The Perlecan Fragment LG3 Is a Novel Regulator of Obliterative Remodeling Associated With Allograft Vascular Rejection

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Rationale: Endothelial apoptosis is increased in association with acute and chronic vascular rejection (VR) of solid allografts. Apoptotic endothelial cells (EC) release LG3, a C-terminal fragment of perlecan of potential importance in vascular remodeling and neointima formation.

Objective: Our 2 goals were to determine whether circulating levels of LG3 are increased in association with acute VR of renal allografts and to evaluate the impact of LG3 on vascular remodeling.

Methods and Results: We conducted a case-control study to compare serum LG3 levels in human renal transplant patients with acute VR, tubulo-interstitial rejection (ATIR) and normal graft function. Aorta transplantation between fully MHC-mismatched mice in association with intravenous LG3 injection was used to characterize the impact of LG3 on vascular remodeling. Scratch assays evaluated the promigratory activity of LG3 on vascular smooth muscle cells (VSMC) in vitro. Serum LG3 levels were significantly elevated in human renal transplant patients with acute VR (n=16) compared to ATIR (n=16) and normal graft function (n=32, P=0.004). In patients with acute VR, graft loss was associated with elevated LG3 levels. Increasing LG3 serum levels in aortic allograft recipients significantly increased neointima formation. LG3 injection fostered accumulation of α-smooth muscle actin-positive cells and decreased the number of CD31 positive EC. LG3 increased the migration of VSMC through extracellular signal-regulated kinases 1/2-dependent pathways.

Conclusion: These results indicate that LG3 is a novel regulator of obliterator vascular remodeling during rejection. (Circ Res. 2012;110:94-104.)

Key Words: apoptosis ■ endothelial cell ■ extracellular matrix ■ neointima ■ kidney

Vascular rejection (VR) is closely associated with the development of obliteratorive changes in the arteries, arterioles, and capillaries of solid organ transplants.1–3 Endothelial apoptosis was shown to be increased in acute VR, and the sustainability of the apoptotic endothelial response is tightly correlated with progressive concentric myointimal thickening, leading to lumen narrowing, downstream ischemic changes, and loss of allograft function.1,3 Although mononuclear leukocytes are present within the neointima, the vast majority of neointimal cells are α-smooth muscle actin (αSMA)-positive cells that stem from the migration and accumulation of recipient and/or donor-derived mesenchymal stem cells (MSC), vascular smooth muscle cells (VSMC), and myofibroblasts.4–6 Recipient-derived MSC have been identified within neointimal lesions in animal models and in human renal transplant recipients with chronic rejection.6 However, nonrecipient-derived αSMA-positive cells also integrate within the neointima, indicating that the migration of donor-derived VSMC or local stem cells is an additional mechanism contributing to neointima formation in transplant vasculopathy (TV).7 In this context, migration of αSMA-positive cells and their acquisition of a prosurvival phenotype are central to progressive lumen narrowing.

Basement membrane degradation is another key hallmark of VR, and mounting evidence suggests that extracellular matrix (ECM) remodeling dramatically alters vascular homoeostasis. ECM proteolysis has been implicated in the production of cryptic ECM fragments, referred to as matricryptins, that display novel functional activity on cells of the vessel wall.8 For example, collagen XVIII degradation by several proteases—including matrix metalloproteinases, elastases, and various cathepsins—triggers production of the
matricryptin endostatin, a C-terminal fragment with potent angiostatic activity.9 Similarly, perlecan, a proteoglycan embedded within the vascular basement membrane, when degraded by bone morphogenetic protein-1/tolloid family of metalloproteinases or cathepsin-L, releases the matricryptic angiostatic C-terminal fragments endorepellin and LG3.10–12

Our group recently uncovered a crucial role of endothelial cell (EC) apoptosis in the production of matricryptic fragments.13–15 This finding is of special relevance in VR, as immune-mediated endothelial injury and sustained endothelial apoptosis are closely correlated with oblitative vascular remodeling in renal and heart transplant recipients.16–18 Caspase-3 activation in apoptotic EC fosters the extracellular release of perlecan-L, which degrades perlecan, thereby liberating LG3.11 In turn, LG3 interacts with α2β1 integrins on MSC and fibroblasts, resulting in a state of resistance to apoptosis.13–15 In MSC, this antiapoptotic phenotype develops downstream of extracellular signal-regulated kinases 1/2 (ERK1/2) activation and is further enhanced by cross-talk with the epidermal growth factor–epidermal growth factor receptor pathway.19 Other groups have shown that LG3–α2β1 integrin interactions on EC significantly inhibit angiogenesis in vitro and in vivo.10,12,19 Collectively, these functions, in contrast to the proangiogenic and antiproliferative activity of undegraded perlecan, point to the possibility that LG3 may contribute to vascular remodeling in conditions associated with EC apoptosis.

In the present work, we sought to determine whether circulating LG3 levels are increased in renal transplant patients with acute VR and whether they correlate with allograft outcome. We also evaluated the functional consequences of increased circulating LG3 levels on vascular remodeling in a pure VR model. Mice were transplanted with a fully major histocompatibility complex (MHC)–mismatched aortic graft in the absence of immunosuppression, followed by intravenous (iv) injection of recombinant LG3. Finally, we characterized the effect of LG3 on migration and ERK1/2 activation in VSMC in vitro.

Methods

Patients, Setting, and Study Design
We performed a retrospective, case-control study in which we compared serum levels of LG3 in kidney transplant recipients who experience acute VR to levels observed in patients with acute tubulo-interstitial rejection (ATIR), or normal graft function. The project was approved by the CHUM ethics review board. All patients who received a kidney graft at the CHUM between January 1, 1990, and August 1, 2009, were identified through an electronic clinical database that has been maintained prospectively from January 1, 1990. Since then, for each renal transplant patient, sera were banked for cause. Each case of acute VR was matched for permanent dialysis. No patient died with a functioning graft.

Analysis of Human Serum Samples
Circulating LG3 levels in serum samples were measured as close as possible to and always before or on the day of diagnostic biopsy in patients with acute VR or ATIR. To evaluate LG3 levels, we ruled out the ELISA technique as it does not allow the discrimination of LG3 fragment from other perlecan derived C-terminal fragments, such as endorepellin. Hence, we adopted an immunoblotting method used to evaluate multimeric forms of serum proteins22 to specifically distinguish the LG3 fragment by molecular weight from other C-terminal perlecan fragments. Undiluted human sera were immunodepleted of IgG and human serum albumin (HSA) with Vivapure HSA/IgG affinity resin (Sartorius Stedim Biotech, Aubagne, France) and reconcentrated in a MWCO5Kd Vivaspin column (Sartorius Stedim). To detect LG3, we separated immunodepleted human sera by SDS-PAGE, transferred onto nitrocellulose membranes and probed with an anti-LG3 antibody (H300, Santa Cruz Biotechnology, Santa Cruz, CA). LG3 levels were quantified by densitometry with Alpha Imager software (Cell Biosciences, Santa Clara, CA). Recombinant LG3 (10–100 ng) was used to establish a standard curve by Western blotting. For all experiments, 1 specific human serum in which the LG3 band was clearly present was used as an internal positive control. This serum was immunodepleted concomitantly with experimental sera and was loaded on all Western blots. Mean LG3 estimation for the internal standard was 7.1 μg/mL with an interexperiment variability (standard deviation) ±0.85 μg/mL. Also, cases and corresponding controls were loaded side by side on the same Western blot, assuring equal LG3 estimation variability for cases (acute VR) and controls (normal and ATIR).

In order to rule out a possible nonspecific signal due to the secondary antibody, the membranes were first probed with the secondary antibody alone followed by detection and then repeating the experiments with anti-LG3 followed by the secondary antibody. In absence of the anti-LG3 antibody, no bands were detected, whereas multiple bands were identified after probing with anti-LG3 (Online Figure IIC, which is available in the Data Supplement at http://circres.ahajournals.org). This experiment established the spec-
ficity of the signal and also confirmed previous reports that multiple C-terminal perlecan bands can be recovered in human serum.22

Statistical Analysis
For the human retrospective case-control study, continuous variables are expressed as means and standard deviations (SD) when normally distributed, and otherwise as medians and interquartile ranges. Categorical variables are summarized as proportions. Between-groups differences in categorical variables were assessed by the χ² or Fisher exact test. We performed a Wilcoxon rank-sum test to assess the differences in LG3 levels between subjects with acute VR and other patients. After transforming LG3 in the natural log, we performed an analysis of variance to assess the difference in LG3 levels between subjects with acute VR who experienced graft loss in the first 6 months after transplantation and those who did not. We performed a linear regression model including kidney function as an independent variable to study various mechanisms of LG3 elevation. For animal and in vitro studies, the results, expressed as mean±SEM, were analyzed by Student t test. P≤0.05 was considered to be significant for all tests.

Production of Recombinant C-Terminal Perlecan Fragment
Recombinant murine LG3 for in vitro experiments and iv injection in mice was produced and purified as described previously.15 Purity of the recovered LG3 protein was assessed by reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue R250 staining. Endotoxins were measured by limulus amebocyte lysate (LAL) test with an Endosafe-PTS spectrophotometer (Charles River Laboratories, Wilmington, MA), and very low levels (1.6 endotoxin units (EU)/mg of rLG3) were detected.

Animals and Aorta Transplantation Procedures
Aortae from BALB/c donors were transplanted in C57Bl/6 recipients as described elsewhere with minor modifications.23,24 In brief, 1 mL of heparinized saline (50 μL/mL) was injected into the vena cava to flush each aorta. A 6-mm segment of abdominal aorta from below the renal arteries to just above the aortic bifurcation was excised and soaked in ice-cold 0.9% normal saline. The grafts were then sutured in the orthotopic position with end-to-end anastomoses, using 11-0 nylon interrupted sutures. Whenever mentioned, the animals received tail vein iv injections of either 50 μg recombinant LG3 or phosphate-buffered saline (PBS, vehicle), and an additional group was treated with 0.08 U lipopolysaccharide (LPS) in PBS. Injections were given every other day during the first 3 weeks posttransplantation for a total of 8 doses. Transplanted and adjacent native aortae were harvested at 3 or 9 weeks posttransplantation, embedded in OCT, and cryosectioned. Immunohistochemistry and Analysis of Mice

Immunohistochemistry and Analysis of Mice

Serum Samples
Paraffin-embedded sections were stained with antibodies against activated caspase-3 (Cell Signaling Technology, Beverly, MA), CD31 (Santa Cruz Biotechnology), phosphorylated ERK1/2 (Abcam, Cambridge, MA), and αSMA (Clone 1A4, Dako North America, Carpinteria, CA). Ultraview Universal DAB detection kit (Ventana Medical Systems, Tucson AZ) was used to detect αSMA, and the avidin–biotin peroxidase ABC staining system (Santa Cruz Biotechnology) for all other antibodies. Online Figure I shows immune and nonimmune (in the absence of primary antibody) staining of all antibodies. Serum LG3 levels were quantified as described for human serum samples.

Cell Culture and Reagents
A7R5 cells (rVSMC) (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 supplemented with 10% fetal bovine serum (FBS; MEDICORP, Montreal, Quebec, Canada). Human primary aortic vascular smooth muscle cells (AOSMC) were obtained from Clonetix (San Diego, CA), grown in SmG-2 culture media, and used at passages 2 to 7. The anti-β1 integrin blocking antibody (4B4) was purchased from Beckman Coulter (Mississauga, Ontario, Canada).

Assessment of Migration and Apoptosis
Migration of confluent rVSMC and AOSMC was measured by an adapted wound assay as described before.28 Unfixed/unpermeabilized adherent cells stained with Hoechst 33342 (20-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2.50-bi-1 to 1H-benzimidazole) (HT) and propidium iodide (PI) were viewed by fluorescence microscopy, as described previously.11,13–15

Immunoblotting
Proteins were extracted, separated by electrophoresis, transferred to nitrocellulose membranes, and probed as described before.11,13–15

Results
Increased Circulating LG3 Levels Are Associated With Acute Vascular Rejection in Kidney Transplant Recipients and Predict Unfavorable Long-Term Outcome
We sought to evaluate, in human renal transplant patients, whether acute VR, a condition classically associated with endothelial injury and apoptosis, is coupled with heightened serum LG3 levels. We performed a retrospective case-control study in which we compared circulating LG3 levels in patients with Banff grade II or III acute VR, Banff grade I ATIR, or normal graft function. Baseline immunosuppression was similar among the 3 groups. In comparison with their controls, subjects with acute VR were younger, and more likely to have positive donor-specific antibodies on historical serum and to receive a second transplant (Table). They also had higher panel reactive antibodies immediately prior to transplantation and a lower GFR at the time of sample collection (Table 1). Median time between serum collection and diagnostic biopsy was 2 days and, in all cases, sera were sampled prior to biopsy.

Circulating LG3 levels were evaluated with an immunoblotting technique to discriminate LG3 from other C-terminal perlecan fragments based on molecular weight (Online Figure IIC). Previous reports and the present results demonstrate that various C-terminal perlecan fragments are present in human serum.22 In the present work we focused on LG3 based on its known association with endothelial apoptosis and on its potential activity of vascular remodeling.13–15 Circulating LG3 levels were significantly higher in subjects with acute VR compared to those with ATIR and normal allografts (Figure 1A and Online Figure IIA). Other C-terminal perlecan fragments did not appear to vary in association with acute VR (data not shown). We also evaluated whether other matricryptins were increased in association with acute VR. Rejecting recipients (acute VR and ATIR) presented higher endostatin serum levels than patients with normal allograft function (Online Figure IID). However endostatin serum levels were not significantly different between acute VR and ATIR recipients (Online Figure IID). These results suggest
that among different matricryptins, LG3 is specifically increased in association with vascular injury. To tease out the potential contribution of renal clearance to LG3 elevation, we performed a multiple linear regression model in which the dependent variable was LG3, and the independent variables were acute VR and estimated creatinine clearance. In univariate analysis, there was a 24-U difference in LG3 levels between subjects with acute VR and other patients (P < 0.003). When graft function was included in the model, this difference was reduced to 7 (P = 0.44). LG3 increased by 4.7 U for each 10 mL/min decrease in GFR (P < 0.007), suggesting that decreased LG3 clearance in association with acute VR is, at least in part, implicated in LG3 elevation.

We also evaluated whether serum LG3 levels were associated with functional outcomes. Median follow-up time was 5.5 years in the renal transplant cohort. In patients with acute VR, those who lost their grafts in the first 6 months after transplantation (n = 9) had higher LG3 levels than patients with preserved allograft function for more than 6 months (n = 7, P = 0.02) (Figure 1B). Subjects with ATIR and normal graft function experienced no graft losses in the first 6 months after transplantation. These results suggest that elevated LG3 levels are associated with the severity of acute VR and with poor graft survival in this group of patients and that LG3 is a biomarker of severe renal allograft vascular injury, which could translate into reduced functional half-life of allografts. These results also demonstrate that, in humans, high LG3 serum levels are present in association with acute VR. On the basis of the known activities of LG3 on angiostasis and acquisition of a neointimal phenotype in MSC,15,22 we sought to determine whether increased LG3 serum levels could alter vascular remodeling. To this end, we turned to an animal model of pure VR.

Kinetics of Vascular Changes Following MHC-Incompatible Aorta Transplantation in Mice

Orthotopic transplantation of aortic grafts was performed between fully MHC-mismatched BALB/c donors and C57Bl/6 recipients in absence of immunosuppression. Intima-media ratios were increased in allogeneic segments at 3 weeks and 9 weeks posttransplantation (Figure 2A). In isogenic allograft recipients, neointima formation was apparent only around sutures at 9 weeks posttransplantation (Figure 2A), suggesting that neointima formation is predominantly, albeit not exclusively, dependent on immune responses triggered by allogeneic MHC. Neointimal cells and medial cells were largely αSMA-positive cells in allogeneic grafts 3 weeks posttransplantation (Figure 2B). At 9 weeks posttransplantation, the vast majority of αSMA-positive cells in allogeneic grafts were identified within the neointima, whereas the media showed evidence of devitalization (Online Figure III). This result is consistent with previous results indicating immune-mediated media devitalization and accumulation of recipient-derived αSMA-positive progenitor cells within the neointima.26

ERK1/2 activation is pivotal to neointima formation in rodent models of mechanical vascular injury or atherosclerosis.27 Similarly, in this model of immune-mediated vascular injury, neointimal ERK1/2 activation was observed in allogeneic transplants at 3 and 9 weeks posttransplantation (Figure 2B). At 9 weeks posttransplantation, the vast majority of αSMA-positive cells in allogeneic grafts were identified within the neointima, whereas the media showed evidence of devitalization (Online Figure III). This result is consistent with previous results indicating immune-mediated media devitalization and accumulation of recipient-derived αSMA-positive progenitor cells within the neointima.26

Table. Characteristics of Patients With Acute Vascular Rejection (AVR), Acute Tubulo-Interstitial Rejection (ATIR), and Stable Graft Function (Reference)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AVR</th>
<th>ATIR</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years (SD)*</td>
<td>39 (9)</td>
<td>50 (12)</td>
<td>47 (10)</td>
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<tr>
<td>Male gender (%)</td>
<td>7 (44)</td>
<td>11 (69)</td>
<td>20 (63)</td>
</tr>
<tr>
<td>Transplantation date (%)</td>
<td>Before 2000: 6 (38)</td>
<td>6 (38)</td>
<td>9 (28)</td>
</tr>
<tr>
<td></td>
<td>2007–2009: 6 (38)</td>
<td>7 (44)</td>
<td>15 (47)</td>
</tr>
<tr>
<td>Donor age in years (SD)</td>
<td>47 (15)</td>
<td>40 (16)</td>
<td>38 (16)</td>
</tr>
<tr>
<td>Previous transplantation (%)*</td>
<td>3 (19)</td>
<td>1 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pretransplant donor-specific antibodies (%)*</td>
<td>4 (22)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Preformed antibodies &gt;20%</td>
<td>5 (31)</td>
<td>0 (0)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Maximal levels (%)</td>
<td>6 (38)</td>
<td>5 (31)</td>
<td>3 (9)</td>
</tr>
<tr>
<td>Positive C4d staining (%)**</td>
<td>6 (75)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Immunosuppression protocol (%)</td>
<td>11 (69)</td>
<td>9 (56)</td>
<td>22 (69)</td>
</tr>
<tr>
<td>Tacrolimus and mycophenolate motefil</td>
<td>1 (6)</td>
<td>2 (13)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Cyclosporine and mycophenolate motefil</td>
<td>4 (25)</td>
<td>5 (31)</td>
<td>9 (28)</td>
</tr>
<tr>
<td>Other combination</td>
<td>12 (9–18)</td>
<td>13 (7–18)</td>
<td>10 (6–15)</td>
</tr>
<tr>
<td>Median time between transplantation and serum collection in days (IQR)</td>
<td>2 (0–9)</td>
<td>2 (1–3)</td>
<td>. . .</td>
</tr>
<tr>
<td>Median time between serum collection and graft biopsy in days (IQR)</td>
<td>5 (0–37)</td>
<td>33 (28–41)</td>
<td>59 (52–72)</td>
</tr>
<tr>
<td>Median creatinine clearance at the time of serum collection in mL/min per 1.73 m² (IQR)*</td>
<td>5 (0–37)</td>
<td>33 (28–41)</td>
<td>59 (52–72)</td>
</tr>
</tbody>
</table>

SD, standard deviation; IQR, interquartile range.

*Information on C4d staining was available for 8 patients with AVR and 9 patients with ATIR.

*P < 0.05 for comparison between AVR and ATIR/reference group.

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Increased Serum LG3 Levels Accelerate Neointima Formation

In this model of progressive VR, there was no detectable increase in serum LG3 levels in allograft recipients compared to isograft recipients 3 weeks posttransplantation (data not shown). These results are consistent with the small surface of allogeneic vascular material transplanted in this model and the subacute nature of the model. However, as shown in human EC,11,13,15 activation of apoptosis in murine aortic EC in vitro triggers cathepsin L extracellular translocation and LG3 production (Online Figure V). Activation of apoptosis in VSMC did not induce LG3 production, confirming the specificity of the association between LG3 release and endothelial apoptosis (Online Figure VI). We then investigated whether raising serum LG3 levels in allogeneic aortic recipients with intravenous injections of recombinant LG3 to levels observed in human acute VR renal transplant patients impacts the severity of allograft remodeling. Recombinant LG3 or vehicle was injected iv every other day for 3 weeks in allogeneic aortic recipients, and the mice were euthanized 3

Figure 1. LG3 circulating levels in renal transplant patients. A, Circulating LG3 levels in patients with normal graft function, acute tubulo-interstitial rejection (ATIR), and acute vascular rejection (AVR). \( *P=0.004 \) for comparison between other AVR patients. Box plots represent the lower range, 1st quartile, median, 3rd quartile, and higher range (HR). HR is excluded for better visual definition in patients with ATIR and AVR. B, Circulating LG3 levels in patients with acute VR who experienced graft loss in the first 6 months after transplantation compared to subjects whose graft survived past 6 months. \( *P=0.02 \). Box plots represent the lower range, 1st quartile, median, 3rd quartile, and higher range (HR).

Figure 2. Progressive neointima formation following allogeneic aorta transplantation. A, Intima/media ratios in isograft (3 weeks \( n=7 \), 9 weeks \( n=4 \)) and allograft (3 weeks \( n=11 \), 9 weeks \( n=5 \)) recipients at 3 and 9 weeks posttransplantation. \( *P<0.05 \). B, Isogenic and allogeneic aortic donor sections stained for \( \alpha \)-SMA and phosphorylated ERK1/2 at 3 weeks posttransplantation. The square insert indicates the magnified area. C, Recipient and donor aortic sections stained for activated caspase-3 and donor aortic section stained for CD31 at 3 weeks posttransplantation. Arrowheads indicate activated caspase-3-positive cells. In, intima; M, media.
weeks posttransplantation. This schedule was based on previous reports demonstrating a significant angiostatic activity of LG3 in vivo in a model of tumor-induced angiogenesis. Western blotting confirmed elevated serum LG3 levels in LG3-injected mice compared to vehicle-injected controls (Online Figure VII). Neointima formation was significantly increased in allogeneic recipients injected with LG3 relative to vehicle (Figure 3A and 3B and Online Figure VIII). Although high LPS levels are known to be required to accelerate rejection, we nonetheless considered the possibility that the very low endotoxin levels present in recombinant LG3 could impact neointima formation. A group of allogeneic recipients was injected with LPS to parallel LG3 endotoxin levels. Neointima formation was not enhanced in these LPS-injected mice compared to vehicle (Figure 3A and 3C and Online Figure VIII). This pattern was comparable to that in noninjected recipients of allogeneic grafts 9 weeks posttransplantation (Online Figures III and X). However, LG3-injected animals did not show evidence of neointima formation in the autologous aorta contiguous to the allograft, and medial αSMA-positive cells were not decreased within the autologous aorta (Online Figure XI). These results suggest that LG3 accelerates vascular remodeling at sites of immune-mediated vascular injury without noticeably altering autologous vascular homeostasis.

LG3 has been reported to inhibit angiogenesis and endothelial migration in other cell systems and animal models. We, therefore, evaluated indices of endothelial repair in LG3-injected mice. Immunohistochemistry for the EC marker CD31 was largely negative in the allogeneic aorta of LG3-injected mice (Figure 3D and 3E). However, the adjacent autologous aorta showed normal endothelial integrity with persistent CD31 staining (Online Figure XI). In vehicle-injected mice, CD31 staining was apparent within the allograft, with only occasional signs of discontinuity in the endothelial lining (Figure 3D and 3E).
This pattern was similar to that of noninjected aortic allografts (Online Figure IV). Collectively, these results indicate that, at sites of immune-mediated vascular injury, increased LG3 serum levels inhibit endothelial repair and foster accumulation of αSMA-positive cells, both mechanisms known to contribute to progressive vascular obliteration.

LG3 Induces ERK1/2-Dependent Promigratory and Prosurvival Phenotypes in VSMC

Migration of αSMA-positive cells and their acquisition of an antiapoptotic phenotype are central to the accumulation of neointimal cells within a hostile environment. We, therefore, explored the functional activity of LG3 in VSMC migration and apoptosis in vitro. Migration of human AOSMC (hAOSMC)
and rat VSMC (rVSMC) within the denuded area of a scratch assay was increased dose-dependently in the presence of LG3 for 24 and 48 hours (Figure 4A and 4B and Online Figure XII). In both cell types, BrdU incorporation was not modulated by exposure to LG3, suggesting that increased proliferation could not be inferred as a cause of increased cellularity within the denuded area (Online Figure XII). Indices of apoptosis were also reduced in VSMC exposed to LG3. Nuclear condensation indicative of apoptosis was reduced in serum-starved rVSMC exposed to LG3 (Figure 4C). This antiapoptotic phenotype occurred in the absence of necrotic features, suggesting that LG3 does not redirect cell death toward a pronecrotic phenotype in rVSMC. PARP cleavage was reduced in hAOSMC exposed to LG3 (Figure 4D). These results suggest a specific promigratory and antiapoptotic activity of LG3 on VSMC.

Having previously found that resistance to apoptosis of MSC induced by LG3 was ERK1/2-dependent and independent of the PI3K pathway,\textsuperscript{15} we assessed ERK1/2 phosphorylation in VSMC exposed to LG3 in vitro. ERK1/2 phosphorylation was enhanced dose-dependently in response to LG3 in hAOSMC and in rVSMC (Figure 5A and Online Figure XII). Inhibition of ERK1/2 activation by PD98059 and U0126 abrogated LG3-induced migration of hAOSMC and LG3 (2 \( \mu \)g/mL) or anti-\( \beta_1 \) integrin antibody (10 \( \mu \)g/mL) or control isotype-matched IgG (10 \( \mu \)g/mL). Lower panels: autoradiographs were quantified by densitometric scanning for the ERK1/2-phosphorylated form relative to the amount of total ERK2 (* \( P < 0.01 \)).

\textbf{Figure 5. Promigratory and prosurvival phenotypes induced by LG3 in VSMC are ERK1/2 dependent.} \textbf{A, Upper panels:} immunoblotting analysis of phosphorylated ERK1/2 and total ERK2 in hAOSMC exposed for 30 minutes to serum starvation (SS) alone or supplemented with LG3 (0.5, 1, 2, and 4 \( \mu \)g/mL). \textbf{Lower panel:} autoradiographs were quantified by densitometric scanning for the ERK1/2-phosphorylated form relative to the amount of total ERK2 (* \( P < 0.01 \)). \textbf{B,} Representative migration assay micrographs of migration of hAOSMC exposed for 24 hours to SS or serum-free medium supplemented with LG3 (2 \( \mu \)g/mL) alone or with PD98059 (50 \( \mu \)mol/L), U0126 (10 \( \mu \)mol/L), or vehicle (DMSO). \textbf{C,} Assessment of migration of hAOSMC exposed for 24 hours to SS or serum-free medium supplemented with LG3 (2 \( \mu \)g/mL) alone or with PD98059 (50 \( \mu \)mol/L), U0126 (10 \( \mu \)mol/L), or vehicle (DMSO) (\( n = 3 \), * \( P < 0.01 \)). \textbf{D,} \textbf{Upper panels:} immunoblotting analysis of phosphorylated ERK1/2 and total ERK2 in hAOSMC exposed for 30 minutes to SS, SS supplemented with LG3 alone, and SS supplemented with LG3 (4 \( \mu \)g/mL) and anti-\( \beta_1 \) integrin antibody (10 \( \mu \)g/mL) or control isotype-matched IgG (10 \( \mu \)g/mL). \textbf{Lower panels:} autoradiographs were quantified by densitometric scanning for the ERK1/2-phosphorylated form relative to the amount of total ERK2 (* \( P < 0.01 \)).
An integrin-blocking antibody (10 µg/mL) or control antibody. Phosphorylation of ERK1/2 in presence of LG3 was abolished by β1-integrin blockade but not by the control antibody (Figure 5D). These results demonstrate the importance of LG3-β1-integrin interaction for activation of ERK1/2 dependent pathways in VSMC.

Discussion

VR is classically associated with EC apoptosis and ECM degradation in various animal models and in human transplant recipients. However, the functional importance of ECM-derived fragments in allograft vascular remodeling remains largely uncharacterized. In the present work, we identify LG3 as a marker of severity in renal transplant patients with acute VR.

We evaluated whether serum LG3 levels rise in association with immune-mediated vascular injury of renal allografts in humans. Using a case-control study design, we determined that median serum LG3 levels, sampled before diagnostic biopsy, were significantly heightened in recipients with acute VR, compared to ATIR or normal allograft function. We chose to exclude patients with C4d-positive staining from the ATIR control group, which would have indicated antibody-mediated rejection, a condition characterized by antibody-dependent microvascular damage. Therefore, the current ATIR group represents tubulo-interstitial injury in the absence of vascular damage, whereas the acute VR cases indicate severe and acute immune-mediated vascular injury. Hence, serum LG3 levels in human renal transplant patients are elevated in conjunction with vascular injury and in this subgroup of patients is associated with reduced allograft survival and loss of function. While this increase is partly explained by decreased renal clearance, increased production due to acute VR is also possible. Other matricryptic fragments of similar molecular weight such as endostatin and other perlecan fragments do not accumulate in association with renal failure in acute VR, which argues against the sole contribution of renal clearance in LG3 accumulation. Kidney function was very different between the groups we studied. There was thus very little overlap in estimated creatinine clearance between groups that allowed for the estimation of the effect of acute VR independent of kidney function. Larger studies will be needed to tease out the various mechanisms of LG3 elevation in patients with acute VR. Nonetheless, the present study demonstrates that increased LG3 levels are present in human renal transplant patients with acute VR. These data are in line with a recent report in which, using an unbiased proteomics approach, urinary LG3 levels were found to be increased in renal transplant patients with biopsy-proven chronic allograft nephropathy.

Having demonstrated the presence of high LG3 serum levels in rejecting human allograft recipients, we sought to characterize the potential impact of LG3 on vascular remodeling. To this end, we tested a well-characterized model of

Figure 6. LG3 produced by apoptotic endothelial cells is a novel regulator of vascular remodeling. A, Upon induction of endothelial apoptosis, cathepsin-L translocates to the extracellular matrix (ECM), where it cleaves perlecan and generates LG3. B, In turn, LG3 interacts with β1 integrins on vascular smooth muscle cells (VSMC), mesenchymal stem cells (MSC), and endothelial cells (EC), leading, respectively, to increased migration, resistance to apoptosis, and angiostasis. Collectively, these responses will enhance neointima formation, inhibit endothelialisation, and favor obliterative changes; αSMA+, αSMA positive cells.
pure VR in mice based on the orthotopic transplantation of a fully MHC-mismatched aortic allograft in the absence of immunosuppression. We injected recombinant LG3 iv in allografted recipients to raise their serum LG3 levels to the range observed in renal transplant recipients with acute VR. LG3-injected allograft recipients showed significantly enhanced vascular remodeling with increased intima-media ratios, neo-intimal accumulation of αSMA-positive cells, and media decellularization. These changes were comparable to those found in noninjected allogeneic recipients 9 weeks posttransplantation, indicating accelerated remodeling in the presence of high circulating LG3 levels. LG3 injections also significantly decreased CD31 expression within allografts but did not alter endothelial integrity in the neighboring autologous aorta. These results are in line with the known angiostatic and antiproliferative activity of LG3 on ECs and fibroblasts, cell types of importance in neo-intima formation.13,15 We extended these findings to VSMC and demonstrated that LG3 significantly enhances the viability of stressed VSMC. More important, we demonstrated that LG3 directly interacts with αSMA-positive cells to activate mechanisms central to neo-intima formation, such as migration and resistance to apoptosis. Our previous work described significant antiapoptotic activity of LG3 on MSC and fibroblasts, cell types of importance in neo-intima formation.13,15 The present results further support a novel role for LG3 in both VSMC and fibroblasts, highlighting the potential importance of LG3 in vascular remodeling.

A healthy endothelium plays an important role in repressing neo-intima formation through the production of antiproliferative factors for αSMA-positive cells. However, we also considered the possibility that LG3 directly interacts with αSMA-positive cells to activate mechanisms central to neo-intima formation, such as migration and resistance to apoptosis. Our previous work described significant antiapoptotic activity of LG3 on MSC and fibroblasts, cell types of importance in neo-intima formation.13,15 We extended these findings to VSMC and demonstrated that LG3 significantly enhances the viability of stressed VSMC. More important, we demonstrated that LG3 significantly increases VSMC migration, which represents a novel function of LG3. Collectively, these results suggest that elevated LG3 levels at sites of vascular injury—whether it is due to increased production, decreased clearance, or both—may contribute to obliterator remodeling through coordinate activities on inhibition of endothelial repair and augmented migration and survival of αSMA-positive cells.

Activation of the prosurvival ERK1/2 signaling pathway has been implicated in neo-intima formation in atherosclerosis and mechanically induced vascular injury27 and transplant vasculopathy.30 The present results further support an important role for ERK 1/2 activation in immune-mediated vascular remodeling as we demonstrated progressive neo-intima formation in association with ERK1/2 phosphorylation in neo-intimal cells of allogeneic aortic allografts. However, mediators inducing ERK1/2 activation in neo-intimal cells during VR are still incompletely defined. Our current results highlight a novel role of LG3 in the activation of ERK1/2 in VSMC. Through interactions with β1 integrins, LG3 increases ERK1/2 activation, leading to increased migration of VSMC. On the basis of these findings, we propose a new mechanistic model in which ECM degradation occurring during vascular injury releases mediators, such as LG3, that inhibit endothelial repair and enhance obliterator remodeling (Figure 6), 2 important mechanisms contributing to reduced allograft function.

In summary, the present work demonstrates that, in renal transplant patients, serum LG3 levels increase in association with acute VR and is associated with poor allograft survival. In a model of VR, heightened circulating LG3 levels accelerate vascular obliteration and repress endothelial repair. The coordinate activities of LG3 on migration and survival of αSMA-positive cells and angiostasis likely contribute to maladaptive and accelerated vascular obliteration. Collectively, these results identify LG3 as a novel regulator of allograft vascular remodeling.

Acknowledgments

Thanks are due to Ms. Katia Hamelin for technical assistance, to Dr. Viktor Kotka for help and advice, and to Mrs. Suzanne Morissette and Mrs. Jo-Ann Fugère for their work on the biological and clinical database in renal transplant patients.

Sources of Funding

This work was supported by research grants from the Canadian Institutes of Health Research (CIHR) (MOP-15447) to M.J.H., from Fonds de la recherche en santé du Québec (FRSQ) to M.J.H. and C.P., and from the Pfizer Cardio-Renal grant program to F.M. M.J.H. is the holder of the Shire Chair in Nephrology, Transplantation, and Renal Regeneration of Université de Montréal. We thank J.-L. Lévesque Foundation for renewed support.

Disclosures

None.

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The Perlecan Fragment LG3 Is a Novel Regulator of Obliterative Remodeling Associated With Allograft Vascular Rejection

Mathilde Soulez, Eve-Annie Pilon, Mélanie Dieudé, Héloïse Cardinal, Nathalie Brassard, Shijie Qi, Shyh-Jong Wu, Yves Durocher, François Madore, Claude Perreault and Marie-Josée Hébert

Circ Res. 2012;110:94-104; originally published online November 10, 2011;
doi: 10.1161/CIRCRESAHA.111.250431

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Material

Supplementary methods

Cell culture

Murine EC (mEC) were isolated from the aorta of Balb/c mice, as described previously\(^1\), \(^2\), and grown in DMEM low glucose culture media supplemented with endothelial cell growth supplements (ECGS), 10% FBS, 10% calf serum, 1% penicillin-streptomycin and 1% fungizone. Media conditioned by either apoptotic or non-apoptotic mEC were generated, as described previously\(^3\)-\(^6\), at passage 4. To produce conditioned medium, cells were pre-incubated for 2 h with DMSO (SSC-DMSO, apoptotic cells) or the cell-permeable and irreversible pan-caspase inhibitor zVAD-fmk (SSC-ZVAD, non-apoptotic cells), then washed once in PBS and exposed to serum-free medium for 4 h. For all experiments, equal volumes of media conditioned by an equal number of cells (apoptotic or not) were compared. We demonstrated in previous work that this system leads to the release of active mediators by apoptotic EC downstream of caspase-3 activation without cell membrane permeabilization\(^6\).

Immunoblotting

Proteins were extracted, separated by electrophoresis, transferred to nitrocellulose membranes, and probed\(^3\)-\(^6\). Proteins from conditioned media were precipitated by the addition of 10 µl per ml of a 2% sodium deoxycholate solution, incubation for 30 min at 4°C, followed by the addition of trichloroacetic acid 9:1 and overnight incubation at 4°C. They were pelleted by centrifugation at 14,000 rpm, washed with cold acetone and solubilized in Laemmli sample buffer, followed by Western blotting against LG3 (H300, Santa Cruz Biotechnology). The other antibodies used for Western blotting were: anti-cathepsin-L (Santa Cruz Biotechnology), anti-PARP (Cell Signaling Technology), and anti-actin (Cedarlane Laboratories, Hornby, ON).

Assessment of proliferation

Measurement of BrdU incorporation during DNA synthesis was performed in subconfluent rVSMC and AOSMC using cell proliferation ELISA BrdU (colorimetric) kit (Roche Diagnostics GmbH, Germany) according to the protocol provided by the manufacturer and as we described previously\(^7\).

Reagents

zVAD-fmk was purchased from R&D Systems (Minneapolis, MN), and ZFF-FMK was from Calbiochem (Gibbstown, NJ).
Online Figure I

Immune and non-immune staining of donor aortic allografts for active caspase-3 (magnification x1,000), αSMA (magnification x200), phosphorylated ERK1/2 (magnification x400) and CD31 (magnification x1,000).

Online Figure II A. Representative Western blot of serum LG3 in renal transplant patients with acute vascular rejection (AVR), acute tubule-interstitial rejection (ATIR) or normal graft function (N). B. Representative Western blot of LG3 in the human serum used as internal control and recombinant LG3 dose-response curve. Recombinant LG3 contains a Histidine tag explaining its molecular weight at 25kD, whereas LG3 recovered in serum samples is estimated at 23kD. C. Representative Western blot of human serum first probed with the secondary antibody alone followed by detection (right panel) and then repeating the experiments with anti-LG3 followed by the secondary antibody (left panel). In absence of the anti-LG3 antibody no bands were detected whereas multiple bands were identified after probing with anti-LG3. D. Circulating endostatin levels in patients with normal graft function, acute tubulo-interstitial rejection (ATIR), and acute vascular rejection (AVR). *p=0.01 for comparison between AVR patients and patients with normal graft function.; *p=0.0465 for comparison between ATIR patients and patients with normal graft function. p=0.73 for comparison between AVR patients and ATIR patients. Box plots represent the lower range, 1st quartile, median, 3rd quartile and higher range (HR). HR is excluded for better visual definition in patients with ATIR and AVR.

Online Figure III

A. Isogenic and allogeneic aortic donor sections stained for αSMA and phosphorylated ERK1/2 at 9 weeks post-transplantation. The square insert indicates the magnified area. In: intima. M: media. B. Donor aortic sections stained for activated caspase-3 and donor aortic section stained for CD31 at 9 weeks post-transplantation. The square insert indicates the magnified area.

Online Figure IV

A. Isogenic and allogeneic aortic sections stained for CD31 at 3 weeks post-transplantation. The square insert indicates the magnified area. B. Isogenic and allogeneic aortic sections stained for CD31 at 9 weeks post-transplantation.

Online Figure V

A. Immunoblotting analysis of PARP cleavage and actin in mEC pre-incubated with either DMSO or zVAD-fmk and then exposed to serum-free medium for 4 h. B. Immunoblotting analysis of LG3 and cathepsin-L in equal volumes of serum-free media conditioned by apoptotic mEC (SSC-DMSO) or non-apoptotic mEC (SSC-ZVAD). C. Immunoblotting analysis of LG3 in equal volumes of serum-free media conditioned by apoptotic mEC in the presence of ZFF-FMK (SSC-ZFF), a cathepsin L inhibitor, or control (SSC-DMSO).

Online Figure VI

Immunoblotting analysis of PARP cleavage and actin in rVSMC exposed to serum-free medium for 8h. B. Percentage of apoptotic rVSMC detected by HT-PI staining after 8h
exposure to normal serum (N), or serum-free medium (SS). C. Immunoblotting analysis of LG3 in equal volumes of serum-free media conditioned by apoptotic VSMC (SSC VSM) or SS.

Online figure VII

A. Immunoblotting analysis of LG3 in sera of mice that received PBS (n=4) or LG3 (n=8 for LG3 level analysis in sera. B. Autoradiographs were quantified by densitometric scanning for LG3 and compared to an internal control. *p≤0.05.

Online Figure VIII

Verhoeff-stained aortic allografts from mice injected iv with vehicle or LG3. Magnification x100. M: media, In: intima.

Online Figure IX

Intima/media ratios in mice injected with LPS (n=4), PBS (n=6) or LG3 (n=8). *p≤0.05.

Online Figure X

Number of αSMA-positive cells (x1,000) per surface (µm²) of media cross sections in allogeneic aortae of mice 3 and 9 weeks post-transplantation and allogeneic aortae of mice 3 weeks post-transplantation injected with vehicle or LG3.

Online Figure XI

Autologous portion of aortae of mice 3 weeks post-transplantation injected with vehicle or LG3 stained for CD31 and αSMA.

Online Figure XII

A. Representative migration assay micrographs of migration of rVSMC exposed for 48 h to serum starvation (SS) or serum-free medium supplemented with LG3 (1, 5 and 10 µg/ml). B. Assessment of migration of rVSMC exposed for 48 h to serum starvation (SS) or serum-free medium supplemented with LG3 (1, 5 and 10 µg/ml). (n= 6, *p<0.005). C. BrdU incorporation in rVSMC exposed 24h to normal medium (N), serum-free medium (SS) alone or supplemented with 10µg/mL of LG3. (n=8, *p<0.001) D. BrdU incorporation in hAOSMC exposed 24h to N, SS alone or supplemented with LG3. (n=12, *p<0.001) E. Upper panels: Immunoblotting analysis of phosphorylated ERK1/2 and total ERK2 in rVSMC exposed for 30 min to SS alone or supplemented with LG3 (1, 5 and 10 µg/ml). Lower panel: Autoradiographs were quantified by densitometric scanning for the ERK1/2-phosphorylated form relative to the amount of total ERK2 (*p<0.05). F. Representative migration assay micrographs and assessment of migration of rVSMC exposed for 48 h to serum-starvation (SS) or serum-free medium supplemented with LG3 (10 µg/ml) alone or with PD98059 (50 µM), U0126 (10 µM) or vehicle (DMSO) (n≥ 6, *p<0.01, †p<0.01).


Online figure I

<table>
<thead>
<tr>
<th>Immune</th>
<th>Non-immune</th>
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<tbody>
<tr>
<td>Active caspase-3</td>
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<tr>
<td>αSMA</td>
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<tr>
<td>Phosphorylated ERK1/2</td>
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<tr>
<td>CD31</td>
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Online figure II

A

B

C

D

Endostatin serum levels (ng/mL)

Normal (n=32)  ATIR (n=16)  AVR (n=16)
Online figure IV

A  3 weeks

B  9 weeks

Isograft  Allograft  Isograft  Allograft

400x

1000x

400x

1000x
Online figure V

A

SS-
DMSO  SS-
ZVAD

PARP

116kD

89kD

Actin

B

SS-
DMSO  SS-
ZVAD

LG3

CathL

C

SS-
DMSO  SS-
ZFF

LG3
Online figure VI

A

B

C

N SS

PARP pro cleaved

Actin

SSC VSM SS LG3 recumb.

LG3

Apoptosis (% of VSMC)

0 5 10 15 20 25 30

N SS

*
Online figure VII

A

SERUM

LG3 injected mice

vehicle injected mice

LG3

ponceau

B

[Graph showing LG3 serum levels (µg/ml) for vehicle and LG3 injected mice. The graph indicates a significant difference (*).]
Online figure IX

Intima/Media ratio (relative units)

LPS  PBS  LG3

*
Online figure X

- Allografts 3 weeks
- Allografts 9 weeks
- Mice injected with vehicle
- Mice injected with LG3

αSMA(+) cell number x 1000 surface (µm²)
Online figure XI

CD31 (x1000)

αSMA (x200)
Online figure XII

A. 

![Images of rat VSMC](SS and SS+LG3 concentrations)

B. 

<table>
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<tr>
<th>Migration (Number of cells in the scratched area)</th>
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<tbody>
<tr>
<td>SS</td>
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<tr>
<td>SS+LG3 (µg/ml)</td>
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C. 

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<th>BrdU incorporation (% of SS)</th>
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<tr>
<td>N</td>
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<td>SS+LG3 (µg/ml)</td>
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D. 

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<th>BrdU incorporation (% of SS)</th>
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<td>SS+LG3 (µg/ml)</td>
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E. 

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<th>Densitometry (arbitrary units)</th>
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<tbody>
<tr>
<td>SS</td>
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<tr>
<td>SS+LG3 (µg/ml)</td>
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F. 

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<th>Migration (Number of cells in the scratched area)</th>
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