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Research Article

Immunohistochemical Distributions of HGF and PCNA in the Kidneys of Diabetic and Non-Diabetic Mice

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Abstract

Diabetes mellitus is a systemic disease that causes functional disorders in various organs and systems. In this study, we investigated the immunohistochemical localization of hepatocyte growth factor (HGF) and proliferating cell nuclear antigen (PCNA) in the kidneys of streptozocin (STZ)-induced diabetic mice. Twenty-four Swiss albino mice were divided into three groups: control, sham and diabetic groups. STZ (100 mg/kg) was administered intraperitoneally (ip) for the development of diabetes. The avidin-biotin-peroxidase complex (ABC) technique was used to determine HGF and PCNA immunoreactivity. In diabetic kidney tissue, there was hydropic degeneration and irregularities on the epithelium of some proximal and distal tubules. Narrowing was observed in some of the Bowman's spaces. HGF and PCNA immunoreactivities were especially intense in the inner cortex and weak in the medulla. More intense HGF and PCNA immunoreactivities were found in the individual epithelial cells of the proximal and distal tubules. Immunoreactivities were stronger in the proximal tubules than in the distal tubules. In addition, HGF and PCNA immunoreactivities were strong in both interstitial regions and papillary ducts. HGF immunoreactivity was weaker in the diabetic group compared to the other groups. PCNA immunoreactivity generally decreased in the diabetic group but increased in the glomeruli of this group. The reason for the latter result was thought to be based on the increase of mesangial cells in the glomeruli.

Keywords: *Diabetes mellitus, HGF, Immunohistochemistry, Kidney, PCNA*

Diyabetik ve Nondiyabetik Farelerin Böbrek Dokusunda HGF ve PCNA'nın İmmunohistokimyasal Dağılımı

Öz

Diyabetes mellitus, çeşitli organ ve sistemlerde fonksiyonel bozukluklara neden olan sistemik bir hastalıktır. Çalışmamızda STZ ile diyabet oluşturulan farelerin böbreğinde HGF ve PCNA'nın immunohistokimyasal lokalizasyonu incelendi. Çalışmada 24 adet swiss albino fare kontrol, sham ve diyabet olmak üzere 3 gruba ayrıldı. Diyabet oluşumu için 100 mg/kg dozunda streptozosin intraperitoneal (ip) uygulandı. HGF ve PCNA immunoreaktivitesini belirlemek amacıyla Avidin-Biotin-Peroksidaz Kompleks (ABC) tekniği uygulandı. Diyabetik böbrek dokusunda bazı tubulus proksimalis ve distalis epitellerinde yer yer düzensizlik ve hidropik dejenerasyon görüldü. Bazı Bowman aralıklarında daralma gözlendi. HGF ve PCNA immunoreaktivitelerinin özellikle iç kortekste yoğun, medulla da ise zayıf olduğu gözlendi. Tubulus proksimalis ve distalis epitellerinde tek tek hücrelerde daha yoğun HGF ve PCNA immunoreaktivitelerine rastlandı. Tubulus proksimalislerdeki immunoreaktivitenin Tubulus distalislere göre daha yoğun olduğu belirlendi. Ayrıca HGF ve PCNA immunoreaktivitelerinin gerek tubüller arası intersitisyel bölgede gerek ise duktus papillariste yoğun olduğu tespit edildi. HGF immunoreaktivitesinin diyabetlilerde diğer gruplara göre daha zayıf olduğu gözlendi. PCNA immunoreaktitivitesinin genel olarak diyabetlilerde azaldığı ancak diyabetiklerin glomeruluslarında artmış olduğu görüldü. Bunun nedeninin ise mezengial hücrelerin artışından kaynaklandığı düşünüldü.

Anahtar sözcükler: *Diyabetes mellitus, HGF, İmmunohistokimya, Böbrek, PCNA*

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease that affects protein, fat and carbohydrate metabolism. It causes damage, dysfunction and deficiencies in various organs and systems in the long term [1-3]. DM also causes glomerular lesions in

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the kidneys, atherosclerosis in the vessels, pyelonephritis and thickening of the basement membranes [4].

Hepatocyte growth factor (HGF) was first identified as a factor that causes hepatocyte growth in the 1980s. It has also been reported to be a pleiotropic cytokine produced in the epithelial cells of various organs, such as pancreatic β cells, lungs and kidneys, and mesangial cells, such as fibroblasts, macrophages and smooth muscle cells [5]. HGF has many effects on renal tubular epithelial cells, including cell proliferation, motility, differentiation and cell stimulation [6]. HGF and its receptor, c-met, also have a role in renal development and maintenance of renal homeostasis [5,7-10]. HGF also plays a role in the mesenchymal-epithelial transition during kidney development [5,11]. In a previous study [12], it was shown that HGF injection increased PCNA gene expression in mice.

Proliferating cell nuclear antigen (PCNA) is a key protein that plays an important role in genomic DNA replication, recombination and repair. PCNA begins to be synthesized in the G1 phase of the cell cycle and reaches its highest level in the S phase [13,14]. PCNA acts as a helper of DNA polymerase δ [15,16]. PCNA also plays a role in the replication and repair mechanisms of nucleic acids [17]. PCNA, which is a protein that determines the rate of cell proliferation [18], coordinates the proteins and regulates their functions in cell division [19]. Few studies [20] have examined PCNA immunoreactivity in diabetic kidneys; however, HGF immunoreactivity in diabetic kidneys was not reported previously.

The aim of this study was to investigate the immunohistochemical distribution of both HGF, which plays an active role in proliferation and differentiation, and PCNA, which is a protein that determines the rate of cell proliferation and repair, in diabetic kidney.

MATERIAL and METHODS

Ethical Approval

Experimental applications in mice were performed with the approval of Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK: 2016/125).

Experimental Animals and Streptozotocin Applications

In this study, 24 Swiss albino mice 8-12 weeks of age were divided into three groups: control (n=8), sham (n=8) and diabetic (n=8). Mice were kept in standard cages at a room temperature of 22±2°C, a 12:12 h light-dark cycle with an average humidity of 50±5%. They were fed standard food and water *ad libitum*. Streptozotocin (STZ; Sigma, St Louis, MO, USA) dissolved in a 0.1 M citrate buffer (pH: 4.5) was intraperitoneally (ip) given to the diabetes group as a single dose of 100 mg/kg [21]. The sham group was treated with 0.1 M citrate buffer in the same way. No application was made to the control group. Blood glucose levels were measured with a handheld glucometer (Accu-Chek-Go, Roche, Switzerland) at 72 h after the STZ administration following an 8-h fast. Mice with a blood glucose level of 200 mg/dL or more were considered diabetic and added to the study [22]. On the 30th day of the experiment, body weight was measured, and kidney tissues were removed following a cervical dislocation under diethyl ether anesthesia [23].

Histological Examinations

Renal tissues were fixed in a 10% formalin solution for histological and immunohistochemical examinations. Tissues were then blocked in paraffin after routine histological procedures [24]. After that, 5-*μm*-thick sections were taken from the paraffin blocks. Crossman's staining (triple staining) and periodic acid-Schiff (PAS) staining were applied to the sections and photographed under light microscopy (Olympus BX51; Olympus Optical Co. Osaka, Japan).

Immunohistochemical Examination

The avidin-biotin-peroxidase complex (ABC) technique was used to determine the immunohistochemical distribution of HGF and PCNA in renal tissue. After deparaffinization and rehydration, the sections were incubated for 10 min in 3% H_2O_2 to prevent endogenous peroxidase activity. After washing with PBS (phosphate buffer solution) to reveal the antigenic sites of tissues, sections were incubated for 10 min in a 0.1 M citrate buffer (pH: 6.0) solution in a microwave oven (800 Watt). After washing again with PBS, sections were incubated in Ultra V Block serum (UltraVision Detection System Large Volume AntiPolyvalent, HRP [RTU], Thermo Scientific TP-125-HL) for 10 min to prevent nonspecific binding. After washing with PBS, the sections were incubated with anti-HGF antibody and anti-PCNA antibody (HGF: Santa Cruz: sc7949, 1:400, PCNA: Abcam: ab18197, 1:1000 dilution) for 1 h at room temperature. The sections were then washed with PBS and incubated for 30 min with biotinylated secondary antibody (UltraVision Detection System Large Volume AntiPolyvalent, HRP [RTU], Thermo Scientific TP-125-HL). After washing again with PBS, sections were incubated for 30 min with streptavidin horseradish peroxidase (UltraVision Detection System Large Volume Anti-Polyvalent, HRP [RTU], Thermo Scientific TP125HL). Sections were washed with PBS and the DAB-H_2O_2 technique was used for chromogen application. For a negative control, the same procedures were performed without adding the primary antibody. Hematoxylin was used for the nuclear counterstain. The prepared slides were examined under a research microscope (Olympus BX51; Olympus Optical Co. Osaka, Japan) and photographed. Grading of the immunohistochemistry results was performed to determine the immunoreaction according to intensity (0: no reaction; 1: mild reaction; 2: medium reaction; 3: very intense reaction). Slides were randomly selected from each subject (n=8 per group) and 20 proximal tubules, 20 distal tubules and 20 Malpighian bodies were also randomly selected from each subject of each group. Selected regions were graded in terms of the immunoreaction intensity.

The cortex and medulla of each subject were also graded in terms of the immunoreaction intensity. Scoring results were analyzed statistically to evaluate the differences between the groups.

Statistical Analysis

The data were analyzed using the SPSS 16.0 program, one-way analysis of variance (ANOVA) tests, multiple comparisons and Duncan's tests. Differences between groups were considered significant when P<0.05.

RESULTS

Blood Glucose and Body Weight

There was no significant difference between the sham and control groups in terms of blood glucose levels. However, the blood glucose level was significantly higher in the diabetic group than in the other two groups (P<0.05; *Table 1)*.

There was a significant decrease in body weight in the diabetic group compared with the other groups (P<0.05). There was also a significant decrease in body weight of the diabetic group on the day 15th and day 30th of the study (P<0.05; *Table 2)*.

Histological Results

The renal tissues had a histologically normal structure in the control and sham groups *(Fig. 1-A)*. In addition to the lymphocyte infiltration *(Fig. 1-B)*, irregular and hydropic degeneration were observed on the epithelial tissues of some proximal and distal tubules of diabetic mice kidneys *(Fig. 1-C)*. In the diabetic group, there was hyperemia in the glomerular capillaries and renal vessels of some Malpighian bodies, and a narrowing in some Bowman's spaces were also seen *(Fig. 1-D)*. In addition, the PAS+ reaction was stronger in the diabetic group than in the control and sham groups *(Fig. 1-E,F)*.

Immunohistochemical Results

Specific HGF immunoreactivity was observed in renal tissues of all groups. Immunoreactivity was observed in the capsule surrounding the kidney. In particular, more intense HGF immunoreactivity was detected in the inner cortex *(Fig. 2-A,B)*. Moderate immunoreactivity was observed in the Malpighian bodies. In addition, HGF immunoreactivity was seen in both the proximal and distal tubules *(Fig. 2-C)*. While HGF immunoreactivity was very weak in the distal tubules, it was stronger in the proximal tubules. Especially in individual cells in the epithelium of proximal tubules, intense cytoplasmic HGF immunoreactivity was detected *(Fig. 2-C,D)*. HGF immunoreactivity, which was observed in individual cells in the proximal tubule, was observed in fewer cells in the diabetic group *(Fig. 2-D)*. HGF immunoreactivity was also observed in the basement membranes of the proximal and distal tubules *(Fig. 2-C)*. However, intense HGF-positive cells were observed in the intertubular region. This immunoreactivity was found to be cytoplasmic in some of the cells and both cytoplasmic and nuclear in a few cells. In addition, intense HGF immuno-reactivity was observed in the blood vessel endothelium. A very weak immunoreactivity was detected in the collecting ducts and Henle's loops in the medulla, while an intense immunoreactivity was observed in the papillary ducts *(Fig. 2-A,B,E)*. In general, HGF immunoreactivity was weaker in the diabetic group than in the control group (P<0.05; *Fig. 2-A,B*; *Table 3)*. No immunoreactivity was detected in the negative control slide *(Fig. 2-F)*.

Table 1. *Statistical analysis of blood glucose level (mg/dL) between groups*

Groups	N	Blood Glucose Level (mg/dL)				
		0. Day	3. Day	15. Day	30. Day	P
Sham	8	109.8±10.7^{aA}	111.5±11.1^{aA}	113.5±11.7^{aA}	116.4±11.2^{aA}	0.617
Control	8	118.6±18.9^{aA}	108.2±10.7^{aA}	111.5±9.5^{aA}	117.2±11.5^{aA}	0.295
Diabetic	8	106.3±7.7^{aA}	291.1±20.7^{bB}	330.7±30^{bcB}	387.4±41.5^{cB}	0.000

^{a,b,c} At the same line, different superscript letters mean significant differences (P<0.05); ^{A,B} At the same column different superscript letters mean significant difference (P<0.05)

Table 2. *Statistical analysis of body weight (gr) between groups*

Groups	N	Body Weight (g)				
		0. Day	3. Day	15. Day	30. Day	P
Sham	8	42.49±2.94^{aA}	40.51±3.20^{aA}	41.76±2.88^{aA}	40.15±3^{aA}	0.271
Control	8	42.11±2.86^{aA}	40.27±2.82^{aA}	40.19±2.85^{aA}	39.09±2.73^{aA}	0.142
Diabetic	8	41.61±2.41^{aA}	39.17±2.13^{aA}	34.85±3^{abB}	28.76±3.9^{bB}	0.000

^{a,b} At the same line, different superscript letters mean significant differences (P<0.05); ^{A,B} At the same column different superscript letters mean significant difference (P<0.05)

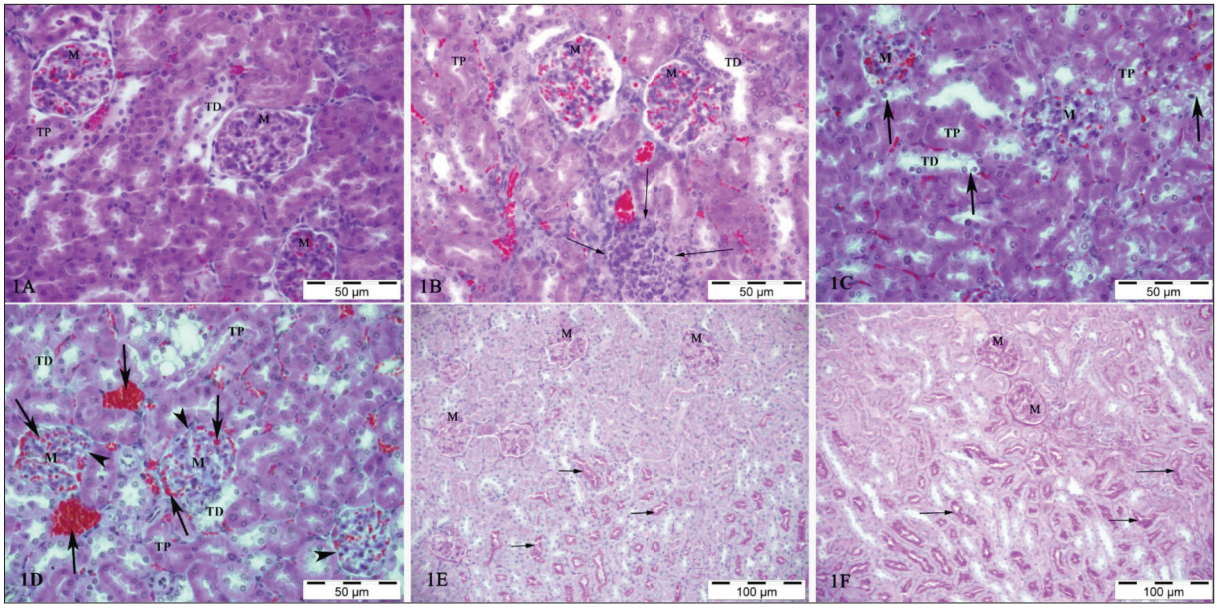

Fig 1. A) General view of kidney tissue in the control group, Triple staining; **B)** General view of kidney tissue in the diabetic group, *Arrows:* lymphocyte infiltration. Triple staining; **C)** General view of kidney tissue in the diabetic group, *Arrows:* hydropic degeneration, Triple staining; **D)** General view of kidney tissue in the diabetic group, *Arrows:* hyperemia in the glomerular capillaries and renal vessels, *Arrowhead:* Bowman's space, Triple staining; **E)** PAS staining in the control group, *Arrows:* weak PAS+ regions; **F)** PAS staining in the diabetic group, *Arrows:* strong PAS+ regions, M: Malpighian body, TP: proximal tubule, TD: distal tubule

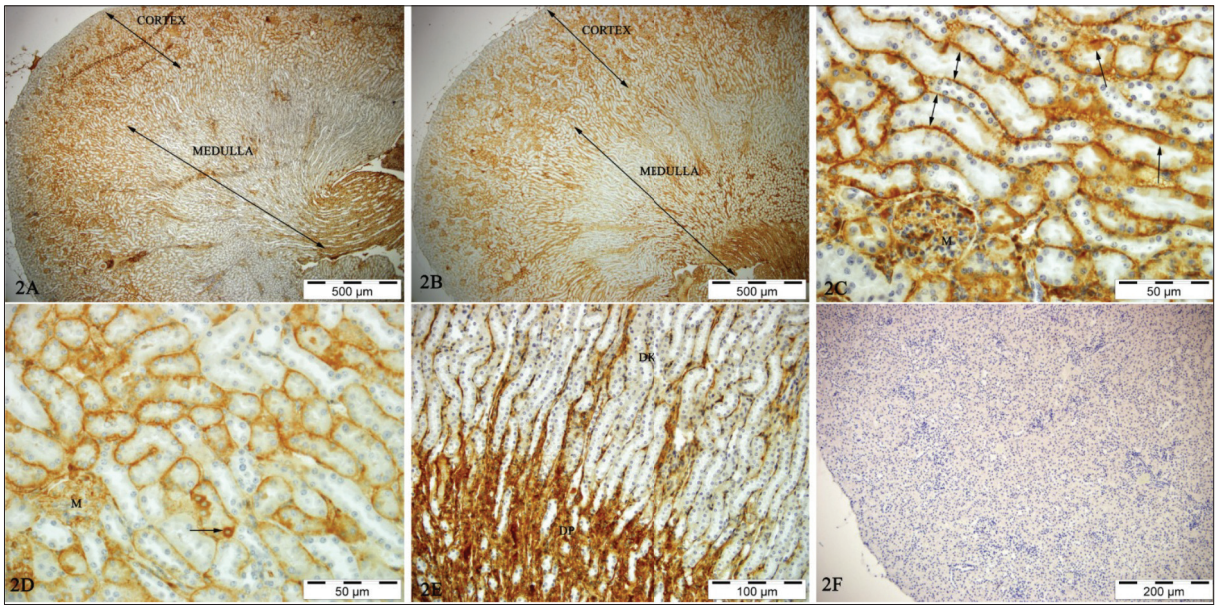

Fig 2. A) General view of HGF immunoreactivity in the control group; **B)** General view of HGF immunoreactivity in the diabetic group; **C)** Control group, M: Malpighian body, *Arrows:* strong immune positive cells, *Bidirectional arrow:* HGF immunoreactivity in the basal membrane; **D)** Diabetic group, M: Malpighian body, *Arrow:* strong immune positive cells; **E)** HGF immunoreactivity in the medulla of the control group, DK: collecting duct, DP: papillary duct, F) negative control

Proliferating cell nuclear antigen immunoreactivity was observed in the kidney capsule in all groups. PCNA immunoreactivity was strong, especially in the inner cortex, and very weak in the medulla *(Fig. 3-A)*. It was also observed that the weak immunoreactivity was found in the distal tubules and more intense immunoreactivity in the proximal tubules. The number of PCNA-positive immunoreactive cells in tubules was less in the diabetic group than in the control and sham groups *(Fig. 3-A,B)*. In addition, intense PCNA immunoreactivity was observed in both the intertubular region and papillary ducts. PCNA immunoreactivity was also observed in the blood vessel endothelium and was more intense in the Malpighian bodies in the diabetic group than in the control and sham groups *(Fig. 3-C,D)*. Although, PCNA immunoreactivity was stronger in the Malpighian bodies of the diabetic group,

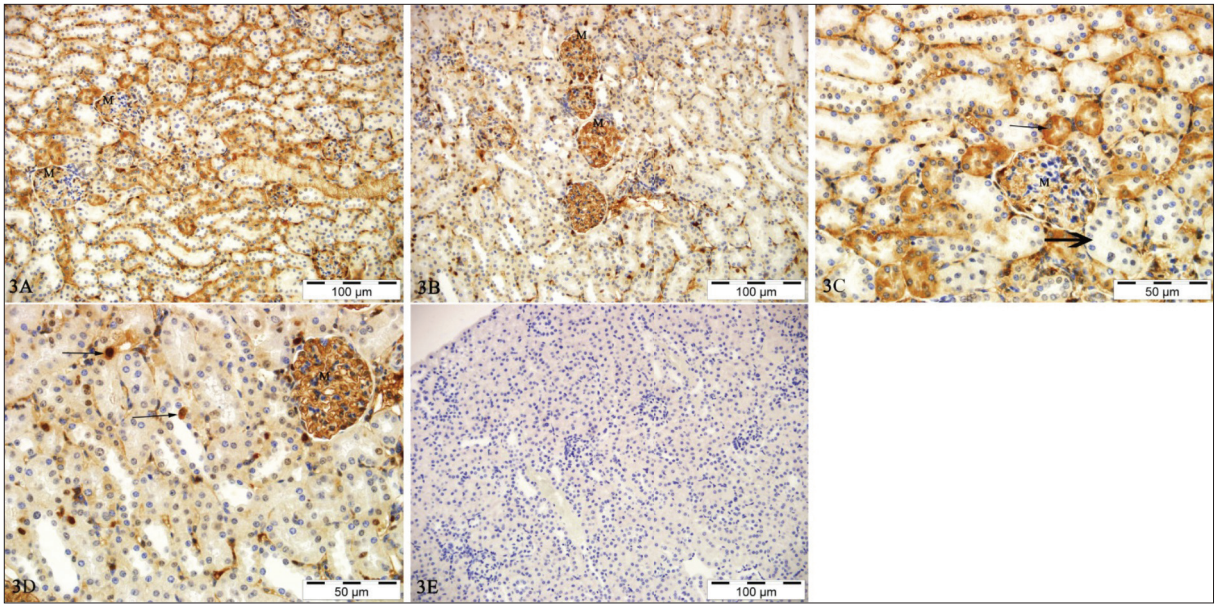

Fig 3. A) General view of PCNA immunoreactivity in the control group, M: Malpighian body; **B)** General view of PCNA immunoreactivity in the diabetic group, M: Malpighian body; **C)** Control group, M: Malpighian body, *Thin arrow:* proximal tubule, *Thick arrow:* distal tubule; **D)** Diabetic group, M: Malpighian body, *Arrows:* strong immune positive cells, **E)** Negative control

Table 3. *Statistical analysis of HGF immunoreactivity between groups*

Parts of Kidney	N	Min.	Max.	Control	Sham	Diabetic	P
				Mean±SD	Mean±SD	Mean±SD	
Cortex	8	1	3	2.70±0.46[a]	2.62±0.49[a]	1.70±0.55[b]	0.000
Medulla	8	0	2	0.58±0.50[a]	0.54±0.53[a]	0.28±0.43[b]	0.000
Malpighian body	160	1	3	2.68±0.55[a]	2.64±0.56[a]	1.65±0.65[b]	0.000
Proximal tubule	160	1	3	2.60±0.60[a]	2.51±0.60[a]	1.79±0.70[b]	0.000
Distal tubule	160	0	3	1.44±0.56[a]	1.44±0.56[a]	0.75±0.46[b]	0.640

[a,b] At the same line, different superscript letters mean significant differences (P<0.05) (0: no reaction, 1: mild reaction, 2: medium reaction, 3: very intense reaction)

Table 4. *Statistical analysis of PCNA immunoreactivity between groups*

Parts of Kidney	N	Min	Max	Control	Sham	Diabetic	P
				Mean±SD	Mean±SD	Mean±SD	
Cortex	8	0	3	2.37±0.51[a]	2.25±0.46[a]	0.62±0.51[b]	0.000
Medulla	8	0	2	0.50±0.53[a]	0.51±0.53[a]	0.49±0.54[a]	1.000
Malpighian body	160	1	3	1.25±0.43[a]	1.12±0.33[a]	2.02±0.76[b]	0.000
Proximal tubule	160	0	3	2.01±0.47[a]	2.00±0.46[a]	0.77±0.41[b]	0.000
Distal tubule	160	0	1	0.23±0.42[a]	0.18±0.39[a]	0.21±0.38[a]	0.462

[a,b] At the same line, different superscript letters mean significant differences (P<0.05); 0: no reaction, 1: mild reaction, 2: medium reaction, 3: very intense reaction)

PCNA immuno-reactivity was generally weaker in the diabetic group than in the control and sham groups in renal tissues (except for the Malpighian bodies). Generally, PCNA immunoreactivity decreased in the diabetes group (P<0.05; *Fig. 3-B,D*; *Table 4*). No immunoreactivity was seen in the negative control slide *(Fig. 3-E)*.

DISCUSSION

In this study, we aimed to investigate the effects of DM on kidney histology and immunohistochemical distribution of HGF and PCNA in diabetic and non-diabetic kidneys.

In STZ-induced diabetes studies [23,25], it has been reported that body weights in diabetic groups decrease compared to control groups. On the other hand, Al-Malki and El-Rabey [23] reported that there was no significant difference in terms of body weights in the first week of a diabetic group, but there was a significant weight loss in diabetic groups compared to the control groups at the 2nd, 3rd and 4th weeks. In the present study, body weights decreased in the diabetic group, similar to other studies [23,25].

In the mouse experimental diabetes model, it has been reported that there are degenerations of Bowman's capsule, the glomerular and tubular basement membranes and tubular dilatation [26]. Al-Malki and El Rabey [23] reported that DM caused constriction of the glomerular basement membrane in the kidney, and a hydropic degeneration within glomerular cells and tubular epithelial cells. The findings of the present study were consistent with previous findings [26,27], as the tubular structures of kidney tissues in the diabetic group exhibited irregular structures and different sizes of cellular nuclei. Al-Malki and El-Rabey [23] reported hydropic degeneration in the glomeruli and tubules of diabetic rats. In our study, hydropic degenerations were observed in some proximal and distal tubules. We thought this may be related to the STZ dose and the duration of the experiment.

Normally, HGF is produced at low levels by the liver, spleen and kidney in our bodies. Tashiro et al.[28] reported that HGF mRNA was expressed in the thymus, kidney, lungs, brain tissue, and the liver of rats. In experimental studies with HGF injections, it has been reported that HGF reduces renal fibrosis in obstructive nephropathy, increases tubular cell proliferation by inhibiting apoptosis, and reduces TGF-β1, which plays a major role in tubulointerstitial fibrosis [29,30]. Previous studies [31,32] have determined that serum HGF levels are affected by the presence and severity of certain diseases and the presence of DM complications. Wolf et al.[33] studied the immunohistochemical localization of HGF in human and rat tissues and reported that HGF immunoreactivity was strong in the distal tubules and collecting ducts in the kidney, and weak in the proximal tubules and Henle's loops. In addition, HGF immunoreactivity in the proximal tubules is mostly concentrated at the margins of the microvilli, and completely negative in the glomeruli [33]. In our study, HGF immunoreactivity was more intense, especially in the inner cortex region, while it was moderate in the collecting ducts in the medulla and intense in the papillary ducts. In addition, HGF immunoreactivity was strong in the Malpighian bodies and cytoplasmic HGF immunoreactivity was observed in very few cells in the proximal and distal tubule epithelium. To our knowledge, no studies have examined HGF immunoreactivity in diabetic kidneys. However, it has been reported that the HGF gene slows the progression of diabetic nephropathy [34], recovers renal functions in chronic renal disease [35] and high glucose levels suppress HGF production in muscle cells [36]. On the other hand, another study [37] has reported that HGF serum levels increase in patients with DM. In our study, HGF immunoreactivity was weaker in the diabetic group compared to the control and sham groups. We thought that decreased HGF immunoreactivity in diabetic kidneys may be due to tissue disorders.

Foley et al.[38] reported that PCNA immunoreactivity is found in the nuclei and cytoplasm of tubular epithelial cells in the kidney tissue. They also reported that PCNA immunoreactivity is present in mesangial cells in Malpighian bodies [38]. Gross et al.[20] have determined that PCNA immunoreactivity is more intense in the glomeruli of diabetic rats than in non-diabetic rats. However, they also reported that the number of PCNA-positive cells in the tubulointerstitial area were significantly higher in the diabetic group [20]. In our study, intense PCNA immunoreactivity was observed in the glomeruli of the diabetic group, whereas the PCNA immunoreactivity in the tubular epithelium was very weak in the diabetic group compared to the non-diabetic group. A decrease in the number of podocytes [39] and an increase in the number of mesangial cells have been reported in DM [40]. PCNA is used as a marker of mesangial cell growth [18]. In our study, we think that the increase in PCNA immunoreactivity in the glomerulus of a diabetic kidney may be based on increasing mesangial cells.

In conclusion, we observed structural disorders in renal tissue and decreased HGF immunoreactivity in diabetic mice. PCNA was generally decreased in diabetic kidneys but increased in the glomeruli of diabetic kidneys. This may be caused by a proliferation of mesangial cells in the glomeruli.

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