Relationship between apoptotic markers (Bax and Bcl-2) and biochemical markers in type 2 diabetes mellitus

Hasnan J, Yusof M I, Damitri T D, Faridah A R, Adenan A S, Norbaini T H

ABSTRACT

Introduction: Bax is essential for apoptosis in normal cells. However, overexpression of Bcl-2 enhances cell survival by suppressing apoptosis in cells subjected to apoptosis-inducing stimuli. The aim of this study was to examine the expression of apoptotic (Bax and Bcl-2) and biochemical markers in type 2 diabetics mellitus.

Methods: A test group comprising 41 type 2 diabetes mellitus patients and a control group comprising 36 non-diabetic patients were enrolled in this study. Skin biopsy tissue samples were stained immunohistochemically for Bcl-2 and Bax expressions. Fasting plasma glucose (FPG), triglycerides, total cholesterol (TC), high-density lipoprotein cholesterol (HDLC) and glycosylated haemoglobin A1c (A1C) were analysed. Low-density lipoprotein cholesterol (LDLC) was calculated.

Results: Bcl-2 expression was significantly higher (p-value is less than 0.001) in the control group. Bax expression was significantly higher (p-value is 0.018) in the diabetic group. Positive Bcl-2 expression was observed in 18 of 36 (50 percent) controls. Positive Bcl-2 expression was found in 5 of 41 (12.2 percent) diabetics. There was a significant difference (p-value is less than 0.001) between the two groups for mean FPG, HDLC and A1C. There was no significant difference for TC, LDLC and triglycerides between the two groups. Positive Bax expression was found in 11 of 35 (31.4 percent) controls. The odds of developing Bcl-2 among non-diabetics were 12.67 times compared to diabetics (p-value is less than 0.001).

Conclusion: Prolonged hyperglycaemia induces apoptosis in the endothelial cells of diabetic ulcers, which aggravates microvasculopathy and delays tissue healing and regeneration.

Keywords: type 2 diabetes mellitus, apoptosis, biochemical markers

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Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian 15200, Malaysia

Hasnan J, MPath
Associate Professor

Damitri TD, BSc
Research Officer

Department of Orthopaedics

Yusof MI, MMed
Associate Professor

Department of Chemical Pathology

Faridah AR, PhD
Associate Professor

Adenan AS, PhD
Lecturer

Department of Biostatistics

Norbaini TH, MMed
Lecturer

Correspondence to:
Dr. Mohd Imran Yusof
Tel: (60) 9 766 3000 ext 4512
Fax: (60) 9 765 3370
Email: drimran@kb.usm.my

INTRODUCTION

Diabetes mellitus (DM) is a major public health problem, and the prevalence of type 2 DM is increasing in the world population. By 2010, it is expected that over 200 million people would suffer from DM.[1] Hyperglycaemia has been documented to contribute to the development of microvascular complications and macrovascular disease.[2] This is an important cause of morbidity and mortality in diabetic patients.[3]

Hyperglycaemia can cause two main complications in DM: microvascular complications which mainly affect the retina, kidney and peripheral nervous system may progress to more overt serious complications, and macrovascular complications, mainly atherosclerosis, may lead to cerebrovascular ischaemia and stroke.[4,5] Diabetic retinopathy and nephropathy are diabetes-specific microvascular complications that share similar pathophysiological features, suggesting common underlying mechanisms at the biochemical level.[6]

Vascular dysfunction is said to be a contributing factor in the aetiology of several clinically important secondary complications of DM, including retinopathy, accelerated atherosclerosis, nephropathy, neuropathy and impaired wound healing.[7] There is evidence suggesting that an early site at which these vascular complications develop is the endothelium. In particular, early abnormalities, such as an increase in the renal and retinal blood flow, impaired vasodilation in response to physiological and pharmacological stimuli, and augmented vascular permeability, have been attributed to endothelial cell damage.[5]

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Table I. Apoptosis markers used in the study.

Antibody	Supplier	Dilution	Incubation time	Positive control
Bax	DAKOcytomation, Germany	1:200	Overnight	Gastric adenocarcinoma
Bcl-2	DAKOcytomation, Germany	1:100	1 hour	Tonsil

Table II. Biochemical markers of the control and diabetic groups.

Test	Mean ± SD		Mean difference (95% CI)	*t*-stat	p-value
	Control (n = 36)	Diabetes (n = 41)			
FPG (mmol/L)	4.41 ± 1.12	11.02 ± 4.25	−6.62 (−8.07, −5.16)	−9.07	< 0.001
TG (mmol/L)	1.37 ± 1.30	1.87 ± 0.95	−0.50 (−1.01, 0.01)	−1.95	0.61
TC (mmol/L)	5.26 ± 1.31	5.04 ± 1.88	0.22 (−0.53, 0.97)	0.59	0.55
LDLC (mmol/L)	3.28 ± 1.38	3.17 ± 1.57	0.35 (−0.58, 0.8)	0.31	0.75
HDLC (mmol/L)	1.47 ± 0.72	1.00 ± 0.38	0.46 (0.2, 0.72)	3.48	0.001
A1C (%)	5.00 ± 0.67	9.50 ± 2.24	−4.50 (−5.36, −3.64)	−10.43	< 0.001

$p < 0.001$ is significant.
CI: confidence interval; SD: standard deviation; FPG: fasting plasma glucose; TG: triglyceride; TC: total cholesterol; LDLC: low-density lipoprotein cholesterol; HDLC: high-density lipoprotein cholesterol; A1C: glycosylated haemoglobin A1c

haemoglobin, better known as HbA1c (A1C), and fasting plasma glucose (FPG), have been used as markers of diabetes control. In individuals with type 2 DM, blood glucose control predicts not only diabetes control but also vascular disease. The underlying mechanism of the association is still unclear. Apoptosis is an energy-dependent, natural, genetically-controlled process by which an organism eliminates unnecessary single cells.[6] The term "apoptosis" has been coined to explain the morphological processes principal to controlled cellular self-destruction, and was first discovered in a publication by Kerr et al.[8] Bax belongs to a family of proteins which share homology with Bcl-2 in several highly conserved regions. The Bcl-2 proteins are involved in the regulation of apoptosis, and act to promote or suppress cell death. An overexpression of Bax promotes cell death. Bax can form homodimers and can heterodimerise with other Bcl-2-related proteins. The formation of heterodimers between Bax and Bcl-2 homologues with death repressor function (Bcl-2 and Bcl-Xl) leads to the inhibition of the death-promoting effects of Bax. It has been proposed that the relative expression of the different Bcl-2 families of proteins controls the sensitivity of cells to apoptotic stimuli.[9]

Bcl-2 is a member of the Bcl-2 family of proteins that regulates apoptosis. The gene which encodes Bcl-2 was first identified at the chromosomal translocation point t(14:18) in human B cell follicular lymphoma.[10] The overexpression of Bcl-2 enhances cell survival by suppressing apoptosis in a number of cells that are subjected to a wide range of apoptosis-inducing stimuli, including nerve growth factor withdrawal, radiation and chemotherapeutic agents.[10] The aim of this study was to analyse the relationship between apoptotic markers (Bcl-2 and Bax) and the biochemical markers of diabetes control (A1C and FPG levels).

METHODS

This cross-sectional study was conducted in the Hospital Universiti Sains Malaysia (HUSM) from August 2003 to August 2005. There were two groups of patients in this study: Group I consisted of patients with type 2 DM, and Group II consisted of non-diabetes patients who were admitted to HUSM for any surgical procedure. There were 41 and 36 patients in Groups I and II, respectively. The skins and blood samples were taken from the patients after written consent was obtained. The skin samples were taken during surgery, while the blood samples for FPG, fasting lipid profile, A1C and urine samples were taken at the time when the patients were recovering from surgery.

The skin biopsy, measuring about 1 cm × 2 cm, was taken by the surgeon at the incision site and immediately placed in a universal bottle containing 10% formaldehyde. The skin sample was subsequently processed using a vacuum filtration tissue processor (Sakura Tissue-Tek VIP E150, Sakura Finetek, Tokyo, Japan). All the prepared tissue sections were then stained with haematoxylin-eosin to assess the vascular morphology.

From each patient, 5 ml of fasting venous blood was obtained. Subsequently, 2.5 ml was aliquoted into a tube containing potassium ethylenediaminetetraacetic acid (EDTA) to determine the A1C level. Another 2.5 ml of blood was aliquoted into a tube containing sodium oxalate to determine the FPG level. The plasma was

Table III. Bax expression of the control and diabetic groups.

Bax expression	No. (%)		Chi-square	Odds ratio	p-value
	Control (n = 35*)	Diabetes (n = 39*)			
Positive	11 (31.43%)	23 (58.97%)	5.636	0.319	0.018
Negative	24 (68.57%)	16 (41.03%)			

$p < 0.05$ is significant.
* One subject in the control and two in the diabetes groups could not be processed due to technical error.

Table IV. Bcl-2 expression between the control and diabetic groups.

Bcl-2 Expression	No.(%)		Chi-square	Odds ratio	p-value
	Control (n = 36)	Diabetic (n = 41)			
Positive	18 (50.00%)	5 (12.20%)	17.607	12.667	< 0.001
Negative	18 (50.00%)	36 (87.80%)			

$p < 0.001$ is significant.

subsequently separated after a 3-min centrifugation at 4000 rpm and analysed for FPG by the automated enzymatic glucose oxidase method using a commercial kit (RANDOX) on the Hitachi 912 autoanalyser (Hitachi, Mannheim, Germany). Lipid profile was also analysed using commercial kits (ROCHE) on the Hitachi 912 autoanalyser. All samples were determined for quantitative glycated haemoglobin concentration using the DiaSTAT haemoglobin A1C programme on the Bio-Rad DiaSTAT analyser (Bio-Rad, Hercules, CA, USA), following the standard recommended procedures. The technique was performed to demonstrate the expression of each apoptotic marker separately: Bax in 1:200 (µl) and Bcl-2 in 1:100 (µl). A standard labelled streptavidin biotin (Dako, Glostrup, Denmark) method was used on formalin-fixed paraffin-embedded tissue sections.

The tissue blocks were trimmed and sectioned with microtome (Leica, Wetzlar, Germany) to obtain 3–5-µm thick sections, and then the tissues sections were deparaffinised in xylene and dehydrated. For the detection of apoptotic markers, slides were pretreated with Tris EDTA buffer (10mM, pH 9.0) for 14 min in microwave oven heating. Subsequently, all the sections were treated for 10 min with peroxidase blocking reagent (DAKO Glostrup, Denmark) to quench the endogenous peroxidase activity, and then incubated with primary antibodies, followed by rinsing with Tris buffered saline (pH 7.2). The sections were incubated for 30 min with optimally diluted biotinylated secondary antibody and for 30 min with horseradish peroxidase to be ready to use. For visualisation, the slides were immersed in diaminobenzidine (DAKO, Glostrup, Denmark) substrate for 5 min, followed by washing in distilled water. The slides were counterstained with Harris haematoxylin, dehydrated and mounted. To assess the specificity of the reactions, gastric adenocarcinoma and tonsillar tissues were used as positive control Bax and Bcl-2 expressions, respectively. Negative controls (incubation without primary antibody) were also used for this purpose. The antibody sources, recommended positive controls and dilutions are shown in Table I.

The Statistical Package for Social Sciences version 11.5 (SPSS Inc, Chicago, IL, USA) was used for the analysis of the biochemical and histopathological data in this study. The distribution of all numerical variables was checked for normality and presented by mean and standard deviation. To analyse the difference between the means, the Student's *t*-test for the two groups (two independent means) was used for variables with normal distribution.

A light microscope (Nikon, Tokyo, Japan) was used to examine the immunohistochemically-stained skin biopsy slides. Initially, the expression and immunostaining of Bax and Bcl-2 were scored according to Galkowska et al's intensity score. Staining intensity was scored on a positive scale (positivity), ranging from 0 to 2 where, 0 = no staining, 1+ = weakly stained, and 2+ = moderately to strongly stained. We then further grouped the scores obtained into two categories (0 = non-expressing [−ve], 1+ & 2+ = expressing [+ve]) to obtain the categorical data for statistical analysis.

RESULTS

There was a significant difference ($p < 0.001$) between the two groups in the mean FPG (control 4.41 ± 1.12 mmol/L; diabetics 11.02 ± 4.25 mmol/L), high-density lipoprotein cholesterol (control 1.47 ± 0.72 mmol/L; diabetics 1.00 ± 0.38 mmol/L) and A1C (control 5.00% ± 0.67%; diabetics 9.50% ± 2.24%) (Table II). However, there was no significant difference in the triglyceride, total

Table V. Differences between the biochemical markers and Bax expression.

	Mean ± SD		Mean difference (95% CI)	*t*-stat	p-value
	Bax positive	Bax negative			
FPG (mmol/L)	8.93 ± 4.87	6.60 ± 3.24	2.33 (0.44, 4.22)	2.46	0.023
TG (mmol/L)	1.76 ± 1.5	1.49 ± 0.76	0.28 (−0.26, 0.81)	1.02	0.84
TC (mmol/L)	4.77 ± 1.92	5.34 ± 1.25	−0.57 (−1.31, 0.17)	−1.54	0.075
LDLC (mmol/L)	2.87 ± 0.73	3.39 ± 1.32	−0.52 (−1.20, 0.17)	−1.51	0.47
HDLC (mmol/L)	1.23 ± 0.73	1.23 ± 0.53	0.00 (−0.3, 0.3)	−0.002	0.516
A1C (%)	8.16 ± 3.08	6.93 ± 2.49	1.22 (−0.14, 2.6)	1.79	0.096

P < 0.05 is significant.
CI: confidence interval; SD: standard deviation; FPG: fasting plasma glucose; TG: triglyceride; TC: total cholesterol; LDLC: low-density lipoprotein cholesterol; HDLC: high-density lipoprotein cholesterol; A1C: glycosylated haemoglobin A1c

Table VI. Differences between the biochemical markers and Bcl-2 expression.

	Mean ± SD		Mean difference (95% CI)	*t*-stat	p-value
	Bcl-2 positive	Bcl-2 negative			
FPG (mmol/L)	4.81 ± 1.87	9.10 ± 4.76	−4.29 (−6.43, −2.15)	−3.99	< 0.001
TG (mmol/L)	1.47 ± 1.56	1.69 ± 0.95	−0.23 (−0.81, 0.36)	−0.77	0.542
TC (mmol/L)	4.77 ± 1.26	5.28 ± 1.74	−0.51 (−1.34, 0.32)	−1.23	0.282
LDLC (mmol/L)	2.76 ± 1.25	3.39 ± 1.53	−0.63 (−1.4, 0.13)	−1.66	0.493
HDLC (mmol/L)	1.49 ± 0.79	1.13 ± 0.50	0.37 (0.06, 0.68)	2.38	0.124
A1C (%)	5.19 ± 0.79	8.38 ± 2.83	−3.19 (−4.58, −1.79)	−4.57	< 0.001

P < 0.05 is significant.
CI: confidence interval; SD: standard deviation; FPG: fasting plasma glucose; TG: triglyceride; TC: total cholesterol; LDLC: low-density lipoprotein cholesterol; HDLC: high-density lipoprotein cholesterol; A1C: glycosylated haemoglobin A1c

cholesterol and low-density lipoprotein cholesterol.

The distribution of Bax were significant (p = 0.018) between the two groups (Table III). Bax expression was positive in 11 (31.43%) and negative in 24 (68.57%) of 35 control patients. In the diabetic patients, Bax was positive in 23 (58.97%) and negative in 16 (41.03%) of the 39 cases. Interestingly, the distribution of Bcl-2 expression was highly significant (p < 0.001) between the two groups (Table IV). Positive Bcl-2 expression was observed in 18 (50.00%) of the 36 controls, while another 18 (50.00%) were negative. Positive Bcl-2 expression was found in only 5 (12.20%) of the 41 diabetics, compared to 36 (87.80%) who showed negative expression.

When comparing the apoptotic marker expressions on the endothelial cells and the serum level of the biochemical markers, it was noted that there was a significant difference (p = 0.023) between the FPG level and the Bax negative and positive cases (Table V). There was also a significant difference (p < 0.001) between the FPG and the Bcl-2 positive and Bcl-2 negative expressions (Table VI). A significant difference between the A1C level and the Bcl-2 negative and Bcl-2 positive expressions was also observed.

DISCUSSION

In the present study, Bax, a pro-apoptotic marker, was increased in the blood vessels of diabetic patients. In contrast, the expression of Bax in the control patients was decreased. Our results showed that the expression of Bax was significantly different when compared between the groups (p = 0.018). We also noted that the Bax expression showed a significant increase with an elevated FPG level (p = 0.023). These observations were consistent with a previous report which showed that Bax levels were increased in human diabetic retinas that had high levels of glucose.[12,13] Another study by Li et al reported that the expression of Bax was increased in the cortex of the kidney of diabetic animal models.[14] Several studies demonstrated that Bax is the major effector in apoptotic ganglion cell death in the retina after ischaemia, excitotoxicity and axotomy, and in retinal degeneration.[15-19] In addition, apoptosis induced by high glucose has been shown in endothelial cells *in vitro*[20,21] and was supported in a report by Sekiguchi et al, which verified the apoptotic phenomenon in human aortic endothelial cell culture with a high glucose concentration.[22]

Bax overexpression in diabetic subjects indicates that there is endothelial cell dysfunction, which in turn triggers endothelial cell death. The overexpression of Bax is believed to aggravate the endothelial dysfunction in a diabetic, which further increases the levels of FPG and A1C. This appears to be a vicious cycle, and unless

prolonged hyperglycaemia is corrected, more blood vessels will undergo dysfunction and death over time.

The present data also revealed that the expression of Bcl-2 in diabetic patients was significantly correlated ($p < 0.001$) with FPG and A1C levels. This expression is significantly low ($p < 0.001$) when compared to the control patients. These observations were consistent with a previous report that indicated that there were low expressions of Bcl-2 in diabetic human adult retinas.[23] Sgarbosa et al, who studied the role of apoptosis in the maintenance of placental homeostasis, also noted that there was a higher apoptosis index and lower Bcl-2 expression in the placentas of diabetic women.[24]

However, some studies have noted an upregulation of Bcl-2 in the microenvironment of hyperglycaemia. In a study of the expression of apoptosis mediators in the retinas of human subjects with DM, Abu-El-Asrar et al found that the glial cells of the retinas of diabetics showed an upregulation of Bcl-2 compared to non-diabetic subjects.[25] Galkowska et al also demonstrated that there was a high expression of Bcl-2 in the edge of diabetic ulcers compared to normal venous ulcers.[11] The differences in immunohistochemistry techniques, sample sizes, types of tissues and antibodies used may account for the discrepancy.

The relationship between hyperglycaemia and apoptosis has been reported in a few studies. Moley showed that hyperglycaemia upregulates p53 and downregulates the glucose transporters, GLUT1, 2 and 3, triggering the mitochondrial death cascade pathway.[26] In addition, the oxidative stress induced by glucose deprivation triggers Bax-associated events, including subsequent caspase activation and the progression of apoptotic cell death.[27] Another study conducted by Gao et al demonstrated that hypoxia stimulates apoptosis and inhibits proliferation in the presence of normal glucose, while hyperglycaemia significantly attenuates the hypoxic-induced growth response.[28] Therefore, high glucose level could lead to endothelial dysfunction by activating the expression of Bax and downregulating Bcl-2 in diabetic patients. In diabetic ketoacidosis, the high production of lactate could further aggravate endothelial dysfunction by reducing oxygen in the tissue microenvironment. In this regard, Bcl-2 inhibits, whereas Bax promotes apoptosis in such situations. The mechanisms by which Bcl-2 and Bax develop their functions are unknown, although much has been said about their localisation in the mitochondrial membrane.

In conclusion, prolonged hyperglycaemia induces apoptosis in the endothelial cells of diabetic ulcers, and this will further aggravate microvasculopathy and delay tissue healing and regeneration of diabetic ulcers.

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