AN ULTRASTRUCTURAL STUDY OF MACROVASCULAR CHANGES IN RATS WITH DIABETES MELLITUS AND HYPERTENSION

by

MICHAEL YUMING SONG

M.D., Capital Institute of Medicine, Beijing, China, 1986

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE in

THE FACULTY OF GRADUATE STUDIES

Department of Anatomy

Faculty of Medicine

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1992

© Michael Yuming Song, 1992
In presenting this thesis in partial fulfillment of the requirements for an advanced degree at The University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Anatomy

The University of British Columbia
2177 Wesbrook Mall
Vancouver, B.C.
Canada V6T 1Z3

Date:  <<29, Sep. 1992>>
ABSTRACT

Numerous investigators have presented evidence of increased mortality in patients with diabetes mellitus due to cardiovascular disease. The reasons for a predisposition to vascular pathology that, in the advanced state, can lead to atherosclerosis are still unclear.

Our hypotheses were: 1) the condition of diabetes mellitus in a streptozocin animal model may show vascular changes similar to early pathology in macrovessels, 2) since the model is normotensive, inducing hypertension will result in early atherogenic pathology, 3) the response to diabetic and/or hypertensive circumstances may be diversified due to the function of blood vessels, and 4) endothelium is the initiator of vascular pathology in diabetes. To test these hypotheses, we carried out a quantitative analysis of the renal and coronary arteries from rats using light, transmission and scanning electron microscopy. Diabetes mellitus was induced with streptozocin and hypertension was induced using deoxycorticosterone acetate. There were four experimental groups: control, control/hypertensive, diabetic, and diabetic/hypertensive.

In the renal artery, the tunica media and luminal areas significantly increased in the hypertensive groups, but proportionately, the vessel dimensions did not alter, with all four groups having similar tunica media/lumen ratios. There was a significant elevation of extracellular matrix surrounding
the smooth muscle cells of the tunica media in the renal artery due to the diabetic condition, although there were no changes in wall dimensions. Renal arteries from the control/hypertensive group had a significantly thickened tunica media as did the diabetic/hypertensive group over control values. The latter also had an even greater significant elevation of the extracellular matrix compared with either the diabetic or control/hypertensive group. This means that the composition of the tunica media had significantly altered, with more connective tissue. In addition, there was marked subendothelial invasion of macrophage-type cells and electron-dense deposits of various shapes and densities. The number of adherent white blood cells, probably monocytes, on the endothelium was significantly increased in the renal artery from the hypertensive groups.

In the coronary artery, in contrast, the pattern of pathological responses was different from that seen in the renal artery. The tunica media and luminal areas were significantly less in the diabetic condition even though there was no alteration in the makeup of the tunica media. The hypertensive groups had an altered composition of the tunica media with more extracellular matrix compared with the controls. Both control/hypertensive and diabetic/hypertensive groups had not only a significant increase in medial and luminal areas, but also both had a proportionately greater
hypertrophy of the media compared with the increase in luminal areas. This resulted in significant differences in media/lumen ratios over normotensive controls. When the two hypertensive groups were compared with each other, the diabetic animals did not increase to the same extent as the nondiabetic did in both tunica media and luminal areas. In addition, in both hypertensive groups the adventitial area significantly increased over that in the respective controls, which was greater than the luminal hypertrophy, giving significant differences in adventitial to luminal ratios. The tunica intima did not show the striking early lesion stage seen in the renal arteries from the diabetic/hypertensive group, and none of the four groups had adherent white blood cells.

We have, therefore, demonstrated different patterns of vascular changes due to the diabetic condition in this animal model. We have also shown that, with hypertension and diabetes combined, the early vascular pathology is exacerbated in the renal artery but not in the coronary artery. The vessel proportions in the coronary artery also changed due to hypertension, in contrast to the renal artery where vessels from animals in all four groups had similar wall/lumen ratios.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER 1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 HISTORICAL REVIEW OF DIABETES MELLITUS:</td>
<td>1</td>
</tr>
<tr>
<td>Definition and classification</td>
<td>2</td>
</tr>
<tr>
<td>1.2 COMPLICATIONS OF DIABETES MELLITUS</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1 Diabetic hypertension</td>
<td>5</td>
</tr>
<tr>
<td>1.3 PATHOGENIC MECHANISMS OF VASCULAR COMPLICATIONS IN THE DISEASE</td>
<td>6</td>
</tr>
<tr>
<td>1.3.1 Hyperglycemia</td>
<td>6</td>
</tr>
<tr>
<td>1.3.2 Hyperlipidemia</td>
<td>8</td>
</tr>
<tr>
<td>1.3.3 Hyperinsulinemia</td>
<td>9</td>
</tr>
<tr>
<td>1.3.4 Hypertension</td>
<td>10</td>
</tr>
<tr>
<td>1.3.5 Other risk factors</td>
<td>11</td>
</tr>
<tr>
<td>1.3.6 Interrelationships among risk factors</td>
<td>11</td>
</tr>
<tr>
<td>1.4 EXPERIMENTAL DIABETES MELLITUS IN RATS</td>
<td>12</td>
</tr>
<tr>
<td>1.5 RESEARCH HYPOTHESES</td>
<td>13</td>
</tr>
<tr>
<td>CHAPTER 2. MATERIALS AND METHODS</td>
<td>14</td>
</tr>
<tr>
<td>2.1 INDUCTION OF DIABETES MELLITUS AND HYPERTENSION</td>
<td>14</td>
</tr>
<tr>
<td>2.2 PERFUSION FIXATION</td>
<td>15</td>
</tr>
<tr>
<td>2.3 PREPARATION FOR ANALYTICAL TRANSMISSION AND TRANSMISSION ELECTRON MICROSCOPY (TEM)</td>
<td>16</td>
</tr>
<tr>
<td>2.4 PREPARATION FOR SCANNING ELECTRON MICROSCOPY (SEM)</td>
<td>17</td>
</tr>
<tr>
<td>2.5 MORPHOMETRIC ANALYSIS OF RENAL AND CORONARY ARTERIES</td>
<td>17</td>
</tr>
<tr>
<td>2.6 STATISTICAL ANALYSIS</td>
<td>18</td>
</tr>
<tr>
<td>CHAPTER 3. RESULTS</td>
<td>19</td>
</tr>
<tr>
<td>3.1 BODY WEIGHT</td>
<td>19</td>
</tr>
<tr>
<td>3.2 BLOOD PRESSURE</td>
<td>19</td>
</tr>
<tr>
<td>3.3 MORPHOMETRIC STUDIES OF THE RENAL ARTERY</td>
<td>19</td>
</tr>
</tbody>
</table>
3.3.1 Medial and lumenal areas and medial to luminal (M/L) ratios ..........19
3.3.2 Percentage of extracellular matrix in the tunica media ..............20
3.3.3 Numbers of adherent monocytes ..........20
3.4 MORPHOLOGY OF THE RENAL ARTERY .........................21
  3.4.1 Light microscopy (LM) and TEM studies ......................21
  3.4.2 SEM studies ...........................................23
3.5 ATEM STUDIES OF ELECTRON DENSE MATERIALS ..........24
3.6 MORPHOMETRIC STUDIES OF THE CORONARY ARTERY ....24
  3.6.1 The medial, luminal and adventitial areas and medial to luminal (M/L), adventitial to luminal (A/L) ratios .........................24
  3.6.2 Percentage of extracellular matrix in the tunica media ..........25
3.7 MORPHOLOGY OF THE CORONARY ARTERY ....................26
  3.7.1 LM and TEM studies ..................................26

CHAPTER 4. DISCUSSION ........................................27
  4.1 ENDOTHELIAL SURFACE .....................................29
  4.2 ADHERENCE OF MONOCYTES ..................................30
  4.3 TUNICA INTIMA ............................................31
    Endothelial cells .........................................31
    Subendothelial space ......................................34
  4.4 TUNICA MEDIA .............................................36
  4.5 TUNICA ADVENTITIA .......................................38

CHAPTER 5. CONCLUSIONS ....................................40

CHAPTER 6. REFERENCES .....................................81
LIST OF TABLES

Table 1. .................................................................42
The body weight of animals in the four groups.

Table 2. .................................................................43
Systolic, diastolic and mean blood pressures of animals in the four groups.

Table 3. .................................................................44
Medial, luminal areas and media to lumen ratios of the renal artery.

Table 4. .................................................................45
Areas of tunica media, of the lumen, and of the adventitia and medial, adventitial to luminal ratios in the coronary artery.
LIST OF FIGURES

Figure 1. ...........................................46
The extracellular matrix percentage in the tunica media of the renal artery in the four groups.

Figure 2. ...........................................47
Number of adherent monocytes on the endothelium counted on cross section and specimens prepared for SEM.

Figure 3. ...........................................48
Light microscopic photographs of the renal artery from the four experimental groups.

Figure 4. ...........................................50
Electron microscopic photographs of the renal artery from the four experimental groups.

Figure 5. ...........................................52
Electron microscopic photograph of the renal artery from DIA group showing the surface of endothelium surface.

Figure 6. ...........................................54
Electron microscopic photograph of the renal artery showing the tunica intima from a DIA/HT rat.

Figure 7. ...........................................56
Electron microscopic photograph of the renal artery showing the tunica media from a DIA/HT rat.

Figure 8. ...........................................58
Electron microscopic photograph from a DIA/HT rat showing white blood cell adherence to the endothelial cells of the renal artery.

Figure 9. ...........................................60
Electron microscopic photographs from DIA/HT rats showing different types of electron-dense materials in the subendothelial space.

Figure 10. ...........................................62
Scanning electron microscopic photographs of the renal artery from the four experimental groups.

Figure 11. ...........................................64
Scanning electron microscopic photograph from DIA group showing the fibrous-like materials on the endothelium.

Figure 12. ...........................................66
Scanning electron microscopic photographs from DIA/HT rats showing the attached monocytes on the endothelium of renal arteries.
Figure 13. Scanning electron micrographs showing different sizes and shapes of monocytes in the renal arteries.

Figure 14. Scanning electron micrograph from a DIA/HT rat showing the macrophage backing into the blood stream.

Figure 15. Analytical transmission electron microscopic photograph of electron-dense materials in the subendothelial space in the renal artery from a diabetic hypertensive rat.

Figure 16. Analytical transmission electron microscopic photograph of the metal content in a red blood cell.

Figure 17. The extracellular matrix percentage in the tunica media of the coronary artery in the four groups.

Figure 18. Transmission electron micrographs of the coronary artery from the four experimental groups.

Figure 19. Transmission electron micrograph showing a fibroblast in the tunica adventitia in the coronary artery from the diabetic hypertensive group.
LIST OF ABBREVIATIONS

ATEM........Analytical electron microscopy
CON..........Control
CON/HT.......Control hypertensive
DIA..........Diabetic
DIA/HT........Diabetic hypertensive
DOCA..........Deoxycorticosterone acetate
HDL..........High density lipoprotein
IDDM..........Insulin-dependent diabetes mellitus
LDL..........Low density lipoprotein
LM..........Light microscopy
NIDDM..........Noninsulin-dependent diabetes mellitus
SEM..........Scanning electron microscopy
STZ..........Streptozocin
TEM..........Transmission electron microscopy
VLDL..........Very low density lipoprotein
ACKNOWLEDGMENTS

I would like to thank the members of my committee, Drs. John H. McNeill, and William K. Ovalle for their direction and guidance in my undertaking of this work.

I would also like to thank Dr. Charles E. Slonecker, Head of the Department of Anatomy, for his help in facilitating my attendance at relevant conferences during the course of study.

I gratefully acknowledge the guidance, patience and encouragement of my supervisor Dr. Mary E. Todd throughout the course of this study.

This master's project was supported by grants from the Heart and Stroke Foundation of British Columbia and Yukon, and personal support in the form of a Fellowship (1991-92) and Traineeship (1992-93). In addition, I am grateful to the University of British Columbia through whom I received a Medical Faculty Summer Studentship (1990), and a University Graduate Fellowship (1990-91).
CHAPTER 1. INTRODUCTION

1.1 HISTORICAL REVIEW OF DIABETES MELLITUS

Knowledge of diabetes dates back to centuries before Christ (Cahill 1985). The Egyptian Papyrus Ebers described an illness associated with the passage of much urine. The renowned Greek physician, Aretacus of Cappadicia made the first complete clinical description, describing it as "a melting down of the flesh and limbs into urine". In the 3rd to 6th centuries A.D., scholars in China, Japan and India wrote of a condition with polyuria in which the urine was sweet and sticky. The name diabetes mellitus (mellitus=honey) was established by Willis in 1674 with the observation "as if imbued with honey and sugar". From the time of the earliest recorded history of diabetes, progress in the understanding of the disorder came slowly until the middle of the 19th century. Within the past century an association was established with a disturbance in the beta cells, clustered as tiny islets of tissue in the exocrine pancreas. These islets were described in mammals by Langerhans in 1869 and have borne his name since then. Soon after, the German scientists, Von Mering and Minkowski, found that surgical removal of the pancreas produced diabetes in the dog. The scientific world was overwhelmed with joy in 1921 when a young surgeon, Frederick Banting, and his graduate student assistant, Charles Best, working in Toronto, prepared active
extracts of pancreas which lowered the elevated glucose levels of diabetic dogs. Within months, children with diabetes who were slowly wasting away due to metabolic starvation as their flesh literally melted into sweet urine, regained strength promptly after starting treatment with insulin. Instead of dying of diabetic ketoacidosis and coma within weeks or months, life returned to almost normal temporarily, except for the need for insulin injections. The survival of the diabetic patient increased steadily. However, in the years 1948 to 1980, cardiovascular disease accounted for well over half of the deaths in a diabetic autopsy series (Legg and Harawi 1985), rather than the diabetes mellitus being the cause.

Definition and classification:
Diabetes mellitus is a grouping of anatomical and chemical problems resulting from a number of factors in which an absolute or relative deficiency of insulin or its function is a primary factor (Cahill 1985). It is a complex syndrome characterized by 1. hyperglycemia, secondary to deranged secretion and/or action of insulin; 2. specific microvascular complications, including thickening of capillary basement membranes along with retinopathy and nephropathy. 3. macrovascular disease, i.e. accelerated atherosclerosis, and 4. a variety of other complications including neuropathy, complicated pregnancy, and increased tendency to infection.

There are two types of diabetes, although in reality they form
INTRODUCTION

a spectrum of insulin deficiency. The individual totally or almost totally lacking insulin is termed an "insulin-dependant" or "type I" diabetic. At the other end of the spectrum is "noninsulin-dependent" or "type II" diabetes. In one broad group, "IDDM" or Type I, there is a correlation with certain inherited histocompatibility antigen types encoded on chromosome 6 which controls 1) alloantigenicity; 2) some components of the complement system; and 3) some immune responses. There are various degrees of both serologic and cell-mediated autoimmunity which produces antibodies to insulin and to cytoplasmic constituents of beta cells. Viral inflammation at or near the time of onset has also been indicated in its pathogenesis, which may initiate damage to the beta cells in a juvenile diabetic and this may lead to some sort of destructive process which eventually causes death to all or nearly all the beta cells. This type almost always ends in total insulin deficiency. The other broad group (NIDDM or Type II) does not have specific correlations either with histocompatibility genes, virus or autoimmunity, and usually has some remaining beta cell function. However, it is clearly genetically influenced since it occurs in identical twins with almost total concordance (Barnett et al. 1981). NIDDM is associated with obesity in more than 80% of patients (Kahn 1985). Insulin resistance in obesity has been found to be correlated with a decrease of insulin receptors on the fat cell
INTRODUCTION

(Archer et al. 1975). These receptors allow the specific recognition of insulin molecules, and trigger a series of intracellular events resulting in increased transport of substrates or alterations of enzyme activity.

1.2 COMPLICATIONS OF DIABETES MELLITUS

Diabetes mellitus is a disease that affects virtually all regions of the body with numerous metabolic and hormonal abnormalities which results in early functional changes as well as morphological evidence of injury in multiple target organs. Particularly, microvascular, macrovascular and neurologic lesions are prominent (Brownlee 1985, Bariety 1989, Jialal and Chait 1989, Mossaz and Assal 1989, Williamson and Kilo 1989). Coronary heart disease and other large-vessel diseases such as atherosclerosis, have long been observed clinically to occur earlier and with greater frequency in patients with diabetes than in the general population (Jarrett 1984). Cardiovascular disease, therefore is the main cause of death in the diabetic population (Marble 1976, West 1978). Early atherosclerotic lesions are characterized by the following (Schwartz et al. 1991): changes in arterial endothelial permeability, adherence of monocytes and transendothelial migration of monocytes, and intimal foam cells and fatty deposits. Later, migration of smooth muscle cells from the arterial media into the intima
occurs with proliferation of the "altered" smooth muscle cells. This is associated with increased synthesis of collagen, elastin, and proteoglycan by these cells. Thus, at least four major mechanisms - changes in endothelial permeability, monocyte activation, smooth muscle cell proliferation, and accumulation of connective tissue - are major features of atherosclerosis.

1.2.1 Diabetic Hypertension

Arterial hypertension is a common problem in patients with both type I and type II diabetes mellitus (Fuller 1985). From the data currently available, it is estimated that hypertension is not only approximately twice as common in the diabetic as in the nondiabetic population (Christlieb 1973, Fuller 1988), but also an additive risk factor for coronary, cerebrovascular, and peripheral vascular disease (Kannel and McGee 1979, Bennett 1988). Systemic hypertension contributes appreciably to cardiovascular morbidity and mortality by acceleration of diabetic micro- and macrovascular complications (Janka and Dirschedl 1985, Fuller 1988). Elevated arterial pressure enhances shear stress on endothelial cells and leads to increased transcapillary pressure gradients. Mechanisms involved in the genesis of diabetic hypertension could be hypervolemia and imbalance of the renin-angiotensin-aldosterone system. Studies have shown that hyperglycemia is associated
INTRODUCTION

with hypervolemia in diabetic rats by the osmotic effect of elevated glucose in the extracellular fluid (Christlieb 1974). Hyperinsulinemia may enhance sodium retention by a direct effect on the proximal tubule in the kidney as well as by indirect mechanisms involving various components of the renin-angiotensin-aldosterone system (DeFronzo et al. 1976, Baum 1987). These factors are directly causative in the development of hypertension.

1.3 PATHOGENIC MECHANISMS OF VASCULAR COMPLICATIONS IN THE DISEASE

1.3.1 Hyperglycemia

Hyperglycemia is believed to be the major cause of diabetic vascular complications including both micro and macrovascular vessels (West 1978). The effects of hyperglycemia include a direct effect on rate of atherogenesis, a deleterious effects on coagulation, vessel-wall metabolism, or nutrition, or osmotic effects. It is uncertain by which mechanism hyperglycemia changes the function of blood vessels, but several postulated mechanisms have been published. The increased polyol (sorbitol) pathway activity in endothelial cells in the diabetic has been shown to result in increased glucose flux and impaired oxygen action in the arterial wall (Gabbay 1973, Morrison et al. 1972). Some researchers have
demonstrated that chemically reactive nonenzymatic glycosylation end-products accumulate on interstitial matrix proteins, as a function of time and glucose concentration, forming covalent cross-links which leads to changes of physical properties of the arterial wall such as its elasticity (Bunn et al. 1978, McVerry et al. 1981, Brownlee 1989). Advanced glycosylation end-products contributed to increased vascular permeability, and alteration of endothelium surface coagulant properties in macrovasculature by binding to a specific macrophage receptor (Esposito et al. 1989). When this process is completed, tumor necrosis factor, interleukin-1, and possibly other cytokines are released from the endothelium and smooth muscle cell. These multifunctional cytokines not only increase vascular permeability but also induce synthetic and/or proliferative response of endothelial and smooth muscle cells (Brownlee 1989). Glycosylation end-products also accumulate continuously on long-lived vessel-wall proteins such as collagen which can result in a further complication by the entrapment of low density lipoproteins (LDL) in the arterial wall. Investigation in cultured cells and in vivo showed that hyperglycemia leads to the development of diabetic vascular complication by enhancing the membranous protein kinase C activity in endothelial cells (Lee et al. 1989). This can result in modulation of growth factor receptor turnover (Hachiya et al. 1987) and smooth muscle contraction (Jiang and
INTRODUCTION

Morgan 1987).

1.3.2 Hyperlipidemia

The lipids (cholesterol, triglycerides, phospholipids) are transported in human plasma as macromolecular complexes with specific proteins (apoproteins), thus forming lipoproteins. About 75% of circulating cholesterol are conveyed in the form of low density lipoproteins. It is possible that high serum levels of LDL may contribute to the process of endothelial injury. Oxidized LDL within the arterial wall may perpetuate endothelial injury, promote cellular lipid uptake and accumulation, and enhance monocyte recruitment (Rajavashisth et al. 1990, Cushing et al. 1990). The space-occupying intracellular (macrophages) and extracellular accumulation of cholesterol and cholesterol esters may contribute significantly to the growth of the atherosclerotic plaque; defects in the process of removal of cholesterol from the arterial wall may favor its accumulation and thus the progression of the disease; the regions of atherosclerotic plaques with a high content of fat are soft and vulnerable to rupture, causing subsequent formation of thrombus with rapid progression of the plaque. Biochemical studies have also demonstrated that many of the changes in lipid and lipoprotein metabolism can be attributed to hyperglycemia. For example, the overproduction of very low density lipoprotein triglyceride, in turn results from the
INTRODUCTION

increased flow of substrates, particularly glucose and free fatty acids, to the liver. In addition to inducing an overproduction of very low density lipoprotein triglyceride, noninsulin-dependent diabetes appears to be associated with a defect in clearance of very low density lipoprotein triglyceride. This decreased fractional catabolic rate returns to normal as glycemia improves (Howard 1989).

1.3.3 Hyperinsulinemia

Hyperinsulinemia reflects resistance to insulin's action and is a complex process representing a compensatory response to diminished target tissue sensitivity to insulin's effects, predominantly on glucose handling. It appears to be a potential factor in the enhanced atherosclerosis observed in diabetes mellitus. Epidemiologic and clinical studies reinforce the relationship between insulin and vascular disease. Atherosclerotic conditions such as macrovascular disease (Sloan et al. 1971) and coronary artery disease (Pyörälä 1979, Ducimetiere et al. 1980) all show strong independent correlations with hyperinsulinemia. Insulin itself can enhance proliferation of cultured smooth muscle cells, (Kuebler et al. 1983, Nelson et al. 1988), so the hyperinsulinemia could contribute to subendothelial vascular smooth muscle cell replication through the altered endothelial barrier. Insulin may also enhance sodium retention by a direct
effect on the proximal tubule as well as by an indirect mechanism involving various components of the renin-angiotensin-aldosterone system (DeFronzo et al. 1976, Petrasek et al. 1988). These factors are directly causative in the development of hypertension. Insulin could also promote atherosclerosis by acting on lipid and lipoprotein metabolism such as overproduction of very low density lipoproteins (Howard 1989).

1.3.4 Hypertension
The relationship between hypertension and atherosclerosis has long been recognized. High blood pressure contributes to the increase of intimal permeability, intimal thickening, and also there is increased adhesion of monocytes to endothelium, as well as migration of these cells to the subendothelium (Todd and Friedman 1972, Todd 1992). Hypertension can also increase smooth muscle proliferation (Friedman et al. 1971, Lee et al. 1983) which is an essential part of, and is intrinsic to the earliest phases of atherosclerosis by actions of growth factors derived from platelets and blood leukocytes (Schwartz et al. 1990). Calcium channel antagonists in experimental models of atherogenesis have shown that extracellular Ca$^{2+}$ entering the smooth muscle components is a major factor in the atherosclerotic damage (Fleckenstein et al. 1990, Weinstein 1990).
1.3.5 Other risk factors

Diabetic macrovascular disease is a multifaceted disorder. Numerous abnormalities in vascular endothelial function, platelet activity, blood clotting, and fibrinolysis have been described in both experimental and human diabetes. Platelet functional abnormalities has been observed in diabetics especially those with vascular complications, including platelet hypersensitivity and increased adhesiveness (Winocour 1989) which may be related to hyperglycemia either directly or through nonenzymatic glycosylation (see under hyperglycemia). Platelets are able to release a mitogen, platelet derived growth factor, that stimulates endothelial and smooth muscle cell proliferation (Ross et al. 1986). Hence, it may exacerbate the damage of arterial wall.

1.3.6 Interrelationships among risk factors

The increased risk of macrovascular disease in diabetes cannot simply be explained as a result of an increased prevalence of known risk factors such as hypertension or hypercholesterolemia. The course of the excess atherosclerosis in diabetics is probably multifactorial. Once diabetes is established, the levels of risk factors increase and perhaps interact with some other factors to augment their effect. Hyperinsulinemia appears to be a potential factor in the enhanced atherosclerosis, and hyperglycemia itself may also
alter vascular wall function. These effects on vasculature could contribute to hypertension, since structural and functional abnormalities in the arterial bed are well-established factors contributing to the development of hypertension. Platelets do not adhere to normal endothelium, therefore the enhanced platelet accumulation in diabetics can result from either a change in the platelets sensitivity or a change in the endothelium (Colwell 1989, Winocour 1989).

1.4 EXPERIMENTAL DIABETES MELLITUS IN RATS

Chemically-induced diabetes in rats has been used as an animal model to investigate the disease. Very little is known about morphological changes of macrovasculature in the early stages of the disease in animal models or in man. Unlike human diabetics, rats with streptozocin (STZ) induced diabetes do not develop hypertension, although they have the characteristic hyperglycemia and hyperlipidemia. Therefore, inducing hypertension with deoxycorticosterone acetate (DOCA) will help to mimic most of the situations of human diabetics. Although earlier reports described hypertension in this model (Kawashima et al. 1978, Buñag et al. 1982, Rodrigues et al. 1986), later work has demonstrated there was no evidence of hypertension in the STZ rat (Kusaka et al. 1987, Yamamoto 1988, Hebden et al. 1989, Todd et al. 1990).
INTRODUCTION

This animal model, therefore, has many of the characteristics of the disease in humans. Since there has been a scarcity of quantitative investigations of macrovessels from diabetic animal models, we chose renal and coronary arteries to carry out a morphometric examination to determine the degree and extent of vascular pathology in vessels from rats with STZ induced diabetes mellitus, and in diabetes with hypertension superimposed.

1.5 RESEARCH HYPOTHESES

1. Macrovessels may show vascular changes in the rat model with STZ induced diabetes mellitus.
2. Since the model is normotensive, inducing hypertension will result in early atherogenic pathology.
3. The type of blood vessels may play an important role in the development of early atherogenic changes.
4. Endothelium is the initiator of vascular pathology in diabetes.
2.1 INDUCTION OF DIABETES MELLITUS AND HYPERTENSION

Male Wistar (200-300 g) and Sprague Dawley rats (200-250 g; Charles River, Montreal) were lightly anaesthetized with halothane then injected via the tail vein with STZ (55 mg/kg, dissolved in saline at a concentration of 55 mg/ml) or with an equivalent volume of saline (1 ml/kg). The presence of glycosuria 48 h after administration of STZ was used to confirm that these animals were indeed diabetic, although at the end of all experiments blood samples were taken for the determination of plasma glucose levels. All rats were housed under identical conditions and allowed free access to food (Purina rat chow) and water. Seven days after administration of saline or STZ, half of the rats were injected subcutaneously with either 25 mg/kg DOCA or an equivalent volume of DOCA vehicle (1.8 g sodium chloride, 1.8 g benzyl alcohol, 1.0 g carboxymethylcellulose, 0.8 g polysorbate 80; made up to 200 ml with distilled water). Those saline-injected rats that received DOCA vehicle were designated CON, whereas those that received DOCA were designated CON/HT. Similarly, STZ injected rats that received DOCA vehicle were designated DIA and those given DOCA as DIA/HT. Rats were injected twice weekly with 25 mg/kg DOCA or an equivalent volume of DOCA vehicle for a total period of 6 weeks. After the first injection of DOCA or its
vehicle, all rats were given 0.9% saline to drink ad libitum rather than water. In the group that was used for scanning electron microscopy, the rats were diabetic for 1 to 3 weeks before DOCA administration was initiated.

2.2 PERFUSION FIXATION

Blood vessel samples were obtained from intra-arterial perfusion fixation at each animal's mean blood pressure. This method provides reproducible results of vascular dimensions and cellular components of samples fixed at representative physiological in situ pressures (Todd et al. 1983, Todd 1990, Todd and Gowen 1991). A cannula was inserted into the left common carotid artery under intra-peritoneal sodium pentobarbitone anaesthesia, (60 mg/kg), and systolic, diastolic and mean blood pressures were recorded (Grass Polygraph, Model 7). A total of 45 ml of fixative was perfused: 3% glutaraldehyde/2% formaldehyde in a glucose containing Krebs-Henseleit solution (Palaty 1971) at ph 7.3. Following this, the left renal artery and right coronary artery were removed and fixed for a total of 2.0 hours in the 3% glutaraldehyde/2%formaldehyde solution.

2.3 PREPARATION FOR ANALYTICAL TRANSMISSION (ATEM) AND TRANSMISSION ELECTRON MICROSCOPY (TEM)
MATERIALS & METHODS

Samples of the left renal and right coronary arteries for 5 animals in each of the four groups of male Wistar rats were postfixed with 1% osmium tetroxide in 0.1 N cacodylate buffer, then stained in saturated aqueous uranyl acetate solution, dehydrated in acetone and infiltrated using epon-araldite (Todd et al. 1983). Complete cross sections (0.5 μm thick) were cut with either a glass or diamond knife on a Reichert Om U3 Ultramicrotome. They were stretched using ethylene dichloride. With eyelash probes and a pickup loop, sections were placed on a glass slide, heat fixed and stained with a 1:1 mixture of 1% Azur II and 1% Toluidine Blue in 1% sodium borate. Sections were examined and photographed with a Leitz Photomicroscope or Zeiss Axiophot Photomicroscope to identify changes in the tunicae intima, media and adventitia and overall dimensional changes. Ultrastructural details were investigated using 70-100 nm thick sections on copper grids, poststained in Reynold's lead citrate stain and with saturated aqueous uranyl acetate. Examination and photography were carried out using a Philips 301 Electron Microscope operated at 60 kV. Sections of arterial walls containing electron-dense materials in the subendothelial space were studied by Analytical Transmission Electron Microscopy using a Hitachi H-800 STEM with an ORTEC EEDS II EDX Energy Dispersive X-Ray Spectrometer to determine the contents of different elements.
2.4 PREPARATION FOR SCANNING ELECTRON MICROSCOPY (SEM)

Following perfusion fixation as previously described, samples of the left renal artery from male Sprague Dawley rats were cut into longitudinal strips. There were 8-10 animals in each group. After rinsing in buffer and dehydration in a graded series of ethanols, they were infiltrated (using a graded series) with 100% 1-1-2 Trichlorotrifluoroethane (Freon TF) in ethanol. Critical point drying was carried out with CO₂ as the transition fluid. Specimens were secured, luminal surface upwards, to the stud with Scotch-brand double-sided tape, surrounded with a conducting silver paint, and coated with gold using a Hummer Sputtering Coating Unit. Examination and photography of the lining endothelial layer was carried out using a Hitachi F-2300 Scanning Electron Microscope.

2.5 MORPHOMETRIC ANALYSIS OF RENAL AND CORONARY ARTERIES

The areas of the tunica media, the lumen and the adventitia were measured using a morphometric software program compatible with the Apple II⁺ and Apple Digitizing Tablet. Tunica media-to-lumen ratios were calculated from different groups using the method of Todd et al. (1983). The areas were measured on three sections from each artery and averaged to give the values from any one animal. The number of adherent cells was counted in
MATERIALS & METHODS

each cross-section. Ultrastructural details of extracellular matrix percentage in the tunica media were calculated by measuring the smooth muscle in four photographs from randomly chosen areas in each of the four quadrants for each artery with the morphometric software program. Each smooth muscle cell profile was traced and the total area of tunica media was measured. The sum of smooth muscle areas over the total tunica media area gave the cellular percentage in the tunica media. Using SEM, an analysis of the numbers and types of cells adhering to the surface of the endothelium in each of the four groups was made. The size of the strip of left renal artery was calculated in mm² and the number of cells per mm² was calculated by counting the cells on the total surface of each sample.

2.6 STATISTICAL ANALYSIS

All of the values are given as the mean ± SE (standard error of the mean). In order to determine if the pathological effects were due to diabetes or hypertension, or the combination of both, Factorial Analysis of Variance (two way ANOVA) was carried out. This method could also study whether the two diseases together are either an additive effect or a synergistic interaction.
CHAPTER 3. RESULTS

3.1 BODY WEIGHT

The initial body weights of animals in the four groups were not different. After seven weeks of STZ administration, the animals in the diabetic groups were significantly lighter than the non-diabetic groups (Table 1).

3.2 BLOOD PRESSURE

The blood pressures of the animals, taken at the time of perfusion fixation, are listed in Table 2. The two hypertensive groups (CON/HT and DIA/HT) had blood pressures that were significantly elevated over their controls in systolic, diastolic and mean blood pressures.

3.3 MORPHOMETRIC STUDIES OF THE RENAL ARTERY

3.3.1 Medial, luminal areas and tunica media to lumen (M/L) ratios:

The mean values for tunica media and luminal areas and the calculated medial to luminal (M/L) ratios are shown in Table 3 for each group. Renal arteries from the two hypertensive groups had significant increases (p<0.01) in both medial and luminal areas over the control values, indicating that the
RESULTS

dimensions of the vessels had increased significantly as a result of the DOCA administration and elevated blood pressure. The M/L ratio, however, did not alter indicating that the proportions of the vessels remained constant. There were no differences between vessels from the normotensive groups or between hypertensive groups.

3.3.2 Percentage of extracellular matrix in tunica media: The ultrastructural analysis of the amounts of extracellular matrix in the renal artery is shown in Figure 1. The increase in the percentage indicates that the amount of extracellular matrix increases significantly in the DIA over CON (p<0.01). Therefore, even though there were no measurable differences in the overall vessel dimensions, the makeup of the tunica media had altered in the samples from animals with diabetes mellitus, with more connective tissue in the wall. When the values from the DIA/HT group are compared with the CON/HT, there again is a further significant elevation (p<0.01) in the amount of extracellular matrix in the tunica media. Therefore, diabetes and hypertension together resulted in significant alteration in the tunica media over hypertension alone.

3.3.3 Numbers of adherent monocytes: The number of adherent white blood cells on the endothelium of the renal artery in the four groups were calculated (Figure 2).
RESULTS

The numbers varied considerably from animal to animal, so that there were no significant differences between groups when numbers were counted from cross-sections. The additional four groups with tissue prepared for SEM showed no differences within groups with 1 or 3 weeks diabetes, prior to development of hypertension, so these groups were pooled. Utilization of the SEM permitted a much greater luminal surface region to be investigated over that analyzed from tissue sections. The hypertensive groups had a significant elevation in the number of adherent white blood cells over their respective controls (Figure 2). There was no significant difference between hypertensives and no significant difference between normotensives in terms of adherent monocytes.

3.4 MORPHOLOGY OF THE RENAL ARTERY

3.4.1 Light microscopy (LM) and TEM studies:
The overall variations in the vascular morphology can be seen in light micrographs (Figure 3) and electron micrographs (Figure 4) of representative examples of sections from each of the 4 groups. In the CON and DIA, the endothelium is a uniform flattened sheet with very little subendothelial space between the cells and the internal elastic lamina except that in the DIA group, there often was fibrous or filamentous material on the luminal surface of endothelial cells (Figure 5). The
RESULTS

Outline of the profiles of the smooth muscle cells in DIA (Figure 4b) is not as smooth as in the CON (Figure 4a). When the two hypertensive groups are compared with the two normotensive groups, the increase in tunica media thickness is evident as is the increase in the extracellular matrix. Only in the DIA/HT were there patchy regions with numerous adherent white blood cells, subendothelial cellular invasion, and deposition of electron-dense materials. Figure 6 illustrates an area of marked subendothelial invasion of the tunica intima. White blood cells (probably monocytes) appear to adhere to the endothelium which often had lipid inclusions. The subendothelial cells (probably monocytes that had converted to macrophages) were interspersed with dense material that in some cases appeared crystalline (see insert, Figure 6). Electron-dense material was also observed within macrophages. In the vessels that had more and larger patches of the modified tunica intima, the tunica media had more areas of smooth muscle necrosis and smooth muscle cells that were in the process of becoming foam cells with lipid droplets (Figure 7). The extracellular matrix was also much more vesicular and heterogeneous in such regions compared with CON, DIA and CON/HT. Since no dense deposits or subendothelial macrophages were observed in vessels from the CON/HT group, this again supports the concept that diabetes and hypertension together cause an increase in the degree of pathogenesis.
3.4.2 SEM studies

The luminal surface of the endothelial cells is generally smooth in the CON group (Figure 10a). The endothelial cells appeared fusiform in shape with the long axis oriented in the direction of blood flow. The cells had scattered surface microvilli and were delineated by marginal folds.

In the DIA group (Figure 10b), many areas of the endothelial surface had a layer of fibrous-like materials (Figure 11). The surface became more irregular or uneven. The cell borders were more prominent and the endothelial cells had more microvilli.

In the CON/HT hypertensive group (Figure 10c), the endothelial surface became very uneven and the fusiform shape of the endothelial cells was lost. In these regions, pores or craters were observed on the surface. Patchy areas covered with fibrous-like materials were also present.

The endothelial cells became polygonal in shape in the DIA/HT group (Figure 10d), and were associated with many patches of adherent monocytes which sent out processes to the endothelial cells against the blood flow. Fibrous-like materials, and pores or craters were also found in this group. The endothelial cells overlapped and this was not typical of control samples. There were two types of adherent monocytes, numerous typical monocytes with upstream tails (Figure 12), and larger cells without tails that may have been free-grazing macrophages, or macrophages (Figure 13) that had returned to
RESULTS

the circulation from the subendothelial space (Figure 14).

3.5 ANALYTICAL TRANSMISSION ELECTRONMICROSCOPIC (ATEM) STUDIES OF ELECTRON-DENSE MATERIALS

The composition of electron-dense materials was studied by ATEM (Figure 15). The metal content of control areas (free of electron-dense materials) and areas with red blood cells were also studied (Figure 16). There was a significant sulphur peak in the electron-dense materials compared with control areas, suggesting that they might be protein in origin. On the other hand, there was no significant iron peak found in the electron dense material areas.

3.6 MORPHOMETRIC STUDIES OF THE CORONARY ARTERY

3.6.1 The medial, luminal and adventitial areas, and medial to luminal (M/L) and adventitial to luminal (A/L) ratios:

The medial, luminal and adventitial areas were measured and medial to luminal (M/L) and adventitial to luminal (A/L) ratios were calculated (Table 4). No dimensional changes resulted due to diabetes in the normotensive group. Coronary arteries from the two hypertensive groups not only had significant increases in medial and luminal areas over their respective controls (Table 4), but also both had a proportionately greater
hypertrophy of the media compared with the increase in luminal areas giving significant differences in M/L ratios over control values (Table 4). When the two hypertensive groups were compared with each other, there was another significantly less increase in both medial and luminal areas due to the diabetic condition, but M/L ratios remain unchanged (Table 4). These results indicate that DOCA administration caused increases of tunica media and lumen, but to a lesser extent when diabetes was superimposed. In addition, in both hypertensive groups, the adventitial area significantly increased over that in the respective controls in almost identical proportions to the tunica media hypertrophy giving significant differences in adventitial to luminal ratios (Table 4).

3.6.2. Percentage of extracellular matrix in the tunica media:
There was no alteration in the makeup of tunica media in the diabetic group compared with the control values. The hypertensive groups had significant increases of extracellular matrix in the tunica media over the CON values (Figure 17) but were not significantly different from each other.
RESULTS

3.7 MORPHOLOGY OF THE CORONARY ARTERY

3.7.1 LM and TEM studies:
Sections of the right coronary artery were examined from the same approximate region in each of the rats (i.e., 5 mm from the junction of the artery with the ascending aorta). The vessels from the CON (Figure 18a) and DIA (Figure 18b) rats were morphologically similar. Both groups showed a well-defined inner layer of circumferentially arranged smooth muscle cells, with more longitudinally orientated smooth muscle cells in groups adjacent to the adventitia (Figure 18). The profiles were regular in outline with small amounts of extracellular matrix. The flattened endothelial cells were regularly arranged adjacent to the internal elastic lamina. In coronary arteries from both the CON/HT (Figure 18c) and DIA/HT (Figure 18d) groups, the tunica media was increased in thickness more than twofold compared with normotensive groups (Table 4), due to marked augmentation of the extracellular matrix and enlarged smooth muscle cell profiles. In addition, the profiles were much more irregular with numerous branching processes. The total thickness of the adventitia was also increased, with hypertrophied fibroblasts and more paracellular collagen being present. These fibroblasts showed evidence of enlarged endoplasmic reticulum (Figure 19), which is consistent with increased synthetic activity.
CHAPTER 4. DISCUSSION

The two supply vessels chosen for this investigation represented organ systems that have a high incidence of pathology in diabetes mellitus, namely nephropathy, coronary artery disease and cardiomyopathy. There were fibrous-like materials on the endothelial surface, and an increase of the extracellular matrix in the tunica media in the renal artery due to diabetic condition. Vessels from hypertensive groups not only had significantly elevated adherent monocytes on the endothelium, but also possessed enlarged lumen and a thickened tunica media over the control values. The DIA/HT group had even greater significant elevation of the extracellular matrix, and, in addition, had marked subendothelial invasion of macrophage type cells and deposits of various shapes and densities (Song et al. 1991a & b, Song et al 1992a & b, Todd et al. 1992).

The reaction of the coronary artery to the diabetic condition was morphologically different from that in the renal artery, perhaps due to the type of blood vessel and different blood flow hemodynamics. Even though there was no alteration in the makeup of the tunica media, the tunica media and luminal areas were significantly less in the DIA group. This suggested that the coronary artery did not hypertrophy to the same extent as that which occurred in the renal artery. When DOCA was administered, both CON/HT and DIA/HT had not only a significant
DISCUSSION

increase in tunica media and luminal areas over their respective controls but also both had a proportionately greater hypertrophy of the tunica media compared with the increase in luminal areas. This gave significant differences in medial/luminal (M/L) ratios, in contrast to the response of renal artery to the experimental conditions. When the two hypertensive groups were compared, the diabetic rats did not increase to the same extent as the hypertensive rats with respect to the tunica media and luminal areas. Since there is a prominent decrease of myocardial weight in diabetic rats (Modrak 1980), the demands of blood supply through coronary arteries could be reduced, and this might lead to a decrease in the degree of hypertrophy in vessel dimensions in diabetic rats. In terms of the kidney, there is a significant increase of renal blood flow and an elevation of kidney weights in diabetes (Jensen et al. 1981). This could be one of the reasons why, even though the total body weight was less, the renal arteries hypertrophied to the same extent in the diabetic hypertensive group. In both hypertensive groups, moreover the adventitial area in the coronary artery increased over the respective controls in almost identical proportions to the tunica media hypertrophy giving a statistically significant elevation of adventitial to luminal ratios. Hypertension caused hypertrophy of the tunica media and an increase in luminal size. Therefore, the combination of diabetes and
DISCUSSION

hypertension in this particular artery had less of an effect due to the summation of the results compared with hypertension alone. This difference in response of the two arteries in short-term diabetes suggests that particular organ systems are targeted differentially in the early stages of the disease.

4.1 ENDOTHELIAL SURFACE

Plasma proteins and platelets do not normally adhere to intact endothelial cells. However, increased vascular permeability resulting from hyperglycemia allows significant amounts of plasma proteins to leak out of the circulation (Zucker et al. 1979). Diabetics have significantly elevated plasma and serum viscosities, which reflects the high concentrations of fibrinogen, fibrinectin and the globulins in the circulation (Musso et al. 1989, Memeh 1991). Because of the "stickiness" of the blood components or endothelium and the decrease of blood flow caused by increase plasma viscosity (Dintenfass 1979), these plasma elements may accumulate on vessel walls (Song et al. 1992a & b), giving the evidence of fibrous-like materials on the endothelial surface in the renal artery both on tissue sections (Figure 5) and with SEM (Figure 11). These areas were also associated with platelets and red blood cells. The altered metabolic state of diabetes contributes to changes in platelet sensitivity (Colwell 1989, Winocour 1989) and red
DISCUSSION

blood cell functions (Jialal and Chait 1989) which could cause this phenomenon. Similar phenomena were also observed in the thoracic aorta (Arbogast et al. 1984, Hadcock et al. 1991, Todd 1992).

4.2 ADHERENCE OF MONOCYTES

Another observation was that there were adherent white blood cells in vessels from diabetic animals as well as in the hypertensive groups. These adherent patches were variable in both size and in number. However, focal adherence of white blood cells is the initial stage of an altered endothelium and the first stage in development of atherogenic pathology (Simionescu 1988). Because of the endothelial patchiness, large areas of the vessel wall need to be examined to determine whether a significant elevation of numbers in adherent cells occurs. Identical cells have been recognized as monocytes by others using immunocytochemical and histochemical techniques (Bowyer and Mitchinson 1989). Even though there was no difference in the number of adherent monocytes between diabetic and control groups in the renal artery, a significant elevation in numbers of these cells in vessels from diabetic rats over controls in thoracic aorta has been observed in the same animals (Todd 1992). Hadcock et al (1991) have also shown that adherent white blood cells on the endothelium had been
DISCUSSION

increased as early as one week after alloxan injection in the rabbit compared with age-matched controls. This is further evidence that the condition of diabetes mellitus has predisposed particular organ systems to develop vascular disease, and the aorta seems to be one of the leading vessels to be involved.

4.3 TUNICA INTIMA

Endothelial cells

There is increasing evidence that the endothelium may be the target and initiator of vascular pathology (Lüscher 1990, Raij 1991, Todd 1992). Lee and coworkers (1989) have found that elevated plasma glucose levels found in diabetes mellitus affects endothelial cells by activation of protein kinase C. This may either cause or contribute to structural and functional changes in blood vessels. Protein kinase C has been shown to stimulate vascular cells resulting in neovascularization, cell growth and modulated receptor turnover (Lee et al. 1989). Alterations of endothelial surface might occur as a result of the "injury" to the cell. This is of particular interest because there are several reports that monocytes adhere to areas of high endothelial cell turnover in experimental atherosclerosis (Gerrity et al. 1979, Walker and
Discussion

Bowyer 1984). Gilcrease and co-worker have demonstrated that high concentrations of glucose increased the nonenzymatic glycosylation of endothelial cell membrane proteins in vitro and in vivo, and also increased monocyte adherence to endothelium. It appears that the high glucose media did not activate monocytes and thereby increase their adhesive properties, in general, but rather, modified the endothelial cell adhesion molecules which increased monocyte adhesion (Gilcrease and Hoover 1991). In order that attached monocytes enter the intima, they must first penetrate the endothelium. Chemotactic factors released by endothelial cells lining arterial walls and smooth muscle cells are presumed to play an important role in this phenomenon (Berliner et al. 1986, Quinn et al. 1987, Kirstein et al. 1990). Monocyte chemoattractant protein 1, a known chemotaxic factor, has been isolated from human and rabbit atherosclerotic lesions (Ylä-Herttuala et al. 1991).

In this study, the evidence of an additive effect of diabetes and hypertension was seen in the focal lesions occurring only in the renal artery from diabetic hypertensive animals (Todd et al. 1992). Here, the endothelial surface became uneven, and in en face views obtained by SEM the fusiform shape of the endothelial cells was lost in the renal artery from hypertensive rats (see Figure 10c). The changes were increased with the DIA/HT group in which polygonally-shaped endothelial
DISCUSSION

cells were seen overlapping instead of abutting with each other (Song et al. 1992a & b). In these regions, many patches of adherent monocytes sent out processes to the endothelium against the blood flow. Pores or craters were also found (Figure 10d). These could be sites where lipid vesicles accumulated but may have collapsed during the processing of the specimen for microscopy. This type of surface modification has been observed previously in the aorta from chronic STZ-induced diabetes in rats (Pieper and Gross 1988). The white blood cells that we presume to be monocytes, also appeared to be passing through the endothelium and apparently phagocytosing subendothelial deposits (Figure 8). Identical deposits were seen as intracytoplasmic inclusions in these macrophages (Todd et al. 1992).

Hemodynamic fluid mechanics have shown that with experimental high-shear stress, endothelial cells are elongated and orientated in the direction of flow, where as in low-shear stress regions, the endothelial cells tend to be rounded up (Nerem et al. 1981, Levesque and Nerem 1985). Since both plasma and serum had significantly elevated viscosities (Memeh 1991) due to diabetes, the disturbed blood flow might result in low-shear stress on the endothelium. This may also affect the shape of endothelial cells in diabetics and, in fact, the cells from diabetic rats observed in our study tended to loose their elongation (Song et al. 1992a & b).
Subendothelial space
When monocytes arrive in the subendothelial space of a blood vessel, they are activated as macrophages, and become "professional phagocytes" (Gerrity 1981a,b) and, as such, can be actively involved in the removal of particulate matter such as necrotic debris (Figure 6). Under varying circumstances, they are known to also synthesize collagen and glycosaminoglycans (Lewis et al. 1985). They also secrete an impressive array of other substances, including complement components and acid hydrolases (Unanue 1976), peroxidase, superoxide dismutase, enzyme inhibitors, and chemotactic factors (Ross 1981). Of special interest with respect to their role in atherosclerosis, is the secretion by these cells of a growth factor which stimulates proliferation of both smooth muscle cells and endothelial cells (Martin et al. 1981, Glenn and Ross 1980), and of fibronectin (Alitalo et al. 1980), a protein involved in cell adhesion and in nonimmune opsonization of particles for phagocytosis. This is the process whereby phagocytosis is enhanced through coating of the foreign particle by an opsonin such as antibody or complement. They are progressively loaded with cholesteryl ester-rich deposits to become foam cells. Quantitative studies show that there was a significant increase of number of adherent monocytes in the hypertensive groups over the control values (Figure 3). Thus they become the macrophages of the early lesions of the tunica
intima (Davies 1987). In these short term studies, we had no evidence of any invasion of smooth muscle into the tunica intima in the renal artery as is characteristic of later lesions (Davies 1987). Since atherosclerosis tends to occur at an earlier age and with greater severity in the diabetic population (Mossaz and Assal 1989), our results provide supporting evidence for abnormal structure with diabetes alone and also, even more so, exacerbation of the atherogenic type of pathology with hypertension (Todd et al. 1992). It is possible that given a longer time frame we would have seen smooth muscle cell invasion of the tunica intima in arteries from the diabetic animals. Hebden et al have demonstrated that these animals have elevated plasma lipid levels (Hebden et al. 1990) which play a crucial role in development of early atherogenic lesions (Simionescu 1988). The nature of the intimal dense deposits requires further study to elucidate the composition of this material. Similar types of deposits have been described as crystallized hemoglobin and fibrin (Kowala et al. 1988). Our analytical transmission electron microscopic studies of the subendothelial space did not support the proposal that these deposits originate from red blood cells. There was no evidence of any increase of Fe^{2+} iron in the electron dense areas compared with regions which would be expected if they were red blood cell in origin. These deposits could have been secreted by activated endothelial cells on their abluminal surfaces, or
somehow be passing through the endothelial layer via a yet undiscovered means as a result of increased permeability. Clearly, there are many avenues of investigation that remain to be examined.

4.4. TUNICA MEDIA

Our results suggest that smooth muscle cells must be synthesizing more connective tissue in the renal artery from diabetic rats than in control animals. The basement membrane of microvessels typically is thickened or becomes layered in diabetes mellitus (Brownlee 1985, Cahill 1985, Williamson and Kilo 1989) indicating an alteration in connective tissue synthesis, although in these instances, it is the endothelium rather than smooth muscle that is most likely involved. Therefore the differences between micro- and macroangiopathy may not be so extreme if synthesis of extracellular connective tissue and matrix is a common component (Mossaz and Assal 1989). The coronary artery in this study did not show an over production of connective tissue in the tunica media. Ditzel et al (1957) have reported that vasoconstriction develops progressively and that some form of vessel wall atrophy occurs in the diabetic patients. Other investigators have also found similar phenomena in intestinal arterioles in streptozocin treated rats (Bohlen and Hankins 1982). It is clear that
DISCUSSION

diabetic rats typically have smaller hearts (Modrak 1980), so that the atrophied coronary artery might be a secondary adaption to the decrease of myocardial weight. Structural modification as a result of elevated blood pressure, particularly a thickened vascular wall, was one of the first responses noted in hypertension and one that has been studied extensively (Folkow 1990). A similar response in both the renal and coronary arteries with significant thickening of the tunica media in the hypertensive groups was observed in our study. Due to the high blood pressure, the luminal areas of the renal artery were also increased proportionately with the tunica media leading to a constant medial to luminal ratios. However, in the coronary artery, possibly because the vessels are embedded in the cardiac tissues, the increase of luminal areas were not proportionate to the increase of medial area. In addition, the coronary artery is muscular in nature compared with the musculoelastic renal artery, This gave a significant elevation of medial to luminal ratios. In particular, what we were assessing was whether the double stimulus of diabetes and hypertension had an additive effect on vascular structure. There appeared to be two areas of clear differences, i.e. in the extracellular matrix percentage in the tunica media and in the presence of focal lesions in the renal artery. The amount of extracellular matrix in the tunica media was significantly increased in the renal arteries from DIA/HT animals confirming
DISCUSSION

that the combination was more deleterious than hypertensive alone. The fact that the media-to-lumen ratios were virtually identical in renal artery in all groups showed a strong bias toward dimensional parameters being very important in relation to the blood pressure levels in this vessel. Again, in coronary artery, this was not the case. Diabetes did not lead to more connective tissue synthesis in the tunica media. Rather, a smaller artery in terms of medial and luminal areas was probably due to the smaller heart (Modrak 1980). When hypertension was induced, the media and lumen areas were significantly less than with hypertension alone.

4.5 TUNICA ADVENTITIA

Connective tissue accumulation in the artery is a feature of the atherosclerotic plaque. In the atherosclerotic lesions the collagen content is increased due to increased biosynthesis (Jialal and Chait 1989). The fibroblasts of the adventitia respond by transformation from the quiescent, finely branching profiles, to metabolically active cells packed with rough endoplasmic reticulum (see Figure 16). This structural change to an actively synthesizing cell correlated with the overall significant increase in the area of the adventitia in the hypertensive groups compared with their respective controls. Since coronary artery is embedded in the myocardium, measuring
DISCUSSION

the adventitial area was possible, whereas the adventitia was damaged during excision of the renal artery. Hypertension not only increases the thickness of tunica media, but also enhances the proliferation of fibroblasts in the tunica media (Todd 1992). Similar phenomena were also seen in the renal and the coronary arteries. In the coronary artery, not only was the adventitial area increased, but also the degree of hypertrophy was greater than the increase of luminal areas, giving a significantly different adventitial/luminal ratio.
CHAPTER 5. CONCLUSION

These studies have demonstrated different patterns of alterations in supply arteries due to the diabetic condition, i.e. significant increase of the extracellular matrix in the tunica media and fibrous-like materials on the endothelial surface with a change to a more vesicular appearance in the renal artery. In the coronary artery, even though there was no change in the composition of the tunica media, the dimension of the vessel was altered, i.e. a decrease in both tunica media and luminal areas. Diabetes resulted in a divergent alteration in these two arteries, suggesting that particular organ systems are targeted differentially in the early stage of the disease in this animal model. These could be explained by different pathophysiologic and hemodynamic changes in both the heart and kidney due to hyperglycemia.

Hypertension caused by DOCA administration leads to an increase in vessel size and tunica media thickness in both supply arteries. The elevation of medial and luminal areas was proportionate to each other in the renal artery compared with normotensive values, giving similar media/lumen ratios. In the coronary artery, in contrast, not only were the medial and luminal areas increased, but also the adventitial areas were elevated. The degree of the hypertrophy of tunicae media and adventitia was greater than the increase of luminal areas. This resulted in significant differences in media/lumen and
adventitia/lumen ratios over normotensive controls. Since the coronary artery is embedded in the cardiac tissue, this could restrain the dilatation of the vessel caused by high blood pressure, resulting in significant difference of media/lumen and adventitia/lumen ratios.

The consequence of DOCA-hypertension and diabetes combined was an additive rather than a synergistic interaction in the morphometric studies. The vascular pathology was particularly evident in the renal artery in DIA/HT rats with marked subendothelial invasion of macrophage type cells and deposits of various shapes and density.
TABLE 1.

The body weight of animals in the four groups.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals</td>
<td>CON</td>
<td>/HT</td>
<td>DIA</td>
<td>/HT</td>
</tr>
<tr>
<td>Wistar</td>
<td>472.1±9.8</td>
<td>461.7±15.0</td>
<td>343.6±13.9**</td>
<td>359.7±18.8**</td>
</tr>
<tr>
<td>Sprague Dawley</td>
<td>477.7±18.2</td>
<td>447.9±14.1</td>
<td>348.1±9.5**</td>
<td>306.2±9.5**</td>
</tr>
</tbody>
</table>

** The body weights of diabetic animals were significantly lighter compared with their respective controls in both Wistar and Sprague Dawley rats. p<0.01.
TABLE 2.

Blood pressures (mmHg) measured using intra arterial cannulation immediately prior to perfusion fixation. SYSTO=Systolic Pressure, DIASTO=Diastolic Pressure, MEAN= Mean Blood Pressure.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>SYSTO</th>
<th>DIASTO</th>
<th>MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CON</strong> Wistar rats CON/HT</td>
<td>138.5±5.8</td>
<td>115.5±5.6</td>
<td>130.8±5.7</td>
</tr>
<tr>
<td><strong>CON</strong> DIA</td>
<td>131.6±4.8</td>
<td>105.5±6.1</td>
<td>123.7±4.6</td>
</tr>
<tr>
<td><strong>CON</strong> DIA/HT</td>
<td>186.8±6.5**</td>
<td>136.0±5.8**</td>
<td>158.8±6.6**</td>
</tr>
<tr>
<td><strong>Sprague</strong> CON</td>
<td>123.3±1.7</td>
<td>75.0±5.1</td>
<td>96.3±3.2</td>
</tr>
<tr>
<td><strong>Sprague</strong> CON/HT</td>
<td>163.0±3.8**</td>
<td>114.7±4.2**</td>
<td>135.8±4.6**</td>
</tr>
<tr>
<td><strong>Sprague</strong> DIA</td>
<td>126.7±4.6</td>
<td>90.7±5.4</td>
<td>106.5±5.2</td>
</tr>
<tr>
<td><strong>Sprague</strong> DIA/HT</td>
<td>181.2±8.4**</td>
<td>140.7±10.0**</td>
<td>157.0±10.0**</td>
</tr>
</tbody>
</table>

There were significant elevations of blood pressures in the two hypertensive groups (HT) compared with normotensive rats, either control (CON) or diabetic (DIA). There were no significant differences between normotensive or between hypertensive groups. *P<0.05, **P<0.01.
TABLE 3.

Areas of the tunica media and of the lumen were measured in complete cross sections of the renal artery, and media to lumen ratios (M/L) were calculated.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Media</th>
<th>Lumen</th>
<th>M/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>67.2±1.2</td>
<td>610.0±41.3</td>
<td>11.3±1.1</td>
</tr>
<tr>
<td>CON/HT</td>
<td>101.8±4.8**</td>
<td>782.5±41.3**</td>
<td>13.2±1.0</td>
</tr>
<tr>
<td>DIA</td>
<td>76.8±12.2</td>
<td>612.2±68.5</td>
<td>12.5±0.9</td>
</tr>
<tr>
<td>DIA/HT</td>
<td>111.9±6.8**</td>
<td>842.0±48.5**</td>
<td>13.6±1.4</td>
</tr>
</tbody>
</table>

** indicates significant increase in tunica medial and luminal values of hypertensive animals over the control values. P<0.01.
Areas of the tunica media, of the lumen and of the adventitia were measured in complete cross sections of the coronary artery. Media to lumen (M/L) and adventitia to lumen (A/L) ratios were calculated.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Media (µm²)</th>
<th>Lumen (µm²)</th>
<th>Adventitia (µm²)</th>
<th>M/L</th>
<th>A/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>12.8±0.4</td>
<td>108.4±6.3</td>
<td>10.4±0.6</td>
<td>0.12±0.01</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>CON/HT</td>
<td>30.0±3.3**</td>
<td>158.2±19.5**</td>
<td>30.3±6.4**</td>
<td>0.19±0.01**</td>
<td>0.19±0.03**</td>
</tr>
<tr>
<td>DIA</td>
<td>9.6±0.5#</td>
<td>83.4±7.4#</td>
<td>8.7±1.7</td>
<td>0.12±0.01</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>DIA/HT</td>
<td>23.6±1.8***#</td>
<td>120.4±9.6***#</td>
<td>24.8±3.5**</td>
<td>0.20±0.01**</td>
<td>0.20±0.01**</td>
</tr>
</tbody>
</table>

#DIA or DIA/HT significantly different from CON or CON/HT. **CON/HT or DIA/HT significantly different from CON or DIA. #P<0.05, **P<0.01.
FIGURE 1.

The extracellular matrix percentage in the tunica media of the renal artery in the four groups.

#DIA or DIA/HT significantly different from CON or CON/HT. **CON/HT or DIA/HT significantly different from CON or DIA. P<0.01.
FIGURE 2.

Number of adherent monocytes, counted from complete cross section of the renal artery, or from specimen prepared for SEM.

**CON/HT or DIA/HT significantly different from CON or DIA.**

\( p < 0.01 \)
FIGURE 3. Light microscopic photographs of portions of the renal artery from the four experimental groups: a=control (CON), b=diabetic (DIA), c=control/hypertensive (CON/HT), d=diabetic/hypertensive (DIA/HT). The CON (a) and DIA (b) vessels are very similar with internal and external elastic laminae (L). The endothelial cells (E) are flattened and there was no obvious increase in the subendothelial region. In contrast, the CON/HT (c) and particularly DIA/HT (d) both show increased wall thickness and amount of extracellular matrix (p). The DIA/HT (d) vessels only, had patchy accumulations of electron-dense deposits (D) in the subendothelial space associated with adherence of white blood cells (W). (1,000X).
**FIGURE 4.** Electron microscopic photographs of portions of the renal artery from the four experimental groups, a=control (CON), b=diabetic (DIA), c=control/hypertensive (CON/HT), d=diabetic/hypertensive (DIA/HT). The smooth muscle cell profiles (S) are more irregular in DIA (b) compared with the CON (a). There was significantly more extracellular matrix (P) in DIA (b) compared with the CON (a) (see Figure 1). The DIA/HT vessels (d) had more vesicular extracellular components (see Figure 1) than the CON/HT vessels (c). In addition, there were electron-dense materials (D) in the subendothelial space in DIA/HT rats. A foam cell (F) is noted in the tunica media (d). (1,900X).
FIGURE 5. Electron microscopic photograph of the renal artery from the DIA group showing the endothelial surface covered by fibrous-like material (arrow heads). Endothelium (E). Internal elastic lamina (L). Smooth muscle cell (S). Platelet (P). (3,900X)
FIGURE 6. Electron microscopic photograph of the renal artery showing the tunica intima from a DIA/HT rat. An endothelial cell (E) is swollen with lipid droplets (L) and is seen adjacent to an adherent white blood cell (W). The subendothelial space contains an increase in electron-dense deposits (D) and macrophages (M). Internal elastic lamina (I). (6,100X). The insert shows details of the electron-dense deposits. (28,000X).
FIGURE 7. Electron microscopic photograph of part of the renal artery from a DIA/HT rat. The tunica media layer contains an increase in the extracellular materials (P) which appear heterogeneous and vesicular. Smooth muscle cells (S) are irregular in shape and intermingle with foam cells (P). Elastic lamina in tunica media (L). (5,000X). The insert shows detail of the extracellular matrix in the tunica media. (28,000X).
FIGURE 8. Electron micrograph of the renal artery from a DIA/HT rat showing white blood cell (W) adherence to endothelial cells (E). One appears to be passing between endothelial cells, with endothelial plasma-membranes on either side. The white blood cell seems to be engulfing (arrows) some of the electron dense-deposits (D) in the subendothelial space. Lumen (L). Internal elastic lamina (I). (9,400X).
FIGURE 9. Electron micrographs from DIA/HT rats showing different types of electron-dense deposits in the subendothelial space with (D) or without (WD) periodicities. Endothelial cell (E). Macrophage (M). Internal elastic lamina (I). (a=12,000X, b=15,600X).
FIGURE 10. Scanning electron microscopic photographs of the renal artery from the four experimental groups: a=control (CON), b=diabetic (DIA), c=control/hypertensive (CON/HT), d=diabetic/hypertensive (DIA/HT). All micrographs are the same magnification. Bar = 20μm. In the CON (a), the luminal surface is generally smooth. The endothelial cells (E) appear fusiform in shape with their long axes oriented in the direction of blood flow (Arrow). The cells possess scattered surface microvilli (arrow heads) and are delineated by marginal folds. In DIA (b), many areas have a layer of fibrous-like material (see also Figure 11). The surface is more irregular than that seen in the control. The cell borders were more prominent and the endothelial cells (E) have more microvilli (arrow heads). In CON/HT (c), the surface is very uneven and the fusiform shape of the endothelial cells (E) is lost. In these regions, pores or craters (C) are seen. Patchy areas covered with fibrin-like materials are also present. In DIA/HT (d), the endothelial cells (E) are now polygonal in shape, and are associated with many patches of adherent monocytes (see Figure 12a). Fibrin-like materials, and pores or craters (C) are seen in this group. The endothelial cells overlapped instead of abutting with each other as was typical of control samples. Red blood cell (R).
FIGURE 11. Scanning electron microscopic photograph of the endothelium of the renal artery from the DIA group showing fibrous-like material (arrows) on the endothelial luminal surface. Bar = 20µm.
FIGURE 12. Scanning views of the endothelial surface of the renal artery from a diabetic hypertensive animal. (a) Monocytes (M) on the surface of the endothelium. Bar = 10μm. (b) A monocyte appears to be sending out processes (arrows) to the endothelial cells. Bar = 5μm. There were two types of adherent monocytes, numerous typical monocytes with upstream tails (arrow heads), and larger cells without tails that may have been free grazing macrophages (see also Figure 13).
FIGURE 13. SEM views of the monocytes/macrophages with different sizes and shapes are seen on the luminal surface of renal arteries from DIA or DIA/HT groups. These appear to be monocytes that have been transformed into macrophages. Red blood cell (R). Both, Bar = 10μm.
FIGURE 14. SEM view of monocyte/macrophage on the endothelial surface in the renal artery from a diabetic hypertensive rat. Some researchers have previously shown that macrophages in the subendothelial space can transport lipid back to blood stream (Daoud et al. 1985). This electron micrograph shows a modified macrophage which seems to be backing into the vessel lumen. Bar = 20µm.
FIGURE 15. Analytical Transmission Electron microscopic record of the electron-dense materials in the subendothelial space in the renal artery from the diabetic hypertensive rat. A significant sulphur peak is noted in the electron-dense materials compared with those elements found in the red blood cell (see Figure 16).
ID: COARSE STRI. GRAN.
ELEMENTS FOUND: CU P S CL K FE
PRESET: OF F
FULL SCALE: 1K LINEAR 0-10 KEV
DEAD TIME: 18%
COUNTS/SECOND 0.030
SCINT.

0.00 2.56 5.12 7.68 10.22
FIGURE 16. Analytical Transmission Electron microscopic record of the metal contents in a red blood cell. Note that there is virtually no sulphur in the red blood cell.
ID: RBC

ELEMENTS FOUND: P  CL K  FE  CO  CU

PRESET: OF F  DEAD TIME: 7%  COUNTS/SECOND: 338

FULL SCALE: 512 LINEAR  0-10 KEV  10 EU/CH
FIGURE 17.

The percentage of extracellular matrix in the tunica media of the coronary artery in the four groups.

**CON/HT or DIA/HT significantly different from CON or DIA. P<0.01.**
FIGURE 18. Electron microscopic photographs of the coronary artery from the four experimental groups, a=control (CON), b=diabetic (DIA), c=control/hypertensive (CON/HT), d=diabetic/hypertensive (DIA/HT). The vessels from the CON (a) and DIA (b) rats were similar with flattened endothelial cells (E). Both groups showed a well-defined inner layer of more circumferentially arranged smooth muscle cells, with more longitudinally orientated smooth muscle cells (S) in groups adjacent to the adventitia (A). The CON/HT (c) and DIA/HT (d) both show increased tunica media thickness (see Table 4) and amount of extracellular matrix (P) (see Figure 17). (4,100X).
FIGURE 19. Electron microscopic photograph of the tunica adventitia (A) of a coronary artery from the diabetic hypertensive group showing the prominent rough-surfaced endoplasmic reticulum (arrows) of a fibroblast (F). (9,350X).


REFERENCES


REFERENCES


Fuller JH. Epidemiology of hypertension associated with diabetes mellitus. Hypertension 7:II3-II7; 1985.


Gerrity RG. The role of the monocyte in atherogenesis II. Migration of foam cells from atherosclerotic lesions. Am J
REFERENCES


Glenn KC, Ross R. Human monocyte-derived growth factor(s) for mesenchymal cells: activation of secretion by endotoxin and concanavalin A. Cell 25:603-615; 1981.


REFERENCES


REFERENCES


REFERENCES


Todd ME, Gowen B. Arterial wall and smooth muscle cell
REFERENCES

development in young Wistar rats and the effects of surgical

Todd ME, Hebden RA, Gowen B, Tang C, McNeill JH. Atrial
structure and plasma ANF levels in rats with chronic diabetes.

Todd ME, Laye GC, Osborne DN. The dimensional characteristics
of smooth muscle in rat blood vessels. A computer-assisted

Todd ME, Song MY, McNeill JH. Coexistence of diabetes and
hypertension results in unique structural alterations in the
renal artery in rats beyond that found with diabetes alone.

Unanue ER. Secretory function of mononuclear phagocytes: A

Walker LN, Bowyer DE. Endothelial healing in the rabbit aorta
and the effect of risk factors for atherosclerosis.

Weinstein DB. Protective effects of calcium channel
antagonists in experimental models of atherogenesis and
vascular disease. In: Hypertension: Pathophysiology,
Diagnosis and Management. Laragh JH, Brenner BM Eds. Roven

West KM. Epidemiology of diabetes and its vascular lesions.

Williamson JR, Kilo C. Basement membrane in diabetes mellitus.
In: Molecular and Cellular Biology of Diabetes Mellitus Vol.
III: Complications of diabetes mellitus. Draznin B, Melmed S,
LeRoith D Eds. Alan R. Liss, Inc., New York, New York, pp:19-
29; 1989.

Winocour PD. The role of platelets in the pathogenesis of
diabetic vascular disease. In: Molecular and Cellular Biology of

Yamamoto J. Blood pressure and metabolic effects of streptozotocin in Wistar-kyoto and spontaneously hypertensive
1083; 1988.

Ylä-Herttuala S, Lipton BA, Rosenfeld ME, Särkioja T, Yoshimura
REFERENCES
