Urol Res (2002) 30: 79–83
DOI 10.1007/s00240-002-0233-x

ORIGINAL PAPER

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Decreased urinary bladder apoptosis in a rabbit model of diabetes mellitus

Received: 24 August 2001 / Accepted: 14 December 2001 / Published online: 1 February 2002

Abstract Urinary bladder dysfunction is a recognised complication of diabetes mellitus (DM). This is thought to be partly related to altered bladder morphology as DM is associated with increased bladder weight. In DM, increased cellular proliferation is well established. However, there is evidence that in other pathological states affecting the urinary tract, altered apoptosis may also play a role. We therefore used a rabbit model to investigate whether there are any changes in bladder apoptosis with DM. Diabetes was induced in adult New Zealand white rabbits. Age-matched controls were also used. After 6 months, the bladders were excised and weighed. The TUNEL technique was used to detect and quantify apoptosis in both DM and age-matched control bladders. Diabetes was confirmed as this group had significantly ($P < 0.001$) elevated serum glucose compared to controls. The bladder weights were also significantly ($P < 0.001$) greater in the DM rabbits. Apoptosis was significantly ($P < 0.001$) decreased in the urothelial cells of the DM bladders. Our results confirm previous findings that DM is associated with increased bladder weight. Although this is associated with increased cellular proliferation, we have demonstrated that decreased apoptosis may also play an important role. Therefore, decreased apoptosis may be important in the pathophysiology of DM cystopathy.

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Keywords Apoptosis · Urinary bladder · Rabbit · diabetes mellitus

Introduction

It has been long established that bladder dysfunction is a recognised complication of diabetes mellitus (DM) [6]. This has been attributed in part to DM-induced peripheral autonomic neuropathy [7]. Morphological, histological and functional alterations in autonomic neurotransmission have been reported in diabetes [9]. In addition, there is evidence that diabetes is associated with increased bladder weight [12]. This is thought to be secondary to diabetes-induced diuresis, which, in turn, leads to increased bladder weight [12]. The increase in bladder weight has, in itself, been attributed to increased cellular proliferation, in particular, urothelial cell proliferation [5]. In addition to this effect, it has been demonstrated in a rat model of diabetes that there is a time-dependent increase in bladder distension/increased micturition volume, which stimulates thymidine incorporation into DNA. This is an indication of increased cellular proliferation, leading in increased bladder weight [12].

Our group has further demonstrated increased bladder cellular proliferation associated with diabetes. Endothelin-1 (ET-1) is a potent vasoconstrictor peptide with mitogenic activity that is known to be elevated in humans with DM [10, 13]. There is growing evidence that ET-1 is associated with cellular hyperplasia. As such, we have shown an increased bladder cellular proliferation that is inhibited by ET-1 antagonists in a rabbit model of diabetes [10].

Bladder outlet obstruction is another pathological condition that is known to be associated with increased bladder weight and cellular content [3]. We have previously demonstrated, in a rabbit model of partial bladder outlet obstruction, increased bladder cellular proliferation that was also inhibited by ET-1 antagonists, implying that ET-1 may play an important role in bladder remodelling associated with partial outlet obstruction

[8]. Santarosa et al., using a similar model of partial bladder outlet obstruction, have demonstrated the hyperplasia of urothelial cells that is associated with increased bladder weight [11]. They have also shown that by relieving the obstruction there is a decrease in the bladder weight towards its pre-obstruction value [11]. This decrease in bladder weight was attributed to increased apoptosis (programmed cell death) of the urothelial cells [11]. They therefore concluded from their study that hyperplasia and apoptosis are opposing cellular processes that mediate the bladder's response to obstructive stimuli [11].

To date, the role of apoptosis in DM bladder remodelling has not been determined. The urinary bladder responds in a similar manner to DM or obstructive stimuli, namely by increasing urothelial cellular hyperplasia and its weight. We, therefore, investigated whether there are any changes in bladder apoptosis that may play a role in bladder remodelling in a rabbit model of DM.

Materials and methods

Induction of DM

Age-matched, 3-kg male adult New Zealand White rabbits ($n = 6$) were injected intravenously with alloxan (via the ear vein; 65 mg/kg). Six age-matched rabbits were used as controls. The DM rabbits were fed ad libitum with SDS standard rabbit plain (SDS, Whitham, UK) and allowed free access to water.

Collection and analysis of samples

Blood was sampled at monthly intervals from the ear vein. This was placed into serum gel bottles to determine serum electrolytes, creatinine, urea and glucose concentrations using the standard methodology for the Hitachi 717 Automatic Autoanalyzer (Boehringer Mannheim, Lewes, Sussex, UK).

Urine was also monitored over the duration of diabetes for glucose, ketone bodies and proteins with Multistix (Ames Division, Miles Laboratories, Stoke Poges, Buckinghamshire, UK).

Preparation of tissues

Following cervical dislocation, the urinary bladders from 6-month DM and age-matched controls were excised and weighed. They were subsequently divided into detrusor and bladder neck at the level of the ureteric orifice. The tissues were immediately stored at –70°C in airtight containers until used. The tissues stored at –70°C were subsequently mounted in AMES OCT embedding compound (BDH Laboratory Supplies, Poole, UK). Transverse sections (10 μm) were cut in a cryostat at approximately –20°C and thaw-mounted onto gelatinised microscope slides. The slides were stored at –70°C in airtight containers until use.

In situ apoptosis detection

In situ apoptosis was performed by enzymatic in situ labeling of apoptosis-induced DNA strand breaks using an In Situ Cell Death Kit (Roche Molecular Biochemicals, Palo Alto, USA). DNA polymerase as well as terminal deoxynucleotidyl transferase (TdT) were used for the incorporation of labelled nucleotides to DNA strand breaks in situ. The tailing reaction using TdT is also described as the TUNEL (TdT-mediated dUTP nick end labelling) technique.

The processes involved are briefly described as follows. First, the tissue sections were fixed with 4% paraformaldehyde for 20 min at room temperature. The sections were then washed with phosphate buffer solution (PBS) for 30 min. This was followed by incubation in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Subsequently, the sections were labelled and analysed. This involved the following steps. The slides were rinsed (×2) with PBS. The area around the tissue was dried and 50 μl of TUNEL reaction mixture was then added. The tissues were then covered with a coverslip to ensure a homogeneous spread across the tissue and to avoid evaporative loss. The TUNEL reaction mixture was prepared as per instructions from the In Situ Cell Death Kit (Roche Molecular Biochemicals). The slides were then incubated in a humidified chamber for 60 min at 37°C in the dark. This was followed by rinsing the slides (×3) with PBS. The tissues were then analysed directly under a fluorescence microscope. Fluorescein labels incorporated into nucleotide polymers were detected and quantified by fluorescence microscopy. Six random areas of 2 mm^2 were used to count the number of apoptotic cells for each tissue and selected tissues were photographed.

Statistical analysis

Comparisons of animal and bladder weights, serum glucose concentrations and apoptosis between the 6-month DM group and the age-matched controls were performed using the Student's unpaired *t*-test.

The principles of laboratory animal care were followed and British Home Office approval was sought prior to starting the study.

Results

Animal weights

The starting weights of the age-matched controls and 6-month DM rabbits were all similar (Table 1). However, in the 6-month DM group the final weights were significantly ($P < 0.04$) decreased. Age-matched controls gained a significant ($P < 0.001$) amount of weight at 6 months when compared to their starting weight (Table 1). At 6 months, the age-matched controls were significantly ($P < 0.001$) heavier than the 6-month DM animals (Table 1).

Table 1. Animal weights and serum glucose in the 6-month diabetic (*DM*) rabbits and age-matched controls. Results are expressed as mean ± SD

Animals	Animal weights (kg)		Serum glucose (mmol/l)	
	Starting	Final	Starting	Final
DM	3.51 ± 0.05[a]	3.28 ± 0.10	7.3 ± 0.54	26.3 ± 1.41[d]
Control	3.40 ± 0.02[c]	4.70 ± 0.04[b]	7.7 ± 0.31	7.5 ± 0.39

[a] 6-month DM starting weight vs 6-month DM final weight (*$P < 0.04$)
[b] 6-month age-matched control final weight vs 6-month DM final weight ($P < 0.001$)
[c] 6-month age-matched control starting weight vs final weight ($P < 0.001$)
[d] 6-month DM serum glucose vs age-matched control serum glucose ($P < 0.001$)

Serum biochemical indices

Serum glucose concentrations (non-fasting) were significantly ($P < 0.001$) elevated in the 6-month DM rabbits when compared to their age-matched controls (Table 1).

Serum electrolytes, creatinine and urea were not significantly different between the age-matched controls and the DM group (data not shown).

Urinary biochemical indices

Urinary ketone and protein concentrations were not significantly different between the age-matched controls and the DM group (data not shown).

Bladder weights

Urinary bladder weights were significantly ($P < 0.001$) greater in the DM rabbits compared to controls (Table 2).

Apoptotic count

Apoptosis was significantly ($P < 0.001$) lower in the DM detrusor urothelial cells compared to controls (Table 3, Figs. 1, 2). Similarly, apoptosis was also significantly ($P < 0.001$) lower in the DM bladder neck urothelial cells compared to controls (Table 3, Figs. 3, 4).

Table 2. Comparison of 6-month diabetic (*DM*) bladder weights with age-matched controls. Results are expressed as mean ± SD

	Control	DM
Bladder weights (g)	2.1 ± 0.2	*4.2 ± 0.3

*$P < 0.001$

Table 3. Comparison of bladder urothelial cell apoptotic counts between 6-month diabetic (*DM*) and age-matched control rabbits. Results are expressed as mean ± SD

Bladder	Control	DM
Detrusor	36 ± 8	6 ± 3*
Bladder neck	58 ± 12	5 ± 2*

*$P < 0.001$

Discussion

Several methods have been described to identify apoptotic cells [1, 2, 4]. Endonucleolysis, resulting in the cleavage of nuclear DNA into oligonucleosome-sized

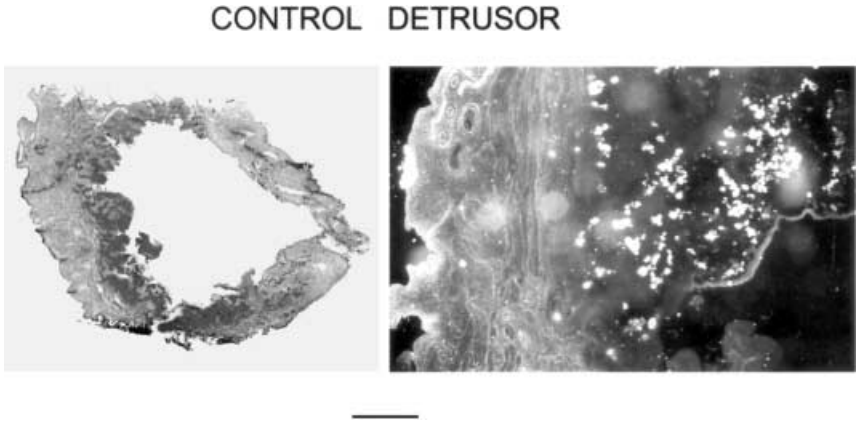

Fig. 1. Level of apoptosis in age-matched control detrusor. *Left panel*: haematoxylin and eosin (h and e) stained section of control detrusor. *Right panel*: consecutive control detrusor section treated with the TUNEL technique and apoptosis detected by fluorescence microscopy. *Scale bar* = 200 μm (*left panel*); 40 μm (*right panel*)

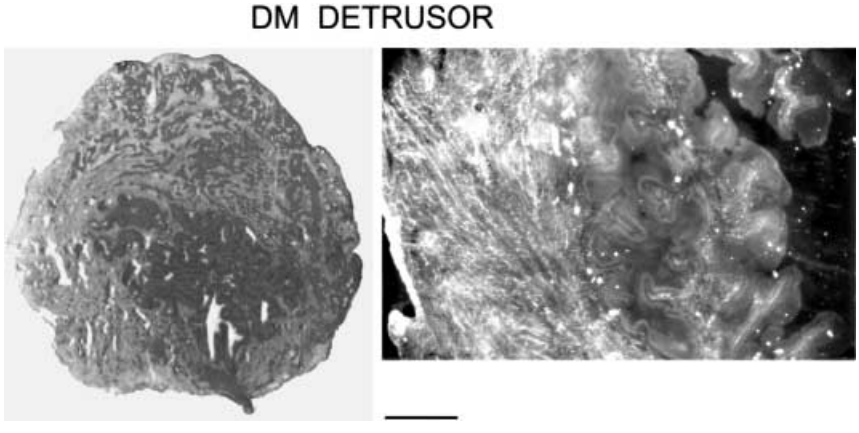

Fig. 2. Level of apoptosis in 6-month diabetic (DM) detrusor. *Left panel*: H&E stained section of DM detrusor. *Right panel*: consecutive DM detrusor section treated with the TUNEL technique and apoptosis detected by fluorescence microscopy. *Scale bar* = 200 μm (*left panel*); 40 μm (*right panel*)

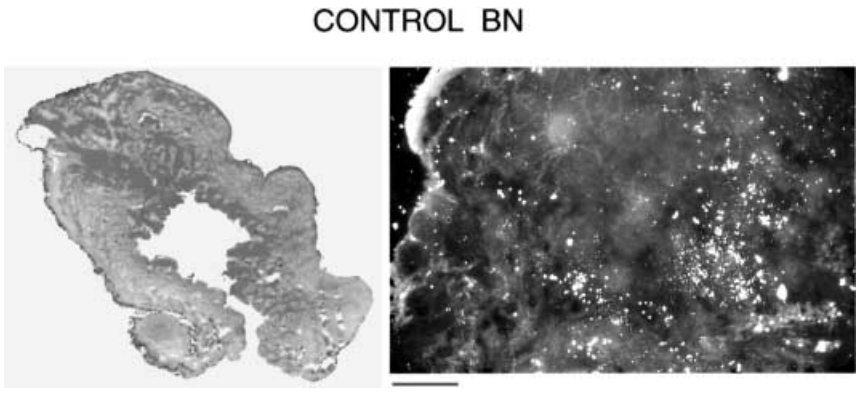

Fig. 3. Level of apoptosis in age-matched control bladder neck. *Left panel*: H&E stained section of control bladder neck. *Right panel*: consecutive control bladder neck section treated with the TUNEL technique and apoptosis detected by fluorescence microscopy. *Scale bar* = 200 μm (*left panel*); 40 μm (*right panel*)

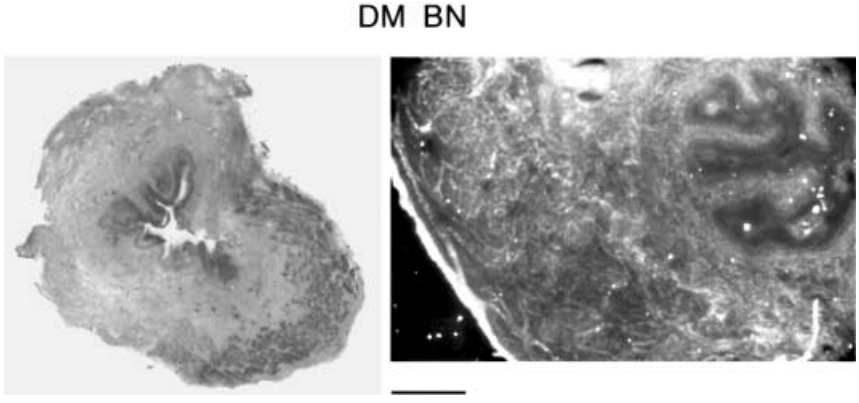

Fig. 4. Level of apoptosis in 6-month DM bladder neck (BN). *Left panel*: H&E stained section of DM bladder neck. *Right panel*: Consecutive DM bladder neck section treated with the TUNEL technique and apoptosis detected by fluorescence microscopy. *Scale bar* = 200 μm (*left panel*); 40 μm (*right panel*)

fragments, is considered as the key biochemical event of apoptosis. Hence, this process is commonly used for the detection of apoptosis by the typical 'DNA ladder' on agarose gels during electrophoresis. Unfortunately, this method does not provide information regarding apoptosis in individual cells. It also fails to relate cellular apoptosis to histological localisation or cell differentiation. However, this can be accomplished by the enzymatic in situ labelling of apoptosis-induced DNA strand breaks. This is done by the enzymatic in situ labeling of apoptosis induced DNA strand breaks, using the TUNEL technique, as in this study. This technique has the advantage of allowing the direct detection of DNA fragmentation and it preferentially labels apoptosis in comparison to necrosis, thereby discriminating apoptosis from necrosis.

Our study has confirmed previous findings, using a rabbit model of DM, that DM is associated with an increase in urinary bladder weight. We have also demonstrated that there is a significant decrease in apoptosis in the DM detrusor and bladder neck urothelial cells compared to age-matched controls. Although a rise in DM bladder weight is thought to be associated with increased cellular proliferation, we have shown that reduced apoptosis may also play an important role. Therefore, it appears that in DM hyperplasia and apoptosis are opposing cellular processes that mediate the bladder's response to diuresis-induced distension. It is not surprising that altered apoptosis was a feature of the urothelium rather than the smooth muscle. This is because it has been previously shown that hyperplasia is restricted to the urothelial cells in DM [5, 11]. Hence, as hyperplasia and apoptosis are closely related opposing cellular processes, it is understandable that if one process has an influence on the urothelial cells so does the other. It is not known why apoptosis was not a factor in the smooth muscle cells. This, in itself, poses an interesting question and merits further investigation, which was beyond the scope of the present study.

Bladder outlet obstruction is another pathological condition for which an imbalance between cellular hyperplasia and apoptosis may play an important role leading to increased bladder weight [11]. Hence, it is possible that this imbalance between hyperplasia and apoptosis is a common bladder pathological pathway, resulting in increased bladder weight irrespective of the underlying stimulus.

There is growing evidence for the role of ET-1 in bladder hyperplasia associated with both DM and bladder outlet obstruction [8, 10]. However, at present it is not known whether this plays a role in apoptosis. On the other hand, there is evidence that other growth factors may be associated with apoptosis. It has been demonstrated, in a rabbit model of partial bladder outlet obstruction, that increased bladder weight is associated

with the increased expression of basic fibroblast growth factor and a suppression of the expression of transforming growth factor-β [11]. Upon release of the partial obstruction it was noted that there was a decrease in the bladder weights associated with increased apoptosis [11]. This change was observed with the background of decreased expression of basic fibroblast growth factor mRNA and an increased expression of transforming growth factor-β mRNA when compared with RNAs from hypertrophied bladders [11]. Hence, it is thought that the local synthesis of these growth-promoting and growth-inhibiting factors may be responsible for the level of apoptosis. It is not known whether these factors are involved in DM-related apoptosis. Despite this, it is reasonable to predict that they may also be involved, as DM and partial bladder outlet obstruction have been shown to exhibit similar cellular and morphological changes.

In conclusion, we have demonstrated in a rabbit model of DM that an alteration in the balance between apoptosis and hyperplasia is associated with an increase in bladder weight. The exact clinical pathological ramifications of these changes is unknown. However, the decreased apoptosis associated with increased bladder weight may, in turn, play a role in the development of DM cystopathy/neuropathic bladder. In addition, although the exact pathophysiological mechanisms underlying the decreased apoptosis in the DM bladder urothelial cells are not known, there is indirect evidence, from work on partial bladder outlet obstruction, that altered expression of basic fibroblast growth factor and transforming growth factor-β may be involved. However, further work is needed to confirm whether this is also the case in the DM bladder.

Acknowledgements We thank the Charles Wolfson Charitable Trust for the funding of this study.

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