RhoA Activation Mediates Phosphatidylinositol 3-Kinase–Dependent Proliferation of Human Vascular Endothelial Cells: An Alloimmune Mechanism of Chronic Allograft Nephropathy

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Abstract. HLA class I ligation on graft endothelial cells (EC) has been shown to promote graft arteriosclerosis and chronic allograft nephropathy. This study investigated transcriptional and functional changes mediated by anti-HLA antibodies (Ab), developed by transplant recipient, on vascular renal EC. For mimicking interactions that occur between alloantibodies and graft endothelium, HLA-typed primary cultures of human EC were incubated in vitro in the presence of monomorphic or polymorphic anti-HLA class I Ab. Gene expression analysis identified the upregulation of several molecules involved in cell signaling and proliferation, including the GTP-binding protein RhoA. It was demonstrated further that HLA class I ligation on EC induced a rapid translocation of RhoA to the cell membrane associated with F-actin stress fiber formation and cytoskeleton reorganization. Western blot analysis showed that anti-HLA class I Ab induced, in addition to RhoA, the activation of phosphatidylinositol 3-kinase, reflected by the phosphorylation of Akt (Ser473) and GSK3β (Ser9), in EC. C3 exoenzyme, an inhibitor of RhoA, inhibited RhoA translocation in response to HLA class I ligation and reduced phosphatidylinositol 3-kinase activation. EC proliferation and cell cycle progression, examined by 5,6-carboxyfluorescein diacetate succinimidyl ester staining, demonstrated that anti–HLA-induced EC proliferation was efficiently prevented by the 3-hydroxy-3-methylglutaryl CoA reductase inhibitor simvastatin (0.1 μmol/L) through inhibition of RhoA geranylgeranylation. Taken together, these findings support the conclusion that RhoA is a key mediator of signaling pathways that lead to cytoskeletal reorganization and EC proliferation in response to alloantibodies that bind to HLA class I and demonstrate the specific and potent inhibitory effect of simvastatin on allostimulated EC growth.

Chronic allograft nephropathy is the major factor limiting long-term survival of renal allografts (1,2). The hallmark of chronic allograft nephropathy is transplant arteriosclerosis, which is characterized by the intimal proliferation of endothelial cells (EC), smooth muscle cells (SMC), and fibroblasts, leading to vessel obstruction and ischemia that causes late graft failure (3). Several risk factors have been identified, including both immune injury to transplant vessels and nonimmunological factors (e.g., ischemia/reperfusion, hypertension, hyperlipidemia). The immunologic mechanisms that induce chronic allograft nephropathy are poorly understood, but it is suspected that the associated vascular changes are a result of early injury to the endothelium of the graft mediated by allogeneic T cells and anti-HLA alloantibodies (2). The incidence of transplant arteriosclerosis is increased in transplant recipients who produce anti-donor HLA antibodies (Ab) after transplantation, suggesting that anti-HLA Ab play a role in the pathogenesis of the disease (4,5). In previous studies, it has been shown that anti-HLA Ab, developed by transplant recipients after transplantation, are capable of transducing signals via HLA class I molecules, which stimulate cell proliferation (6). Furthermore, ligation of class I molecules with Ab also results in increased tyrosine phosphorylation of several intracellular proteins on EC (7,8). Treatment of cells with IFN-γ and TNF-α upregulated MHC class I expression and potentiated anti-HLA Ab–mediated proliferative responses (9). These findings support a role for anti-HLA Ab in the transduction of proliferative signals, which stimulate the development of intimal hyperplasia associated with chronic allograft nephropathy and renal transplant loss.

We previously demonstrated that natural, preformed, antidoer Ab mediated changes in EC gene expression according to their specificity (10,11). In the present study, we investigated the effect of HLA class I ligation mediated by anti-HLA Ab on endothelial gene expression. Primary cultures of HLA-
typed human vascular EC, isolated from cadaveric transplant donors, were incubated with anti-HLA mAb directed to either monomorphic or polymorphic HLA class I regions. RNA differential display reverse transcription–PCR (RT-PCR) was used to compare gene expression between resting and anti-HLA–treated EC and to identify genes and molecular mechanisms upregulated upon HLA class I ligation. Among the candidate genes found to be overexpressed, several cell-cycle regulators were identified, including the GTP-binding protein RhoA. These changes in transcription suggested that anti-HLA class I Ab could trigger EC proliferation via a Rho-dependent pathway. Thus, our study further examined the implication of RhoA protein in signaling pathways that lead to EC proliferation and transplant arteriosclerosis. The fluorescent dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) has been used to monitor EC division and proliferation and therefore was used to demonstrate that control of Rho GTPase activation could modulate EC proliferation.

Materials and Methods

Reagents

Simvastatin was supplied by Merck, Sharp & Dohme. Thrombin, inhibitors of phosphatidylinositol 3-kinase (PI3-K) LY294002 and wortmannin, and isoprenoid compounds farnesyl pyrophosphate (FP) and geranylgeranyl pyrophosphate (GGPP) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). 

Primary EC Isolation and Culture

Human arterial EC were isolated from renal artery patches taken before kidney transplantation, as described previously (12). EC were grown in Endothelial Cell Growth Medium (ECGM) supplemented with 10% FCS, 0.004 ml/ml ECGS/Heparin, 0.1 ng/ml human EGF, 1 ng/ml human bFGF, 1 μg/ml hydrocortisone, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B (G3-22010; PromoCell, Heidelberg, Germany) at 37°C in a humified 5% CO2 atmosphere.

Gene Expression Analysis

RNA Differential Display RT-PCR. EC were incubated for 2 h at 37°C in medium that contained 100 U/ml recombinant human TNF-α (provided by Prof. Müller Neuman, Ludwigshafen, Germany) or 10 μg/ml anti-HLA class I mAb. Treatment of EC was performed using either monomorphic (anti–HLA-A,B,C mAb:W6/32, IgG2a) or polymorphic (anti–HLA-A2, anti–HLA-B51, and anti–HLA-Bw4) anti-HLA class I purified Ab (OneLambda, Canoga Park, CA). EC that were incubated with isotypic control mouse IgG1 or IgG2a (10 μg/ml; Sigma-Aldrich) or medium alone were used as controls.

RNA isolation and RNA differential display RT-PCR were performed as described previously (10) using the RNA image kit (GenHunter, Brookline, MA). Selection, cloning, and sequencing of cDNA fragments and bioinformatic analysis were performed as previously reported (11,13).

Quantitative Real-Time RT-PCR. The ABI PRISM 7700 sequence detection application program (PE Applied Biosystems, Foster City, CA) was used to measure fluorescence emitted during PCR amplification of targeted sequences in a 96-well reaction plate. Real-time detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green (PE Applied Biosystems) to DNA. A standard curve using serial dilutions of the purified target sequence (10⁵, 10⁴, 10³, 10², and 10¹ copies/well) allowed quantification. Normalization was obtained by the concomitant quantification of hypoxanthine-guanine phosphoribosyl transferase transcripts in each sample. Each sample was analyzed in duplicate.

Oligonucleotide primers pairs for hypoxanthine-guanine phosphoribosyl transferase (sense, 5'-TGGAAAACGCAATACAAAGCCT-3'; antisense, 5'-CATGCAAAGCTCTACTAAGCAG-3') and for RhoA (sense, 5'-TTAGTCCACGGTGTCGTCTC-3'; antisense, 5'-TATGACAGCATGTGTTCTCA-3') generated PCR products of 140 and 177 bp, respectively.

Semiquantitative RT-PCR. Primer pairs were as follows: RhoA (sense, 5'-CAGTITCCAGAGGTGTATG-3'; antisense, 5'-AGAAGCGGACACCAGATTTT-3) and β-actin (sense, 5'-AATCTGGGCACACCACCTTCTACA-3'; antisense, 5'-CGACGTAGCAGCTTCTCTTA-3). The PCR conditions were 18 cycles for β-actin and for RhoA, at a denaturation temperature of 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. PCR products were run on 12.5% agarose gels and stained with ethidium bromide.

Immunofluorescence Microscopy

EC were grown on four-well glass slides (Lab-Tek; Nunc, Naperville, IL), and confluent monolayers were treated with anti–HLA-A,B,C mAb (W6/32: 10 μg/ml), thrombin (1 U/ml), or TNF-α (100 U/ml) after a 24-h deprivation period. EC were treated with culture medium alone or an isotypic mouse IgG2a were used as controls. After treatment, EC were fixed in 4% paraformaldehyde-PBS for 20 min and permeabilized in 0.1% Triton X-100 at room temperature for 15 min. For F-actin staining, cells were incubated with 2 μg/ml TRITC-phallidin (Sigma-Aldrich) for 20 min. For immunofluorescent detection of RhoA, assessed on resting or treated confluent EC, cells were stained with 2 μg/ml FITC-labeled anti-RhoA mAb. The slides were examined with a fluorescence microscope (Eclipse E600 Y-FL, Epifluorescence, Nikon, Japan). Images were acquired using ACT-1 software (Nikon).

Western Blotting and Pull-Down Assays

Lysis of the EC membrane was performed on ice in 5 mmol/L MgCl2, 100 mmol/L NaCl, 1% NP-40, 1 mmol/L PMSF, 0.2 U/ml aprotinin, and 100 μg/ml leupeptin. Total cell lysates were obtained using 10 mmol/L Tris-HCl (pH 7.4), 125 mmol/L NaCl, 1% SDS, 1 mmol/L PMSF, 0.2 U/ml aprotinin, and 100 μg/ml leupeptin. Cell membrane fractions were obtained after ultracentrifugation and solubilization as described previously (14). For Western blot analysis, proteins (10 to 20 μg per lane) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (ECL Hybond; Amersham Biotech UK, Little Chalfont, England). Membranes were washed for 30 min with TBS 0.1% Tween 20 (TBST) and preblocked for 2 h in TBST that contained 5% BSA (blocking solution; Sigma). Incubation with primary Ab diluted in blocking solution was performed overnight at 4°C. After extensive washing with TBST, the bound antibody was detected by a peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (CST). After 45 min of washing, the blots were developed using the ECL Western blotting detection system (Amersham, Les Ulis, France). Ab used in this study were rabbit polyclonal IgG anti-Akt, anti-Phospho-Akt (Ser473), anti-Phospho-Akt (Thr380), anti-phospho GSK3β (Ser9), anti-PTEN, anti-IκBα, and anti-phospho-IκBα (Ser36), all were from Cell Signaling Technology (CST, Beverly, MA). Mouse anti–glyceraldehyde-3-phosphate dehydroge-
nase mAb was from Chemicon (Temecula, CA) and anti-RhoA specific (26C4) from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit and anti-mouse IgG, horseradish peroxidase–linked Ab (CST) were used as secondary Ab in chemiluminescent Western blot assays. RhoA activation was determined by affinity precipitation of the active GTP-bound RhoA using a glutathione S-transferase (GST)-fusion protein of the Rho-binding domain of the Rho effector rhotekin (GST-RBD) using the EZ-Detect Rho Activation kit (Pierce Biotechnology, Rockford, IL). The active or GTP-Rho pulled down from lysate was detected by Western blot using a specific anti-RhoA antibody (26C4; Santa Cruz Biotechnology).

CFSE Staining and EC Proliferation Analysis
EC monolayers were grown to confluence and quiescent cells were cultured with 2% FCS in the absence of growth supplements for 12 h before treatment. EC were then incubated for 24 h with 10 μg/ml anti-HLA mAb (anti–HLA-A,B,C: W6/32 or anti–HLA-A2: HB-117), 10 μg/ml control IgG1 (for HB-117) or IgG2a (for W6/32), 10 ng/ml bFGF, or medium alone. After treatment, cells were harvested with trypsin/EDTA, washed twice in culture medium, and incubated in the presence of 5 μmol/L of CFSE (Molecular Probes, Eugene, OR) in PBS for 10 min at 37°C. After washing, labeled cells were plated onto six-well culture plates and cultured for the indicated period of time (24 or 48 h) in ECGM supplemented with 2% FCS. Cells were then harvested, washed three times in PBS, and fixed in PBS that contained 1% paraformaldehyde. Fluorescence was measured on 10,000 cells/sample using a FACScalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using CellQuestPro and ModFitLT software (Becton Dickinson Immunocytochemistry Systems, San Jose, CA). Cell proliferation was calculated using the Proliferation Wizard Model of the CellQuestPro and ModFitLT software (Becton Dickinson Immu- nocytometry Systems, San Jose, CA). The proliferation index is the sum of the cells in all generations divided by the computed number of original parent cells present at the start of the experiment. Results are representative of at least three independent experiments.

Statistical Analyses
Data are shown as mean ± SD. All data were evaluated with two-tailed, unpaired t test or compared by one-way ANOVA. P < 0.05 was considered significant for all tests.

Results

HLA Class I Ligation on Vascular EC Increases Expression of Genes Related to Cell Proliferation
For mimicking the Ab-mediated allospecific interactions involved in the chronic allograft nephropathy, cultured HLA-typed primary EC were incubated with monomorphic (HLA-A,B,C: W6/32) or polymorphic (HLA-A2, HLA-Bw4, HLA-B51) anti–HLA class I Ab. EC that were incubated with culture medium or stimulated with TNF-α were used as negative and positive controls, respectively. After a 2-h treatment, total RNA was extracted and subjected to RNA differential display RT-PCR analysis. Among the 45 cDNA fragments found to be overexpressed in response to anti-HLA Ab binding, several encoded proteins related to cell-cycle signaling, regulation, and proliferation, including the GTP-binding protein RhoA, the cytoplasmic microtubule motor protein dynein, the microtubule-associated protein 1B, the nibrin protein, the ribonucleoprotein A2/B1, and the syntaxin 3A (Table 1). For further exploring the signaling pathway involved in EC proliferation–mediated graft arteriosclerosis, a particular focus was given to determine the role of the GTP-binding protein RhoA. For providing additional evidence that RhoA could be overexpressed by EC in response to class I HLA ligation, primary cultures of these cells were assessed for RhoA expression at the level of mRNA by using real-time quantitative RT-PCR. Up-regulation of the transcript level for RhoA was first confirmed by quantitative RT-PCR analysis, performed independently on three different primary EC cultures incubated with anti-HLA mAb, alone or cross-linked with anti-mouse IgG (Figure 1A). RhoA mRNA expression was observed further in response to both monomorphic and polymorphic anti-HLA binding with a maximal increase obtained for anti–HLA-A,B,C (W6/32) and anti–HLA-A2 (HB-117) mAb (Figure 1B), both directed to the largest number of determinants on vascular EC. Concomitant to enhanced mRNA level for RhoA, mRNA for RhoB and RhoC but not Rac1 and Cdc42 were also increased at 2 h after anti-HLA class I Ab binding to vascular EC (data not shown).

Table 1. Genes upregulated in endothelial cells in response to anti-HLA class I antibody binding

<table>
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<tr>
<th>Gene Product (NCBI Accession No.)</th>
<th>Functions</th>
<th>Length of cDNA Fragment (bp)</th>
<th>% of Sequence Homology</th>
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<td>GTP binding protein RhoA (XM-003287)</td>
<td>Signal transduction, cell proliferation, cytoskeletal remodeling</td>
<td>450</td>
<td>100</td>
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<td>Nijmegen breakage syndrome 1 (Nibrin, NBS1) (XM-005310)</td>
<td>DNA double-strand-break repair</td>
<td>200</td>
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<td>Development and cellular regeneration</td>
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<td>95</td>
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<td>Ribonucleoprotein A2/B1 (BC000506)</td>
<td>Transcription, pre-mRNA processing, cytoplasmic mRNA translation</td>
<td>180</td>
<td>100</td>
</tr>
<tr>
<td>Syntaxin 3A (XM-004177)</td>
<td>Membrane fusion during cell division</td>
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<td>100</td>
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<tr>
<td>Dynein, cytoplasmic, intermediate polypeptide1 (DNCI-1) (XM-004944)</td>
<td>Spindle orientation and cytoskeletal reorientation during mitosis</td>
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HLA Class I Ligation Induces RhoA Translocation on EC and Cytoskeleton Reorganization

To assess activation of RhoA in response to HLA class I ligation, we investigated RhoA translocation by immunofluorescence and immunoblot detection. Intracellular localization of RhoA was first analyzed by immunofluorescent labeling of RhoA in confluent EC monolayers (Figure 2A). Our data indicated that, in the resting state, RhoA is mainly localized in the Golgi-ER (endoplasmic reticulum) system. Incubation of EC in the presence of anti-HLA Ab for 8 min leads to the translocation of RhoA protein from the cytosolic part to the inner side of the cell membrane. The translocation was also confirmed by Western blots performed on membrane fractions. EC that were stimulated with thrombin were used as positive controls and with irrelevant mouse IgG as negative controls. Figure 2B shows the increase in protein level for RhoA corresponding to the 23-kD band detected by immunoblotting on cell membrane lysates. In contrast, the overall amount of RhoA did not change. RhoA plays an important role in temporal and spatial determination of the actin cytoskeletal control (15). Figure 3 shows that anti-HLA binding to EC surface induced the formation of cytoplasmic stress fibers and accumulation of a pronounced ring of F-actin. Time-course analyses indicated that cytoskeleton reorganization begins 15 min after HLA class I binding and is reflected by an increase in fluorescence intensity as compared with resting and control cells. EC that were stimulated with thrombin, used as controls, also exhibited a rapid increase in F-actin staining and stress fiber appearance (maximal at 15 min).

Figure 1. Quantitative analysis of RhoA mRNA expression by reverse transcription–PCR (RT-PCR). (A) RhoA transcript levels were analyzed by real-time quantitative RT-PCR as described in Materials and Methods and expressed as arbitrary units after normalization to hypoxanthine-guanine phosphoribosyl transferase. *P < 0.05 versus controls. RT-PCR was performed on RNA from three independent, HLA-typed cultures of primary endothelial cells (EC; #1147, #11500, and #8186) that were treated for 2 h with an isotypic control IgG2a (IgG control), anti-HLA class I antibodies (Ab; W6/32, 10 μg/ml) alone or cross-linked with anti-mouse IgG (W6/32: CX, 10 μg/ml each). (B) RT-PCR was performed on RNA from EC (#8186) that were treated for 2 h with 10 μg/ml of different anti-HLA class I Ab (anti–HLA-A,B,C:W6/32 alone or cross-linked with anti-mouse IgG: W6/32:CX, anti–HLA-A2 or anti–HLA-Bw4). EC that were incubated with culture medium alone or TNF-α (100 U/ml) were used as controls.

Figure 2. RhoA translocation in response to HLA class I ligation on EC. (A) Representative immunofluorescence for RhoA localization was performed on confluent EC monolayers that were incubated for 8 min with 10 μg/ml irrelevant mouse IgG2a (a) or anti-HLA class I W6/32 mAb (b). Photographs are representative of three different experiments. (B) Western blot analysis performed on EC membrane fractions (top) and total lysates (bottom) after treatment for 10 min with medium alone, thrombin, an isotypic control IgG, or anti-HLA class I (10 μg/ml for both). Magnification, ×600 in A.
RhoA Activation in Response to HLA Class I Ligation Precedes RhoA Upregulation at mRNA and Protein Levels

The activation of RhoA subsequent to HLA class I cross-linking was characterized further by pull-down assays. As shown in Figure 4A, an increase in GTP-bound RhoA protein was observed in response to HLA class I cross-linking. Time-course analysis showed that active (GTP-bound) RhoA requires 10 to 20 min to reach a maximum and then declined before returning to baseline at 60 min. During the same period, the amount of RhoA in whole lysates did not change significantly. Thus, concomitant to membrane translocation, RhoA activation, in response to HLA class I ligation, was confirmed by an increase in GTP-bound RhoA. In additional experiments, the effect of HLA ligation was examined on RhoA mRNA and protein levels after 1, 2, and 4 h. RT-PCR for 18 cycles

Figure 3. F-actin staining and stress fiber formation. EC were grown to confluence in four-well glass slides and cultured without growth factors in the presence of 2% FCS (fetal calf serum) for 12 h. EC monolayers were then incubated with 1 U/ml thrombin or anti-HLA class I mAb (W6/32, 10 μg/ml) for the indicated period of time. After treatment, EC were fixed, permeabilized, and stained with 2 μg/ml TRITC-phalloidin. Photographs are representative of three different experiments. Magnification, × 600.

Figure 4. RhoA activation and upregulation at mRNA and protein levels. EC were incubated in the presence of anti-HLA class I mAb (10 μg/ml) for the indicated period of time. (A) Pull-down assay. RhoA activation was determined by affinity precipitation of the active GTP-bound RhoA using a glutathione S-transferase (GST)-fusion protein of the Rho-binding domain of the Rho effector rhotein (GST-RBD). The GTP-Rho pulled down from lysate was detected by Western blot using a specific anti-RhoA antibody. The total amount of RhoA in cell lysates was used as a control for the comparison of RhoA activity. Blots were reprobed with anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Ab to ensure equal loading. (B) Semiquantitative RT-PCR analysis of RhoA mRNA levels. Total RNA was extracted from EC and subjected to RT-PCR (18 cycles of amplification). The specific PCR bands were separated in 1.2% agarose gels and stained with ethidium bromide. β-Actin mRNA was amplified as a control. (C) Western blot analysis of RhoA protein level in cell lysates. Immunoblot analysis was performed with anti-RhoA Ab, detected with horseradish peroxidase (HRP)-conjugated anti-mouse Ab, and visualized with enhanced chemiluminescence. Bottom panel shows GAPDH levels in samples from a blot reprobed with anti-GAPDH Ab. Results shown are representative of three separate experiments.
PTEN expression and GSK3 and reduces Akt phosphorylation (Figure 5B) as well as which selectively inactivates RhoA/B/C proteins (16), effi-
clostridial cytotoxin. Pretreatment of EC with C3 exoenzyme,
PI3-K activation, Rho GTPases were selectively inhibited by
–
more directly the role of RhoA protein in anti-HLA
–
translocation. Taken together, these data strongly suggest
that PI3-K/Akt is a downstream target of Rho GTPases in
this process.

RhoA Mediates PI3-K/Akt Pathway Activation in EC
Western blot analysis was performed to explore signaling
events related to RhoA activation in response to alloantibodies
binding on human EC. EC that were stimulated with 1 U/ml
thrombin or 100 U/ml TNF-α were used as controls. By
Western blotting, we further examined PI3-K activity and
found that, concomitant to RhoA activation, HLA class I
ligation promotes Akt activation through phosphorylation at
Ser473 (Figure 5A) and at Thr308 (data not shown). In con-
trast, the total amount of Akt protein showed no difference.
Immunoblots also indicated that anti-HLA class I Ab mediate
the induction of PTEN and the phosphorylation of GSK3β at
Ser9 in EC, both maximal 20 min after treatment. IkBα phos-
phorylation at Ser36 was also observed but, in contrast with
TNF-α–induced phosphorylation of IkBα, was not correlated
with a decreased level of constitutive IkBα. For addressing
more directly the role of RhoA protein in anti-HLA–mediated
PI3-K activation, Rho GTPases were selectively inhibited by
clostridial cytotoxin. Pretreatment of EC with C3 exoenzyme,
which selectively inactivates RhoA/B/C proteins (16), effi-
ciently reduced RhoA translocation to the cell membrane and
reduces Akt phosphorylation (Figure 5B) as well as PTEN expression and GSK3β phosphorylation (data not
shown). However, the specific PI3-K inhibitors wortmannin
and LY294002 also prevent Akt without decreasing RhoA
translocation. Taken together, these data strongly suggest
that PI3-K/Akt is a downstream target of Rho GTPases in
this process.

CFSE Analysis of Anti-HLA–Induced EC Proliferation
Increased proliferation of EC is the major functional conse-
quence attributed to anti-HLA class I Ab (7,9,17). For further
linking the signaling pathway to the cell proliferation, CFSE
staining has been used to document further EC division and
proliferation promoted by anti-HLA class I alloantibodies. In
contrast with cell proliferation assays that measure bulk cell
division over a narrow window of time or can identify cells that
have recently divided without determining how many genera-
tions have occurred, CFSE staining allows the clear resolution
and quantitative analysis of eight to 10 sequential cell divisions
(18). The data in Figure 6 show the CFSE staining profiles of
EC over a 24-h period. Cells that were treated with culture
medium or 10 ng/ml bFGF were used as negative and positive
controls, respectively. The proliferative effect of HLA ligation
on EC was reflected by a higher proliferation index (8.97 and
7.55 for anti–HLA-A,B,C and -A2, respectively, versus 6.4 for
medium). Moreover, a higher percentage of EC committed to
proliferate was observed in response to both polymorphic
(HLA-A2) and monomorphic (HLA-A,B,C) anti-HLA class I
mAb compared with medium-treated cells.

![Image](image_url)
Simvastatin Prevents HLA Class I–Induced Proliferation of EC by Inhibiting Geranylgeranylation of RhoA

To provide a functional link between anti-HLA–induced proliferation and signaling pathways, we compared the effect of RhoA and PI3-K inhibitors. On the basis of our results, we examined the possibility that clinically relevant inhibitors of RhoA, such as 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductases inhibitors or statins (14,19,20), may prevent RhoA-mediated EC proliferation in response to anti-HLA Ab. CFSE staining showed that a low, clinically relevant dose (0.1 \( \mu \text{mol/L} \)) of simvastatin efficiently abrogates the proliferative effect of HLA class I ligation on vascular EC (Figure 7). Similar inhibition was achieved at 0.5 \( \mu \text{mol/L} \) without affecting cell viability. As shown in Figure 7A, pretreatment of EC with wortmannin before treatment with anti-HLA class I mAb also reduced EC proliferation assessed by CFSE staining. The inhibitory effect was reflected by both a decreased proliferation index and a lower percentage of the last cell generation.

HMG-CoA reductase is a proximal rate-limiting enzyme in the cholesterol synthesis pathway from acetyl CoA. This pathway also generates several metabolic compounds that have important cellular functions, including FPP and GGPP that contribute to lipid modifications of various proteins. To determine whether the inhibitory effect of simvastatin on EC proliferation in our model results from a reduction in lipid intermediates, we made attempts to reverse simvastatin’s effect by the addition of the downstream byproducts FPP and GGPP at the onset of simvastatin treatment. As shown in Figure 7, A and B, GGPP abrogates the inhibitory effect of simvastatin (relative proliferation index: 1.25 ± 0.1 versus 1.3 ± 0.1 \( [P > 0.1] \) and 0.87 ± 0.15 \( [P < 0.05] \) for EC treated with anti-HLA in the absence or presence of simvastatin, respectively). In contrast, FPP was not able to reverse the inhibitory effect of simvastatin on cell division, suggesting that simvastatin prevents anti-HLA–mediated EC proliferation through inhibition of RhoA geranylgeranylation. To explore further the impact of RhoA activation on the subsequent RhoA mRNA upregulation, we treated EC with or without simvastatin (0.1 \( \mu \text{mol/L} \)) before cross-linking of HLA class I with specific Ab. Cells that were incubated with irrelevant IgG were used as controls. As expected, simvastatin completely prevents the induction of active GTP-bound RhoA in response to HLA class I ligation (Figure 8A). Effect of simvastatin on RhoA transcription was then examined at later time points by RT-PCR. In the presence of simvastatin, no increase of RhoA mRNA was observed as compared with resting levels (Figure 8B). These results demonstrate that blocking RhoA activation with simvastatin also prevents upregulation of RhoA transcription.

**Discussion**

HLA class I signaling pathways have been shown to be involved in the proliferation of vascular EC and SMC associated with transplant arteriosclerosis (6,7,9). However, molecular mechanisms and signaling pathways implicated in HLA class I–mediated EC proliferation remain unclear. Allospecific Ab-mediated ligation of class I molecules expressed on the surface of EC have been shown to trigger intracellular signaling events reflected by phosphorylation of Src, paxillin, and focal adhesion kinase (8). Activation of the PI3-K/Akt pathway also occurs in response to class I ligation on EC (21). Together, these findings indicate that multiple pathways may act in...
concert to promote EC proliferation. However, upstream signaling events and the hierarchical activation cascade remain to be established. The present work demonstrates that HLA class I ligation on vascular EC induces transcriptional changes, including the upregulation of genes implicated in the cell cycle and proliferation, and identified RhoA as a key target for this angiogenic process.

Differential gene expression analysis is a powerful tool to investigate transcriptional changes in cells or tissues. However, the change in the transcription level of a gene is not always correlated with the causal role of that gene. Moreover, changes in gene expression are not invariably associated with changes in protein synthesis. Thus, gene expression analysis may be viewed as a guiding tool to initiate functional investigations. Rho GTPases, including RhoA, play a central role in eukaryotic cells, coordinately controlling the organization of the actin cytoskeleton with other cellular activities such as gene transcription, cell-cycle progression, and migration (22). The Rho GTPases also function as key regulators of important signaling pathways. This study demonstrated that RhoA was rapidly activated and then upregulated at the transcriptional level in response to HLA class I ligation on vascular EC. To our knowledge and as reported in recent publications (23,24), regulation of RhoA at a transcription or protein level is almost unknown. These studies showed an increase of RhoA mRNA associated with enhanced RhoA protein level. It is extremely interesting that, in EC, intercellular adhesion molecule-1 cross-linking also induced upregulation of RhoA at both mRNA and protein levels (25). Consistent with these data, our results may suggest that mRNA upregulation may reflect RhoA activation and consumption and may constitute a feedback response to RhoA activation, providing more RhoA for subsequent activation.

Although other GTPases can be upregulated, our attention was focused on determining the role of the signaling protein RhoA in class I-mediated signaling leading to angiogenesis in
Figure 8. Inhibitory effect of simvastatin on RhoA activation and transcription induced by HLA class I cross-linking. EC were pre-treated for 18 h with or without simvastatin (0.1 μmol/L) before incubation with anti-HLA class I Ab (W6/32, 10 μg/ml) or irrelevant isotype control IgG for the indicated period. (A) Pull-down assays. The GTP-Rho pulled down from lysate was detected by Western blot using a specific anti-RhoA antibody. (B) Semiquantitative RT-PCR analysis of mRNA expression for RhoA. Total RNA was extracted from EC and subjected to RT-PCR (18 cycles of PCR amplification). The specific PCR bands were separated in 1.2% agarose gels and stained with ethidium bromide. β-Actin mRNA was amplified as a control. Results are from a representative experiment of three performed.

Inhibitory effect of simvastatin on RhoA activation and transcription induced by HLA class I cross-linking. EC were pre-treated for 18 h with or without simvastatin (0.1 μmol/L) before incubation with anti-HLA class I Ab (W6/32, 10 μg/ml) or irrelevant isotype control IgG for the indicated period. (A) Pull-down assays. The GTP-Rho pulled down from lysate was detected by Western blot using a specific anti-RhoA antibody. (B) Semiquantitative RT-PCR analysis of mRNA expression for RhoA. Total RNA was extracted from EC and subjected to RT-PCR (18 cycles of PCR amplification). The specific PCR bands were separated in 1.2% agarose gels and stained with ethidium bromide. β-Actin mRNA was amplified as a control. Results are from a representative experiment of three performed.

The PI3-K/Akt signaling pathway is a key regulator of the angiogenic phenotype in EC (28). Activated PI3-K and Akt have been shown to stimulate angiogenesis through increased expression of vascular endothelial growth factor mRNA in EC (29). Akt has several downstream targets that are involved in the regulation of the cell cycle, including E2F, forhead transcription factor, S6 protein kinase, and GSK3β (30). A recent study documented the signaling pathways involved in angiogenesis by demonstrating that phosphorylation of GSK3β, one of the many substrates for Akt, is essential for EC survival and migration in vitro and angiogenesis in vivo (31). Akt down-regulates GSK3β through site-specific phosphorylation at Ser9. GSK3β is a cyclin D1 protein kinase (32). Inactivation of GSK3β through Akt-mediated phosphorylation, as observed in response to HLA class I ligation on EC, has been shown to decrease turnover and to stabilize cyclin D1 (32). Consequently, we can hypothesize that modulation of cyclin D1 through GSK3β inactivation could provide a second target for PI3-K in the regulation of the cell cycle. Supporting this hypothesis, we observed by flow cytometer analysis that a preincubation (18 h) with simvastatin, which in our study efficiently inhibits EC proliferation, significantly reduced cyclin D1 expression in EC (data not shown).

PTEN and PI3-K have opposing functions in the control of cell-cycle progression (30). Indeed, PTEN overexpression inhibits cell growth in a variety of normal and transformed cells. PTEN is primarily expressed, in parallel to Akt, in the mid to late G1 phase during cell-cycle progression before pRb hyper-phosphorylation (33). However, coexpression of PTEN with activated PI3-K or Akt, as reported in our study, efficiently antagonizes PTEN-mediated growth suppression (33). Therefore, PTEN induction may suggest the existence of a negative feedback loop that occurs after Rho-dependent PI3-K activation. Nevertheless, the respective role and downstream targets of GSK3β and PTEN have to be investigated.

Inhibitors of HMG-CoA reductase, or statins, have been shown to be useful in the reversal of endothelial dysfunction, an effect that may be independent of the reduction in cholesterol levels. Although their contribution to angiogenesis could vary according to the type of statin and dose levels (34), statins have been showed to have direct beneficial effects, including inhibition of SMC and EC proliferation (14) and preproendothelin-1 gene expression (19). Most of these actions resulted from RhoA inactivation (35,36). Recently, it was shown that simvastatin prevents thrombin-induced translocation of RhoA to the plasma membrane in EC (36). In accordance, we now demonstrate that simvastatin also inhibits activation of RhoA and further regulation mediated by HLA class I cross-linking. In repressing allogeneic-induced angiogenesis through RhoA and subsequently PI3-K inhibition, statins therefore provide a new type of immunomodulation that could prevent chronic transplant nephropathy. The clinical relevance of statins is supported by recent data showing the improvement in long-term graft survival in heart (37) and kidney (38) transplant recipients under statin therapy.

In this study, RNA differential display RT-PCR also identified several other molecules involved in cell-cycle progres-
tion and regulation (Table 1). Although their precise regulation at mRNA and protein levels remains to be analyzed, the functions of these proteins further indicate that anti-HLA alloantibodies alter EC cell cycle and proliferation. In addition, our data do not exclude roles for other Rho GTPases, most notably RhoB and RhoC.

Collectively, our data suggest that RhoA-dependent activation of the PI3-K/Akt signaling pathway promotes cell-cycle progression and proliferation of vascular EC that are treated with anti-HLA alloantibodies. Our data established a specific progression and proliferation of vascular EC that are treated with anti-HLA alloantibodies. In addition, alloantibodies alter EC cell cycle and proliferation. In addition, alloantibodies alter EC cell cycle and proliferation.

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