Human Vascular Smooth Muscle Cells From Diabetic Patients Are Resistant to Induced Apoptosis Due to High Bcl-2 Expression

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An emerging body of evidence suggests that vascular remodeling in diabetic patients involves a perturbation of the balance between cell proliferation and cell death. Our aim was to study whether arteries and vascular smooth muscle cells (VSMCs) isolated from diabetic patients exhibit resistance to apoptosis induced by several stimuli. Internal mammary arteries (IMAs) were obtained from patients who had undergone coronary artery bypass graft surgery. Arteries from diabetic patients showed increasing levels of Bcl-2 expression in the media layer, measured by immunofluorescence and by Western blotting. Human IMA VSMCs from diabetic patients showed resistance to apoptosis, measured as DNA fragmentation and caspase-3 activation, induced by C-reactive protein (CRP) and other stimuli, such as hydrogen peroxide and 7β-hydroxycholesterol. The diabetic cells also exhibited overexpression of Bcl-2. Knockdown of Bcl-2 expression with Bcl-2 siRNA in cells from diabetic patients reversed the resistance to induced apoptosis. Consistent with the above, we found that pretreatment of nondiabetic VSMCs with high glucose abolished the degradation of Bcl-2 induced by CRP. Moreover, cell proliferation was increased in diabetic compared with nondiabetic cells. This differential effect was potentiated by glucose. We conclude that the data provide strong evidence that arterial remodeling in diabetic patients results from a combination of decreased apoptosis and increased proliferation. Diabetes 55:1243–1251, 2006

**V**ascular complications are the leading cause of death in diabetic patients, and at least some of these are related to functional and structural alterations in large arteries (1,2). With respect to functional changes, we have shown previously that vascular reactivity was altered in arteries isolated from diabetic patients (3), whereas structural alterations are characterized by wall thickening (2). Increase in intima-media thickening of the carotid artery has been suggested to be a useful predictor of a high-risk group of cardiovascular events (4,5). In general, three different patterns of arterial remodeling have been identified: inward, characterized by a decrease in lumen diameter; outward, characterized by an increase in lumen diameter; and compensatory, characterized by a lumen diameter preservation despite changes in intima-media thickness. Clinical data showed that type 2 diabetic patients had greater intima-media thickening than nondiabetic patients (6). It is generally accepted that increased cell proliferation is a key feature of intima-media thickening in diabetes (7,8). However, an emerging body of evidence suggests that an increase in intima-media thickening involves a perturbation in the balance between cell proliferation and cell death (9,10). Based on this postulate, it has been described that hyperglycemia reduces vascular smooth muscle cell (VSMC) apoptosis by a mechanism mediated by protein kinase C and overexpression of the antiapoptotic protein Bcl-2 (11,12). Moreover, other studies have shown that the Fas/Fas-ligand pathway is impaired in patients with type 2 diabetes (13), supporting the idea that the rate of apoptosis of VSMCs is decreased in diabetic patients. However, the majority of studies showing the role of Bcl-2 in the antiapoptotic effect of high glucose were conducted either in animals or in nondiabetic human VSMCs. For this reason, we investigated whether the impairment of VSMC apoptosis observed in animal models can be extended to human arteries and VSMCs isolated from diabetic patients. To this purpose, we measured apoptosis and cell proliferation induced by different stimuli in VSMCs from diabetic and nondiabetic patients. We also focused our work on the expression of Bcl-2 and Bcl-xL, in both groups of patients and corroborated our findings in VSMCs in culture with those in isolated arteries.

**RESEARCH DESIGN AND METHODS**

Patients were recruited from those undergoing coronary artery bypass graft surgery at the Cardiac Surgery Service (Hospital Clínico, San Carlos, Madrid, Spain). Diabetes was defined following the criteria established by the American Diabetes Association (14) as fasting serum glucose concentration ≥126 mg/dl and use of antidiabetic oral drugs or insulin. Patient data included age, sex, active smoker, obesity; total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, glucose, and blood pressure. Table 1 shows the clinical and biochemical characteristics of the patients studied.

Exclusion criteria of the patients included age >80 years, pathologies affecting inflammatory status (renal failure, liver disease, etc.), and cancer.
Internal mammary arteries (IMAs) were collected by the surgeons during the surgical procedure, labeled, and used within the next few minutes after the operations. The study was conducted according to the Declaration of Helsinki and informed consent was obtained from all subjects before sampling. C-reactive protein (CRP) was purchased from Calbiochem (Bionova Científica). Hydrogen peroxide and 7β-hydroxycholesterol were purchased from Sigma. The stock solution of 7β-hydroxycholesterol was dissolved in ethanol at 1,000× concentrated. All other reagents were obtained from Sigma unless otherwise stated.

**Immunofluorescence staining.** Expression of Bcl-2 in human IMA was measured by immunofluorescent staining. Arteries from diabetic and nondiabetic patients were fixed with 4% paraformaldehyde (in 0.2 mol/l phosphate buffer, pH 7.2–7.4) for 2 h and washed with PBS. Arteries were then immersed in PBS 0.1 mol/L sucrose 30% at 4°C for 3 h and embedded in optimal cutting temperature (Tissue Tek; Bayer) for 30 min. The arteries were snap frozen at −80°C for the analysis. Frozen transverse sections 7 μm thick were obtained (Cryostat HM500; Microm International, Düsseldorf, Germany), dried at 37°C, and washed with PBS + 0.3% Tween 20 (PBS-T). Nonspecific binding was blocked by incubating the samples for 1 h in 3% bovine albumin in PBS-T. Frozen transverse sections were incubated with mouse monoclonal anti–Bcl-2 antibody (Neomarker) at a 1:100 dilution overnight at 4°C. Excess of the primary antibody was removed by washing with blocking solution, and samples were incubated with Alexa-568 goat anti-mouse secondary antibodies (Molecular Probes) at a 1:500 dilution for 2 h at 37°C. After washing, images were captured using a Leica TCS SP2 inverted microscope. Quantification of Bcl-2 expression was performed by using the Image J 1.33 software (National Institutes of Health) (15). Data are presented as fold increase of Bcl-2 expression with respect to each negative control. Analysis of Bcl-2 intensity was done by two independent researchers and the concordance analyzed with the interclass correlation coefficient (16). The value obtained for this parameter was 0.78, within the acceptable range (17).

The specificity of the immunostaining was evaluated by omission of the primary antibody and processing as above. Under these conditions, no staining was observed in the vessel wall of either diabetic or nondiabetic patients.

**Analysis of collagen and elastin in human arteries.** In large vessels, elastin is organized into concentric rings of elastic lamellae around the arterial lumen, whereas collagen is present in all three layers of the vascular wall. Segments of IMA were immediately fixed in 4% paraformaldehyde in PBS for 3 h, transferred to a cryomold containing optimal cutting temperature and frozen at −80°C for the analysis. Frozen transverse sections 7 μm thick were obtained (Cryostat HM500; Microm International, Düsseldorf, Germany), dried at 37°C, and washed with PBS + 0.3% Tween 20 (PBS-T). Nonspecific binding was blocked by incubating the samples for 1 h in 3% bovine albumin in PBS-T. Frozen transverse sections were incubated with mouse monoclonal anti–Bcl-2 antibody (Neomarker) at a 1:100 dilution overnight at 4°C. Excess of the primary antibody was removed by washing with blocking solution, and samples were incubated with Alexa-568 goat anti-mouse secondary antibodies (Molecular Probes) at a 1:500 dilution for 2 h at 37°C. After washing, images were captured using a Leica TCS SP2 inverted microscope. Quantification of Bcl-2 expression was performed by using the Image J 1.33 software (National Institutes of Health) (15). Data are presented as fold increase of Bcl-2 expression with respect to each negative control. Analysis of Bcl-2 intensity was done by two independent researchers and the concordance analyzed with the interclass correlation coefficient (16). The value obtained for this parameter was 0.78, within the acceptable range (17).

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### TABLE 1
Clinical and biochemical characteristics of the patients studied

|                | Nondiabetic | Diabetic | \(P\)  |
|----------------|-------------|----------|--------|
| n              | 10          | 9        | 0.84*  |
| Age (years)    | 61.9 ± 11.4 | 65.7 ± 11.7| 0.67†  |
| Sex (male/female) | 8/2         | 5/4      | 0.1*   |
| BMI (kg/m²)    | 27.8 ± 3.3  | 25.1 ± 4.3| 0.03*  |
| Total cholesterol (mg/dl) | 210.8 ± 44.6 | 164.0 ± 36.6| 0.1*   |
| LDL cholesterol (mg/dl) | 129.3 ± 41.1 | 92.8 ± 26.7| 0.03*  |
| HDL cholesterol (mg/dl) | 47.3 ± 8.1   | 36.0 ± 8.3 | 0.037* |
| Triglycerides (mg/dl) | 150.8 ± 52.6 | 174.0 ± 57.0| 0.4*   |
| Glucose (mg/dl) | 97.8 ± 34.3  | 158.0 ± 42.9| 0.01*  |
| HbaA1c (%)     | NA          | 7.3 ± 0.8 | NA     |
| Hypertension   | 3 (33)      | 6 (66)   | 0.15‡  |
| Smokers        | 5 (50)      | 3 (33)   | 0.56‡  |

Data are means ± SD and n (%). *Student’s t test, †Fisher’s exact test, and ‡χ² test. NA, not analyzed.
embedding medium (Tissue Tek; Bayer), and frozen in liquid nitrogen. Frozen transverse sections (7 μm) were incubated with picrosirius red (0.1% wt/vol) (Sirius red 3FB in saturated aqueous picric acid) for 30 min with gentle agitation for collagen staining (18). Color images were captured with a microscope (Nikon Eclipse TE 2000-S; ×20 objective) using a digital camera (Nikon DXM 1200F). The collagen was measured in the whole media layer using Image J 1.33 software (National Institutes of Health) and showed as arbitrary units. To analyze the intima-media thickness, the arteries were incubated with 0.01 mg/ml Hoechst 33342 to stain cell nuclei (blue). Intima-media thickness in arteries was measured (in micrometers) using Image J software. The value of each artery was obtained by the mean of five random samples measured in the cross section.

The content of elastin was studied with fluorescent confocal microscopy based on the autofluorescent properties of elastin (Ex 488 nm/Em 500–560 nm) using a Leica TCS SP2 confocal system. The number of elastin lamellae was obtained by counting.

Western Blotting. To determine the expression of Bcl-2, cells from diabetic and nondiabetic patients were plated onto 60-mm Petri dishes and allowed to attach for 24 h. At the time of harvest, the cells were washed with ice-cold PBS, lysed on ice with 200 μl lysis buffer (10% glycerol, 2.3% SDS, 62.5 mmol/l Tris-HCl, pH 6.8, 150 mmol/l NaCl, 10 mmol/l EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml chymostatin, 1 μg/ml aprotinin, and 1 mmol/l phenylmethylsulphonyl fluoride), and boiled for 5 min. Equal amounts of protein were run on 12.5% SDS polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride membranes (Immobilon-P; Amersham, Madrid, Spain) and blocked overnight at 4°C in blocking solution (5%

Analysis of caspase-3 activity. Human VSMCs (nondiabetic and diabetic) were plated on 96-well plates and allowed to attach for 24 h. The cells were then treated with 10 μg/ml CRP for 8 h. Caspase-3 activity was measured spectrophotometrically using a commercially available kit (Calbiochem) following the manufacturer’s instructions. Data are represented as fold increase of the control values.

Transfection of Bcl-2 siRNA. Human VSMCs from diabetic patients were plated onto 96-well plates at 60% confluency. Cells were allowed to attach for 24 h and then transected with Bcl-2 siRNA (Cell Signaling, Izasa S.A., Spain) following the manufacturer’s instructions. In addition, a fluorescein-labeled nontargeted siRNA control allowed us to monitor the transfection specificity. After transfection (48 h), cells were treated with CRP (10 μg/ml for 12 h) or H2O2 (10 μmol/l for 12 h) and apoptosis analyzed by DNA fragmentation as above stated.

Measurement of cell proliferation. To determine cell proliferation, diabetic and nondiabetic VSMCs were plated onto 96-well plates and allowed to attach for 24 h. The cells were then treated with glucose (5–25 mmol/l) or mannitol (25 mmol/l) as osmotic control 48 h before the induction of apoptosis.

Results are expressed as means ± SD, n = 3 patients. *P < 0.05 vs. 5 mmol/l glucose.

Cell cultures. Human IMA VSMCs were cultured from explants in RPMI (Life Technologies, Barcelona, Spain) containing 10% FCS. The cells exhibited typical “hill and valley” smooth-muscle morphology by phase-contrast microscopy, and the cultures were stained positively with a monoclonal anti-smooth α-actin antibody. Experiments were performed with VSMCs between passages 3 and 5. For the analysis of cell death by apoptosis, the cells (diabetic and nondiabetic) were treated with different apoptotic stimuli: 1–10 μg/ml CRP for 4–12 h and 10 μmol/l H2O2 for 12 h or 100 μmol/l 7-b-hydroxycholesterol for 48 h. To test the effect of glucose in the apoptosis of VSMCs, the cells were pretreated with glucose (5–25 mmol/l) or mannitol (25 mmol/l) as osmotic control 48 h before the induction of apoptosis.

Measurement of cellular DNA fragmentation. VSMCs from nondiabetic and diabetic patients were plated on 96-well plates and allowed to attach for 24 h. Cellular DNA fragmentation was measured with a commercially available cellular DNA fragmentation enzyme-linked immunosorbent assay kit (Roche-Boehringer) following the manufacturer’s instructions. DNA fragmentation was expressed as fold increase of the control values.

### Results

**Effect of glucose on Bcl-2 expression.** Western blotting showing the expression of Bcl-2 in nondiabetic VSMCs incubated with increasing concentrations of glucose. Bar graph shows the mean ± SD, n = 3 patients. *P < 0.05 vs. 5 mmol/l glucose.

**Statistical analysis.** Results are expressed as means ± SD and are accompanied by the number of observations. Statistical analyses were carried out using Student’s t test or one-way ANOVA when necessary. Differences with a P value < 0.05 were considered statistically significant.
RESULTS

Overexpression of Bcl-2 in diabetic VSMCs. We analyzed the basal expression of the antiapoptotic proteins Bcl-2 and Bcl-xL, both in arteries and in VSMCs in culture isolated from diabetic and nondiabetic patients. Figure 1A shows the expression of Bcl-2 in human arteries analyzed in immunofluorescence-stained samples, and Fig. 1B shows arteries analyzed by Western blotting. The expression of Bcl-2 in the media layer was significantly higher in diabetic than in nondiabetic patients. We also analyzed the content of Bcl-2 in VSMCs, as shown in Fig. 1C. VSMCs isolated from diabetic patients exhibited increased levels of Bcl-2 expression in comparison with those from nondiabetic subjects (Fig. 1C, upper panel). In the case of Bcl-xL, we found a slight increase in levels both in arteries (Fig. 1B, bottom panel) and in cells in cultures (Fig. 1C, bottom panel) from diabetic patients. However, this increase was not statistically significant. Preliminary results show that when we correlated the level of Bcl-2 expression in arteries with levels of glucose in the serum of the patients, we found that increasing levels of glycemia correlate with increasing levels of Bcl-2 in the media layer ($r^2 = 0.370$). As an extension of this finding, we performed an experiment in which nondiabetic VSMCs were treated with increasing concentrations of glucose (5–25 mmol/l) for 48 h (Fig. 2). Under these circumstances, the Bcl-2 protein levels increased in parallel with the glucose levels.

Apoptosis in VSMCs from diabetic and nondiabetic patients. Because we observed an overexpression of Bcl-2 in diabetic patients, we tested whether this overexpression was reflected by resistance of VSMCs to cell death by apoptosis in diabetic patients. Apoptosis was induced by CRP because this risk marker has been found in increasing concentrations in plasma from diabetic patients. We first performed a concentration- and time-dependence curve for DNA fragmentation induced by CRP. We established the concentration of 10 μg/ml and the time point of 12 h as most effective for DNA fragmentation (Fig. 3A). Under these circumstances, CRP produced an increase in DNA fragmentation in nondiabetic human VSMCs, although no effect was observed in diabetic VSMCs. To confirm that CRP induced apoptosis rather than necrosis, we analyzed the activation of caspase-3, a well-known marker of apoptosis. Yet again, CRP induced caspase-3 activation selectively in nondiabetic human VSMCs (Fig. 3B). Because some of the effects of CRP might be due to the sodium azide present in the commercial preparation (19), we also examined the effect of sodium azide (0.05% for 12 h) or lipopolysaccharide (0.8 ng/ml for 12 h) or a combination of both, on apoptosis of human VSMCs. We did not find any effect of sodium azide, lipopolysaccharide, or both on DNA fragmentation in this cell type, irrespective whether the cells were derived from diabetic or nondiabetic patients (Fig. 3C).

Effect of CRP on Bcl-2 degradation. Because diabetic patients showed an overexpression of Bcl-2, we aimed to determine whether this overexpression was responsible for the resistance of diabetic VSMCs to induced apoptosis. Figure 4A shows that CRP induced Bcl-2 degradation in nondiabetic but not in diabetic VSMCs. To determine this
whether hyperglycemia might be the key determinant of this resistance to CRP, we tested whether nondiabetic cells exposed to high glucose levels might develop characteristics similar to those of diabetic VSMCs. For this purpose, Bcl-2 degradation and DNA fragmentation induced by CRP were investigated in nondiabetic VSMCs pretreated with high glucose. The effect of CRP on both parameters was either abolished (Bcl-2 degradation, Fig. 4B) or diminished (DNA fragmentation, Fig. 4C) in the presence of high glucose conditions.

Apoptosis induced by other stimuli. To determine whether the resistance to induced apoptosis in diabetic human VSMCs was exclusive for CRP, we tested for apoptosis induced by two other stimuli: hydrogen peroxide and 7β-hydroxycholesterol, a component of oxidized LDL. In normoglycemic cell medium (glucose 5 mmol/l), hydrogen peroxide and 7β-hydroxycholesterol induced a greater apoptotic effect, measured as DNA fragmentation, in nondiabetic than in diabetic VSMCs. Interestingly, when nondiabetic VSMCs were preincubated with an increasing concentration of glucose (5–25 mmol/l), the apoptotic effect of both compounds progressively decreased (Fig. 5).

Transfection of Bcl-2 siRNA. To determine whether the overexpression of Bcl-2 was indeed responsible for the resistance to apoptosis observed in VSMCs from diabetic patients, we performed an experiment in which VSMCs from diabetic patients were transfected with Bcl-2 siRNA or with fluorescein-labeled nontargeted siRNA as control. Figure 6B shows that the expression of Bcl-2 was blocked in cells transfected with Bcl-2 siRNA compared with those treated with nontargeted siRNA. Accordingly, the lack of apoptosis induced by CRP and hydrogen peroxide in human VSMCs from diabetic patients was reversed in cells transfected with Bcl-2 siRNA; under these circumstances, cells from diabetic patients behaved like those from nondiabetic patients. However, VSMCs from diabetic patients transfected with nontargeted siRNA did not show apoptosis induced by such stimuli (Fig. 6C).

Analysis of VSMC proliferation. Cell proliferation was determined by the analysis of BrdU incorporation into cellular nuclei, following stimulation with well-known mitogens. Under normoglycemic conditions (glucose 5 mmol/l), 10% FCS increased BrdU incorporation in both nondiabetic and diabetic VSMCs, although this effect was greater in diabetic VSMCs. This differential effect was potentiated when the glucose concentration was increased in the extracellular medium. Similar results were obtained when proliferation was induced with norepinephrine. However, when we added the mitogen angiotensin II, we did not find any difference between diabetic and nondiabetic VSMCs even though hyperglycemia significantly increased BrdU incorporation (Fig. 7).

Analysis of media thickness and cholesterol content in human arteries. Since diabetic VSMCs in culture were characterized by attenuated apoptosis and augmented proliferation, we determined whether these features were reflected by changes in the structure of the arteries. For this purpose, we analyzed media thickness in both groups
of diabetic and nondiabetic patients and found that diabetic patients showed a significant increase over nondiabetic patients in media thickness (Fig. 8). This might be due to an increase in the proliferation rate and/or a decrease in the apoptosis, but could possibly also be due to an increase in the content of the extracellular matrix. To test for the latter possibility, we studied the content of collagen and the number of elastin lamellae in the media. We found no differences in either of these parameters between diabetic and nondiabetic patients (Fig. 8).

**DISCUSSION**

The most relevant findings of this study of isolated arteries and VSMCs of diabetic patients are upregulation of the antiapoptotic protein Bcl-2, increased resistance to apoptosis, increased VSMC proliferation, and enhanced media thickness. We have found an increase in Bcl-2 expression in the media layer as well as in VSMCs obtained from diabetic patients. Previous studies have shown that hyperglycemia inhibited rat VSMC apoptosis in vitro by a mechanism that involved protein kinase C activation (11) and Bcl-2 upregulation. However, to our knowledge, this is the first report to show the upregulation of Bcl-2 in diabetic patients and the resistance to apoptosis in VSMCs obtained from diabetic patients.

CRP is an independent risk factor for cardiovascular diseases and has been found in increasing concentrations in diabetic patients (20). Moreover, CRP has been found to induce apoptosis in VSMCs (21). We observed that treatment of human VSMCs with CRP induced apoptosis (measured by three independent methods: DNA fragmentation, Bcl-2 degradation, and caspase-3 activation) exclusively in cells obtained from nondiabetic patients. The reason for this selective effect may be due to increased levels of the antiapoptotic protein Bcl-2 in diabetic human VSMCs, which would protect them from induced apoptosis. This hypothesis was corroborated by the fact that nondiabetic VSMCs treated with high glucose showed increasing levels of Bcl-2 (Fig. 2) and that the degradation of Bcl-2 initiated by CRP was abolished when the cells were pretreated with high glucose (Fig. 4). It has been suggested that hyperglycemia may induce phosphorylation of Bcl-2 via protein kinase C, which would protect Bcl-2 from degradation (11). This fact might explain, at least in part, why CRP only induced Bcl-2 degradation in nondiabetic patients. It is important to point out that diabetic cells are resistant to apoptosis even in normoglycemic conditions, indicating that Bcl-2 overexpression is a maintained feature in dia-
glucose could protect Bcl-2 degradation by promoting its phosphorylation via ERK1/2. Further research will achieve new insight into this issue.

To rule out that the selective apoptotic effect of CRP was exclusive for this protein, we also analyzed apoptosis in diabetic and nondiabetic VSMCs induced by other stimuli. In this context, nondiabetic VSMCs in cultures incubated with hydrogen peroxide and 7β-hydroxycholesterol were more sensitive to apoptosis than diabetic VSMCs. However, when nondiabetic VSMCs were preincubated with increasing concentrations of glucose for 48 h, they became resistant to apoptosis, similar to the diabetic cells. To support the hypothesis that the overexpression of Bcl-2 in VSMCs from diabetic patients was responsible for the resistance to apoptosis, we knocked-down Bcl-2 expression with a Bcl-2 siRNA. Under these circumstances, VSMCs from diabetic patients showed apoptosis induced by CRP and hydrogen peroxide to the same extent as the cells from nondiabetic patients.

Because there is a delicate balance within the vessel wall between apoptosis and proliferation, its perturbation may contribute to vascular remodeling. We also analyzed VSMC proliferation in diabetic and nondiabetic cells. We found greater stimulation of cell growth induced by FCS in diabetic versus nondiabetic cells. This effect was potentiated when the cells were preincubated with increasing concentrations of glucose. The mechanism responsible for the accelerated VSMC proliferation in diabetes remains unclear. However, it has been reported that high glucose upregulates AT1 receptor expression (24). Although the study of AT1 receptors is not within the scope of this work, we also found that the proliferative effect of angiotensin II was enhanced in high glucose conditions in both diabetic and nondiabetic patients, which may corroborate the latter results. Other mitogens involved in cardiovascular complications are catecholamines. Norepinephrine has been shown to induce increasing VSMC growth in cells isolated from animals exposed to risk factors like diabetes (25) and contributes to flow-mediated arterial remodeling (26). In our experiments, norepinephrine only induced VSMC proliferation under high-glucose conditions, and this effect was more prominent in diabetic than in nondiabetic VSMCs.

The decrease in apoptosis in VSMCs along with the increase in VSMC growth observed in diabetic patients is reflected in an increasing media thickness in these patients, as shown in Fig. 8. Interestingly, we also observed a positive correlation between media thickness and Bcl-2 expression in the arterial wall, which strongly supports the idea that Bcl-2 overexpression is important in the vascular remodeling in diabetic patients. Another feature that could contribute to vascular remodeling is an increase in the content of matrix protein within the medial layer. To test for this possibility, we analyzed the collagen content and the number of elastin lamellae in isolated arteries by a method previously validated (27) but did not find any differences between diabetic and nondiabetic patients, suggesting that the increase in the media thickness is most likely due to an increase in the cellularity of the medial layer.

In conclusion, the above findings of upregulation of Bcl-2, reduced apoptosis, and enhanced VSMC proliferation in diabetic patients contribute to our understanding of the mechanisms that underlie vascular remodeling in diabetic patients and will hopefully lead to discovery of new targets for future therapy of the vascular complications in type 2 diabetes.
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REFERENCES
1. Terry JG, Tang R, Espeland MA, Davis DH, Vieira JL, Mercuri MF, Crouse JR: Carotid structure in patients with documented coronary artery disease and disease-free control subjects. Circulation 107:1146–1151, 2003
2. Henry RM, Kostense PJ, Spijkerman AM, Dekke JM, Nijpels G, Heine RJ, Kamp O, Westendorf N, Bouter LM, Stehouwer CD: Arterial stiffness increases with deteriorating glucose tolerance status: the Hoorn Study. Circulation 107:2089–2095, 2003
3. Okon E, Chung A, Rauniyar P, Padilla E, Tejerina T, McManus B, Luo H, van Breenen C: Compromised arterial function in human type 2 diabetic patients. Diabetes 54:2415–2423, 2005
4. Kawamori R, Yamasaki K, Matsushima H, Nishizawa H, Nao K, Hougaku H, Maeda H, Handa N, Matsumoto M, Kamada T: Prevalence of carotid atherosclerosis in diabetic patients: ultrasound high-resolution B-mode imaging on carotid arteries. Diabetes Care 28 (Suppl. 1):S37–S42, 2005
5. Ambrose JA, Tannenbaum MA, Alexopoulos D, Hjendahl-Monsen CE, Leavy J, Weiss M, Borrico S, Gorlin R, Fuster V: Angiographic progression of coronary artery disease and the development of myocardial infarction. J Am Coll Cardiol 12:56–62, 1988
6. Henry RM, Kostense PJ, Dekker JM, Nijpels G, Heine RJ, Kamp O, Bouter LM, Stehouwer CD: Carotid arterial remodeling: a maladaptive phenomenon in type 2 diabetes but not in impaired glucose metabolism; the Hoorn study. Stroke 35:671–676, 2004
7. Kunjathoor VV, Wilson DL, LeBoeuf RC: Increased atherosclerosis in streptozotocin-induced diabetic mice. J Clin Invest 97:1767–1773, 1997
8. Koya D, King GL: Protein kinase C activation and the development of diabetic complications. Diabetes 47:850–866, 1998
9. Bennett MR, Evan GI, Schwartz SM: Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. J Clin Invest 95:2266–2274, 1995
10. Gibbons GH, Dzau VJ: The emerging concept of vascular remodeling. N Engl J Med 330:1431–1438, 1994
11. Hall JL, Matter CM, Wang S, Gibbons GH: Hyperglycemia inhibits vascular smooth muscle cell apoptosis through a protein kinase C-dependent pathway. Circ Res 87:574–580, 2000
12. Li H, Telemague S, Miller RE, Marsh JD: High glucose inhibits apoptosis induced by serum deprivation in vascular smooth muscle cells via upregulation of Bcl-2 and Bcl-xl. Diabetes 54:540–545, 2005
13. Cosson E, Bringuier AF, Parias J, Guillot R, Vaysse J, Attali JR, Feldmann G, Valensi P: Fas/Fas-Ligand pathway is impaired in patients with type 2 diabetes: influence of hypertension and insulin resistance. Diabete Metab 31:47–54, 2005
14. American Diabetes Association: Diagnosis and classification of diabetes mellitus (Position Statement). Diabetes Care 28 (Suppl. 1):S37–S42, 2005
15. Ahramoff MD, Magelhaes PJ, Ram SF: Image processing with Image J. Biophotonics Int 11:36–42, 2004
16. Shout PE, Fleiss JL: Intraclass correlations: uses in assessing rater reliability. Psychol Bull 86:420–428, 1979
17. Kramer MS, Feinstein AR: The biostatistics of concordance: clinical biostatistics. Clin Pharmacol Ther 29:111–123, 1981
18. Izzard AS, Graham D, Burnham MP, Heerkens EH, Dominiczak AF, Heagerty AM: Myogenic and structural properties of cerebral arteries from the stroke-prone spontaneously hypertensive rat. Am J Physiol Heart Circ Physiol 285:H1489–H1494, 2003
19. Taylor K, Giddings J, van den Berg C: C-reactive protein-induced in vitro endothelial cell activation is an artefact caused by azide and lipopolysaccharide. Arterioscler Thromb Vasc Biol 25:1225–1230, 2005
20. Bahceci M, Tuzcu A, Ogun C, Canoruc N, Itlimur K, Aslan C: Is serum C-reactive protein concentration correlated with HbA1c and insulin resistance in type 2 diabetic men with or without coronary heart disease? J Endocrinol Invest 28:145–150, 2005
21. Blaschke F, Bruenmer D, Yin F, Takata Y, Wang W, Fishbein MC, Okura T, Higaki J, Graf K, Fleck E, Isueh WA, Law EE: C-reactive protein induces apoptosis in human coronary vascular smooth muscle cells. Circulation 110:579–587, 2004
22. Yang Y, Yu X: Regulation of apoptosis: the ubiquitous way. FASEB J 17:790–799, 2003

FIG. 8. Analysis of the intima-media thickness and collagen content in human IMA. A: Representative confocal images showing transversal sections of IMA. Intima-media thickness and number of elastin lamellae were analyzed as indicated in the RESEARCH METHODS section. B: Representative images of collagen staining with picrorousin red of transversal sections from IMA. Bar graph shows the mean ± SD of n = 9 patients. *P < 0.05 vs. non-diabetic patients.
23. Sakuma H, Yamamoto M, Okumura M, Kojima T, Maruyama T, Yasuda K: High glucose inhibits apoptosis in human coronary artery smooth muscle cells by increasing bcl-xL and bfl-1/A1. *Am J Physiol Cell Physiol* 283: C422–C428, 2001

24. Sodhi CP, Kanwar Y, Sahai A: Hypoxia and high glucose up regulate AT1 receptor expression and potentiated ANG II-induced proliferation in VSM cells. *Am J Physiol Heart Circ Physiol* 284: H846–H852, 2003

25. Bauch HJ, Grunwald J, Vischer P, Gerlach U, Hauss WH: A possible role of catecholamines in atherogenesis and subsequent complications of atherosclerosis. *Exp Pathol* 31:193–204, 1987

26. Erami C, Zhang H, Tanoue A, Tsujimoto G, Thomas SA, Faber JE: Adrenergic catecholamine tropic activity contributes to flow-mediated arterial remodeling. *Am J Physiol Heart Circ Physiol* 289: H744–H753, 2005

27. Briones AM, Gonzalez JM, Somoza B, Giraldelo J, Vila E, Gonzalez MC, McGrath JC, Arribas SM: Role of elastin in spontaneously hypertensive rat small mesenteric artery remodelling. *J Physiol* 562:185–195, 2003