EXPRESSION OF APOPTOSIS MARKERS IN THE SKIN MICROVASCULATURE OF PATIENTS WITH DIABETES MELLITUS TYPE 2 AT HOSPITAL UNIVERSITI SAINS MALAYSIA

by

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LIST OF ABBREVIATIONS, TERMINOLOGIES and SYMBOLS

Abbreviation	Full
µg	microgram
%	percent
A2O	Tumor necrotic factor, alpha-induced protein 3
ABC	Avidin-Biotin Complex
ADA	American Diabetes Association
A1C	Hemoglobin A_{1c} in %
AGEs	Advanced glycation end-products
Apaf-1	Apoptotic protease activating factor 1
BAX	BCL2-associated X gene
Bax	BCL2-associated X protein
Bax-α	21 kDa BCL2-associated X protein alpha
Bax-β	24 kDa BCL2-associated X protein beta
Bax-γ	5 kDa BCL2-associated X protein gamma
Bax (anti-bax)	BCL2-associated X protein (pro-apoptotic)
B-cell	Lymphocytes
Bcl-2	B-cell lymphoma protein 2 (anti-apoptotic)
Bcl-xl	BCL2-associated X protein XL
BMI	Body mass index in kg/m^2
BVC	Blood vessel counting
BBP	Biochemical Blood Profile
Ca^{2+}	Calcium ion
C. elegens	*Caenorhabditis elegans*

Caspase	Cysteine proteases
Caspase- 3	Cysteine protease -3
Caspase -7	Cysteine protease -7
CHD	Coronary heart disease
CI	Confidence Interval
c-Myc	Protein oncogene
DCCT	Diabetes Control and Complications Trial
DEVD	caspase-3 inhibitor
df	degree of freedom
DM	Diabetes mellitus
DM 2	Diabetes melitus type 2 or type 2 diabetes
DNA	Deoxyribonucleic acid
ECC	Endothelial cell counting
ECT	Endothelial cell thickness in μm
ESRD	End-stage renal disease
FasL	Fas ligand
FITC	Fluorescein isothiocyanate
FLP	Fasting lipid profile
FFAs	Free fatty acids
FPG	Fasting plasma glucose in mmol/L
h	hour
24h	24 hours
HDL-c	High density lipoprotein cholesterol in mmol/L
H&E	Hematoxylin & Eosin (stain)
HIER	Heat induced epitope retrieval

HRP	Horseradish peroxidase
HUSM	Hospital Universiti Sains Malaysia
ICE	Interleukin-1–converting enzyme
IFG	Impaired fasting glucose
Ig	Immunoglobulin
IgG	Immunoglobulin G
IGT	Impaired glucose tolerance
IHC	Immunohistochemistry
ISS	Immunohistochemistry scoring system
kDa	kiloDalton
L	Litre
LDL	Low density lipoproteins
LDL-c	Low density lipoprotein cholesterol in mmol/L
LSAB	Labelled streptavidin biotin (complex)
MI	Myocardial infarction
min	minute
mg	milligram
Mg^{2+}	Magnesium ion
mmol	millimole
NHANES	National Health and Nutrition Examination Survey
NO	Nitric oxide
NOS	NO synthase (enzyme)
OGTT	Oral glucose tolerance test

OMIM	Online Mendelian Inheritance in Man (www.ncbi.nlm.nih.gov)
$p<0.05$	Standard level of significance where the p-value is less than 0.05
PO	Peroxidase
p53	Tumor supressor
PAS	Periodic acid schiff (stain)
PKC	Protein kinase C
RNA	Ribonucleic acid
s	seconds
t(14:18)	Portion of chromosome 14 and 18 that undergo reciprocal translocation
ROS	Reactive oxygen species
T-cell	Thymus cell (group of white blood cells)
TC	Total cholesterol in mmol/L
TEM	Transmission electron microscopy
TcR	T-cell receptor
TG	Triglycerides in mmol/L
TNF	Tumor necrosis factor
TUNEL	Transferase-mediated dUTP nick-end labeling
UKPDS	United Kingdom Prospective Diabetes Study
VLDLC	Very low density lipoprotein cholesterol in mmo/L
vWF	von Willebrand Factor (old term Factor VIII)
WHO	World Health Organization

19q13.33	BAX gene locus on chromosome 19 on the long arm at cytogenetic band 19q13.33
18q21.33	BCL-2 gene locus on chromosome 18 on the long arm at cytogenetic band 18q21.33
14q32	Ig heavy chain locus on chromosome 14 on the long arm at cytogenetic band 14q32
12p13.2	Ig heavy chain locus on chromosome 12 on the long arm at cytogenetic band 12p13.2

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ABSTRAK

Perubahan salur darah pada pesakit diabetes jenis 2 berkait rapat dengan kesan jangka panjang komplikasi diabetes. Sehubungan dengan itu, kajian ini bertujuan untuk mengkaji hubungan antara parameter histologi seperti penanda apoptotsis, Bax (penanda pro-apoptotsis), Bcl-2 (penanda anti-apoptotsis) dan perubahan morfologi salur darah menggunakan teknik immunohistokimia (IHC), haematoxylin dan eosin (H&E) dan pewarnaan khas bagi perubahan salur darah yang terdiri daripada ketebalan sel endotelium (ECT), pengiraan sel endotelium (ECC) dan pengiraan salur darah (BVC) dengan parameter biokimia kawalan diabetes untuk membolehkan parameter histopatologi digunakan sebagai prediktor vaskulopati. Kajian hirisan lintang ini bermula dari Ogos 2003 hingga November 2005. Subjek kajian terdiri daripada 41 orang pesakit diabetes jenis 2 dan 36 orang bukan pesakit diabetes (kontrol), berumur antara 20 hingga 70 tahun dari Hospital Universiti Sains Malaysia. Biopsi kulit dan sampel darah diambil daripada setiap pesakit diabetes dan kontrol. Tisu biopsi kulit diwarnakan dengan pewarnaan histokimia untuk mengkaji ekpresi Bax dan Bcl-2 yang dilakukan di makmal Patologi, Pusat Pengajian Sains Perubatan (PPSP), USM. Ujian darah yang dibuat adalah glukosa plasma semasa berpuasa (FPG), trigliserida (TG), kolesterol total (TC), kolesterol lipoprotein ketumpatan tinggi (HDL-c) dan kolesterol lipoprotein ketumpatan rendah (LDL-c) yang telah dilakukan di makmal Patologi Kimia, PPSP, USM. Manakala ujian darah hemoglobin A1c

terglikosil (HbA1c) telah dilakukan di makmal Endokrin, PPSP, USM. Didapati bahawa taburan ekspresi Bax meningkat dengan signifikan ($p<0.05$) apabila dibandingkan di antara pesakit diabetes dan kontrol. Manakala ekspresi Bcl-2 didapati meningkat di kalangan pesakit kontrol dengan sangat signifikan ($p<0.001$) apabila dibandingkan dengan pesakit diabetes. Didapati bahawa min ECT, ECC dan BVC pula menunjukkan perbezaan yang signifikan ($p<0.05$) di kalangan pesakit diabetes dan kontrol yang menunjukkan ekspresi Bax dan Bcl-2 positif atau negatif. Seterusnya, min FPG menunjukkan perbezaan yang signifikan dengan ekspresi Bax positif dan negatif. Walaubagaimanapun, tidak ada perbezaan nyata di antara bacaan A1C, TG, TC, HDL-c dan LDL-c dengan ekspresi Bax. Menariknya, FPG dan HbA1c menunjukkan perbezaan signifikan yang tinggi ($p<0.001$) dengan ekspresi Bcl-2. FPG dan HbA1c juga menunjukkan perbezaan signifikan yang tinggi ($p<0.001$) di antara pesakit diabetes dan kontrol. Perubahan salur darah menunjukkan perbezaan min ECT, ECC dan BVC yang amat signifikan di antara pesakit diabetes dan kontrol, tetapi tidak ada perbezaan nyata bagi TG, TC dan LDL-c. Kesimpulannya, kajian ini telah mendapati bahawa ahli famili Bcl-2 seperti Bax dan Bcl-2 memainkan peranan penting dalam penghasilan salur darah yang baru tetapi tidak normal. Penemuan baru adalah ECT, ECC dan BVC memainkan peranan dalam mengenalpasti perubahan salur darah yang tidak normal. Perubahan salur darah juga berkait dengan kandungan biokimia darah pesakit diabetes seperti FPG dan HbA1c, manakala kandungan lipid profilnya tidak konsisten.

EXPRESSION OF APOPTOSIS MARKERS IN THE SKIN MICROVASCULATURE OF PATIENTS WITH DIABETES MELLITUS TYPE 2 AT HOSPITAL UNIVERSITI SAINS MALAYSIA

ABSTRACT

The microvascular changes in diabetes are directly related to the long term complications of diabetes. Therefore, this research is proposed to study the relationship between the histopathological parameters such as apoptotic markers namely Bax (pro-apoptotic marker) and Bcl-2 (anti-apoptotic maker) and morphology of microvasculature which are endothelial cell thickness (ECT), endothelial cell counting (ECC) and blood vessel counting (BVC) using immunohistochemistry (IHC), haematoxylin & eosin (H&E) and special stain of vasculopathy with biochemical parameters of diabetes control, in order to use the histopathology parameters as predictors of diabetes vasculopathy. A cross-sectional study was conducted from August 2003 to November 2005. Forty one type 2 diabetes patients and 36 non diabetes (control) patients (20 to 70 years old) from Hospital Universiti Sains Malaysia (HUSM) were included in this study. Skin biopsy and blood samples were taken from each diabetes and control patients. The skin biopsy tissue samples were stained with immunohistochemistry stain for Bax and Bcl-2 expression in the Pathology laboratory, School of Medical Sciences, USM. Blood samples were collected for fasting plasma glucose (FPG), triglycerides (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-c) and low density lipoprotein cholesterol (LDL-c) analyses in the Chemical Pathology laboratory. The glycosylated hemoglobin A1c (HbA1c) was analysed in the Endocrine laboratory. Bax expression was significantly increased in diabetes patients ($p<0.05$). However, Bcl-2 expression was significantly increased ($p<0.001$) in control patients when

compared with diabetes patients. There were significant differences ($p<0.05$) of mean ECT, ECC and BVC between diabetic and control patients with positive or negative Bax and Bcl-2 expressions. FPG showed significant difference of mean ($p<0.05$) with expression of positive and negative Bax. However, there were no significant differences for HbA1c, TG, TC, HDL-c and LDL-c with Bax expression. Interestingly, FPG and A1C showed highly significant differences ($p<0.001$) with Bcl-2 expression. Between diabetic and control patients, there was a significant mean difference ($p<0.001$) for FPG and HbA1c, but there were no significant differences for TG, TC, and LDL-c. The mean difference of microvasculature was significant ($p<0.05$) for ECT, ECC and BVC between diabetes and control patients. From this study we found that the expression of Bcl-2 family such as Bax and Bcl-2, play an important role in contributing to abnormal blood vessel proliferation in diabetic patients. A new finding is that ECT, ECC and BVC are indicative of blood vessel abnormality in diabetic patients. These microvasculatures in diabetes patients were also associated with biochemical changes such as in FPG and HbA1c, while the lipid profile fraction associations were inconsistent.

CHAPTER 1

INTRODUCTION

1.1 Type 2 Diabetes

Type 2 Diabetes is a common chronic disorder and represents a serious disease in the world nowadays. DM referred to as diabetes, is characterized by the presence of an excess of glucose in the blood and tissues of the body. Diabetes in Greek means siphon, referring to the release of excess urine, and mellitus is honey in Latin, implying the presence of glucose in urine. Thus, diabetes mellitus means the passage of large amount of glucose enriched urine. There are a number of different types of DM. All of these have different aetiologies and course of treatment but subjects with diabetes have a common problem, whereby the body has little or no ability to absorb glucose from the bloodstream into cells. Subsequently, the cells cannot get hold of their primary fuel source, glucose and the blood glucose level rises. Usually the blood glucose level is controlled by the hormone called insulin. It helps cells to absorb glucose from the bloodstream and use it for energy production. Consequently, the failures of production or the action of insulin cause diabetes.

The prevalence of DM is on the increasing and an estimated 239 million people worldwide are expected to develop DM by the year 2020. Data shows that in 1995, about 1,380,000 adults had confirmed DM, and it is has been estimated that about 1,000,000 more people have diabetes which has not been diagnosed (www.diabetes.org.uk, 2002). Diabetes prevalence increases with age, where in United Kingdom, one in twenty people over the age of 65 years

have type 2 diabetes and this increases to one in five people over the age of 85 years (www.doh.gov.uk/nsf/diabetes, 2002).

The factors influencing the increase in type 2 diabetes are varied and as yet have not been fully understood. Nevertheless, these factors are expected to consist of an increase in the number of people at risk of developing type 2 diabetes due to refusal of physical activity, increasing dietary energy intake, survival of people with type 2 diabetes and recognition of previously undiagnosed type 2 diabetes. Early detection of type 2 diabetes is a crucial factor in preventing the development of diabetic complications. There are now some good evidence that changes in lifestyle may help to delay and possibly prevent the onset of type 2 diabetes.

After taking so long to gain recognition, the awareness in diabetes is now rising speedily (Zimmet, 1999) and it is a stimulating time for researchers and clinicians to be involved in the study and treatment of the disease. According to the report from Mafauzy *et al.* (1999) the prevalence of DM and impaired glucose intolerance (IGT) were 10.5 % and 16.5 % respectively in Kelantan, a north-east state of Malaysia (Mafauzy *et al.,* 1999). Meanwhile 0.3 % and 4.4 % were Orang Asli and 4.7 % and 11.3 % were Malays (Ali *et al.*, 1993; Choi & Shi, 2001).

Type 2 diabetes is more prevalent in the general population and the increasing prevalence (8%-45%) of type 2 diabetes in children and adolescents may be reversed within one to two decades (Fagot-Campagna et al., 2000; Fagot-Campagna & Narayan, 2001).

1.2 Distribution of Type 2 Diabetes

The worldwide prevalence of diabetes (>90% of cases of diabetes) is subject to increase from the present estimate of 150 million to 220 million in 2010, and 300 million (5.4% of the world population) in 2025 (Amos *et al.,* 1997; King *et al.,* 1998). The prevalence of type 2 diabetes in America is the highest among Pima Indians, followed by Hispanics, Blacks and Whites (Ismail & Gill, 1999). The diabetes epidemic around the world has been most marked in non-European populations, as evidenced by studies from Native American and Canadian communities, Pacific and Indian Ocean island populations (de Courten *et al.,* 1997), groups in India (Ramachandran *et al.,* 1997) and Australian Aboriginal communities (O'Dea, 1991). In the Pacific island of Nauru, where diabetes was almost unknown 50 years ago, it is now present in approximately 40% of adults (Zimmet *et al.,* 1990).

The potential for increase in the number of cases of diabetes is greatest in Asia (Amos *et al.,* 1997). Data from Mauritius demonstrated the highest yet reported prevalence in people of Chinese pulling out, in addition to demonstrating a high diabetes prevalence and a distinguished worldly increase between 1987 and 1998 in Asian Indians and Creoles (Zimmet, 1999). Together with evidence of prevalence of type 2 diabetes which has doubled

between 1984 and 1992 in Singaporean Chinese (Tan *et al.,* 1999), and the high prevalence in Taiwan (Chou *et al.,* 1992), these data provide alarming indicators of the size in future epidemic in the People's Republic of China. Here, the overall prevalence of type 2 diabetes was, until recently, less than 1%. Recent studies showed a threefold increase in prevalence in certain areas of China within the past two decades (Pan *et al.,* 1997).

The Third National Health and Nutrition Examination Survey (NHANES III), 1988-1994 data show that diabetes (diagnosed and undiagnosed combined) affects 7.8% of adults aged 20 years and above. In the U.S., the rate is 18.8% for adults aged 60 years and above (Harris *et al.,* 1998).

The high prevalence of undiagnosed diabetes and the proportion of cases with evidence of complications at diagnosis undoubtedly create a very important need for screening. Between 35-50% of diabetes cases are undiagnosed at any one time.

Most cases will be of type 2 diabetes, which is strongly associated with a sedentary lifestyle and obesity (Zimmet, 1999). This trend of increasing prevalence of diabetes and obesity has already imposed a huge burden on healthcare systems and this will continue to increase in the future (Zimmet, 2000; ADA, 1998).

The new prevalence of diabetes cases were 0.2% (0% to 1.4%) and 2.8% (1.6% - 4.7%) in patients whose individual risk factor was age over 45 and in patients aged over 45 with one or more additional risk factors for diabetes, respectively (Lawrence *et al.,* 2001). The higher diabetes prevalence was associated with ethnic group, age (≥ 40 years), dietary intake, obesity and lack of physical activity.

1.3 Diagnosis of Type 2 Diabetes

Diagnosis of type 2 diabetes depends on the measurement of glucose levels in the blood. Oral glucose tolerance testing (OGTT) was introduced when the measurement of blood glucose was much less accurate than it is today. It involves measuring the rise and subsequent fall of blood glucose values after drinking 75 g of anhydrous glucose from the start to 2 hours after drinking (the defining glucose values vary according to which type of sample is used). The test is often performed with an overnight fasting and the 2 hours values which are generally the most informative. Indeed, with specific blood glucose measurement the fasting value alone is very rational of glucose tolerance.

When the symptoms are clear cut, random or fasting glucose levels will be remarkably raised and diagnostic. The correct defining levels are a matter of opinion and vary from time to time. It is most useful to refer to the recommendations from the current World Health Organization (WHO, 1999) (**Table 1.1**). The differences between plasma and whole-blood glucose concentrations and between capillary and venous level, are too frequently unnoticed. Whole-blood values are about 10 to 15 percent lower than those of plasma and capillary values are 7 percent higher than venous values in the fasting state and 8 percent higher after a glucose load. These differences are important because clinical laboratories may use venous plasma whereas bedside monitoring techniques use capillary whole blood or plasma.

Table 1.1 Level of glucose for fasting period and after 2 hours taking glucose for DM diagnostic and for hyperglycemia categories

Categories	Intake Time	Concentration of Glucose, mmol/L (mg/dl)			
		Whole blood		Plasma	
		Venous	Capillary	Venous	Capillary
Diabetes mellitus	Fasting	≥ 6.1 (110)	≥ 6.1 (110)	≥ 7 (126)	≥ 7 (126)
	2 hours after taking glucose*	≥ 10 (180)	≥ 6.1 (200)	≥ 11.1 (200)	≥ 12.2 (220)
Impaired Glucose Tolerance (IGT)	Fasting	< 6.1 (110)	< 6.1 (110)	< 7 (126)	< 7 (126)
	2 hours after taking glucose*	≥ 6.7- < 10 (120-180)	≥7.8 - <11.1 (140-200)	≥7.8 -<11.1 (140-200)	≥8.9-<12.2 (160-220)
Impaired Fasting Glucose (IFG)	Fasting	≥ 5.6-<6.1 (100-110)	≥ 5.6-<6.1 (100-110)	≥ 6.1-<7 (110-126)	≥ 6.1-<7 (110-126)
	2 hours after glucose load*	< 6.7 (120)	< 7.8 (140)	< 7.8 (140)	< 8.9 (160)

Source: Consultant report WHO (1999).
*75 g oral glucose load

1.4 Complications of Type 2 Diabetes

Diabetic complications are more frequent in type 2 diabetes patients with about 50% suffering from one or more complications at the time of diagnosis. The complications of diabetes, often called late complications are damage and loss of function in organs that have glucose transporters which are not sensitive to insulin and thus do not require insulin for glucose entry (Vinik *et al.,* 2003). Diabetic complications are divided into microvascular and macrovascular complications. Microvascular complications include damages to the retina of the eye leading to loss of vision (Bosco *et al.,* 2005), the nephrons of the kidney leading to chronic renal failure and the blood supply of cells of the peripheral nervous system with loss of nerve function. Whereas in macrovascular complications there are disorders of lipid metabolism and blood coagulation leading to narrowing of vessels and altered blood flow in the cardiovascular system resulting in heart attack, stroke and limb amputation (Cameron *et al.,* 2001). Dyslipidaemia, hypertension, obesity, smoking and lack of exercise are the other factors contributing to the risk of diabetic complications (Mwendwa *et al.,* 2005).

Development of diabetic complications in type 2 diabetes begins with repeated acute, but reversible, changes in cellular metabolism caused by increased levels of glucose. Alterations in target tissue function and structure follow and progress to the clinically recognised conditions referred to above as microvascular complications. Atherosclerosis is a result of macrovascular complications. Both microvascular and macrovascular disease can affect the coronary artery and cause cardiac dysfunction. Furthermore, in epidemiological

studies, the microvascular and macrovascular complications are linked. The morbidity risk associated with all types of diabetes mellitus is listed in **Table 1.2.**

Table 1.2 List of risks mobility complications in all types of DM

Complications	**Relative risk***
Amputation	40
End-stage renal disease (ESRD)	25
Blind	20
Myocardial infarction (MI)	2-5
Stroke	2-3

*Compared with non-diabetes patients (Donnelly *et al.,* 2000)

1.4.1 Microvasculature

Most of the long-term complications of diabetes mellitus stalk from failure of the microvasculature. It has long been documented that microvascular disease underlies diabetic retinopathy as well as nephropathy and diabetic foot (Wild et al., 2004). Recently, damage to the microvasculature has been a concern in diabetic cardiopathy (Poornima *et al.,* 2006) as well as the pathogenesis of diabetic neuropathy (Kim and Robinson, 2006). Microangiopathy is not a singular process but involves different stages of development. In the development of diabetes, changes of microcirculation function are marked which may be reversible if the metabolic abnormality is normalised. Progressive increasing duration of diabetes causes structural adaptive changes to occur, most obvious of which is basement membrane thickening, owing to buildup of extravascular matrix proteins (fibronectin). The thickening of the basement membrane is a novel discovery and have been demonstrated in the microvasculature of the eye (Fong *et al.,* 2004), kidney (ADA, 1998), muscle (Klein *et al.*, 1987), and skin (Yasuda *et al.,* 1990).

The importance of structural adaptive changes of the underlying microvasculature that affect its functions has been discussed. It is adequately prevalent after prolonged duration of diabetes and is regarded as the ultrastructural hallmark of diabetes mellitus. Eventually, in many microvascular networks, complete failure of transfer function occurs possibly precipitated by microvascular occlusion. Such terminal event outcome is most well accepted in areas of underperfusion such as the retinal microcirculation. These areas of poor tissue nutrition may activate reparative mechanisms which in the case of

retinopathy may be damaging in themselves as the new vessels may develop into the vitreous of the eye and subjected to the risk of bleeding and hence obscuring the light path to the retina.

Although it is usual to believe that the microangiopathic complications occur frequently in type 2 diabetes, current clinical observation reveals that maculopathy is the major problem faced by those with type 2 diabetes (Jaap & Tooke, 1995). Nephropathy tends to run a slower course in type 2 diabetes.

Furthermore, it is well known that the prevalence of hypertension (in the absence of nephropathy) is much greater in type 2 diabetes, as is the overall risk of arterial disease, possibly reflecting the expression of the insulin resistance syndrome, of which type 2 diabetes is simply part of. As it becomes clearer that type 2 diabetes is a heterogeneous collection of conditions resulting from various degrees of insulin-resistance and β-cell failure, it may not be sufficient to fully understand the nature of diabetic microangiopathy.

The microvascular changes in diabetes are associated with endothelial dysfunction. Moreover, vascular endothelial cells form the inner lining of all blood vessels. During both normal and pathological development, the formation of new vessels and the regression of preexisting ones are dependent on the balance between endothelial cell proliferation and endothelial cell apoptosis. In mature vessels, endothelial cell turnover is also under the control of these tightly regulated phenomena. Since the vascular endothelium is involved in various physiological processes, endothelial cell apoptosis may represent an

initial step in a variety of pathological situations such as atherosclerosis and hypertension. As for other cell types, it has been hypothesized that interactions of endothelial cells with their microenvironment may be critical for their survival (Mallat & Tedgui, 2000).

However, another study noted that oxidative stress and the accumulation of advanced glycation end-products (AGEs) appears to promote the apoptosis of retinal microvascular cells and that antioxidants or AGEs inhibitors might ameliorate diabetic retinopathy (Yatoh *et al.,* 2006).

1.4.2 Diabetic foot

The burden of diabetic foot disease is set to raise in the future since the contributory factors, such as peripheral neuropathy and vascular disease are observed in more than 10% of people who are diagnosed with type 2 diabetes and the first year after diagnosis of diabetes is a stage of danger for foot ulcers and amputations. Furthermore, the prevalence of type 2 diabetes is increasing in the developing countries such as Africa, Asia and South America (Wild *et al.,* 2004). Peripheral neuropathy can cause symmetric sensory failure in the feet and legs, ensuing in the loss of protective sensation and the combination with vascular injury (diminished blood flow) and infection can lead to development of the diabetic foot. Neuronal and vascular complications can cause small lesion ulcers to develop on the foot from dry skin. These may go unseen by the patient (due to damage in sensory nerve function) until severe infection or gangrene becomes recognized. Patients with diabetes must be evaluated for risk of developing foot lesions (ADA, 1998).

Diabetic patients with sensory loss or vascular disease possibly with structural, skin or nail deformities are at high risk of developing foot ulcers and should be seen at regular intervals by a qualified professional in order to control diabetic foot problems (Ekere *et al.,* 2005). These patients need to be taught on the role of the loss of sensory protection in foot injury and trained how to perform daily foot care. They should keep away from recurring weight-bearing exercise such as jogging, step exercise and extended walking. Diabetic patients who are not at high risk should be educated to understand basic preventative measures for foot care and have their feet inspected routinely.

Basic preventative measures include foot hygiene, well-fitted footwear, daily inspection of feet, avoidance of foot trauma and seeking professional help if problem occurs such as avoiding self-care of ingrown toenails, corns or athlete foot (Knowels *et al.,* 1997). Those who develop new swelling, redness, discolourations, pain or ulceration of their feet should be referred urgently to a multidisciplinary foot care service (www.nice.org.uk, 2005).

1.4.3 Diabetic Neuropathy

Diabetic neuropathies are a family of nerve disorders caused by diabetes. Diabetic neuropathies are among the most common of all the long-term complications of diabetes affecting up to 50% of patients (Dyck *et al.,* 1993). People with diabetes face the risk of possible damage to nerves throughout the body. Neuropathies lead to numbness and sometimes pain and weakness in the hands, arms, feet and legs (Macleod, 1997). Problems may occur in every organ system, the digestive tract, heart and as well as sex organs. People with diabetes can experience nerve problems at any time, but the duration of diabetes, probably leads to high risk. An estimated 50% of those with diabetes have some form of neuropathy, but not all with neuropathy have symptoms. The highest rates of neuropathy are among people who have diabetes for at least 25 years.

Diabetic neuropathy is also more frequent in people who have had problems controlling their blood glucose levels, in those with high levels of blood lipids and blood pressure, in overweight people, and in people over the age of 40. The most common type is peripheral neuropathy, also called distal symmetric neuropathy, which affects the arms and legs (Abbott *et al.,* 1998).

The causes are possibly different for different varieties of diabetic neuropathy. Researchers are studying the effect of glucose on nerves to find out exactly how prolonged exposure to high glucose causes neuropathy (Oyibo *et al.,* 2002). Nerve damage is due to metabolic circumstances, such as high blood glucose, long duration of diabetes, possibly low levels of insulin and

abnormal blood lipid levels (Boulton *et al.,* 2005). Other factors that contribute to diabetic neuropathy such as neurovascular factors which damage the blood vessels that carry oxygen and nutrients to the nerves, autoimmune factors that cause inflammation in nerves, mechanical injury to nerves, such as carpal tunnel syndrome, inherited traits that increase susceptibility to nerve disease and lifestyle factors such as smoking or alcohol use.

Symptoms depend on the type of neuropathy and which nerves are affected. Some people have no symptoms at all. For others, numbness, tingling, or pain in the feet is often the first sign (Oyibo *et al.,* 2002). A person can experience both pain and numbness. Often, symptoms are minor at first, and since most nerve damage occurs over several years, mild cases may go unnoticed for a long time. Symptoms may involve the sensory or motor nervous system, as well as the involuntary (autonomic) nervous system (Vinik *et al.,* 2003).

1.4.4 Diabetic Nephropathy

Diabetic nephropathy is caused by damage to the kidney and is the single leading cause to end-stage renal disease (ESRD). It is a major cause of mortality in diabetic patients (ADA; 1998d; 2002a). Mortality rate from cardiovascular disease in people with diabetes who also had nephropathy are up to eight times higher than in people who do not have diabetic nephropathy. The incidence of nephropathy in type 2 diabetic patients varies with ethnic origin and ranges from 25% (Europeans) to 50% (African-Caribbean, South Asian and Japanese). The higher incidence in African-Caribbean and South Asian may be due to higher frequency of arterial hypertension and a younger age of onset of type 2 diabetes, respectively (Viberti, 1997).

Diabetic nephropathy is defined as the presence of albuminuria from urine test. The clinical symptoms of diabetic nephropathy develop over several years. Early changes in renal function include glomerular hyperfiltration, increased renal blood flow and hypertrophy of the kidney. Microalbuminuria (albumin excretion rate 30-299 mg/24hours) is the first sign of diabetic nephropathy. Macroalbuminuria develops in 20-40% of type 2 diabetic patients with microalbuminuria over a period of 10-15 years. In these patients with macroalbuminuria, the glomerular filtration rate falls over a period of several years. Once overt nephropathy (macroalbuminuria) occurs, ESRD develops only in 20% of type 2 diabetic patients after 20 years (ADA, 2002a). Patients with macroalbuminuria (clinical nephropathy) have an albumin excretion rate >300 mg/24hours. There are three methods for microalbuminuria screening. The first method is measurement of albumin to creatinine ratio in a random spot

urine collection. Secondly, 24-hr urine collection with creatinine, allowing the simultaneous measurement of creatinine clearance and finally timed urine collection (**Table 1.3**).

Table 1.3 Definitions of abnormalities in albumin excretion

Category	24-hr collection (mg/24 hr)	Timed collection (µg/min)	Spot collection (µg/mg creatinine)
Normal	<30	<20	<30
Microalbuminuria	30-299	20-199	30-299
Clinical albuminuria	≥300	≥200	≥300

Because of variability in urinary albumin excretion, two of three specimens collected within a 3 to 6 month period should be abnormal before considering a patient to have crossed one of diagnostic threshold. Exercise within 24 hr, infection, fever, congestive heart failure, marked hyperglycemia, marked hypertension, pyuria and hematuria may elevate urinary albumin excretion over baseline values (ADA, 2002a).

In people with diabetes who already have nephropathy, tight blood glucose control and tight control of high blood pressure can significantly reduce the decline in renal function.

1.4.5 Diabetic Retinopathy

Estimates of the incidence of diabetic retinopathy range widely and are increasing around the world. About 135 million individuals are diagnosed with diabetic retinopathy. Data from ADA demonstrated that in America diabetic retinopathy affects over 18.2 million people or 6.3% of the total population. Retinopathy results from damage to the capillaries supplying the retina and is a major cause of blindness in people with diabetes (Donald *et al.,* 2004; ADA, 1998a; 2001; 2002b). The risk of developing retinopathy increases with duration of diabetes (ADA, 1998a; 2001; 2002b) and poor blood glucose control (Donald *et al.,* 2004). Other risk factors for retinopathy include hypertension, dyslipidaemia (ADA, 1998a; 2001; 2002b) and pregnancy (www.doh.gov.uk/nsf/diabetes, 2002). The main types of retinopathy associated with visual loss are proliferative retinopathy in which new blood vessels develop, thus leading to haemorrhage and scarring. Mean while, maculopathy occurs where there is capillary blood vessel leakage into the retina. If untreated, 6-9% of people with proliferative retinopathy become blind each year and 10% of people with maculopathy develop moderate visual loss each year.

A recent study (Donald *et al.,* 2004) proposed that there is a link between hyperglycemia and biochemical pathway leading to microvascular complications. This includes activation of protein kinase C (PKC), polyol pathway, formation of AGEs and oxidative stress. These processes are thought to modulate the disease process through effects on cellular metabolism,

signaling and growth factors. In addition, it is important to keep the blood glucose in normal range in order to prevent diabetic retinopathy.

1.5 Apoptosis

1.5.1 Definition and Causes

Apoptosis is energy-dependent, natural, genetically controlled process by which the organism eliminates unnecessary single cells (Mallat & Tedgui, 2000). The term apoptosis had been coined in order to explain the morphological processes principal to controlled cellular self-destruction and was first discovered in a publication by Kerr, Wyllie and Currie (Kerr, 1972). Apoptosis is of Greek origin, meaning “falling off or dropping off”, in analogy to leaves falling off trees or petals dropping off flowers. This analogy emphasizes that the death of living matter is an essential and compulsory part of the life cycle of any organism.

The apoptotic form of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and preservation of the cell populations in tissues in physiological and pathological conditions. It should be stressed that apoptosis is a well-defined and possibly the most common form of programmed cell death, but that other, non apoptotic types of cell death also might be of biological significance (Leist, 2001).

Apoptosis is an orderly and tightly controlled series of events in gene expression and protein activities. It plays a major role in normal growth and differentiation of organ systems and appears to be involved in a variety of human disorders (Li *et al.,* 2004).

Apoptosis is associated with the onset and development of diabetes. It has been demonstrated that free fatty acids (Lupi *et al.,* 2002), protooncogene (c-Myc) (Laybutt *et al.,* 2002), oxidative stress (Gorogawa *et al.,* 2002), high glucose (O'Brien *et al.,* 1997), and Fas (Savinov *et al.,* 2003) induce apoptosis of pancreatic β-cells, leading to the development of diabetes. Furthermore, high glucose, AGEs, glycated LDL and oxidative stress have been revealed to induce cell apoptosis in retina (Mizutani *et al.,* 1996), kidney, neuron, myocardial cells and vascular endothelial cells, which is related to the acceleration of diabetic complications and atherosclerosis (Podesta *et al.,* 2000; Mohr *et al.,* 2002 & Artwohl *et al.,* 2003).

There are two gene families that are mostly important in the control of apoptosis; the genes encoding the interleukin-1–converting enzyme (ICE) family of cysteine proteases (caspases) and those related to the proto-oncogene Bcl-2. Both of these families are homologous to cell death genes in Caenorhabditis elegans (C. elegans) (Hale *et al.,* 1996). Proteolytic activity is implicated in many apoptotic systems, the ICE family being of special importance, since it seems to be central in Fas-mediated and tumor necrosis factor (TNF)-induced apoptosis.

Proteolysis is probably a common incident in the apoptotic process, with different proteases involved and several proteins having been shown to be the subject of proteolytic activity.

New evidence suggests that Bcl-2 protein has two different functions: *1*) as an ion channel protein and *2*) as an adaptor/docking protein through its binding to several other proteins which are important in modulating the apoptosis. However, the precise way in which these proteins modulate apoptosis is unclear and conflicting theories have been proposed (Hale *et al.,* 1996). Further, the gene product of Bcl-2 does not prevent apoptosis under all conditions (e.g., does not protect target cells from apoptosis induced by cytotoxic T cells [Vaux *et al.,* 1992]). Finally, genes involved in cell differentiation and proliferation are also important in modulating the apoptotic process (e.g., the c-Myc, the p53, and the apoptosis suppressor gene A20). Both c-Myc and p53 are implicated in the induction of apoptosis under certain conditions, whereas A20 is a cytokine-induced primary response gene involved in the inhibition of the apoptotic process (Hale *et al.,* 1996).

1.5.2 Morphology

Apoptotic cells can be recognized by morphological changes which are the cell shrinks, shows deformation and looses contact with its neighbouring cells (**Figure 1.1**). Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organelles. The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response (Kerr *et al.,* 1972).

Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most particularly the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles (Saraste, 2000). Apoptosis is in contrast to the necrotic mode of cell death in which case the cells suffer a major assault, resulting in a loss of membrane integrity, swelling and disruption of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Leist, 2001).

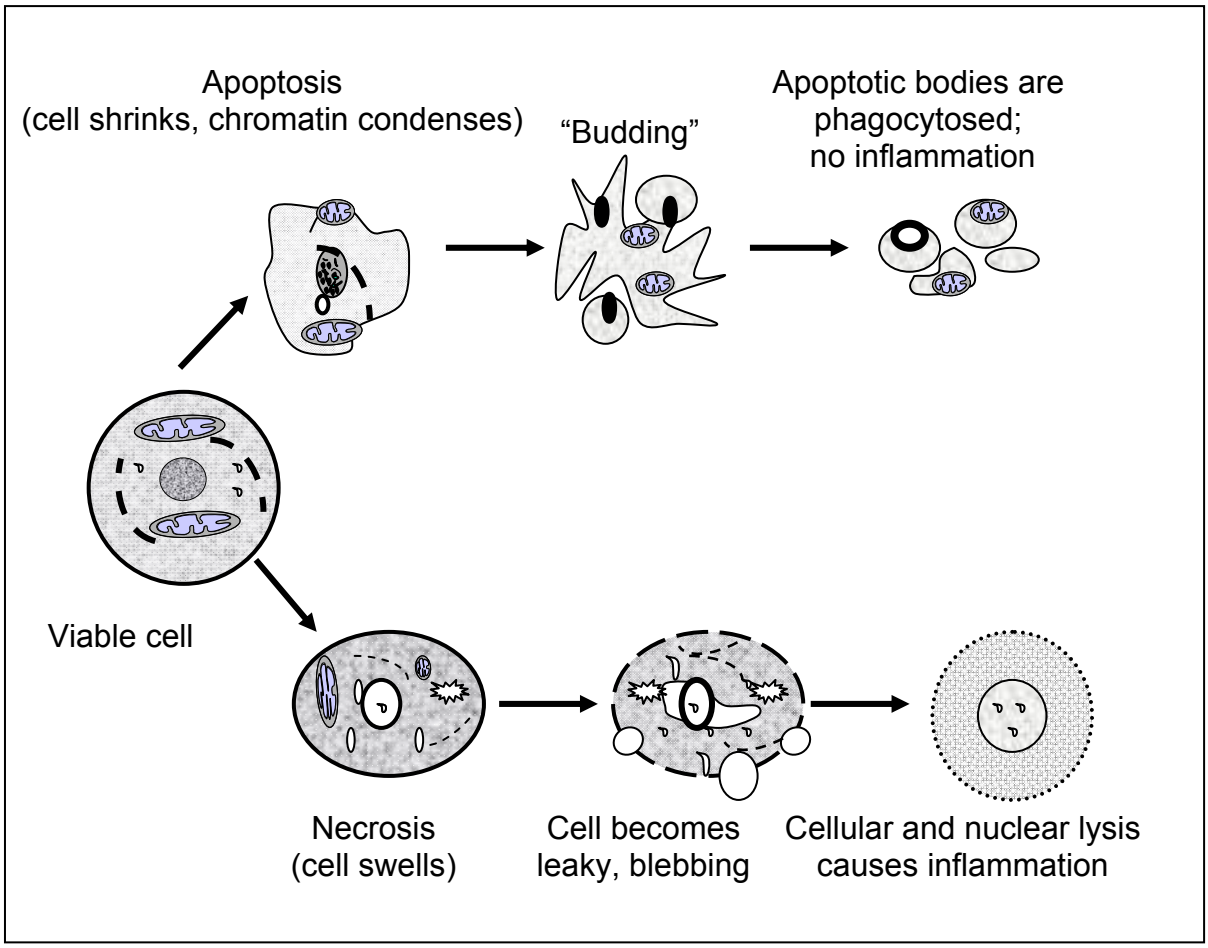

Figure 1.1 Hallmarks of the apoptotic and necrotic cell death process.

Apoptosis includes cellular shrinking, chromatin condensation and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies that contain organelles, cytosol and nuclear fragments and are phagocytosed without triggering inflammatory processes. The necrotic cell swells, becomes leaky and finally is disrupted and releases its contents into the surrounding tissue resulting in inflammation (taken from Van Cruchten, 2002).