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EFFECT OF METABOLIC SYNDROME ON THE RESPONSE TO ARTERIAL INJURY

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Abstract

Background—Metabolic syndrome is now an epidemic in the US population. Intimal hyperplasia remains the principal lesion in the development of restenosis after vessel wall injury.

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Contribution Statements

By submitting this manuscript, each author certifies that they have made a direct and substantial contribution to the work reported in the manuscript by participating in each of the following three areas: (1) conceiving and designing the study; or collecting the data; or analyzing and interpreting the data; (2) writing the manuscript or providing critical revisions that are important for the intellectual content; and (3) approving the final version of the manuscript. In the table below, we have listed all of the authors and use an "X" to indicate all of the substantive contribution(s) of each author. For more information, see the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication" (http://www.icmje.org/index.html), section II.A on Authorship.

Author Name	Conception and design	Analysis and interpretation	Data Collection	Writing the article	Critical revision of the article	Final approval of the article	Statistical analysis	Obtaining funding	Overall Responsibili
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Mark G. Davies	x	x	x	x	x	x	x	x	X

Disclosure Statement

Author Name	
Enrico A. Duru	Nothing to disclose
Yuyang Fu	Nothing to disclose
Mark G. Davies	Nothing to disclose

The aim of this study is to characterize the changes induced in wall morphology in the developing intimal hyperplasia within a murine model in the presence of diabetes (type 1) and metabolic syndrome.

Methods—Control (wild-type B6), Diabetic Type 1 (NOD) and Metabolic Syndrome (RCS-10) mice were used. The murine femoral wire injury model was employed in which a micro wire is passed through a branch of the femoral and used to denude the common femoral and iliac arteries. Specimens were perfusion-fixed and sections were stained with H&E and Movat's stains such that dimensional and compositional morphometry could be performed using an ImagePro system. Additional stains for proliferation and apoptosis were employed.

Results—In control mice, the injured femoral arteries develop intimal hyperplasia, which is maximal at 28 days and remains stable to day 56. Sham-operated vessels do not produce such a response. In diabetic mice, the intimal response increased 5-fold with a 2-fold increase in proteoglycan deposition, while in the metabolic syndrome mice there was a 6-fold increase in the intimal response and a similar increase in proteoglycan deposition. Collagen deposition was different, with a 22-fold increase over control in collagen deposition in diabetes and a 100-fold increase over control in collagen deposition in metabolic syndrome as compared to the control injury mice. Maximal vascular smooth muscle cell (VSMC) proliferation was decreased in both diabetes and metabolic syndrome compared to controls, while early cell apoptosis in both diabetes and metabolic syndrome was sustained over a longer period of time compared to wild-type mice.

Conclusions—These data demonstrate that development of intimal hyperplasia is markedly different in diabetes and metabolic syndrome compared to controls, with an increase in collagen deposition, a reduction in VSMC proliferation and an increase in early VSMC apoptosis. These findings suggest that preventative strategies against restenosis must be tailored for the diabetic and metabolic syndrome patients.

Keywords

vascular smooth muscle; response to injury; metabolic syndrome

INTRODUCTION

Diabetes and metabolic syndrome are considered the disease of the 21st Century. Metabolic syndrome is a pre-diabetic state, which is a constellation of well-defined metabolic risk factors: insulin resistance, atherogenic dyslipidemia, central abdominal obesity, hypertension and development of vascular pro-thrombotic and pro-inflammatory states (1, 2). It is characterized by high plasma levels of free fatty acids in association with high blood glucose levels. These patients present frequently for revascularization due to atherosclerotic occlusive disease (2). The presence of metabolic syndrome can be correlated with increased carotid intima-media thickness in both men (3) and women (4). Metabolic syndrome has also been demonstrated to amplify vascular wall thickness and stiffness (5). Diabetes and insulin resistance have been shown to be independent predictors of early restenosis after coronary stenting (6-9). After controlling for age, sex, previous myocardial infarction, stent length, current smoking, and statin therapy in a population of patients from the GENetic DEterminants of Restenosis (GENDER) study, metabolic syndrome was also associated

with a greater target vessel revascularization (TVR) and the combined endpoint of death, myocardial infarction, and target vessel revascularization after percutaneous coronary intervention (10). We recently reported our results with lower extremity intervention in both diabetes and metabolic syndrome and demonstrated poorer anatomic outcomes in the presence of either diabetes or metabolic syndrome (12-15). Animals models of diabetes and metabolic syndrome show changes consistent with an exaggerated intimal hyperplasia response (11). Given these clinical findings, this study is designed to test the hypothesis that both diabetes and metabolic syndrome enhanced intimal hyperplasia through different cellular means. To test this hypothesis we employed the murine femoral wire injury model and examined the changes in vessel morphology and cell kinetics under normal, diabetic and metabolic syndrome conditions.

MATERIALS AND METHODS

Experimental Design

Control (wildtype, B6), diabetic (NOD) and metabolic syndrome (RCS-10) mice were used. The murine femoral wire injury model was employed in which a micro wire is passed through a branch of the femoral and used to denude the common femoral artery. Specimens were perfusion-fixed and sections were stained with Hematoxylin/Eosin (H&E) and Movat's stains such that morphometry could be performed using an ImagePro system. Animal care and procedures were conducted at Houston Methodist Research Institute in accordance with state and federal laws and under protocols approved by the Houston Methodist Research Institute Animal Care and Use Committee. Animal care complied with the "Guide for the Care and Use of Laboratory Animals" issued by the Institute of Laboratory Animal Resources.

Mouse Strains

Control wild-type mouse: Mice with a C57BL/6J background served as the primary mouse in our control group. NOD mouse: Diabetes in NOD/LtJ mice is characterized by insulitis, a leukocytic infiltrate of the pancreatic islets. Marked decreases in pancreatic insulin content occur in females at about 12 weeks of age and several weeks later in males. Onset of diabetes is marked by moderate glycosuria and by a non-fasting plasma glucose higher than 250 mg/dL. Diabetic mice are hypoinsulinemic and hyperglucagonemic, indicating a selective destruction of pancreatic islet beta cells. NOD/LtJ females are more widely used than males because the onset of IDDM symptoms occurs earlier and with a higher incidence (90-100% by 30 weeks of age). NOD/LtJ males develop IDDM at a frequency of between 40-60% by 30-40 weeks of age.

RCS-10 mouse: In the NONcNZO10/LtJ mouse, the onset of hyperglycemia occurs between 12-16 weeks on a 6% fat diet, with greater than 85% being diabetic by 24 weeks. Males exhibit increased serum triglycerides, moderate to severe liver steatosis and pancreatic islet atrophy similar to NZO/HlLt males. Serum insulin and leptin values are significantly lower than in NZO/HlLt, and are only moderately elevated above those recorded in NON/Lt males.

Blood glucose and lipid level measurement

All mice were monitored weekly for the development of diabetes by blood glucose measurement with blood glucose monitoring system Fast Draw® test strips (J&J LifeScan, U.S.A.). Two consecutive non-fasting glucose measurements >200 mg/dL constituted a diagnosis of diabetes. All mice were monitored twice monthly for high lipid levels by blood lipid level measurement with Cardiochek P.A. analyzer Cardiochek Cholesterol Test Strips (Polymer Technology Systems, U.S.A.). Total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol and triglyceride levels were measured. Non-fasting total cholesterol measurements >200 mg/dL, LDL >150 mg/dL or triglyceride levels >200mg/dL are consider higher than normal range.

Femoral wire injury

Endoluminal injury to the common femoral artery was produced by 3 passages of a 0.25-mm-diameter angioplasty guidewire (Advanced Cardiovascular Systems) as previously described (16, 17). Control sham-operated arteries underwent dissection, temporary clamping, arteriotomy, and ligature, without passage of the wire. Non-operated normal femoral arteries were used as additional controls. The vessels were harvested for morphometry and histology.

Morphometry

Following perfusion fixation, each vessel was bisected and central segments from each of these halves were embedded in paraffin and cross sections were cut (10 sections, 100 μm apart). Standard morphometric measurements were performed on histological cross sections stained with H&E and Movat's stains using SPOT camera system (Diagnostic Instruments, Inc., Sterling Heights, MI). The luminal, intimal and medial areas of each vessel are determined using an ImagePro system (Media Cybernetics, Inc., Rockville, MD). Following determination of dimensions of each vessel, a ratio of the intimal and medial areas (I/M ratio) was calculated.

Determination of Extracellular Matrix (ECM) components

Slides stained with modified Movat's stain were analyzed using an ImagePro system. The stain shows proteoglycans as blue, collagens as yellow, nuclei as black, muscle as red, fibrin as bright red and elastic fibers as dark purple**.** The area occupied by each component in a cross section was determined and expressed as a percent of the cross-sectional area.

Determination of in vivo proliferation and apoptosis

Labeling of proliferating VSMC in specimens was performed using the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich, St Louis MO; administered by Intraperitoneal injection 24 hours prior to sacrifice, 60 μg/g body weight). The number of BrdU-stained VSMC nuclei was counted and the BrdU labeling index (% of unlabeled cells) calculated on 10 slides (100 μm apart). Labeling of apoptotic VSMC on adjacent sections from each vessel specimens were evaluated using caspase 3 (Cell Signal, Danvers, MA) after processing the tissue according to the manufacturer's guidelines on 10 slides (100 μm

apart). Stained VSMC nuclei were counted and the caspase 3 labeling index (% of unlabeled cells) was calculated.

Data and Statistical Analysis

All data are presented as the mean ± standard error of the mean (s.e.m.) of six observations and statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate. A p-value less than 0.05 was regarded as significant. Non-significant p-values were expressed as p=ns.

RESULTS

Blood sugar and lipids levels

Control wild-type animals had normal levels of glucose and cholesterol and triglycerides. Diabetic mice (NOD) had high levels of glucose and triglycerides; cholesterol was normal. Metabolic syndrome animals (RCS10) had high levels of glucose, cholesterol and triglycerides (**Table 1**).

Wall morphology

In normal B6 wild-type mice, the injured femoral arteries develop intimal hyperplasia, which is maximal at 28 days and does not change substantially between day 28 and day 56. Sham operated on vessels did not produce such a response. In diabetic mice, the intimal response increased 5-fold, while in the metabolic syndrome mice there was a 6-fold increase in the intimal response (**Fig 1**).

Extracellular matrix

In normal wild-type mice, the intimal hyperplasia was composed of proteoglycans, collagen and cells. In diabetic mice, associated with the increase in the intimal hyperplasia, there was 2-fold increase in proteoglycan deposition. Collagen deposition in diabetic mice was 22-fold greater than that seen in control animals. In the animals with metabolic syndrome there was a similar 2-fold increase in proteoglycan deposition but a 100-fold increase in collagen depositions compared to the control wild-type injury mice (**Fig 1**).

Cell kinetics

In normal B6 mice, cell apoptosis peaked early within 3 days and cell proliferation peaked at 3-5 days. Maximal cell proliferation was decreased in both diabetes and metabolic syndrome compared to control wild type mice while early cell apoptosis was sustained over a longer period of time over wild type mice in both diabetes and metabolic syndrome (**Fig 2**).

Inflammatory Infiltrate

Both neutrophils and macrophages were seen within the vessel wall in the control wild-type animals, with neutrophils peaking at day 3 and macrophages from day 8 to day 29. In diabetic mice, the neutrophil peak was greater but occurred at the same day 3 time point, while in metabolic syndrome the pattern was similar to that of control wild-type mice. The peak neutrophil infiltration in diabetic animals was 2.5-fold over control and metabolic

syndrome. In the animals with metabolic syndrome, macrophage infiltration occurred much earlier than in control wild-type mice, beginning at day 3 and continuing until day 15. In comparison, diabetic mice showed an enhanced macrophage infiltration, which was sustained until day 29. The peak macrophage infiltration in metabolic syndrome was 3.3-fold greater than control wild-type mice while macrophage infiltration in diabetic animals was 2.5-fold over control wild-type mice (**Fig 3**).

DISCUSSION

We had previously demonstrated that under normal circumstances, murine injured femoral arteries developed intimal hyperplasia, which is maximal at 28 days and does not change substantially between day 28 and day 56 (18, 19). Sham operated vessels did not produce such a response (18, 19). Cell apoptosis peaked within 3 days and cell proliferation peaked at 7 days after injury. In contrast, this study has demonstrated that there are substantial changes in the development and the composition of intimal hyperplasia after arterial injury in mice with diabetes and metabolic syndrome. The contribution of acute and chronic inflammation and of connective tissue is enhanced.

In vitro, diabetes appears to enhance vascular smooth muscle cell proliferation and migration through alterations in proteases, integrins, glycoproteins and formation of Advanced Glycation Endproducts (AGEs) (20, 21). In vivo, elevated blood glucose levels have been shown to induce a series of alterations within the vasculature including endothelial dysfunction, cellular proliferation, changes in extracellular matrix conformation and impairment of LDL receptor-mediated uptake decreasing the *in vivo* clearance of LDL concentrations (22-26). Multiple animal studies of intimal hyperplasia formation induced by balloon-denuding injury in diabetic animal models have demonstrated conflicting data as to the roles of hyperglycemia and hyperinsulinemia (27-32). True diabetic models must be also differentiated by type 1 - lack of insulin - and type 2 - insulin resistance. Studies in type 1 diabetic models (Alloxan-induced diabetic rabbit (33) and BB Wistar diabetic rat (34)) have demonstrated increased intimal thickening after balloon injury compared with non-diabetic animals. Streptozotocin-induced type 1 diabetic Sprague-Dawley rats subjected to aortic stenting developed thicker intimal hyperplasia with decreased lumen area 14 days after stenting compared with controls. (31). In contrast, Park et al. have shown that there was no difference in neointimal area in the streptozotocin (STZ)-treated rats compared with controls, irrespective of insulin administration or dose of STZ (28). The majority of the studies have not used mice as their animal model.

This study in NOD mice, which lack insulin, has demonstrated that compared to non-diabetic wild-type mice, the intimal response after arterial injury in type 1 diabetes is increased 5-fold. In the diabetic NOD mice, the enhanced intimal hyperplastic response was associated with a 2-fold increase in proteoglycan deposition but with a decrease in VSMC proliferation. Apoptosis was sustained for a longer period of time. This produced a relatively acellular intimal hyperplastic response. Moreover, compared to wild-type controls, there was a 22-fold increase in collagen deposition in diabetes and a 100-fold increase in collagen deposition. Persistently elevated serum glucose has been shown to induce an increase in selected matrix gene transcription that persists for weeks after restoration of euglycemia in

vivo (35). This results in an increased synthesis of extracellular matrix components such as collagen type IV, fibronectin and laminin. Abnormalities in the synthesis and metabolism of heparan sulfate have also been reported in association with both experimental and human diabetes (36-38). (20, 21)(22-26)(39, 40)

Metabolic syndrome is characterized by hyperinsulinemia due to insulin resistance at the cellular level, high glucose levels, a pro-inflammatory and a prothrombotic state (41). In this study, the intimal response after arterial injury of mice with metabolic syndrome was increased 6-fold over similar wild-type mice but had an increase in proteoglycan deposition equivalent to that seen in the diabetic mice and a lower cell proliferation response to that observed in wild-type mice. Haudenschild et al. reported that following arterial injury there was increased aortic intimal thickening observed in the obese Zucker type 2 diabetic rats compared with the low-dose STZ-treated, insulin-treated Wistar diabetic rats (42). In a report by Park et al. (28), intimal hyperplasia development at 21 days following carotid balloon injury was increased 2-fold in obese Zucker rats (a metabolic syndrome model) compared with lean Zucker rats. In both obese and lean Zucker rats, cell proliferation peaked in the media at 3 days (28). In obese Zucker rats, carotid artery balloon injury was associated with increased intimal thickness and increased VSMC proliferation (43). (43) Furthermore, increased intimal hyperplasia correlated with increased reactive oxygen species (ROS) production, as demonstrated by dihydroethidium staining (39). This study did not demonstrate enhanced cell proliferation in metabolic syndrome. It did show increased cell apoptosis and chronic inflammation, which would be consistent with a heightened pro-inflammatory state. This study confirms for the first time that in metabolic syndrome there is increased intimal hyperplasia and that this is the result of deposition of extracellular matrix with a predominantly collagen, rather than proteoglycan, composition.

Inflammation in the diabetic mice was characterized by an early intense neutrophil response. Diabetic mice also showed an enhanced macrophage infiltration into the wall, which was sustained until day 29. The macrophage response in the wild-type mice was not as intense and subsided around day 15. The pattern of the acute inflammatory infiltrate in the mice with metabolic syndrome was also similar to that of control wild-type mice and much less than the diabetic mice. In contrast, however, the mice with metabolic syndrome demonstrated an earlier and enhanced macrophage infiltration into the wall, which subsided around day 15. In obese Zucker rats, carotid artery balloon injury was associated with increased adhesion molecule expression and inflammatory cell infiltration, compared to controls (43).

Conclusions

These data demonstrate that development of intimal hyperplasia is markedly different in diabetes and metabolic syndrome compared to controls, with an increase in collagen deposition, a reduction in VSMC proliferation and an increase in early VSMC apoptosis. This data would suggest that these lesions are not very amenable to anti-proliferative interventions in the immediate post-injury period and that in later periods the greater composition of extracellular matrix will need alternative strategies to interrupt the wound

healing response. These findings would also suggest that preventative strategies against restenosis must be tailored for the diabetic and metabolic syndrome patients.

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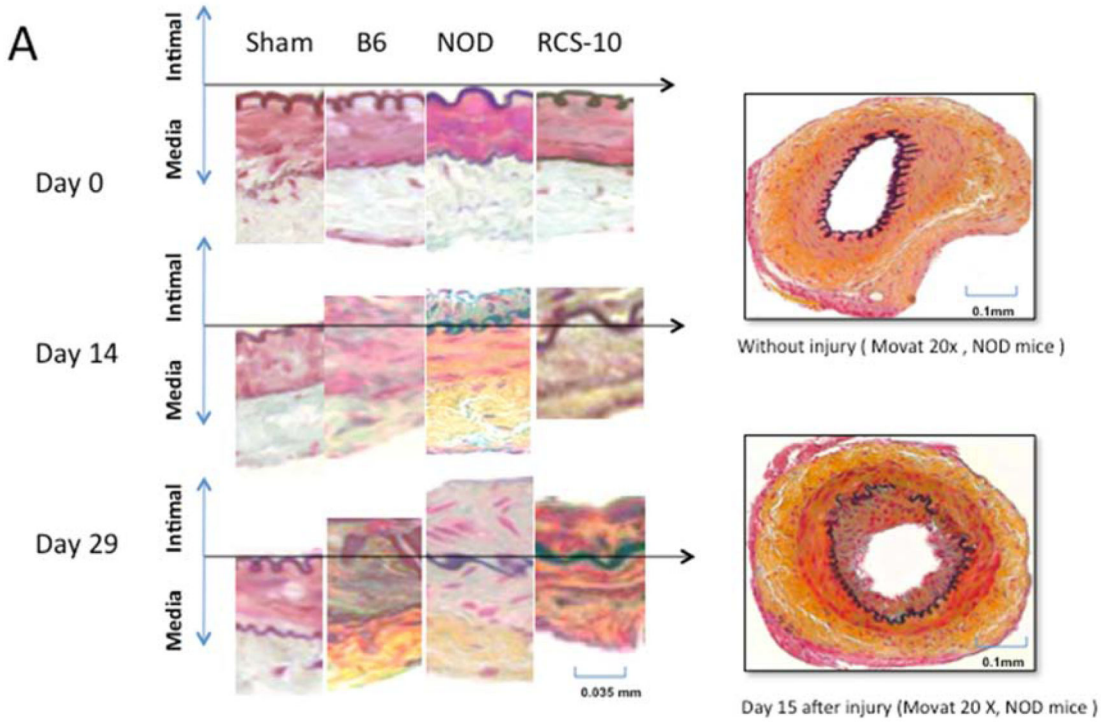

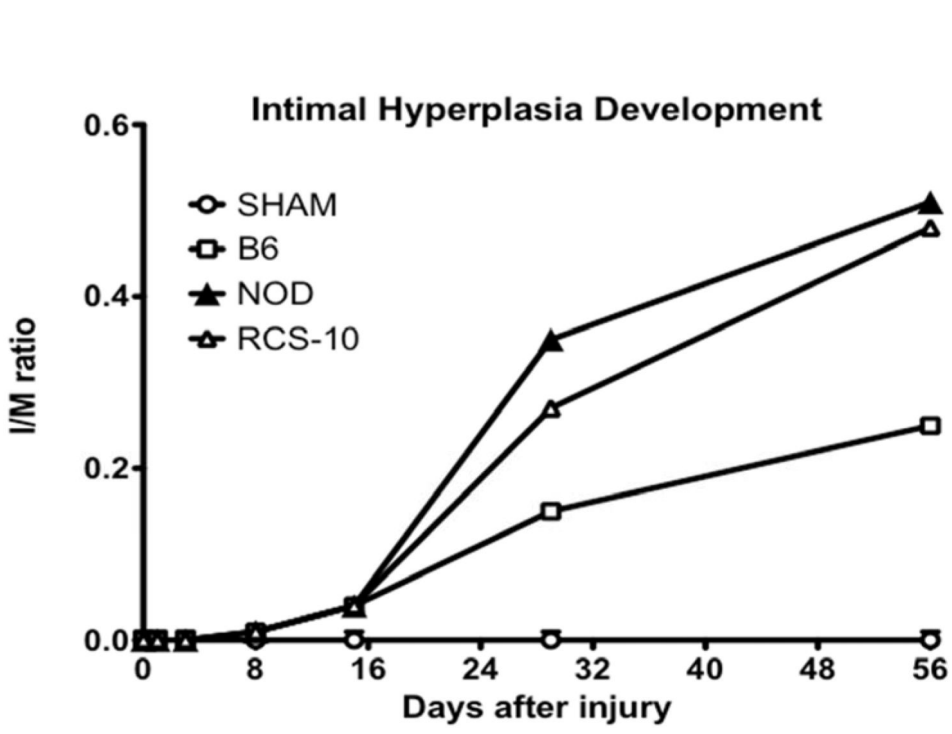

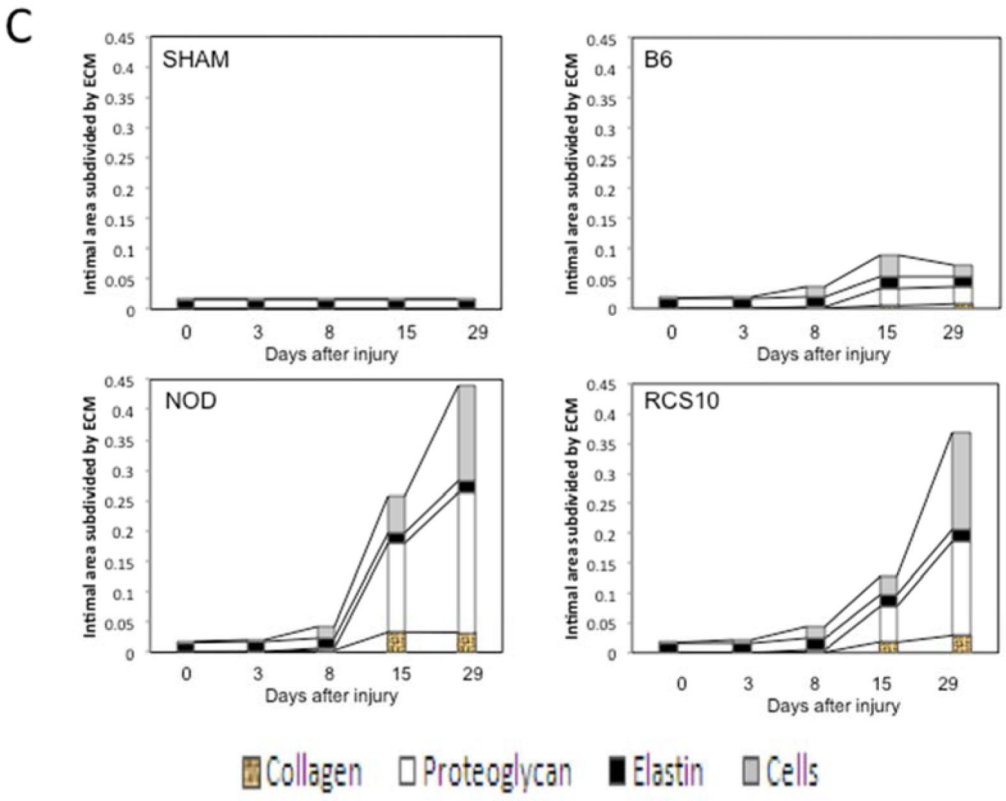

Figure 1.

Changes in the intimal hyperplasia response in wire-injured femoral vessels in sham operated (sham), control B6, diabetic (NOD) and metabolic syndrome (RCS-10) groups. (**A**) shows histological cross sections of the vessel wall stained with Movat's stain in the left panel and representative full cross-sections of the vessel in the right panel. The stain shows proteoglycans as blue, collagens as yellow, nuclei as black, muscle as red, fibrin as bright red and elastic fibers as dark purple. (**B**) shows the changes in Intima / Media ratio over the time course of the experiment. Values are the mean± s.e.m. (n=6 per group). (**C**) shows the comparison of the area of cells, collagen, proteoglycan and elastin in wire-injured femoral vessels in sham, B6, diabetic (NOD) and metabolic syndrome (RCS-10) groups**.** Values are the mean± s.e.m. (n=6 per group). Statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate.

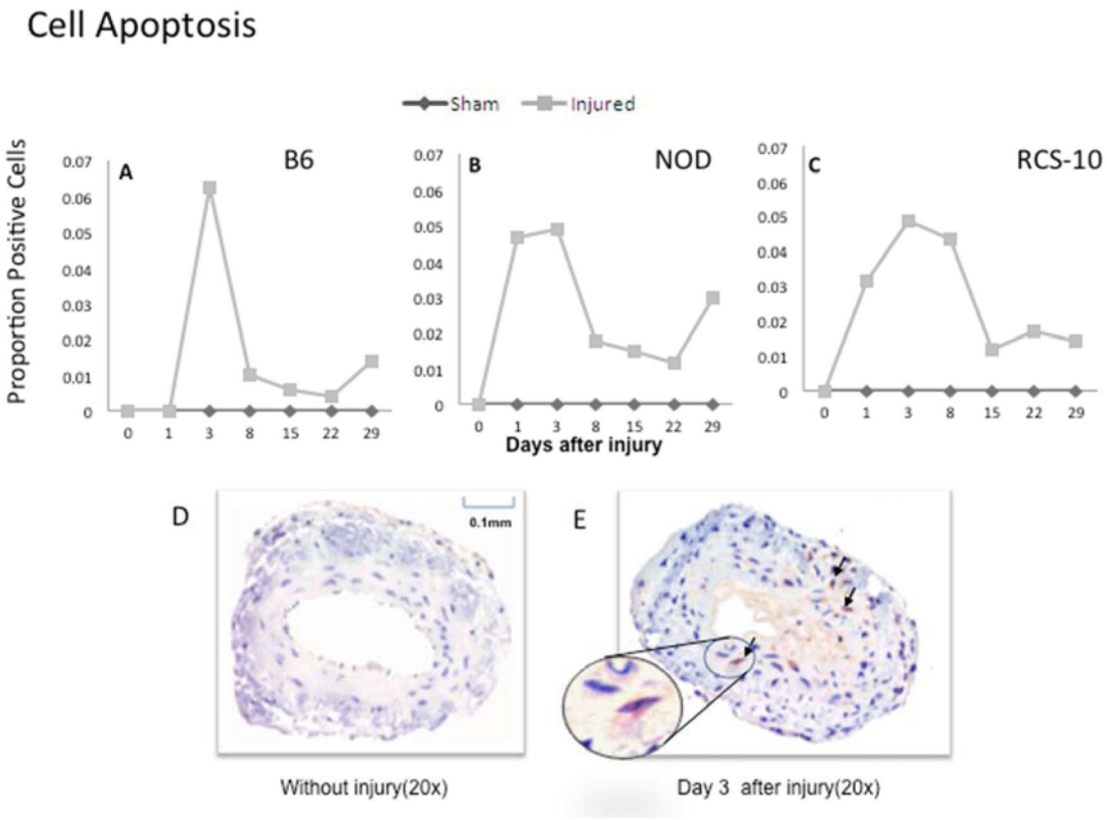

Figure 2.

Changes in cell apoptosis in wire-injured femoral vessels from the control B6 (**A**), diabetic (NOD) (**B**) and metabolic syndrome (RCS-10) (**C**) groups. Labeling of apoptotic VSMC on adjacent sections from each vessel specimen are evaluated using caspase 3. Values are the mean± s.e.m. proportion of positive cells (n=6 per group). **D** and **E** show representational cross-sections of the uninjured vessel in the left panel (**D**) and an injured vessel in the right panel (**E**) which also has an inlay demonstrating a positively stained cell. Statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate.

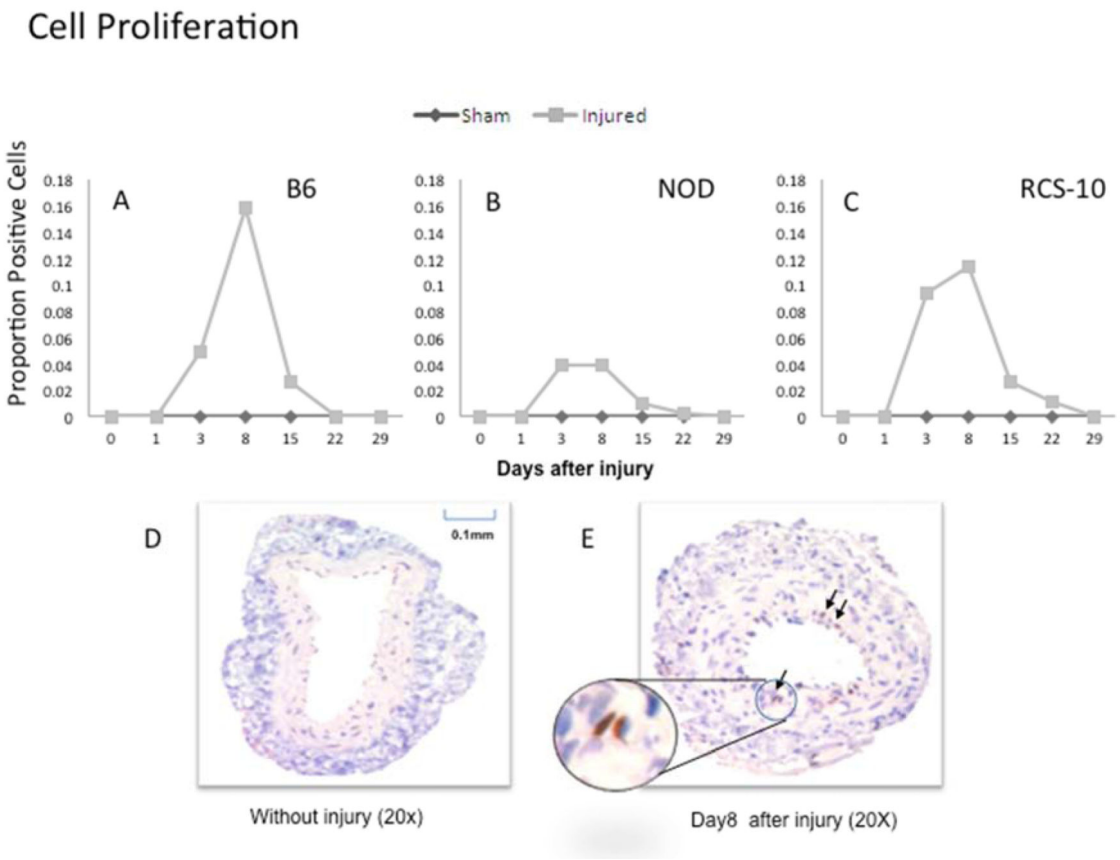

Figure 3.

Changes in cell proliferation in wire-injured femoral vessels from the control B6 (**A**), diabetic (NOD) (**B**) and metabolic syndrome (RCS-10) (**C**) groups. Labeling of proliferating VSMC in specimens is performed using the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU). Values are the mean± s.e.m. proportion of positive cells (n=6 per group). **D** and **E** show representational cross-sections of the uninjured vessel in the left panel (**D**) and an injured vessel in the right panel (**E**) which also has an inlay demonstrating a positively stained cell. Statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate.

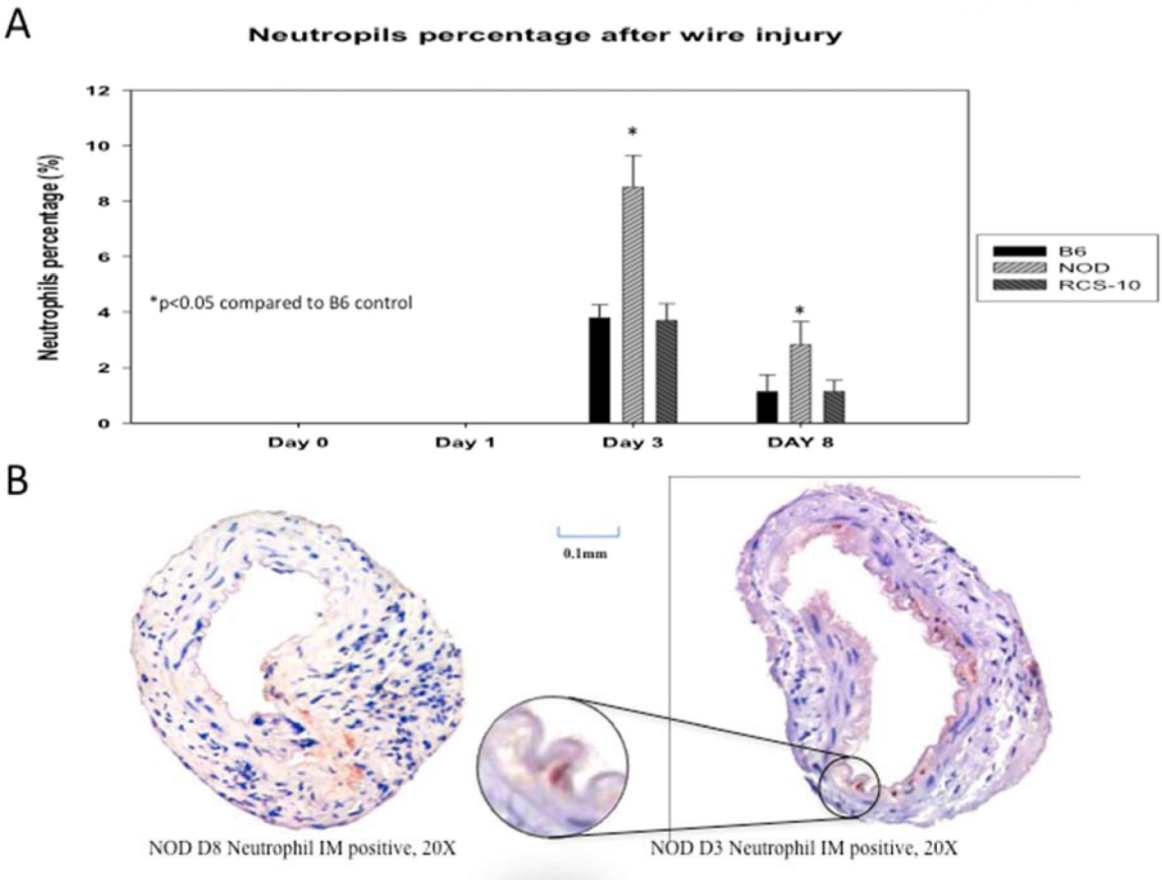

Figure 4.

Comparison of the neutrophil responses seen in wire-injured femoral vessels from the control B6, diabetic (NOD) and metabolic syndrome (RCS-10) groups **(A)**. Values are the mean± s.e.m. % positive cells (n=6 per group). (**B**) shows representational cross-sections of the vessels with the right panel having an inlay demonstrating a positively stained cell. Statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate.

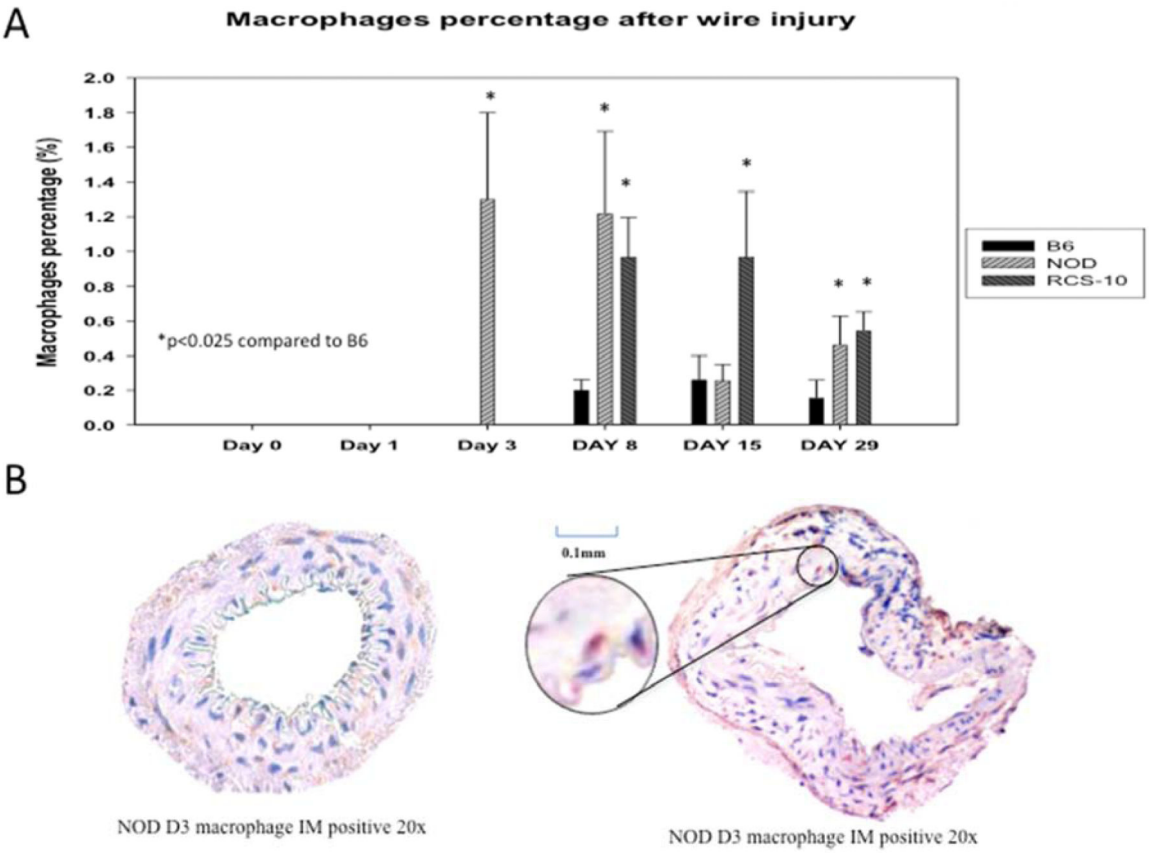

Figure 5.

Comparison of the macrophage responses seen in wire-injured femoral vessels from the control B6, diabetic (NOD) and metabolic syndrome (RCS-10) groups **(A)**. Values are the mean± s.e.m. % positive cells (n=6 per group). (**B**) shows representational cross-sections of the vessels with the right panel having an inlay demonstrating a positively stained cell. Statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate.

Table 1

	Blood Sugar (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Wildtype	145±20	109±18	75±23
RCS10	382±40**	226±50*	448±71**
NOD	343±56**	130±30	316±34 **

* $p<0.05$

** $p<0.01$ compared to wildtype