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Modified LDL is a major cause of injury to the endothelium in diabetes. In the present study, we analyzed the effects on endothelial cells of LDL recovered from type 2 diabetic patients (dm-LDL) or from nondiabetic subjects (n-LDL). Treatment of human umbilical vein endothelial cells with dm-LDL, but not n-LDL, led to the accumulation of cells in G1. To dissect the molecular mechanisms of this effect, we analyzed the expression and function of the cyclin-dependent kinase inhibitor p21\textsuperscript{waf}, a cell cycle regulator known to be a target of the signal transducers and activators of transcription (STATs). dm-LDL led to transient STAT5 phosphorylation and the formation of a STAT5-containing complex and activated p21\textsuperscript{waf} expression at the transcriptional level. Expression of the dominant-negative form of STAT5B, but not of STAT5A, significantly decreased both p21\textsuperscript{waf} expression and the fraction of cells in G1. Finally, immunofluorescence analysis demonstrated that activated STAT5 is expressed in newly formed intraplaque vessels and in endothelial cells lining the luminal side of the plaque. Similarly, p21\textsuperscript{waf} immunoreactivity was found in the neointimal vasculature. Our results suggest a role of STAT5B as a regulator of gene expression in diabetes-associated vascular disease.

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Diabetic LDL inhibits cell-cycle progression via STAT5B and p21waf

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Introduction

Atherosclerosis is a multistep chronic inflammatory disease that involves the interplay between soluble mediators and vascular/inflammatory cells (1). Multiple risk factors, including diabetes, are involved in this pathological process (2, 3). Several lines of evidence indicate that either qualitative or quantitative abnormalities of LDL in the diabetic setting may contribute to its atherogenicity, and in particular to the macrovascular complications that it causes (4–7). Although clinically significant complications of atherosclerosis, such as plaque ulceration, rupture, and thrombosis occur in established atherosclerotic lesions, understanding of the mechanisms of early lesion formation offers the possibility to delay or prevent further progression. A select set of transcriptional factors may be critical in both the initiation and the expansion of lesions. One of these, NF-κB, has been linked to the onset of atherosclerosis (8). It has been reported that both native and modified LDL activate a series of NF-κB–dependent genes that are relevant to the pathophysiology of the vessel wall (8). A similar set of genes is known to be a target of the signal transducers and activators of transcription (STATs) (9). STATs are a family of latent cytoplasmic proteins that, upon activation, acquire DNA-binding activity, translocate into the nucleus, bind to specific promoter elements, and control the expression of target genes (9). Two different but highly homologous STAT5 genes have been isolated, and defined as STAT5A and STAT5B (10). These STAT proteins undergo activation in response to different stimuli, and exert transcriptional activation on a number of genes that are involved mainly in the control of cell proliferation (11).

A large body of evidence indicates that the cell cycle is controlled by a series of regulatory molecules known as cyclins, cyclin-dependent kinases (Cdk’s), and Cdk inhibitors (CKIs) (12). Among these latter molecules are p21waf (13) and p27kip1 (14), dual inhibitors of Cdk’s and of the replication factor known as proliferating cell nuclear antigen (PCNA). The effects of p21waf and p27kip1 lead to cell-cycle arrest and inhibition of DNA replication, respectively (15–17). Evidence is accumulating that supports the idea that certain regulators of cell proliferation modulate cyclin/Cdk activity by affecting CKI expression (18–20). Recent data also support the role of p21waf in mediating inhibition of cell proliferation that is associated with laminar shear stress (21). Although no data are available on the molecular mechanisms involved in shear stress–induced p21waf expression, it has been reported that p21waf is a target of STAT5 during megakaryocytic differentiation (22).

The vast majority of studies aimed at elucidating the role of LDL in diabetes-associated vascular complications have been performed with artificially glycated and/or oxidized LDL (ox-LDL) (23). The aim of the present study was to evaluate the effects of the natural
plasma constituent recovered from type 2 diabetic patients (dm-LDL) on endothelial cells. We found that dm-LDL affects cell-cycle progression via STAT5-mediated p21waf induction. Moreover, activated STAT5 and p21waf immunoreactivity was present in human intraplaque neovessels.

Methods

Patients and controls. Blood was withdrawn from nine blood donors and from nine type 2 diabetic patients in bad metabolic control (fasting plasma glucose ≥10 mM and hemoglobin A1c ≥10%). None of them was under insulin, and all were treated with sulphpylurea agents. The mean age was 68 ± 4 years in the diabetic group, and 62 ± 3 years in controls. Each group consisted of five men and four women.

Reagents. M199 medium (endotoxin tested), BSA, and protein A-Sepharose were from Sigma Chemical Co. (St. Louis, Missouri, USA). Bovine calf serum (endotoxin tested) was obtained from HyClone Laboratories Inc. (Logan, Utah, USA). Trypsin was purchased from Difco Laboratories Inc. (Detroit, Michigan, USA). Nitrocellulose filters, horseradish peroxidase–conjugated protein A, the molecular weight markers [α-32P]dCTP, [γ-32P]ATP, and [α-32P]UTP, the chemiluminescence reagent enhanced, and the Poly(dIdC):poly(dIdC) were obtained from Amersham Pharmacia Biotech Italia (Milano, Italy). Histone H1 was from Roche Diagnostics S.P.A. (Monza, Italy). The presence of endotoxin contamination of LDL preparations was tested by the Limulus amebocyte assay; the concentration was 0.05 ng/ml. Anti-STAT5A (L-20), anti-STAT5B (G-2 and C-17), anti-p21waf, and anti-p27kip1 antisera were obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Anti-phospho-STAT5, anti-CD105, and anti-CD45 were from New England Biolabs Inc. (Beverly, Massachusetts, USA).

Isolation, characterization, and oxidation of LDL. Blood was centrifuged for 15 minutes at 12,000 g at 4°C. LDL was separated according to the method of Redgrave and Carlson (24). Oxidation of n-LDL was performed in a cell-free system as described (25). Oxidation was confirmed by agarose gel electrophoresis. Purity of LDL was assessed by capillary electrophoresis at 200 nm. Thiobarbituric acid–reactive substances assay and capillary electrophoresis. LDL subfractions were pooled, and peroxida
tion was measured using a thiobarbituric acid–reactive substances (TBARS) assay (26). TBARS concentrations were calculated from a calibration curve that was prepared using 1,1,3,3-tetramethoxypropane as a standard. Diene conjugate quantification was performed by capillary electrophoresis as described (27). LDL mobility in capillary electrophoresis was calculated as described by Cruzo et al. (28).

Flow cytometry. HUVECs or ECV304 cells were stimulated with n-LDL (100 µg/ml) or dm-LDL (100 µg/ml) for 12 hours, and then fixed with 70% ethanol. After digestion with RNase, DNA was stained with propidium iodide and analyzed with a flow cytometer.

Western blot analysis and immunoprecipitation studies. HUVEC monolayers were processed as described (30) and incubated with or without 100 µg/ml of either n-LDL or dm-LDL for the indicated time periods. Protein concentrations of cell lysates were obtained and samples were eluted and processed as previously described (30).

Preparation of nuclear extracts and gel retardation assay. Nuclear extracts from untreated or LDL-treated HUVECs were prepared as described by Sadowski and Gilman (31). The double-stranded p21SIE2 oligonucleotide sequence 5′-GATCCCTTTCTGAGAAATGG-3′ (22) was used. Gel retardation reactions were performed as previously described (30).

Cdk2 kinase assay. Cdk2 immunoprecipitated from dm-LDL–stimulated (100 µg/ml) STAT transfected ECV304 clones was divided into two aliquots. Cdk2 immunoprecipitates were resuspended in kinase buffer consisting of 20 mM Tris/HCl (pH7.4), 10 mM MgCl2, and 1 mM DTT containing 50 µM [γ-32P]ATP and 5 µg/ml of histone H1, and incubated for 30 minutes at 30°C.
Agarose beads were removed by centrifugation, and the supernatants were fractionated by 8% SDS-PAGE.

**Nuclear run-off transcription.** Approximately $2 \times 10^7$ HUVECs were stimulated with dm-LDL (100 µg/ml) and lysed in Nonidet P-40 lysis buffer (32). Nuclei were collected and stored as previously described (32). Elongation of nascent RNA was performed as previously described (32). Labeled RNA was purified as described by Gariglio et al. (33), and processed as previously described (32).

**Detection of reactive oxygen species.** 5,6-carboxy-2′,7′-dichlorofluorescein-diacetate (DCF-DA) (20 mM final concentration) was added to HUVECs in the various culture conditions at time 0. At the times indicated, the cells were subjected to flow cytometric analysis as described (34). TNF-α was used as positive control (35).

**Immunofluorescence microscopy.** Carotid specimens from diabetic or hypercholesterolemic patients and healthy carotid specimens were fixed and embedded in paraffin and processed as described (36). Sections were stained with the anti–phospho-STAT5, the anti-p21waf, and the anti-CD105 antibodies. Indirect immunofluorescence analysis was performed as previously described (36).

**Results**

**LDL preparation and characterization.** In diabetic subjects, the highest cholesterol was found in the LDL subfractions with densities ranging from 1.037 g/ml to 1.044 g/ml. In healthy subjects, the highest level of cholesterol was found in the LDL subfractions with densities ranging from 1.028 g/ml to 1.035 g/ml. dm-LDL was consistently smaller and denser than normal LDL. This pattern was confirmed by the density-gradient ultracentrifugation profile (Figure 1a) and by the lower cholesterol/apoB ratio (Table 1). Lipid peroxidation was assessed by TBARS assay and capillary electrophoresis. The results demonstrated the absence of TBARS and of conjugated dienes (determined by measuring absorbance at 234 nm in capillary electrophoresis) in both n-LDL and dm-LDL preparations. It is widely acknowledged that changes in electrophoretic mobility are the most reliable indicator of LDL modifications (27). Indeed, the results reported in Figure 1b demonstrated the same electrophoretic mobility pattern for both n-LDL and dm-LDL. These results, which are consistent with data obtained by Jenkins et al. (37), rule out the possibility that a significant degree of lipid peroxidation was present in the LDL preparations. Moreover, the finding that dm-LDL eluted as a single sharp peak (Figure 1b) led us to exclude the presence of copurified substances in our preparations. After characterization of each sample, pilot experiments were performed using single preparations; in subsequent experiments, pooled sera from normal donors or diabetic patients were used.

**dm-LDL affects cell-cycle progression by regulating p21waf gene expression.** The effects of n-LDL and dm-LDL on the cell cycle were evaluated by flow cytometric analysis. The flow cytometric analysis for the DNA content in HUVECs indicated that the cell populations in the S and G2/M phases decreased after the cells were subjected to dm-LDL. Indeed, cells in the S phase were reduced to 8% of the total compared with the control value of 24.6% and the n-LDL value of 25.5%. Values are for the S phase only. This effect, induced by dm-LDL, was accompanied by an increase to 85.8% in the percentage of cells in G0 or G1 (Figure 2). In order to dis-

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**Table 1**

**Characteristics of n-LDL and dm-LDL**

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Chol (µg/µl)</th>
<th>ApoB (µg/µl)</th>
<th>Chol/apoB</th>
<th>HbA1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(d)</td>
<td>1.61</td>
<td>1.26</td>
<td>1.3</td>
<td>12%</td>
</tr>
<tr>
<td>2(d)</td>
<td>0.65</td>
<td>0.50</td>
<td>1.3</td>
<td>11.8%</td>
</tr>
<tr>
<td>3(d)</td>
<td>1.50</td>
<td>1.06</td>
<td>1.4</td>
<td>10.8%</td>
</tr>
<tr>
<td>4(d)</td>
<td>1.93</td>
<td>1.41</td>
<td>1.4</td>
<td>10.9%</td>
</tr>
<tr>
<td>5(d)</td>
<td>1.65</td>
<td>1.16</td>
<td>1.4</td>
<td>12.4%</td>
</tr>
<tr>
<td>6(d)</td>
<td>2.00</td>
<td>1.50</td>
<td>1.3</td>
<td>13.8%</td>
</tr>
<tr>
<td>7(d)</td>
<td>1.03</td>
<td>0.75</td>
<td>1.4</td>
<td>11.3%</td>
</tr>
<tr>
<td>8(d)</td>
<td>1.10</td>
<td>0.81</td>
<td>1.35</td>
<td>10.1%</td>
</tr>
<tr>
<td>9(d)</td>
<td>0.71</td>
<td>0.49</td>
<td>1.4</td>
<td>12.1%</td>
</tr>
<tr>
<td>10(n)</td>
<td>0.97</td>
<td>0.50</td>
<td>1.9</td>
<td>13.2%</td>
</tr>
<tr>
<td>11(n)</td>
<td>1.04</td>
<td>0.61</td>
<td>1.7</td>
<td>14.8%</td>
</tr>
<tr>
<td>12(n)</td>
<td>1.41</td>
<td>0.90</td>
<td>1.56</td>
<td>16.3%</td>
</tr>
<tr>
<td>13(n)</td>
<td>0.93</td>
<td>0.57</td>
<td>1.6</td>
<td>17.2%</td>
</tr>
<tr>
<td>14(n)</td>
<td>1.19</td>
<td>0.73</td>
<td>1.6</td>
<td>18.1%</td>
</tr>
<tr>
<td>15(n)</td>
<td>1.66</td>
<td>0.98</td>
<td>1.7</td>
<td>19.1%</td>
</tr>
<tr>
<td>16(n)</td>
<td>1.36</td>
<td>0.85</td>
<td>1.6</td>
<td>20.1%</td>
</tr>
<tr>
<td>17(n)</td>
<td>1.88</td>
<td>1.04</td>
<td>1.8</td>
<td>21.1%</td>
</tr>
<tr>
<td>18(n)</td>
<td>0.89</td>
<td>0.56</td>
<td>1.6</td>
<td>22.1%</td>
</tr>
</tbody>
</table>

HbA1c, hemoglobin A1c; n, n-LDL; d, dm-LDL; chol, cholesterol. *Used for pilot experiments.

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**Figure 2**

Effects of dm-LDL and n-LDL on the cell cycle. HUVECs, unstimulated (a) or stimulated for 12 hours with n-LDL (b) or dm-LDL (c), were harvested and fixed with ethanol. DNA was stained with propidium iodide, and fluorescence was evaluated by flow cytometry using FACScan equipment (Becton Dickinson Immunocytometry Systems, San Jose, California, USA). Percentage of cells in each phase is shown, as determined by ModFit LT software (Verity Software House Inc., Topsham, Maine, USA). Similar results were obtained in three different individual experiments.
respective reactions were hybridized against p21waf and
minutes. Equal cpm of the purified radioactive RNAs from the
scription. RNAs were obtained from nuclei prepared from
ed for p21waf as above. Similar results were obtained in three differ-
18 hours with dm-LDL alone (lane 2) or in combination with n-LDL
Figure 3
involved in p21waf expression was dependent upon gene
mechanisms, including increased RNA transcription
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was clearly induced upon dm-LDL treatment, showing
against p21waf and p27kip1. As shown in Figure 3a, and consistent with
the expression of p27kip1 was not affected by either n-LDL or dm-LDL (Figure 3a).
DNA dot-blotted on the same filter. Similar results were obtained
in two different experiments. (b) p21waf gene tran-
subjected to 15% SDS-PAGE, and electrophoretically transferred to
nitrocellulose filters. The filters were immunoblotted with an anti-
p21waf antibody or an anti-p27kip1 antisemur. Five different experi-
ments were performed with similar results. (b) p21waf gene tran-
scription. RNAs were obtained from nuclei prepared from
unstimulated HUVECs or HUVECs stimulated with dm-LDL for 90
minutes. Equal cpm of the purified radioactive RNAs from the
respective reactions were hybridized against p21waf and β-actin
cDNAs dot-blotted on the same filter. Similar results were obtained
in two different experiments. (c) n-LDL does not compete with
dm-LDL. HUVECs were unstimulated (lane 1) or were stimulated for
18 hours with dm-LDL alone (lane 2) or in combination with n-LDL
(50- and 100-fold excess, lanes 3 and 4, respectively), and evaluat-
ed for p21waf as above. Similar results were obtained in three differ-
ent experiments. IB, immunoblot.
sect the molecular mechanisms involved in this effect,
we analyzed the expression of two CKIs: p21waf and p27kip1. As shown in Figure 3a, and consistent with
flow cytometric analysis, treatment with dm-LDL but
not n-LDL led to an increase in p21waf expression. In
contrast, the expression of p27kip1 was not affected by
either n-LDL or dm-LDL (Figure 3a). Sustained
increased levels of p21waf can be mediated by various
mechanisms, including increased RNA transcription
(12). Therefore, to evaluate whether the mechanism
involved in p21waf expression was dependent upon gene
transcription, nuclear run-off experiments were per-
formed. As depicted in Figure 3b, while nuclear p21waf
mRNA was barely detectable in unstimulated cells, it
was clearly induced upon dm-LDL treatment, showing
that a transcriptional mechanism regulated dm-
LDL-mediated p21waf expression.
It is known that modified LDL may exert its effects
by binding to membrane structures other than the
canonical LDL receptor (38, 39). The possibility that a
receptor other than the native LDL receptor was
engaged by dm-LDL was indirectly evaluated by com-
petition experiments. HUVECs were stimulated with
dm-LDL alone or in combination with 50- and 100-
fold excesses of n-LDL, and then assayed for p21waf
expression. As shown in Figure 3c, n-LDL was unable
to abrogate the effect of dm-LDL on p21waf expression,
suggesting that a receptor other than the natural LDL
receptor is recruited by dm-LDL.
Figure 4
dm-LDL triggers STAT5 activation. STAT5A and
STAT5B are pleiotropic regulators of many genes,
including p21waf (22). To assess the role of the STAT5
pathway in regulating dm-LDL-mediated p21waf
expression, the effect of dm-LDL on STAT5 activation
was analyzed. The results shown in Figure 5a demon-
strate that, unlike n-LDL, dm-LDL was able to trigger
STAT5 tyrosine phosphorylation. It is known that
hydrogen peroxide is able to activate STAT1 and
STAT3 (41). However, neither n-LDL nor dm-LDL was
able to induce STAT3 activation (data not shown), and
when ox-LDL was used to stimulate HUVECs, no
STAT5 activation could be detected (Figure 5b). To
assess whether dm-LDL–elicited STAT5 activation
was involved in p21waf induction, an electrophoretic mobi-
licity shift assay was performed using the p21SIE2
sequence, which is a potential STAT binding site in the
p21waf promoter region (22). As shown in Figure 5c,
nuclear extracts from dm-LDL–treated, but not from
untreated or n-LDL–treated, endothelial cells were able
to form a p21SIE2-binding complex that was compet-
et for by an added excess of unlabeled p21SIE2 probe. The
presence of STAT5 in the DNA-protein complex
induced by dm-LDL was demonstrated by the ability of
the antibody to STAT5 to supershift the p21SIE2-bind-
ing complex (Figure 5d). Consistent with the inability
of dm-LDL to trigger STAT3 phosphorylation, the
antibody to STAT3 did not supershift the dm-
LDL–induced p21SIE2 binding complex (Figure 5d).
Expression of ΔSTAT5 proteins in the ECV304 cell line.
Modified forms of STAT5 acting as dominant-nega-
tive proteins were obtained by removing most of the
(40). Accordingly, we examined the effects of n-LDL
and dm-LDL on intracellular ROS production. As
shown in Figure 4, dm-LDL, but not n-LDL, induced a
burst of ROS that peaked at 15 minutes as measured by
DCF fluorescence. TNF-α was used as a positive con-
control. Similar results were obtained with the lucigenin-
enhanced chemiluminescence assay using a 125I LKB
luminometer (Perkin-Elmer Instruments, Bad-Wild-
bad, Germany) (data not shown).

Overview

dm-LDL increases p21waf expression via a transcriptional mecha-
nism. (a) p21waf and p27kip1 expression. Proteins from lyzed HUVECs
were incubated with n-LDL or dm-LDL for different time periods,
subjected to 15% SDS-PAGE, and electrophoretically transferred to
nitrocellulose filters. The filters were immunoblotted with an anti-
p21waf antibody or an anti-p27kip1 antisemur. Five different experi-
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minutes. Equal cpm of the purified radioactive RNAs from the
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(50- and 100-fold excess, lanes 3 and 4, respectively), and evaluat-
ed for p21waf as above. Similar results were obtained in three differ-
ent experiments. IB, immunoblot.
C-terminal tyrosines (11). To assess the biological relevance of STAT5 protein activation in dm-LDL-mediated p21<sup>wafr</sup> expression, ∆STAT5A and ∆STAT5B constructs were used to transfect ECV304 cells. The ECV304 cell line, recently shown to be identical to the T24 cell line derived from a human bladder carcinoma (42), shares several features with endothelial cells (43, 44), including the expression of receptors for native and modified LDL (45). Moreover, in our preliminary experiments, ECV304 cells and HUVECs exhibited a similar response to dm-LDL (data not shown). Cells expressing the ∆STAT5 proteins were selected on the basis of their ability to inhibit activation of endogenous STAT5 proteins. To this end, two stable clones denoted as ∆STAT5A and ∆STAT5B, and a clone expressing the Neo vector were stimulated with IL-3, which is able to activate both STAT5 proteins in these cells (M.F. Brizzi, unpublished data). As shown in Figure 6a, ∆STAT5 expression affected the ability of IL-3 to trigger the activation of endogenous STAT5. In contrast, IL-3 triggered STAT5 tyrosine phosphorylation in Neo-expressing cells.

Expression of ∆STAT5B, but not ∆STAT5A, drastically reduces dm-LDL-mediated p21<sup>wafr</sup> expression. To assess whether the effect of dm-LDL on p21<sup>wafr</sup> expression was directly mediated by STAT5, the two different ∆STAT5 clones were stimulated for 18 hours with dm-LDL or n-LDL, and p21<sup>wafr</sup> expression was assayed. Figure 6b shows that only in ∆STAT5B-expressing cells, not in Neo vector–expressing or ∆STAT5A-expressing cells, dm-LDL failed to induce p21<sup>wafr</sup> expression. The presence of endogenous STAT5B may explain the increased level of p21<sup>wafr</sup> observed in the ∆STAT5A clone. Moreover, the role of STAT5B in regulating p21<sup>wafr</sup> expression was confirmed by the observation that no DNA-binding activity could be detected in the nuclear extract from the dm-LDL–stimulated ∆STAT5B clone (Figure 6c).

Expression of ∆STAT5B rescues Cdk2 activity and prevents accumulation of cells in G1. p21<sup>wafr</sup> is known to regulate Cdk2 activity (12). The finding that dm-LDL was unable to upregulate p21<sup>wafr</sup> in the ∆STAT5B-expressing cells led us to investigate whether this effect was associated with an increase in Cdk2 activity. To this end, Cdk2 kinase assays of cells expressing the different constructs were performed. As shown in Figure 6d, consistent with the role of p21<sup>wafr</sup> in regulating Cdk2 activity, we found that, upon dm-LDL treatment, Cdk2 activity was restored by transfection of cells with ∆STAT5B but not with ∆STAT5A or Neo vector.

The role of STAT5B in regulating cell-cycle arrest in response to dm-LDL was further examined. Ectopically transfected ECV304 cells were stimulated with dm-LDL, and assessed for their ability to progress in the cell cycle. In cultures of cells expressing the Neo or the ∆STAT5A construct, treatment with dm-LDL led to a marked reduction of the percentage of cells in S phase, and to an increase in the percentage of cells in G0 or G1 (Figure 7). In contrast, dm-LDL treatment of cells expressing the ∆STAT5B construct led to an increase in the percentage of cells in S phase (40.9%) and to a corresponding reduction in the percentage of cells in G0 or G1. These observations indicate that increased p21<sup>wafr</sup> expression and the resulting decrease in Cdk2 activity may account for dm-LDL–mediated cell-cycle arrest.

*Endothelial cells from atherosclerotic plaques are immunoreactive to activated STAT5 and p21<sup>wafr</sup>.* To explore the in vivo expression of activated STAT5 and p21<sup>wafr</sup>, nonatherosclerotic human carotid specimens and atherosclerotic human carotid specimens were immunostained with anti-activated STAT5 and anti-p21<sup>wafr</sup> antibodies. As shown in Figure 8, the percentage of cells coexpressing activated STAT5 and p21<sup>wafr</sup> was significantly higher in the atherosclerotic compared to the nonatherosclerotic specimens.
ic plaques from human carotid arteries were analyzed for activated STAT5. The images shown in Figure 8, a–d, demonstrate that both endothelial cells lining the intraplaque vessels and the plaque luminal side, and smooth muscle cells from media express activated STAT5. No STAT5 activation was found in endothelial cells lining nonatherosclerotic vessels, while only a few fibers in the media were positive. Similarly, a positive immunoreactivity for p21waf was found in intraplaque neovessels, but not in other intraplaque cellular components (Figure 8, e–g).

Discussion

Regulation of the cell cycle is achieved through a complex and ordered sequence of events controlled by Cdk’s, the activation of which depends on their association with protein subunits and the cyclins, and on regulatory phosphorylation (12). The activation of Cdk’s is negatively regulated by several CKIs. The four major mammalian CKIs fall into two classes. p21waf and p27kip1 are related proteins with a preference for Cdk2- and Cdk4-cyclin complexes, whereas p16INK4a and p15INK4b are closely related CKIs that are specific for Cdk4- and Cdk6-cyclin complexes (12). In the present study, we demonstrate that dm-LDL treatment leads to an accumulation of cells in G1 by increasing the level of p21waf. Although a sustained increase in levels of p21waf resulting from different stimuli could be mediated by various mechanisms (decreased protein degradation, increased mRNA translation rate, or increased mRNA half-life), a major mode of regulation is transcriptional (12). Accordingly, our run-off experiments strongly support a role for transcription in regulating p21waf expression upon dm-LDL stimulation.

p27kip1 was originally identified as a Cdk2 inhibitor whose activity, but not total protein amount, increased throughout the cell cycle (20). This seemed to be associated with the release from intracellular compartments of p27kip1 (46, 47), and a similar mechanism might account for the stable expression of p27kip1 observed in our experiments.

The members of the STAT family undergo phosphorylation, dimerization, and nuclear translocation to activate target genes (9). Among these, cell cycle-related genes are known to be transcriptionally regulated by STAT5 (48). Moreover, it has been reported that during megakaryocyte differentiation, STAT5 regulates p21waf expression (22). In the present study, we found that dm-LDL, but not n-LDL or ox-LDL, triggers STAT5 activation. Moreover, in nuclear extracts from dm-LDL–treated cells, the formation of a STAT5-containing p21SIE2-binding complex suggests that dm-LDL promotes p21waf transcription through STAT5 activation. The molecular mechanisms underlying the activation of the STAT5 pathway by dm-LDL remain to be defined. In cytokine- or growth factor–mediated STAT activation, a ligand-dependent phosphorylation of the receptor creates a docking site for the Src homology 2 domain of STAT, thus recruiting STAT into the receptor complex (9). It is known that modified LDL interacts with target cells through different receptors, such as the scavenger receptors (38) and/or the receptor for the advanced glycated end products (39). Indeed, our experiments showing that n-LDL was unable to compete with dm-LDL, to activate STAT5, or to induce ROS production suggest that n-LDL and dm-LDL bind to different receptors. Although we were unable to detect an immunoprecipitable complex between STAT5 and scavenger receptors for the advanced glycated end products (data not shown), it is possible that their association occurs either indirectly or through a
low-affinity interaction. However, regardless of the mechanisms and of the receptor engaged by dm-LDL to elicit STAT5 activation, the findings that ectopic expression of ΔSTAT5B was able to alter dm-LDL–mediated upregulation of p21 waf expression, to rescue Cdk2 activity, and to prevent accumulation of cells in G1 strongly suggests a role for STAT5B in regulating this effect on endothelial cells.

Qualitative changes of LDL, such as glycation and/or oxidation, may account for the increased atherogenic risk in diabetes (2). Indeed, dm-LDL did not show a significant degree of lipid peroxidation, but was characterized by a decreased cholesterol/apoB ratio and by a density profile showing a subtraction distribution corresponding to the LDL subclass defined as pattern B (49). It is commonly accepted that, because of their susceptibility to glycation and/or oxidation, these small, dense particles are responsible for the increased atherogenic risk in diabetes (49). Since dm-LDL did not bind to the canonical LDL receptor, did not activate a STAT3-mediated pathway (as shown by the inability of anti-STAT3 antiserum to modify the mobility shift of the p21SIE2-binding complex), and was recovered from patients in poor metabolic control, it is reasonable to assume that glycation, rather than oxidation, represents the qualitative change in dm-LDL that accounts for our results.

In conditions such as atherosclerosis, intimal angiogenesis occurs as part of the adaptive changes known as vasculature remodeling (50). Recent clinical studies suggest that risk factors for coronary artery disease may modify an individual’s capacity for angiogenesis and vascular remodeling. Specifically, hypercholesterolemia and diabetes have been shown to be associ-
ated with a significant impairment in adaptive vascular growth of both capillary-like tube vessels and collateral vessels (51–53). The observation that, through a STAT5B/p21waf1-mediated pathway, dm-LDL can affect the ability of endothelial cells to progress in the cell cycle adds further insight into the molecular mechanisms involved in the impaired vasculature remodeling in diabetes. Moreover, the evidence that activated STAT5 and p21waf1 were highly expressed in endothelial cells lining both the luminal side of the plaque and/or the intimal neovessels supports the possibility that a similar mechanism may be operative in vivo.

In conclusion, the results presented here demonstrate that the natural plasma constituent LDL, from type 2 diabetic patients, can maintain endothelial cells in a quiescent state in G1 through STAT5B-mediated p21waf1 expression. Moreover, the presence of a positive immunoreactivity for activated STAT5 and p21waf1 in intraplaque neovessels supports the possibility that induction of STAT5-dependent genes may exert substantial atherogenic effects on the vessel wall, and specifically, may account for the deranged adaptive vascular growth observed in this pathological condition. Finally, the recent observation that NF-kB and STAT5 regulate the expression of the same gene in T cells (54) raises the possibility that these transcriptional factors may also exert concerted effects on atherogenesis-related genes. However, further studies are required to elucidate the in vivo role of the STAT5 regulatory system.

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