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J Clin Invest. 1996;**97**(7):1767-1773. <https://doi.org/10.1172/JCI118604>.

Research Article

Premature and extensive atheroscleroses involving renal, peripheral, and cardiovascular sites remain major complications of diabetes mellitus. Controversy exists as to the contribution of hyperglycemia versus elevated local or systemic concentrations of insulin to atherosclerosis risk. In this report, we developed the first murine model susceptible to both atherosclerosis and diabetes to determine which diabetogenic factors contribute to vascular disease. C57BL/6 and BALB/c mice were treated with multiple low-dose streptozotocin (STZ) or control citrate buffer and fed rodent chow or an atherogenic-promoting (Ath) diet for 12-20 wk. STZ treatment resulted in sustained hyperglycemia (250-420 mg/dl) and a modest reduction in plasma insulin levels for both strains regardless of diet. Citrate-treated C57BL/6 mice fed the Ath diet showed extensive oil red O-staining fatty streak aortic sinus lesions (20,537 \pm 2,957 μm^2), the size of which did not differ for Ath-fed mice treated with STZ (16,836 \pm 2,136 μm^2). In contrast, hyperglycemic BALB/c mice fed the Ath diet showed a 17-fold increase in atherosclerotic lesion area (7,922 \pm 2,096 μm^2) as compared with citrate-treated mice fed the Ath diet (467 \pm 318 μm^2). Correlations between lesion size and plasma glucose levels were significant for BALB/c ($r = 0.741$, $P < 0.009$), but not C57BL/6 ($r = 0.314$, $P < 0.3$) mice. Lesion size correlated significantly with plasma cholesterol for C57BL/6 ($r = 0.612$, $P < 0.03$) but not BALB/c ($r = 0.630$, $P < 0.1$) mice. Immunohistochemistry [...]

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Increased Atherosclerosis in Streptozotocin-induced Diabetic Mice

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Abstract

Premature and extensive atheroscleroses involving renal, peripheral, and cardiovascular sites remain major complications of diabetes mellitus. Controversy exists as to the contribution of hyperglycemia versus elevated local or systemic concentrations of insulin to atherosclerosis risk. In this report, we developed the first murine model susceptible to both atherosclerosis and diabetes to determine which diabetogenic factors contribute to vascular disease. C57BL/6 and BALB/c mice were treated with multiple low-dose streptozotocin (STZ) or control citrate buffer and fed rodent chow or an atherogenic-promoting (Ath) diet for 12–20 wk. STZ treatment resulted in sustained hyperglycemia (250–420 mg/dl) and a modest reduction in plasma insulin levels for both strains regardless of diet. Citrate-treated C57BL/6 mice fed the Ath diet showed extensive oil red O-staining fatty streak aortic sinus lesions ($20,537 \pm 2,957 \mu\text{m}^2$), the size of which did not differ for Ath-fed mice treated with STZ ($16,836 \pm 2,136 \mu\text{m}^2$). In contrast, hyperglycemic BALB/c mice fed the Ath diet showed a 17-fold increase in atherosclerotic lesion area ($7,992 \pm 2,096 \mu\text{m}^2$) as compared with citrate-treated mice fed the Ath diet ($467 \pm 318 \mu\text{m}^2$). Correlations between lesion size and plasma glucose levels were significant for BALB/c ($r = 0.741$, $P < 0.009$), but not C57BL/6 ($r = 0.314$, $P < 0.3$) mice. Lesion size correlated significantly with plasma cholesterol for C57BL/6 ($r = 0.612$, $P < 0.03$) but not BALB/c ($r = 0.630$, $P < 0.1$) mice. Immunohistochemistry showed that aortic sinus lesions from both strains contained macrophages, but smooth muscle cells were clearly present in lesions of BALB/c mice. In summary, we present the first small animal model showing accelerated atherosclerosis in response to hyperglycemia. Fatty streaks resembled those of human type II lesions in that both macrophages and smooth muscle cells were evident. In addition, our results support the concept that hyperglycemia as opposed to hyperinsulinemia contributes

heavily to risk of atherosclerosis. (*J. Clin. Invest.* 1996. 97: 1767–1773.) Key words: hyperglycemia • genetics • fatty streaks • diabetic complications • vascular disease

Introduction

Premature and extensive atheroscleroses involving renal, peripheral, and cardiovascular sites remain major complications of diabetes mellitus (1–4). About 70–80% of deaths in diabetic patients are due to vascular disease. Since hypertension and hypercholesterolemia, well-known atherosclerosis risk factors, explain just a minor part of the excess incidence of vascular disease among diabetic patients, diabetogenic factors themselves must contribute to the development of arterial disease. In particular, hyperglycemia, the primary clinical manifestation of diabetes, is thought to contribute to diabetic complications by altering vascular cellular metabolism, vascular matrix molecules, and circulating lipoproteins. For instance, hyperglycemia increases diacylglycerol levels and activates protein kinase C activity in the aorta of streptozotocin (STZ)¹-induced diabetic rats (5) and dogs (6). Thickening of the basement membranes in renal glomeruli and peripheral capillaries has been observed in STZ-induced diabetic rats (7) and diabetic patients (4). Hyperlipidemia is a feature of drug-induced diabetes in rats (8) and rabbits (9, 10), as well as poorly controlled diabetes in humans (11). Alterations in lipoprotein–cell interactions are also seen in vitro upon glycation of circulating lipoproteins (12). The role of each of these mechanisms in the pathogenesis of macro- and microangiopathy needs to be clarified.

In addition to hyperglycemia, systemic or local elevations in insulin may contribute to aberrant lipid metabolism (13) and vascular wall function (14). Imperfect normalization of glucose metabolism by replacement insulin therapy may alter the concentrations and compositions of potentially atherogenic lipoproteins (13). Changes in the ratio of apolipoproteins A-I to A-II in HDL have been observed (15), possibly interfering with the protective role of these lipoproteins in vascular disease (16). At the vascular wall, insulin may contribute directly to increasing the levels of cellular cholesterol via its ability to increase cellular sterol synthesis, induce LDL receptors, and inhibit HDL-mediated cholesterol removal (17). Although population studies have shown positive correlations between hyperinsulinemia and increased incidence and mortality rates of cardiovascular disease (3), it remains to be established whether the hyperinsulinemia per se is most detrimental to

Part of this work was presented in abstract form (1995. *FASEB [Fed. Am. Soc. Exp. Biol.] J.* 9:766a).

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Received for publication 10 November 1995 and accepted in revised form 24 January 1996.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/96/04/1767/07 \$2.00

Volume 97, Number 7, April 1996, 1767–1773

1. Abbreviations used in this paper: Ath, atherogenic diet; STZ, streptozotocin; TBS, Tris-buffered saline.

vascular health in diabetes. The alternative possibility is that hyperglycemia is associated with atherosclerosis.

In this report, we test the hypothesis that hyperglycemia can directly contribute to increased risk of large vessel disease. We used STZ to induce hyperglycemia in mice which normally exhibit resistance (BALB/c) or susceptibility (C57BL/6) to high fat diet-induced atherosclerosis (18). We show that STZ-induced hyperglycemia alone did not induce fatty streak lesions in either mouse strain. C57BL/6 mice showed atherosclerotic lesions upon feeding a high fat/high cholesterol diet, but fatty streak lesion formation was independent of hyperglycemia. However, BALB/c mice exhibited significant aortic fatty streak lesions upon combined treatment with STZ and the atherogenic diet. Further, the fatty streak lesions were complicated, containing both macrophages and smooth muscle cells. These data suggest that both dietary and genetic factors can influence lesion formation as a result of hyperglycemia. Since these mice were not hyperinsulinemic, our results support the idea that hyperglycemia alone is a risk factor for cardiovascular disease in diabetes.

Methods

Animals and diets. Female BALB/cByJ (BALB/c) and C57BL/6J (C57BL/6) mice from 6 to 8 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were fed a pelleted rodent chow diet (Wayne Rodent BLOX 8604; Teklad Test Diets, Madison, WI) for 1–2 wk before initiation of studies. Mice were maintained in a temperature-controlled (25°C) facility with a strict 12-h light/dark cycle and given free access to food and water. Blood was collected every 4 wk from the retroorbital sinus into tubes containing anticoagulant (1 mM EDTA) and plasma was stored at –70°C before analysis. Mice were killed with Nembutal (80 mg/kg) given intraperitoneally. Hearts were perfusion-fixed with 4% buffered formalin. Livers were directly placed into liquid nitrogen and stored at –70°C. Pancreata were fixed in 10% neutral buffered formalin. This project was approved by the Animal Care and Use Committee of the University of Washington (Protocol No. 2140-02).

Diets and drug treatment. Two diets used in this study were pelleted rodent chow and an “atherogenic” (Ath) diet known to elicit fatty streak lesions in strain C57BL/6 (18). The rodent chow diet contained ~ 4% fat, 24% protein, and 4.5% crude fiber. The Ath diet provided 30% kcal from fat (primarily cocoa butter) and contained 1.25% cholesterol and 0.5% sodium cholate (18).

BALB/c and C57BL/6 mice were randomly divided into four groups which were mice treated with STZ (mixed anomer; Sigma Chemical Co., St. Louis, MO) and fed either rodent chow or the Ath diet, or mice treated with the vehicle, citrate buffer (0.05 M sodium citrate, pH 4.5), and fed rodent chow or the Ath diet. STZ was dissolved in sterile citrate buffer and injected intraperitoneally into mice (40 mg/kg, ~ 20 μ l) within 5 min of preparation. STZ or citrate buffer was administered for five consecutive days during the first week of this study. 4 wk later, half of the mice in each treatment group were placed on the Ath diet for the duration of the study (12–20 wk). To maintain hyperglycemia in the STZ treatment groups, the multiple low-dose STZ treatment was repeated during week 7, and control mice were injected with citrate buffer. Drug and diet treatments were staggered to allow mice to adjust to the Ath diet.

Body weight. During the 24 wk of this study, mice were healthy as evidenced by coat conditions and body weight gain. Initial body weights for BALB/c and C57BL/6 mice were 21 \pm 1 and 18 \pm 1 grams (mean \pm SEM), respectively. Final body weights for all treatment groups were ~ 25–26 grams for BALB/c and 23–24 grams for C57BL/6 mice.

Analytical tests. Total and HDL cholesterol concentrations were

determined using a colorimetric kit (diagnostic kit No. 236691; Boehringer Mannheim, Indianapolis, IN) with cholesterol standards (Preciset No. 125512; Boehringer Mannheim) as described (19). HDL cholesterol values were measured after selective precipitation of VLDL/LDL by polyethylene glycol (20). Triglyceride concentrations in plasma were determined after removal of free glycerol (diagnostic kit No. 450032; Boehringer Mannheim). Plasma glucose concentrations were determined colorimetrically (glucose trinder kit No. 315; Sigma Diagnostics, St. Louis, MO). Hepatic lipids were extracted using the method of Folch et al. (21), modified to contain Triton X-100 as described by Carr et al. (22). Insulin was quantified using a rat insulin radioimmunoassay kit as described by the manufacturers (No. RI-13K; Lingo Research Inc., St. Charles, MO).

Aortic sinus lesion area. Quantification of atherosclerotic fatty streak lesions was done by evaluation of lesion size in the aortic sinus as described (23) with modifications. Briefly, the heart and upper section of the aorta were removed from animals, cleaned of peripheral fat under a dissecting microscope, and sectioned directly under and parallel to the atrial leaflets. The upper section was embedded in OCT medium and frozen. Every other section (10 μ m thick) throughout the aortic sinus (400 μ m) was taken for analysis. The distal portion of the aortic sinus is recognized by the three valve cusps which are the junctions of the aorta to the heart. Sections were evaluated for fatty streak lesions after staining with oil red O and counterstaining with hematoxylin and eosin. Lesion areas per section were counted and areas estimated using a Compaq 286 computer (Compaq Computer, Houston, TX) equipped with an FG-100 image acquisition board, a high resolution video camera, and a Sony video monitor. Area measurements were done using the Optimas Image Analysis Software Package (BioScan Inc., Edmonds, WA).

Evaluation of pancreata. Pancreata were embedded in paraplast and 10- μ m sections were stained with eosin and counterstained with hematoxylin. Nine sections per pancreas were examined.

Immunohistochemistry. Immunohistochemical staining of cryostat cut sections (10 μ m thick) of the aortic sinus of the heart was done as described previously (24) with some modifications. Briefly, sections were fixed in cold acetone for 10 min, air-dried, and washed two times in Tris-buffered saline (TBS). Endogenous peroxidase activity was blocked by incubating slides in a solution of 3% hydrogen peroxidase for 5 min. All incubations were performed at room temperature in humidified chambers. Slides were incubated with the appropriate primary antibody overnight, followed by 4–6 h of incubation with secondary horseradish peroxidase-labeled goat anti-rat immunoglobulin G antibody (1:200 dilution in TBS). Sections were visualized by chromogenic detection (AEC substrate system No. K0697; Dako Corp., Carpinteria, CA). AEC was used as a chromogen. After treatment, sections were washed with TBS and counterstained with hematoxylin. Smooth muscle cells were identified using a mouse monoclonal antibody against human alpha-actin directly coupled to horseradish peroxidase (antibody dilution was 1:6; No. U7033; Dako Corp.). Macrophages were identified using a rat monoclonal antibody to the mouse and human Mac-1 antigen (No. 1118-129; Boehringer Mannheim). VCAM-1 was detected using a rat monoclonal antibody against murine VCAM-1 (antibody dilution was 1:100, No. 1510-01; Southern Biotechnology Associates, Inc., Birmingham, AL). Anti-Mac-1 and anti-VCAM-1 antibodies bound to tissue were visualized after the binding of anti-rat immunoglobulin coupled to horseradish peroxidase. Control slides were incubated with nonspecific primary antisera, or in some cases without the primary antibody. In no case did control slides show a positive signal.

Statistics. Data are reported as mean \pm SEM. Statistical differences were determined by ANOVA using SYSTAT for the Macintosh (SYSTAT, Inc., Evanston, IL). Pearson's correlation coefficients were used to assess correlations. Three-way ANOVA was used to determine interactions between strain, diet, and drug treatment with diet as the covariant. Post-hoc analyses of significance were made using Tukey's test for additivity. The Student's *t* test was used to compare independent means. *P* < 0.05 was accepted as statistically significant.

Results

Hyperglycemia occurs in STZ-treated mice. Our goal was to test the role of hyperglycemia on atherosclerosis development without the complications of concomitant administration of insulin to maintain animal viability. Further, hyperglycemia had to be maintained for at least 14 wk to provide time for fatty streak formation in C57BL/6 mice (19). Thus, our strategy was to use a multiple low-dose injection regimen for STZ with a repeat series of injections to ensure 18 wk of hyperglycemia in both strains of mice. By repeating the 5-d injection regimen during week 7, STZ-treated mice maintained hyperglycemia for at least 16 of the total 24 wk of study regardless of diet (Fig. 1). During weeks 8–24, plasma glucose levels for BALB/c mice ranged from ~ 250–360 mg/dl as compared with ~ 150–190 mg/dl for citrate-injected controls. C57BL/6 mice showed plasma glucose levels ranging from 310 to 420 mg/dl after STZ treatment as compared with 160–230 mg/dl for citrate-treated mice. Overall, plasma glucose levels nearly doubled for mice treated with STZ as compared with citrate-treated animals.

A three-way ANOVA showed a main effect of drug treatment on plasma glucose ($P < 0.001$) and that the three parameters (strain, diet, treatment) accounted for 85% of plasma glucose levels ($r^2 = 0.859$). Thus, STZ treatment alone and not diet or strain influenced glucose levels. Plasma glucose levels for mice treated with STZ were significantly higher than citrate-treated mice at each time point ($P < 0.001$ – 0.005). Plasma glucose and lipid values within each diet and strain group were not significantly different during weeks 12–24. Thus, for clarity of analysis and presentation, plasma glucose and lipid levels are given as mean values evaluated during weeks 12–24 (Table I).

Hyperglycemia was accompanied by a marked reduction in pancreatic islet number of STZ-treated BALB/c and C57BL/6 mice as compared with control animals, which is consistent with previous reports (25). The loss of islets most likely contributed to the decreased plasma insulin levels observed for STZ-treated mice. Upon STZ treatment, insulin levels for BALB/c mice decreased from 1.50 ± 0.01 to 0.7 ± 0.08 ng/ml ($P < 0.001$), and values for C57BL/6 mice decreased from 0.96 ± 0.01 to 0.65 ± 0.02 ng/ml ($P < 0.001$). Insulin levels for both strains were unaffected by diet.

Plasma lipids. Previous studies have shown that plasma triglyceride levels decrease for mice fed the Ath diet (19) and that STZ leads to hypertriglyceridemia in rodents and rabbits (8, 9). In the current study, the Ath diet decreased plasma triglyceride levels markedly in citrate-treated BALB/c mice and in C57BL/6 mice regardless of drug treatment (Table I). Triglyceride levels tended to be higher for hyperglycemic mice as compared with citrate-treated animals, but the differences did not reach statistical significance. The Ath diet caused marked increases in plasma total (greater than threefold) and VLDL/LDL (greater than eightfold) cholesterol levels. Cholesterol levels were not significantly different between strains, and in general hyperglycemic mice showed greater levels of plasma and VLDL/LDL cholesterol than citrate-treated mice. Changes in HDL levels due to diet and drug treatments were strain dependent. For citrate-treated mice, the Ath diet tended to decrease HDL levels for C57BL/6 but not BALB/c mice as seen previously (26). STZ given to chow or Ath diet-fed mice tended to increase HDL cholesterol levels, although changes did not reach statistical significance.

Hepatic lipids. A three-way ANOVA in terms of strain, diet, and drug treatment showed main effects of diet ($P < 0.001$) and strain ($P < 0.002$ – 0.023) on hepatic lipid levels. For instance, mice fed the Ath diet exhibited significant elevations in triglyceride and cholesterol levels as compared with rodent chow-fed mice, and triglyceride levels for C57BL/6 were consistently higher than levels for BALB/c. Hepatic lipid levels were unaffected by STZ treatment. Thus, changes in plasma lipids due to STZ treatment were not driven by STZ effects on gross hepatic lipid metabolism.

Atherosclerosis development. It is well established that C57BL/6 but not BALB/c mice develop fatty streaks and more advanced atherosclerotic plaques in the aortic sinus within 16 wk of feeding diets rich in fat and cholesterol (18, 27, 28). We tested the possibility that the combination of STZ-induced hyperglycemia and diet-induced atherosclerosis susceptibility would lead to accelerated lesion formation in C57BL/6 mice.

In our study, citrate-treated C57BL/6 mice fed the Ath diet for 20 wk developed extensive aortic fatty streaks with a wide range among animals of $3,300$ – $66,500 \mu\text{m}^2$, with a mean lesion area of $20,537 \pm 2,957 \mu\text{m}^2$ (\pm SEM) (Fig. 2). In contrast, no significant lesions were seen for BALB/c mice fed the Ath diet

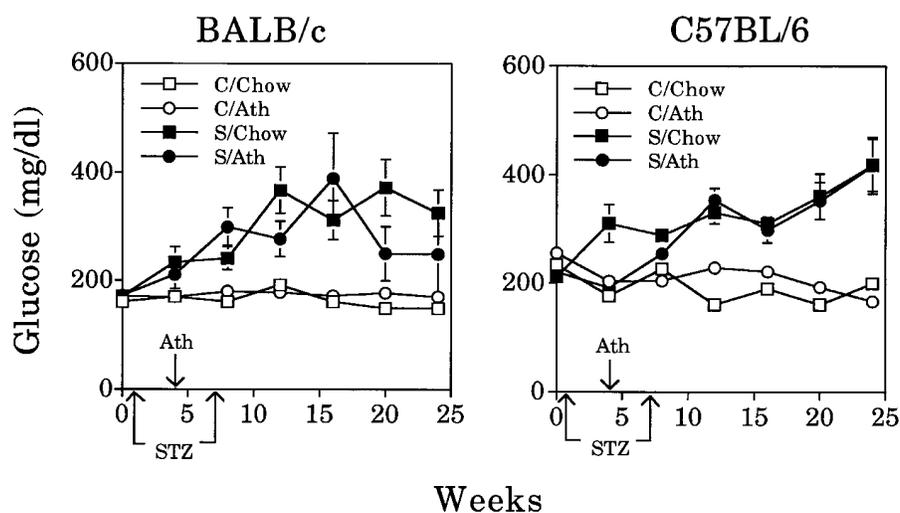


Figure 1. Plasma glucose concentration in response to diet and drug treatments for BALB/c and C57BL/6 mice. Animals were treated with STZ (S, filled symbols) (40 mg/kg, 5 d) or citrate buffer (C, open symbols) during weeks 0 and 7. Mice from both treatment groups were fed either an atherosclerosis-promoting diet (Ath, circles) rich in saturated fat (30% calories primarily as cocoa butter) and cholesterol (1.25% by weight) starting at week 4, or were continually fed rodent chow (Chow, squares). Plasma glucose was determined colorimetrically as described in Methods. Values are reported as mean \pm SEM for $n = 9$ –18 mice.

Table I. Plasma and Hepatic Parameters for BALB/c and C57BL/6 Mice Fed Rodent Chow or Ath Diets and Treated with STZ or Citrate Buffer

| Parameters | Treatment | BALB/c | | C57BL/6 | |
|---------------------------------|-----------|--------|----------------------|-------------------|--------------------|
| | | Chow | Ath | Chow | Ath |
| Plasma (mg/100 ml) | | | | | |
| Glucose | Citrate | 163±10 | 174±2 | 178±10 | 203±14 |
| | STZ | 344±15 | 291±33 | 355±24 | 356±24 |
| | <i>P</i> | 0.001 | 0.005 | 0.001 | 0.001 |
| Triglyceride | Citrate | 46±12 | 18±1* | 34±5 | 7±1*‡ |
| | STZ | 54±9 | 50±11* | 62±14 | 15±5*§ |
| | <i>P</i> | NS | NS | NS | NS |
| Total cholesterol | Citrate | 72±3 | 220±20* | 66±2 | 236±13* |
| | STZ | 79±2 | 377±83 | 75±5 | 290±10* |
| | <i>P</i> | NS | NS | NS | 0.005 |
| VLDL/LDL cholesterol | Citrate | 18±3 | 144±9* | 17±4 | 202±16* |
| | STZ | 18±3 | 265±56* | 18±4 | 237±7* |
| | <i>P</i> | NS | NS | NS | NS |
| HDL cholesterol | Citrate | 54±3 | 80±8 | 49±15 | 35±3 [§] |
| | STZ | 62±2 | 95±15 | 57±3 | 52±10 [§] |
| | <i>P</i> | NS | NS | NS | NS |
| Hepatic lipids (mg/gram tissue) | | | | | |
| Triglyceride | Citrate | 3±0.6 | 11±1* | 11±1 [§] | 15±1 |
| | STZ | 3±0.3 | 14±2* | 7±1 [‡] | 21±2* |
| | <i>P</i> | NS | NS | NS | NS |
| Cholesterol | Citrate | 3±0.2 | 48±3* | 2±0.1 | 64±4* |
| | STZ | 3±0.3 | 52±10* | 2±0.3 | 75±8* |
| | <i>P</i> | NS | NS | NS | NS |

Data are presented as mean±SEM averaged over 12–24 wk for $n = 9$ –20 mice. *P* values in each category compare citrate and STZ treatment groups. * $P < 0.005$ and ^{||} $P < 0.05$ denote comparisons between groups fed rodent chow (*Chow*) and Ath diet. [§] $P < 0.005$ and [‡] $P < 0.05$ denote comparisons between mouse strains.

for up to 20 wk ($467 \pm 318 \mu\text{m}^2$). STZ-induced hyperglycemia combined with the Ath diet did not induce larger lesions in C57BL/6 mice ($16,836 \pm 2,136 \mu\text{m}^2$). However, BALB/c mice treated with STZ and fed the Ath diet showed a 17-fold increase in lesion size ($7,992 \pm 2,096 \mu\text{m}^2$). No lesions were seen for chow-fed mice regardless of drug treatment. Thus, increases in lesion size due to feeding the high fat diet and induced hyperglycemia were strain dependent, suggesting that both diet and genetic background may contribute to accelerated atherosclerosis as seen in diabetes.

To determine whether levels of plasma cholesterol, glucose, or both contributed to lesion formation, correlations between lesion sizes and these plasma parameters among individual mice fed the Ath diet were calculated. For STZ-treated BALB/c mice, plasma glucose ($r = 0.741$, $P < 0.009$) but not plasma total cholesterol ($r = 0.630$, $P < 0.1$) levels correlated with lesion size. In contrast, plasma cholesterol ($r = 0.612$, $P < 0.03$) but not plasma glucose ($r = 0.314$, $P < 0.3$) levels determined lesion sizes for STZ-treated C57BL/6 mice. Thus, hyperglycemia in mice fed the high fat diet is important for lesion development in BALB/c mice.

The character of lesions seen in BALB/c and C57BL/6 was distinct (Fig. 3). While fatty streaks of both strains showed oil red O staining of intra- and extracellular lipid, immunohistochemical staining showed distinct cellularities. Cells within

lesions of C57BL/6 mice reacted only with antibody to Mac-1 showing that the primary cell type in these fatty streaks was macrophages. In contrast, oil red O staining regions of BALB/c mice contained cells immunoreactive to Mac-1 and alpha-actin. Immunoreactive signal to VCAM-1 was present in lesions from both strains, and no qualitative differences in location or signal intensity were seen between strains. Thus, BALB/c fatty streaks were complex, consisting of at least two cell types, macrophages and smooth muscle cells, both of which were lipid laden. This is the first report of smooth muscle cells appearing early in fatty streak development in mice and suggests that the etiology of lesion development is distinct between these strains.

Discussion

In this report, we tested whether hyperglycemia in the absence of concomitant hyperinsulinemia could contribute to enhanced atherosclerosis formation in mice. One of the earliest features of developing atherosclerotic plaques is the appearance of fatty streaks in the artery wall. BALB/c mice made diabetic by STZ treatment and fed a high fat/high cholesterol diet exhibited significant fatty streak formation. Since these mice had low levels of circulating insulin, hyperglycemia was the main diabetogenic factor contributing to increased lesion for-

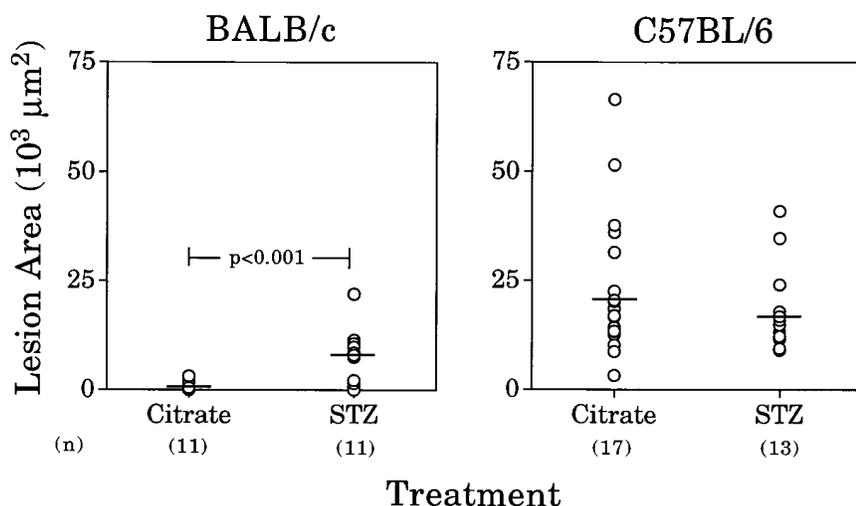


Figure 2. Aortic sinus fatty streak lesion sizes for BALB/c and C57BL/6 mice. Mice were treated during weeks 0 and 7 with STZ (40 mg/kg, 5 d) or citrate buffer as described in Methods. Mice were fed an atherosclerosis-promoting diet rich in saturated fat (30% calories primarily as cocoa butter) and cholesterol (1.25% by weight) starting at 4 wk and continuing for 12–20 wk. Aortic sinuses were collected, sectioned, and stained with oil red O as described in Methods before quantification of fatty streak regions. Horizontal lines indicate mean lesion area for each group.

mation. Strengthening this conclusion is the observation that plasma glucose but not lipid levels correlated with lesion formation in BALB/c mice.

The development of fatty streaks in response to hyperglycemia was strain specific, as both STZ and high fat diet treatments were required to produce fatty streaks in BALB/c mice, but C57BL/6 mice showed extensive lesion development with fat feeding alone. Our results with C57BL/6 mice are comparable to those of Nishina et al. (29) who showed that C57BL/6 and C57BL/Ks strains carrying mutations which predispose them to diabetes and/or obesity do not show enhanced lesion formation upon high fat/high cholesterol diet feeding. This suggests that diabetes does not contribute to arterial disease in the C57BL genetic background.

What could account for the differences in diabetes-accelerated lesion formation we observed in BALB/c and C57BL/6 mice? Previous studies have indicated that among inbred mouse strains fed the Ath diet, the extent of lesion formation is positively correlated with the expression of genes associated with inflammation and oxidative stress (30). In particular, hepatic mRNA levels of several inflammatory and oxidative stress responsive genes were markedly induced in C57BL/6 mice, but less so in BALB/c mice. Further, oxidized LDL were shown to stimulate the expression of these same genes. Glucose promotes the oxidation of serum and arterial wall proteins (31, 32) and thereby contributes to the induction of vascular and lipoprotein changes associated with aberrant lipoprotein uptake, monocyte binding, and inflammatory events. We hypothesize that hyperglycemic BALB/c mice fed the high fat diet experience an increase in oxidized lipoproteins which increase oxidative stress at the vascular wall over a threshold needed to accelerate the development of early atherosclerotic lesions. For C57BL/6 mice, this threshold is surpassed by diet alone. We are currently addressing this hypothesis by examining inflammatory gene expression which is expected to increase in Ath diet-fed BALB/c mice treated with STZ as compared with citrate.

A distinct feature of the aortic fatty streaks in BALB/c mice was their complexity, characterized by a mixture of lipid-laden macrophages and smooth muscle cells reminiscent of type II lesions in humans (33). Although smooth muscle cells have been seen in aortic sinus lesions of specific strains chronically (> 7 mo) fed fat and cholesterol (18, 34), or in genetically

engineered mice with severe hypercholesterolemia (35), smooth muscle cells are not a general feature of early mouse fatty streaks. The fact that these cells were seen in BALB/c but not in C57BL/6 mice suggests that diabetogenic factors contribute to smooth muscle cell proliferative events. Many biochemical pathways linking hyperglycemia to vascular changes have been recognized, including the increased synthesis of specific growth factors (36). Smooth muscle cells appeared early in lesion development in BALB/c mice (16 wk) and thus factors influencing the proliferation of smooth muscle cells can be examined rather quickly in this model.

The role of hyperglycemia in BALB/c lesion development is still unclear. Apart from direct effects due to inflammation, lipoproteins may be altered via glycation (31) making them more readily taken up by scavenger receptors on cells of the artery wall (12, 14). Glycation of matrix molecules occurs, which could provide a stronger trapping network for lipoproteins penetrating vascular spaces. In addition, a direct role of STZ on the vasculature cannot be ruled out. Treatment of mice with STZ resulted in modest or no significant changes in plasma and hepatic lipids as compared with citrate-treated mice within each diet group. Also, pancreas damage was limited and plasma insulin levels were decreased to approximately half normal levels. Thus, evidence for severe STZ toxicity is lacking, making it unlikely that STZ was responsible for the acceleration of lesion formation in BALB/c mice. Further study of these processes and the effects of insulin treatment on lesion development in BALB/c mice is underway.

Our study provides the first system in which to carefully examine the role of hyperglycemia as opposed to hyperinsulinemia in atherosclerosis development. Overall, animal models simulating the interactions between diabetes and atherosclerosis have been poorly developed. Still et al. (8) demonstrated that rats recovered from the diabetic effects of alloxan showed increased atherosclerosis in the aorta when fed a high fat diet, but characterization of lesions and atherogenic mechanisms was not performed. Alloxan-induced diabetic rabbits are largely protected against diet-induced atherosclerosis probably because of an accumulation of large triglyceride-rich lipoproteins which may be excluded from the artery wall (9). Primate models of diabetes which are susceptible to atherosclerosis may be available but are prohibitively expensive for research. Thus, manipulation of BALB/c mice with STZ and high fat di-

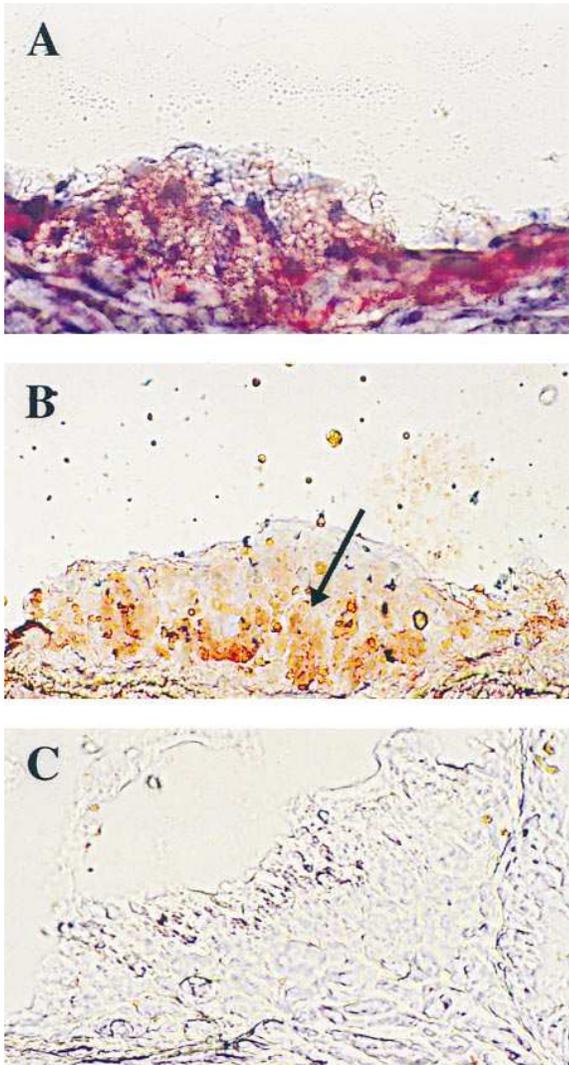


Figure 3. Characterization of early atherosclerotic lesions in the proximal sinus. BALB/c and C57BL/6 mice were fed an atherosclerosis-promoting diet containing excess fat and cholesterol for 12–20 wk and treated with multiple low doses of STZ as described in Figs. 1 and 2. *A* and *B* are serial sections from one BALB/c mouse which were stained for neutral lipid with oil red O (*A*) and treated with antibody to human alpha-actin (*B*) as described in Methods. The location of one of several positively reacting smooth muscle cells is indicated with an arrow. *C* is a section from a C57BL/6 mouse which was treated with antibody to human alpha-actin and illustrates the absence of positively reacting cells in lesions from this mouse strain.

ets will allow identification of dietary and genetic factors affecting vascular disease in diabetes.

In summary, our results support the concept that hyperglycemia as opposed to hyperinsulinemia contributes heavily to risk of atherosclerosis. Both diet and genetic factors contribute to responsiveness of lesion formation in the environment of diabetes and crosses of these strains treated with STZ and high fat diets can be used to identify diabetogenic factors contributing to the accelerated atherosclerosis. The hyperglycemic BALB/c mouse provides a convenient model in which to identify genetic, dietary, and diabetogenic factors contributing to accelerated fatty streak lesions of complex cellularity.

Acknowledgments

We thank Dr. Åke Lernmark and Dr. John Oram (Department of Medicine, University of Washington) for helpful comments and suggestions when reviewing this manuscript, and Dr. Emil Chi and Dr. Ying-Tzang Tien (Department of Pathology, University of Washington) for their excellent technical support.

This work was supported by National Institutes of Health grant DK-02456.

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