression of matrix protein synthesis and TGF-β expression in experimental glomerulonephritis.[18] However, it is still unknown whether the decrease of TGF-β mRNA expression and ECM production is the direct effect of an LPD or is perhaps some epiphenomenon of improved kidney damage. To clarify the ameliorative effect of dietary protein restriction on experimental kidney disease, we investigated the effects of short-term (2 weeks) or long-term (8 weeks) LPDs on the synthesis and gene expression of fibronectin or TGF-β in Adriamycin-induced nephropathy.

METHODS

Experimental design. Male Sprague-Dawley rats that weighed 220 to 250 gm were used in this study. Experimental FGS was induced by the intravenous injection of Adriamycin, 0.25 mg/100 gm body weight, dissolved in 0.9% saline solution and administered twice at 20-day intervals in Sprague-Dawley rats.[1] All of the rats were fed ad libitum with standard chow that contained 20% protein and 0.3% NaCl until an NPD (20%) or an LPD (5%) was started. In the long-term diet experiment (8 weeks), an LPD or NPD was fed to rats for 8 weeks after the second injection of Adriamycin. In the short-term diet experiment (2 weeks), an LPD or NPD was fed to the Adriamycin rats at 4 weeks (Adriamycin-4w group), 8 weeks (Adriamycin-8w group) or 16 weeks (Adriamycin-16w group) after the second injection of Adriamycin, whereas the same diets were given to the control rats at week 10 for 2 weeks (see figure legends for the number of rats used in each experiment). The diets were arranged as isocaloric food and contained identical quantities of calcium (1.4%), phosphorus (1.2%), sodium chloride, potassium, vitamins, electrolytes, and other minerals. Plasma protein, serum cholesterol, blood urea nitrogen, and serum creatinine levels were examined after a 2-week LPD and NPD feeding in the short-term diet experiment or after an 8-week LPD and NPD feeding in the long-term diet experiment. Urinary protein levels from 24-hour urine samples were measured with use of the sulfosalicylic acid method before LPD or NPD feeding and at the end of the experiment. The rats were killed at the end of the experiment. The kidneys were perfused in situ via the aorta with phosphate-buffered saline solution, pH 7.4, and were then excised. The kidney capsules were removed and the cortex trimmed off with scissors for a cortical culture. A part of cortex from either the control rats or the Adriamycin rats was fixed in 7% neutral formalin or saved in liquid nitrogen for cortical RNA extraction.

Histologic examination. Kidney specimens were fixed in neutral buffered formalin and embedded in paraffin for the microscopic study, and sections 2 μm thick were stained with periodic acid–Schiff. A semiquantitative score was used to evaluate the degree of glomerular sclerosis according to the method described by Raij et al.[19] The severity of the lesion was examined in 50 glomeruli that were selected at random, graded from 0 to 4 points according to the percentage of morphologic changes on each glomerulus, and assigned a score as follows: 0, 0%; 1+, 1% to 25%; 2+, 26% to 50%; 3+, 51% to 75%; and 4+, 76% to 100%. The number of glomeruli that showed a lesion of 0 was set as n_0, $1 + n_1$, $2 + n_2$, $3 + n_3$, and $4 + n_4$, respectively. Fifty glomeruli were examined independently, and the sclerosis index was then obtained with use of the following formula: $[(0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4)/50] \times 100$. To estimate the relative interstitial volume of the kidney, tissue sections were examined with a 121-point (100 square) eyepiece micrometer.[20] Representative sections from the entire cortex were then analyzed by means of a point-counting technique to obtain the relative interstitial volume. A minimum of five sections (605) were selected at random and counted in all cases. Any statistical differences between the LPD and NPD groups were calculated with use of the unpaired *t*-test.

Cortical culture and electrophoretic technique. For the preparation of cortical conditioned media, pieces of the cortical tissue were weighed in a petri dish and minced with a sharp blade into small pieces less than 1 mm in diameter, rinsed, and suspended in methionine-free Roswell Park Memorial Institute medium (GIBCO, Grand Island, N.Y.) at a concentration of 20 mg/ml in six-well multiple plates. After 6 hours of incubation, the cortical cultures were biosynthetically labeled by the addition of 200 μCi/ml of ^{35}S-methionine (American Radiolabeled Chemicals, St. Louis, Mo.) for 24 hours. The culture media were harvested and centrifuged for 5 minutes. The pellet was discarded and the supernatant was collected, aliquoted, and stored frozen at −20° C. The samples for sodium dodecylsulfate–polyacrylamide gel electrophoresis were mixed with a sample buffer containing 3% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, and 10% β-mercaptoethanol and heated for 5 minutes at 100° C.[21] Aliquots (15 μl) were equally applied to 4 to 20 gradient gels (Daiichi Medical Co. Ltd., Tokyo, Japan). The molecular size markers were from R&D Systems (Minneapolis, Minn.). Fluorography was performed by incubating the gels in Enlightening (New England Nuclear Nuclides & Sources, Boston, Mass.). Immunoprecipitation of fibronectin was performed by adding 100 μl of polyclonal rabbit anti-rat fibronectin antibody (Chemicon International, Temecula, Calif.) to 500 μl of conditioned medium as previously described.[2]

TGF-β bioassay. As previously described, pieces of the cortical tissue were suspended in serum-free Roswell Park Memorial Institute 1640 medium (GIBCO) at a concentration of 50 mg/ml. After 24 hours of incubation, these conditioned media were harvested and centrifuged for 5 minutes at 4° C. The pellet was discarded and the supernatant was collected, aliquoted, and stored frozen at −20° C.

Mink lung epithelial cells were maintained in DMEM (GIBCO) with 5% fetal calf serum. Subconfluent cells were used in the TGF-β growth inhibition assay as described by Danielpour et al.[22] with a few modifications. The cells were trypsinized, washed with DMEM, and suspended in DMEM supplemented with 2% fetal calf serum, 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, pH 7.4, penicillin (25 U/ml), and streptomycin (25 μg/ml). The cells were seeded at 2×10^4 cells per 200 μl in each well of 96-well dishes. After 1 hour, the conditioned media were added in dilutions of 1:10. After 22 hours of incubation, the cells were pulsed with 1.0 μCi tritiated thymidine per well for 3 hours at 37° C. The cells were then washed twice with 200 μl of phosphate-buffered saline solution and trypsinized and harvested with use of a microculture harvesting device and counted in a liquid scintillation counter to measure tritiated thymidine incorporation. To neutralize TGF-β activity, a rabbit anti–TGF-β antibody (Genzyme, Cambridge, Mass.) was added at a concentration of 10 μg/ml. To measure total TGF-β activity, 1 N HCl was added to the conditioned media until the pH decreased to 2.0 to 2.5. After 30 minutes at room temperature, the transiently acidified media were brought to pH 7.4 with 1 N NaOH. Each sample was assayed in the presence and absence of anti–TGF-β antibody. The TGF-β activity index was obtained by dividing the difference in tritiated thymidine incorporation between two conditions (with and without antibody) by the amount of thymidine incorporation for the same sample in the presence of antibody and then multiplied by 100.

RNA extraction and Northern blot analysis. Cortical tissue samples were isolated and purified as previously described. Total RNA was isolated from cells with use of guanidine isothiocyanate, according to the method described by Chirgwin et al.[23] Ten microgram of poly (A)$^+$ RNA from cortex were subjected to electrophoresis in a 2.2 mol/L formaldehyde-1% agarose gel, transferred to Hybond-N nylon membranes (Amersham Corp., Arlington Heights, Ill.) and then fixed by baking at 80° C for 2 hours. The cDNA probes used were for rat TGF-β1 (provided by Dr. T. Nakamura, Kyushu University, Fukuoka, Japan),[24] rat fibronectin (provided by Dr. R. O. Hynes, Massachusetts Institute of Technology, Cambridge, Mass.),[25] human TGF-β type II receptor (provided by Dr. R. A. Weinberg, Massachusetts Institute of Technology),[26] and chicken glyceraldehyde-3-phosphate dehydrogenase, which was used as an internal control probe. The membranes were prehybridized for at least 2 hours at 37° C in hybridization solution (5 × SSC, 5 × Denhardt's solution, 0.1 mg/ml of salmon sperm DNA, 0.1% SDS, and 50% formamide). The cDNA probes were labeled with ^{32}P-deoxycytidine triphosphate (American Radiolabeled Chemicals, Inc.) with use of the random primer method and hybridized in the hybridization solution at 42° C overnight. The membranes were washed twice in 2 × SSC, 0.1% SDS, and twice in 1 × SSC, 0.1% SDS at 42° C for 15 minutes. Autoradiography was performed by the standard methods. The relative intensity of the bands on autoradiograms was quantified by the scanning Shimadzu CS-9000 densitometer (Shimadzu Corp., Kyoto, Japan).

RESULTS

Clinical findings and kidney weight. In a previous report, rats given Adriamycin developed massive proteinuria after the second Adriamycin injection, which then led to end-stage kidney failure. In the long-term diet experiment (Table I), body weight, daily proteinuria, kidney weight, total protein, total cholesterol, blood urea nitrogen, and serum creatinine levels were reduced by an 8-week LPD, which was started at week 0 after the induction of nephropathy with Adriamycin. Table II shows the short-term effect of an LPD on daily proteinuria, kidney weight, and the degree of the blood sample. Body weight, daily proteinuria, and serum total protein levels all significantly decreased in the Adriamycin rats fed a 2-week LPD compared with those fed a 2-week NPD, which was started at weeks 4, 8, or 16 after the second Adriamycin injection. The kidney weight and total cholesterol level increased in the Adriamycin rats at every stage compared with

Table I. Clinical findings of the Adriamycin-induced rats (long-term effect)

		BW (gm)		Proteinuria (mg/day)	KW/BW	TP	TC	BUN	Creatinine
		Before	After	After	(%)	(gm/dl)	(mg/dl)	(mg/dl)	(mg/dl)
Adriamycin 0-8 wk	NPD	278 ± 7	323 ± 14	118 ± 30	1.78 ± 0.3	5.7 ± 0.1	807 ± 49	34.8 ± 4.2	1.1 ± 0.1
	LPD	285 ± 9	200 ± 10*	38 ± 20†	0.81 ± 0.0†	4.1 ± 0.1*	416 ± 56†	17.2 ± 2.6‡	0.7 ± 0.1§

BW, Body weight; *BUN*, blood urea nitrogen; *KW*, kidney weight; *TC*, total cholesterol level; *TP*, total protein level.
The number of rats in both groups is four.
Each value is expressed as the mean ± SEM.
*$p < 0.001$ compared with the NPD group.
†$p < 0.005$ compared with the NPD group.
‡$p < 0.05$ compared with the NPD group.
§$p < 0.01$ compared with the NPD group.

Table II. Clinical findings of the Adriamycin-induced rats (short-term effect)

		BW (gm)		Proteinuria (mg/day)		KW/BW	TP	TC	BUN	Creatinine
		Before	After	Before	After	(%)	(gm/dl)	(mg/dl)	(mg/dl)	(mg/dl)
Control	NPD	361 ± 12	383 ± 11	13 ± 1	12 ± 5	0.68 ± 0.0	6.1 ± 0.1	78.7 ± 1.8	15.4 ± 1.1	0.4 ± 0.0
	LPD	364 ± 12	346 ± 8*	23 ± 2	5 ± 1	0.61 ± 0.0	5.7 ± 0.2*	77.4 ± 3.9	8.2 ± 1.5†	0.5 ± 0.1
ADR—4 wk	NPD	353 ± 11	358 ± 11	189 ± 28	168 ± 24	1.06 ± 0.1	5.7 ± 0.1	310 ± 84	18.0 ± 2.3	0.8 ± 0.1
	LPD	359 ± 10	292 ± 9‡	185 ± 22	53 ± 8‡	0.92 ± 0.1	4.9 ± 0.1‡	207 ± 39	15.6 ± 2.5	0.9 ± 0.1
ADR—8 wk	NPD	388 ± 11	405 ± 13	392 ± 37	296 ± 39	1.02 ± 0.1	6.2 ± 0.3	415 ± 90	19.8 ± 3.6	0.7 ± 0.1
	LPD	391 ± 8	320 ± 8‡	406 ± 52	88 ± 15‡	0.87 ± 0.1	5.4 ± 0.2§	298 ± 65	13.6 ± 2.1	0.8 ± 0.1
ADR—16 wk	NPD	461 ± 13	426 ± 15	489 ± 50	241 ± 50	1.20 ± 0.1	6.2 ± 0.1	355 ± 30	31.3 ± 3.1	1.2 ± 0.2
	LPD	466 ± 9	367 ± 9‡	402 ± 40	93 ± 14‖	0.90 ± 0.1	5.1 ± 0.1‡	238 ± 22‖	17.7 ± 6.3	1.3 ± 0.4

ADR, Adriamycin; *BUN*, blood urea nitrogen; *BW*, body weight; *KW*, kidney weight; *TC*, total cholesterol level; *TP*, total protein level.
The number of rats in each group is as follows: four for the control rats and 10 for all ADR rats.
Each value is expressed as the mean ± SEM.
*$p < 0.05$ compared with the NPD group of control rats.
†$p < 0.005$ compared with the NPD group of control rats.
‡$p < 0.001$ compared with the NPD groups of ADR 4-week, 8-week, and 16-week rats.
§$p < 0.05$ compared with the NPD group of ADR 8-week rats.
‖$p < 0.01$ compared with the NPD group of ADR 16-week rats.

the normal control rats, whereas there were no differences between the Adriamycin rats fed a 2-week LPD and NPD except for total cholesterol levels at week 16. Blood urea nitrogen and serum creatinine were both significantly high in the Adriamycin rats at week 16 compared with the normal control rats, but no significant differences in blood urea nitrogen or serum creatinine levels were observed in the Adriamycin rats fed a 2-week LPD and NPD at any stage.

Histologic findings. The Adriamycin rats demonstrated progressive focal glomerulosclerosis and tubulointerstitial damage with flattened epithelial cells, round cell infiltration, and fibrosis. A significant decrease in the degree of interstitial volume was found in the Adriamycin rats fed an 8-week LPD compared with those fed an 8-week NPD (NPD, 30.1% ± 1.2%; LPD, 18.9% ± 2.7%, $p < 0.01$), but no significant difference was found in the degree of glomerular matrix score (NPD, 120.0 ± 6.5; LPD, 108.7 ± 8.7) (Fig. 1). In contrast, Fig. 2 shows a progressive increase in the degree of glomerular sclerosis and interstitial fibrosis in the Adriamycin rats fed a 2-week LPD or NPD, which was started at weeks 4, 8, or 16 after the second Adriamycin injection. Although the 2-week LPD slightly decreased the glomerular matrix score and the interstitial volume at each stage, there was no significant difference in the degree of kidney lesions between the Adriamycin rats fed a 2-week LPD and NPD.

Cortical fibronectin biosynthesis. The Adriamycin rats were killed after an 8-week LPD and NPD feeding in the long-term diet experiment or after a 2-week LPD and NPD feeding in the short-term diet experiment. The cortex was placed in culture and biosynthetically labeled to identify any newly synthesized fibronectin, which is the most prominent glycoprotein found in the ECM. In the cortical culture from the Adriamycin rats fed an 8-week LPD, the

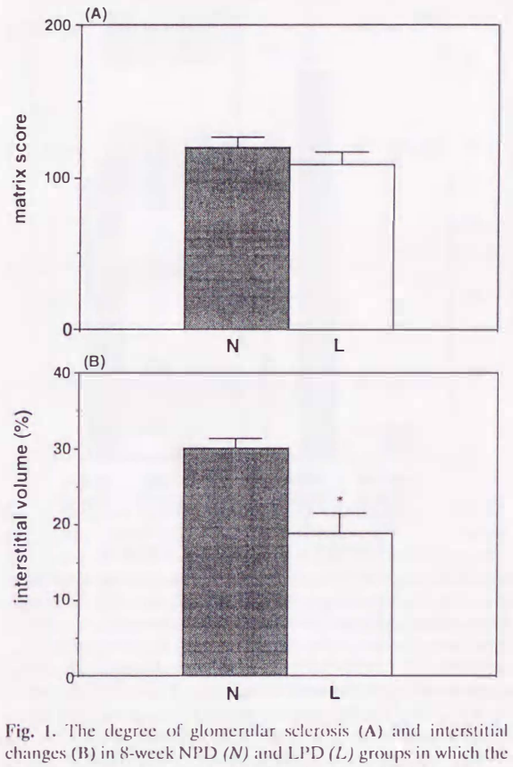

Fig. 1. The degree of glomerular sclerosis (**A**) and interstitial changes (**B**) in 8-week NPD *(N)* and LPD *(L)* groups in which the diets were started immediately after the second Adriamycin injection. The values are the semiquantitative scores from four rats and expressed as the mean ± SEM. *$p < 0.01$ compared with the NPD group.

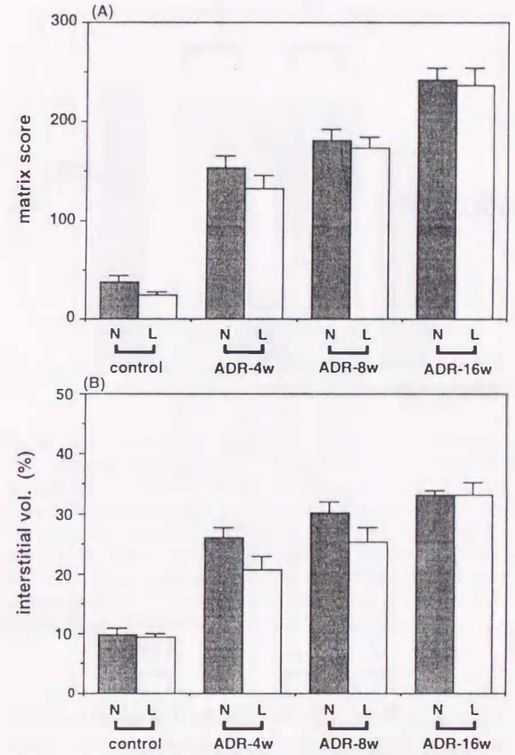

Fig. 2. The degree of glomerular sclerosis (**A**) and interstitial changes (**B**) in 2-week NPD *(N)* and LPD *(L)* groups in which the diets were started at week 4 *(ADR-4w)*, week 8 *(ADR-8w)*, and week 16 *(ADR-16w)* after the Adriamycin injection. The values are the semiquantitative scores from four control rats or 10 Adriamycin rats at each point and expressed as the mean ± SEM.

biosynthesis of fibronectin showed a significant decrease compared with that in the Adriamycin rats fed an 8-week NPD (Fig. 3, *A*). On the other hand, in the Adriamycin rats fed a 2-week NPD, the biosynthesis of fibronectin showed a progressive increase, which reached a peak at week 16. In contrast, remarkable decreases of fibronectin synthesis were also observed in the Adriamycin rats fed a 2-week LPD at each stage (Fig. 3, *B*).

TGF-β bioassay. The conditioned media from the cortical cultures were assayed for their ability to inhibit the proliferation of mink lung epithelial cells in the short- and long-term diet experiments to elucidate the effect of an LPD on the secretion of total (active + latent) TGF-β. To confirm the specificity of the assay, a rabbit monoclonal anti–TGF-β antibody, which neutralizes both TGF-β1 and -β2, was used.

Fig. 4, *A* showed the TGF-β activity in the conditioned media in the long-term diet experiment. Before acidification, the cortical conditioned media from the Adriamycin rats fed an 8-week LPD or NPD had little inhibitory effect on mink lung epithelial cells. The acidified conditioned media from the Adriamycin rats fed an 8-week LPD or NPD had a significant inhibitory effect on mink lung epithelial cells, whereas the amounts of total TGF-β in the conditioned media from the Adriamycin rats fed an 8-week LPD were lower ($p < 0.001$) than those from the Adriamycin rats fed an 8-week NPD.

Before acidification, the cortical conditioned media from the Adriamycin rats fed a 2-week LPD or NPD at week 8 after the Adriamycin injection had little inhibitory effect on mink lung epithelial cells compared with each medium added with anti–TGF-β antibody. The acidified same conditioned media from the Adriamycin rats fed a 2-week LPD or NPD had a significant inhibitory effect on mink

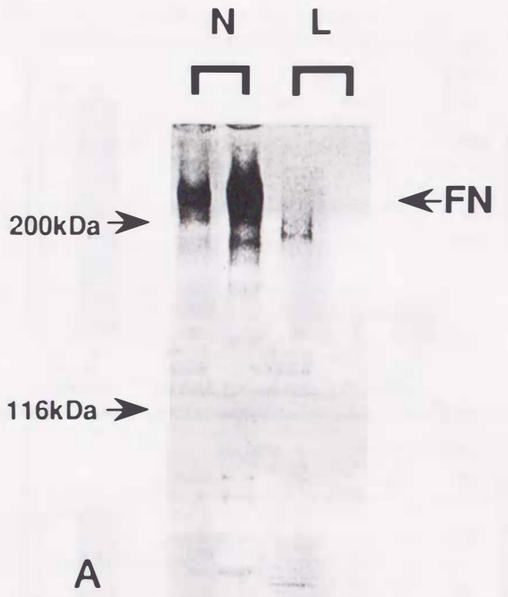

Fig. 3. A. Cortical fibronectin *(FN)* synthesis after an 8-week LPD. The fibronectin biosynthesis from the conditioned media of the Adriamycin rats fed an 8-week LPD *(L)* remarkably decreased compared with that of the Adriamycin rats fed an 8-week NPD *(N)*. **B**, Cortical fibronectin synthesis after a 2-week LPD. Equal amounts of minced cortex from either control rats *(C)* or Adriamycin rats fed 2-week NPDs *(N)* and LPDs *(L)* that were started at weeks 4 *(4w)*, 8 *(8w)*, or 16 *(16w)* after the second Adriamycin injection were cultured for 24 hours and biosynthetically labeled with ^{35}S-methionine. The conditioned media were then immunoprecipitated with anti-fibronectin antibody for analysis of sodium dodecylsulfate–polyacrylamide gel electrophoresis with autoradiography.

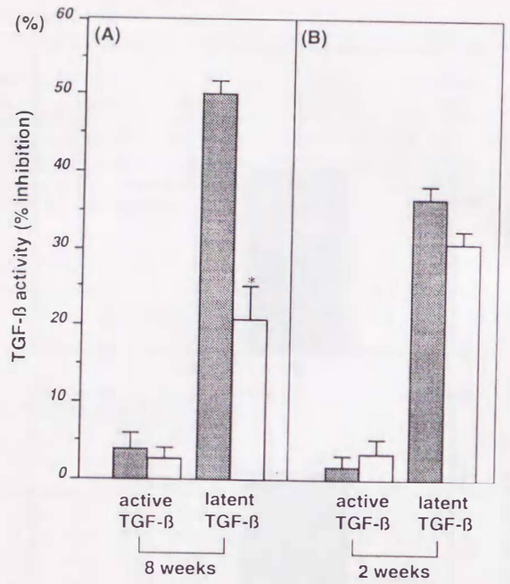

Fig. 4. TGF-β activity in the cortical conditioned media from the Adriamycin rats (A) after an 8-week NPD (▨) or LPD (□) that was started immediately after the second Adriamycin injection and (B) after a 2-week NPD (▨) or LPD (□) that was started at week 8 after the second Adriamycin injection. Each sample was assayed in either the presence or absence of anti–TGF-β antibody. The TGF-β activity index was calculated as described in the Methods section in the text. Each value represents the mean of six samples from three rats and is the mean ± SEM. There was no difference in active or latent TGF-β activity between a 2-week NPD or LPD, whereas latent TGF-β activity in the Adriamycin rats fed an 8-week LPD was lower than that in those fed an 8-week NPD. $^*p < 0.001$.

lung epithelial cells, but there was no significant difference in the TGF-β activity of the conditioned media between the Adriamycin rats fed a 2-week LPD and NPD (Fig. 4, *B*).

Gene expressions of fibronectin, TGF-β1, and TGF-β type II receptor. In the long-term diet experiment, the gene expression of TGF-β1 in the Adriamycin rats fed an 8-week LPD significantly decreased compared with that in the Adriamycin rats fed an 8-week NPD, whereas the gene expression of fibronectin in the Adriamycin rats fed an LPD tended to decrease; however, the decrease was not significant. The gene expression of TGF-β type II receptor showed no significant difference between the Adriamycin rats fed an 8-week LPD and NPD (Figs. 5 and 6). Fibronectin, TGF-β1, and TGF-β type II receptor mRNA expressions in the Adriamycin rats fed a 2-week NPD were higher than those in the control rats fed a 2-week NPD (TGF-β1, 4.7-fold; fibronectin, 5.7-fold; and TGF-β type II receptor, 1.7-fold, at week 4), and the gene expressions of fibronectin or TGF-β1 showed a slowly progressive increase that reached a peak at week 16. In the Adriamycin rats fed a 2-week LPD, fibronectin, TGF-β1, and TGF-β type II receptor mRNA expressions increased compared with those in the control rats fed a 2-week NPD (TGF-β1, 4.7-fold; fibronectin, 5.4-fold; and TGF-β type II receptor, 1.5-fold, at week 4), whereas the peak of the gene expressions was found at week 8 in the Adriamycin rats fed a 2-week LPD (TGF-β1, 8.8-fold; fibronectin, 10.0-fold; and TGF-β type II receptor, 2.3-fold). No significant differences were found in the expressions of fibronectin or TGF-β1 mRNA between the Adriamycin rats fed a 2-week LPD and NPD at any stage, although both gene expressions seemed to be moderately upregulated at week 8 in the Adriamycin rats fed a 2-week LPD (Figs. 7 and 8).

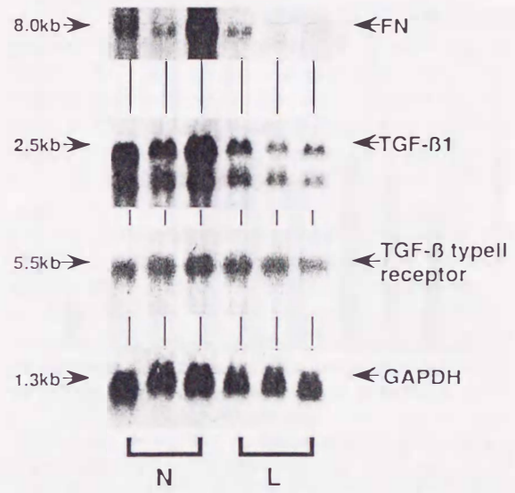

Fig. 5. Northern blotting of fibronectin *(FN)* mRNA, TGF-β1 mRNA, and TGF-β type II receptor mRNA from the renal cortex after an 8-week LPD. Ten micrograms of poly (A)$^+$ RNA from the cortex of the Adriamycin rats fed an 8-week NPD *(N)* or LPD *(L)* immediately after the second Adriamycin injection were loaded into each lane. Poly (A)$^+$ RNA was analyzed with rat fibronectin cDNA, the *Eco*RI/BglI fragment of rat TGF-β1 cDNA, and human TGF-β type II receptor cDNA. The blots were rehybridized with GAPDH cDNA to confirm that approximately equal amounts of RNA were loaded into each lane. *Arrows* indicate the sizes of the major transcripts for fibronectin (8.0 kb), TGF-β1 (2.5 kb), TGF-β type II receptor (5.5 kb), and GAPDH (1.3 kb). *GAPDH*, Reduced glyceraldehyde-phosphate dehydrogenase.

DISCUSSION

Our experiments revealed that the histologic consequences of Adriamycin-induced nephropathy were ameliorated by an 8-week dietary protein restriction. These results correlated with our previous report in which the beneficial effects were found to be proportional to the degree of dietary protein restriction.[27] The effect of the protein restriction on progressive kidney injury in experimental animal models has been proposed to be mediated through hemodynamic mechanisms.[28] In rats with a remnant kidney or experimentally induced diabetes, protein restriction increases afferent arteriolar resistance, resulting in decreased intraglomerular pressure and glomerular plasma flow and a reduced transmission of systemic pressure to the glomerulus.[9,10,29] However, results of a limited number of studies evaluating the glomerular hemodynamic effects of a high-protein diet suggest that dietary protein restriction influences the progression of glomerulosclerosis independent of effects on glomerular hemodynamics.[30,31]

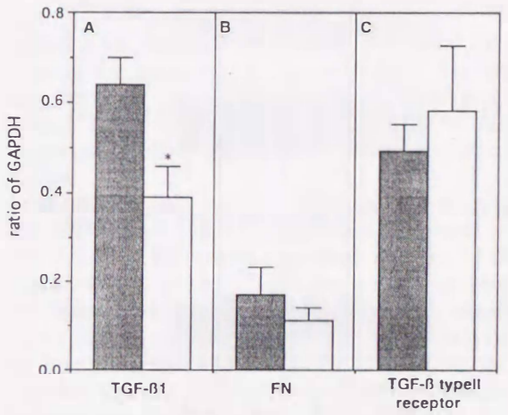

Fig. 6. The quantitation of mRNA for TGF-β1 (A), fibronectin *(FN)* (B), and TGF-β type II receptor (C) in the cortex of the Adriamycin rats after an 8-week NPD (▨) or LPD (□). The diet was started immediately after the second Adriamycin injection. The values are expressed as the mean of the ratio to the expression of GAPDH mRNA and are the mean ± SEM ($N = 3$ in both NPD and LPD groups). $^*p < 0.05$, compared with the gene expression of TGF-β1 in the normal protein diet group. *GAPDH*, Reduced glyceraldehyde-phosphate dehydrogenase.

Dietary protein restriction also influences the immune mechanism, the production of vasoactive substances, or kidney growth. The protective effect of an LPD may be the result of impaired T-cell mediated injury.[11] It has been suggested that eicosanoids may play a key role in the protein synthesis and gene expression of ECM.[32–35] Rats fed a high-protein diet have increased glomerular production and increased urinary excretion of thromboxane compared with rats fed an LPD.[12,13] It has been proposed that

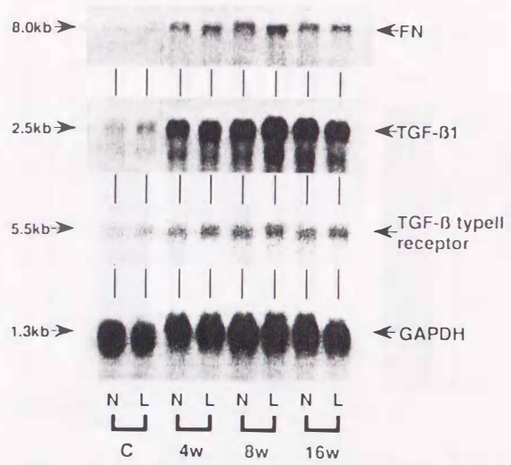

Fig. 7. Northern blotting of fibronectin *(FN)* mRNA, TGF-β1 mRNA, and TGF-β type II receptor mRNA from the renal cortex after a 2-week LPD. Ten micrograms of poly $(A)^+$ RNA from either the control rats *(C)* or the Adriamycin rats fed 2-week NPD *(N)* and LPD *(L)* that were started at weeks 4 *(4w)*, 8 *(8w)*, or 16 *(16w)* after the second Adriamycin injection were loaded into each lane. Poly $(A)^+$ RNA was analyzed with rat fibronectin cDNA, the *Eco*RI/BglI fragment of rat TGF-β1 cDNA, and human TGF-β type II receptor cDNA. The blots were then rehybridized with GAPDH cDNA to confirm that approximately equal amounts of RNA were loaded into each lane. *Arrows* indicate the sizes of the major transcripts for fibronectin (8.0 kb), TGF-β1 (2.5 kb), TGF-β type II receptor (5.5 kb), and GAPDH (1.3 kb). *GAPDH,* Reduced glyceraldehyde-phosphate dehydrogenase.

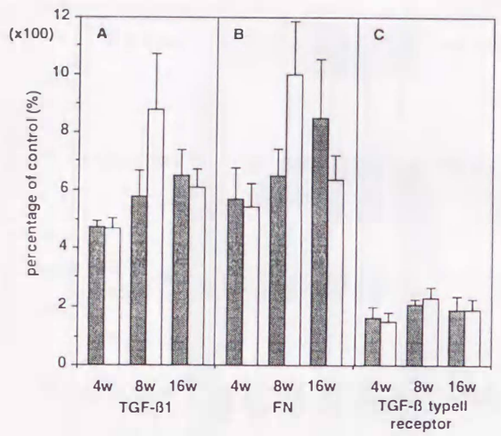

Fig. 8. The quantitation of mRNA for TGF-β1 (A), fibronectin *(FN)* (B), and TGF-β type II receptor (C) in the cortex of the Adriamycin rats after a 2-week NPD (▩) or LPD (□). The diet was started at weeks 4 *(4w)*, 8 *(8w)*, or 16 *(16w)* after the second Adriamycin injection. The values are expressed as the percentages of the control, with the mean control value being designated as 100%, and are the mean ± SEM ($N = 3$ at each point in both NPD and LPD groups). No significant differences between NPD and LPD groups were found in the gene expressions of TGF-β1, fibronectin, or TGF-β type II receptor.

kidney kallikrein and kinins participate in mediating the kidney vasodilatory effect of dietary protein.[15] Plasma renin activity has also been demonstrated to vary directly with the level of dietary protein intake.[16] Although these vasoactive substances may contribute to kidney injury, recent interest has focused on the influence of growth factors on the evolution of kidney disease, particularly the forms of kidney disease associated with kidney hypertrophy.

Protein restriction has been shown to reduce kidney hypertrophy in remnant nephrons.[9] When hypertrophic stimuli were imposed on the kidney, the sclerosing process was found to be accelerated in experimental glomerulonephritis,[36] FGS,[37,38] and diabetic nephropathy.[39] The development of glomerular sclerosis is generally seen in association with an enlargement of the glomerular size.[40] Although the mechanism responsible for the effect of an LPD on kidney hypertrophy is still unknown, the level of protein intake may influence critical growth factors.[41] A causal relationship between the rise in kidney IGF-1 and kidney hypertrophy has been suggested by El-Nahas et al.,[42] who demonstrated that a similar degree of compensatory kidney growth after uninephrectomy was accompanied by a rise in the remnant kidney IGF-1 levels in growth hormone-deficient rats as nongrowth hormone-deficient animals. In another study, they showed that compensatory hypertrophy could be abrogated by an LPD and that this phenomenon was accompanied by a reduction in kidney IGF-1 levels compared with the significantly elevated levels seen in animals fed a high-protein diet.[17]

TGF-β is a main factor in regulating sclerosis and fibrosis because of its widespread effect on ECMs, although many cytokines may be implicated in the sclerotic and fibrotic responses consequent to Adriamycin-induced kidney injury. In our previous study of Adriamycin-induced nephropathy, the progressive increase in latent TGF-β synthesis resembled the pattern of cortical or glomerular fibronectin production.[4] Fibronectin is a key component found in the ECMs of the interstitium. Fibronectin first appears before other matrix protein in scarring tissues[43] and thus provides a scaffold for the deposition and fibrogenesis of interstitial collagens.[44] An increased amount of the fibronectin synthesis in glomerular or cortical culture correlated with either glomerulosclerosis or interstitial fibrosis in this model.[4] TGF-β1 mRNA is augmented throughout the entire process of the disease. The gene expressions of TGF-β type II and III receptors increase progressively in a similar manner to kidney histologic changes. TGF-β was found in both tubular epithelial cells and macrophages dispersed in either the damaged interstitium or glomeruli. It was also noteworthy that the elevation of TGF-β1 mRNA preceded the increase in the fibronectin synthesis and mRNA and that the fibronectin synthesis was reduced by anti–TGF-β antibody added to the cortical tissue culture (unpublished data). These findings suggest that TGF-β stimulates fibronectin production and thus resulted in sclerotic kidney changes in this model. In the long-term diet experiment, TGF-β synthesis and TGF-β1 mRNA were reduced in the Adriamycin rats fed an 8-week LPD in parallel with the amelioration of kidney destruction. These results may suggest that an LPD reduced the fibronectin production through the suppression of the TGF-β synthesis, resulting in the amelioration of kidney injury of Adriamycin-induced nephropathy.

On the other hand, in the short-term diet experiment, the gene expression of TGF-β was not significantly reduced at any stage in the Adriamycin rats fed a 2-week LPD, although a slight decrease in latent TGF-β activity was observed in the Adriamycin rats fed a 2-week LPD. A 2-week LPD did not significantly improve the kidney damage either. Thus two weeks might not be long enough for the LPD to suppress the kidney deterioration and TGF-β overexpression in this model. However, a 2-week LPD remarkably reduced fibronectin synthesis without any reduction in TGF-β expression in this model. This finding suggested that an LPD did not reduce the fibronectin synthesis through the suppression of TGF-β alone but also exerted some direct effect on fibronectin synthesis. The reduction of fibronectin synthesis by a 2-week LPD might be not sufficient to improve chronic kidney destruction. Our previous study demonstrated that the LPD, which was started at weeks 4, 8, or 16 after the Adriamycin injection, was found to be effective in preventing the kidney lesions after a long period of observation,[45] and a longer suppression of fibronectin synthesis also might be needed for an LPD to improve the kidney lesions in this model. Furthermore, the suppression of fibronectin synthesis might be not sufficient for preventing sclerotic tissue damage without inhibiting TGF-β expression. TGF-β modulates matrix accumulation by inhibiting the degradation of matrix protein and augmenting the expression of matrix receptor besides stimulating the matrix protein synthesis. These functions of TGF-β may therefore be critical for the development of either glomerulosclerosis or interstitial fibrosis.

The discrepancy between fibronectin synthesis and fibronectin mRNA expression observed in this 2-week LPD study suggested that an LPD might exert a quicker influence on the protein synthesis of fibronectin than on the transcriptional events of fibronectin, although the mechanism of the posttranscriptional suppression of fibronectin synthesis is still unclear. Posttranscriptional regulation is a genetic term that refers to numerous processes such as capping, splicing, transport of mature mRNA molecules from the nucleus to the cytoplasm, and degradation of mRNA, whether in the nucleus or cytoplasm. Although the primary regulation of fibronectin expression occurs at the transcriptional level,[46-48] a previous study has demonstrated that fibronectin mRNA accumulates in high amounts in polysomes and cannot be translated into protein in the presence of antiproliferative agents such as interferons.[49] Moreover, a long sequence rich in A and U nucleotides in the 3′ untranslated region of tumor necrosis factor-α mRNA[50] and interleukin-1β mRNA[51] can regulate translational efficiency. Fibronectin mRNA with a similar A and U rich 3′ untranslated region may also be modulated in either the stability or translational efficiency.[52] We could not fully elucidate the mechanism in which a 2-week LPD decreased fibronectin synthesis without affecting the expressions of fibronectin mRNA. It might be speculated that a 2-week LPD had some effects on the posttranscriptional regulation of fibronectin mRNA or the destabilization of the message.

In summary, long-term dietary protein restriction reduced the synthesis of fibronectin or TGF-β and the mRNA expression of TGF-β1 in parallel with the improvement of kidney histology. In contrast, the short-term LPD affected the fibronectin synthesis but not the mRNA expressions of fibronectin, TGF-β1, or TGF-β type II receptor. This finding suggested that short-term dietary protein restriction might exert a quicker influence on the protein synthesis of fibronectin than on the transcriptional events of fibronectin.

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