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Role of Fibrinogen-Like Protein 2 Prothrombinase/Fibroleukin in Experimental and Human Allograft Rejection[1]

Qin Ning,[2]* Yi Sun,[3]* Meifang Han,* Li Zhang,* Chuanglong Zhu,* Weijie Zhang,† Hui Guo,† Jinwen Li,† Weiming Yan,* Feili Gong,* Zhonghua Chen,† William He,§ Cheryl Koscik,§ Robert Smith,§ Reginald Gorczynski,§ Gary Levy,§ and Xiaoping Luo‡

Immune coagulation is a major contributor to the pathogenesis of xenograft rejection, viral-induced hepatocellular injury and cytokine-induced fetal loss syndrome. In this study, we investigated the contribution of the novel gene product, fibrinogen-like protein 2 (fgl2) prothrombinase, in mediating immune injury in experimental and human acute allograft rejection. Using a mouse heterotopic cardiac transplant model, mouse *fgl2* (mfgl2)/fibroleukin mRNA transcripts and protein were highly expressed in macrophages, CD4- and CD8-positive T lymphocytes, and endothelial cells in rejecting cardiac allografts in association with deposits of fibrin. Although mfgl2-deficient mice rejected allografts at similar rates to littermate controls, survival of grafts from mfgl2-deficient mice were prolonged and deposition of intravascular fibrin was diminished. Treatment of wild-type mice with a neutralizing anti-fgl2 Ab ameliorated histological evidence for allorejection and intravascular fibrin deposition, and resulted in an increase in graft survival. To address further the relevance of fgl2 in acute allograft rejection, we examined kidney biopsies from patients who had undergone renal transplantation. Human *fgl2* mRNA transcripts and protein were markedly expressed mainly in renal tubule cells, infiltrating lymphoid cells including macrophages, $CD8^+$ T cells, mature B cells (plasma cells), and endothelial cells. Dual staining showed fibrin deposition was localized mainly to blood vessels, in the glomerulus and interstitium and the lumen of tubules, and occurred in association with human fgl2 expression. These data collectively suggest that fgl2 accounts for the fibrin deposition seen in both experimental and human allograft rejection and provide a rationale for targeting fgl2 as adjunctive therapy to treat allograft rejection. ***The Journal of Immunology,*** **2005, 174: 7403–7411.**

Fibrinogen-like protein 2 (fgl2)[4]/fibroleukin, also known as *fgl2* prothrombinase, has recently been cloned and identified and shown to belong to the fibrinogen family of proteins. Mouse *fgl2* (*mfgl2*) and human *fgl2* (*hfgl2*) have been localized to chromosomes 5 and 7, respectively (1–3). *fgl2* prothrombinase has been shown previously to have the attributes of a serine protease capable of directly cleaving prothrombin to thrombin leading to fibrin deposition (2). Several studies indicate that *mfgl2* is involved in experimental xenograft rejection by mediating "immune coagulation," fibrin deposition, and microthrombus formation, leading to classical pathological changes of acute vascular rejection (4). The role of *fgl2* in allorejection has not been examined in detail. Previously, Hancock et al. (5) have suggested that *mfgl2* expression is correlated with allograft rejection, and strategies known to prevent allorejection including infusion of anti-CD154 prevent expression of mfgl2. However, this group recently also suggested that disruption of mfgl2 did not alter type 1 immunity or fibrin deposition associated with allograft rejection (6).

In this present study, we investigated the expression of fgl2 in acute allograft rejection, first using a mouse heterotopic cardiac transplant model, and subsequently extending our studies to examine *hfgl2* expression in rejecting human renal allografts. Expression of fgl2 was studied by both in situ hybridization and immunochemistry. *mfgl2* transcripts were highly expressed in macrophages, T lymphocytes, and endothelial cells in rejecting cardiac allografts in association with deposits of fibrin. Treatment of mice with a high-titered neutralizing anti-fgl2 polyclonal Ab ameliorated the pathological injury and resulted in measurably increased graft survival. In rejecting human renal allografts, hfgl2 mRNA transcripts and protein expression correlated with the presence of rejection. Collectively, these studies suggest that fgl2 expression may be critical to the pathogenesis of both experimental and human allograft rejection and provide a rationale for targeting the *fgl2* gene in an attempt to modulate allograft rejection.

Materials and Methods

All animal studies were conducted according to the guidelines of the Chinese Council on Animal Care and approved by the Tongji Hospital of Tongji Medical School Committees on Animal Experimentation. Informed consent was obtained from human study participants, and the research protocol was reviewed and approved by the hospital institutional review board of Tongji Hospital, Huazhong University of Science and Technology.

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[4] Abbreviations used in this paper: fgl2, fibrinogen-like protein 2; mfgl2, mouse fgl2; hfgl2, human fgl2; Dig, digoxigenin; AP, alkaline phosphatase; MHV-3, murine hepatitis virus strain 3.

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Table I. *Categories of mice undergoing cervical heterotopic heart transplantation*[a]

Groups	Days Posttransplantation (No. of Animals)			
	1	3	5	7
A Isogeneic/no Ab C57BL→C57BL	A1 (5)	A2 (4)	A3 (5)	A4 (7)
B Allogeneic/no Ab BALB/c→C57BL	B1 (5)	B2 (5)	B3 (5)	B4 (7)
C Allogeneic/fg12 Ab BALB/c→C57BL		C1 (4)	C2 (4)	C3 (5)
D Allogeneic/control Ab BALB/c→C57BL		D1 (3)	D2 (3)	D3 (4)

[a] Animals in each group were sacrificed on day posttransplantation indicated, and hearts were recovered and assessed for histology, characterization of infiltrate, fibrin deposition, and mfgl2 expression and grade of rejection.

Mice and cardiac grafting

BALB/cJ and C57BL/6 were purchased from Hubei Provincial Institute of Science and Technology. Mice with a targeted disruption of *mfgl2* gene were housed at the University of Toronto and generated as described previously (7). The cervical heterotopic cardiac allotransplantation model used was described earlier, and is a modification of the technique published by Chen (8). BALB/cJ mice were used as donors, and C57BL/6 mice were used as recipients. C57BL/6 mice were used as both donor and recipient in an isogeneic transplant control group (group A).

For histologic studies, animals were divided into four groups (A–D) as shown in Table I. At predetermined times, animals were sacrificed, and hearts were collected and stored at −80°C until processed for histo- or immunopathology.

In a second series of independent studies, isogeneic transplants or allotransplants were performed, and cardiac graft survival was monitored using a BL-410 Biology Life Signal Analysis System (Chengdu Taimong Electric). A modification of the Banff criteria was used to define pathology grades of rejection of cardiac allografts as shown in Table II (9). Mice were treated with 500 μg of a neutralizing anti-fgl2 polyclonal Ab (in group C); 500 μg of a control rabbit Ab (Sigma-Aldrich) (group D); or purified Fab′ of an anti-fgl2 polyclonal Ab 24 and 72 h posttransplant by tail vein injection (group E). The Fab′ were generated by papin digestion of purified fgl2 Ab followed by separation on AffinityPak Columns of immobilized protein A (Pierce). Fab′ were shown by polyacrylamide electrophoresis and Western blotting to be >95% pure.

In situ hybridization

The method used has been described previously (1). A digoxigenin-11-UTP (Dig-UTP) (Roche)-labeled cDNA probe was cut by *Eco*RI following subcloning of a 169-bp fragment of mfgl2 cDNA, representing nt 756 (ACTGTGACA. . . .) to 924 (. . . . GAGTAAGGA), into pCR2.1 vector (Invitrogen Life Technologies). The Dig-UTP-labeled probe concentration was determined by immunoenzymatic reaction with chemiluminescent detection, and the probes were stored at −80°C. Tissue sections were deparaffinized in 100% xylene and 100% alcohol, followed by prehybridization in 50% formamide and 2× SSC at room temperature for 1 h. The hybridization mixture consisted of 50% deionized formamide, 5% dextran sulfate, 250 μg of salmon sperm DNA per milliliter, and 2 μg of Dig-labeled cDNA probe per milliliter in 2× SSC. The hybridization mixture with the

Table III. *Patient characteristics*[a]

No.	Gender	Age (Years)	Kidney Biopsy (Time From Transplant (mo))	Diagnosis (Banff 97 Criteria)
1	Male	39	4	I A(i2,t2,v0,g0)
2	Male	27	1	I A(i2,t1,v0,g0)
3	Female	54	24	I A(i1,t2,v0,g0)
4	Male	31	36	I A(i1,t1,v0,g0)
5	Male	30	5	I A(i2,t2,v0,g0)
6	Male	42	18	I A(i1,t1,v0,g0)
7	Female	55	24	I A(i1,t1,v0,g0)
8	Female	35	3	I A(i2,t1,v0,g0)
9	Male	32	12	I B(i2,t3,v0,g0)
10	Male	64	120	I B(i2,t3,v0,g0)
11	Male	29	5	I B(i3,t2,v0,g0)
12	Female	41	6	I B(i1,t1,v0,g0)
13	Female	61	17	I B(i2,t1,v0,g0)
14	Female	61	18	I B(i1,t2,v0,g0)
15	Female	42	7	I B(i1,t1,v0,g0)
16	Female	46	72	I B(i2,t3,v0,g0)
17	Male	23	12	II A(i2,t1,v1,g0)
18	Male	49	12	II A(i2,t1,v1,g0)
19	Male	55	12	II A(i3,t2,v1,g1)
20	Male	35	6	II A(i1,t0,v1,g0)
21	Male	51	4	II A(i1,t1,v1,g0)
22	Male	49	4	II A(i3,t2,v1,g0)
23	Male	51	24	II A(i1,t1,v1,g1)
24	Female	34	1	II B(i3,t2,v2,g2)
25	Male	55	1	II B(i3,t2,v1,g0)
26	Male	28	1	II B(i1,t1,v2,g0)
27	Male	37	6	II B(i3,t3,v2,g1)
28	Male	63	2	II B(i2,t1,v2,g0)
29	Male	44	2	II B(i3,t1,v3,g0)
30	Female	50	2	II B(i1,t1,v3,g1)
31	Male	34	2	II B(i3,t1,v3,g1)

[a] t, Tubulitis; i, mononuclear cell interstitial inflammation; g, allograft glomerulonephritis; ah, arteriolar hyaline thickening; v, initial arteritis; cg, allograft glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; cv, fibrosis intimal thickening; mm, mesangial matrix increase; 0, absent; 1, 10–25%; 2, 25–50%; 3, >50%.

Table II. *Histologic grading of cardiac rejection*[a]

Old Term	Grade	Comments
No rejection	0	Biopsy specimens with very sparse lymphoid infiltrates should be included in this grade.
Mild rejection	1	Focal perivascular or interstitial infiltrates. The mild intensity and lack of myocyte damage distinguish this from higher grades.
Focal moderate rejection	2	One focus only with aggressive infiltration and/or focal myocyte damage.
Low moderate rejection	3	Multifocal aggressive infiltrates and/or myocyte damage. The multiple foci may be present in only one fragment or scattered throughout several fragments.
		Diffuse inflammatory process. The intensity of the lymphoid infiltrate varies considerably and is associated with the presence of myocyte damage. This damage must be present in at least two fragments, but some degree of infiltration is present in most fragments.
Severe acute rejection	4	A diffuse and polymorphous infiltrate with or without edema, hemorrhage, and vasculitis. The infiltrate is more intense and more widespread than 3B, and myocyte damage is conspicuous. There are often neutrophils and/or h emorrhage, although neither is essential for classification as this grade.

[a] Modified from Billingham et al. (9).

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Table IV. *Effect of Ab to Fg12 treatment on grade of cardiac graft rejection*

Groups	*n*	Pathology Grades Individual	Mean	±SD
Isogeneic/no Ab				
(A1)	5	0, 0, 1, 0, 0	0.2	0.45
(A2)	4	0, 0, 0, 1	0.25	0.50
(A3)	5	1, 0, 0, 1, 0	0.4	0.55
(A4)	7	1, 0, 1, 1, 0, 0, 1	0.5714	0.53
Allogeneic/no Ab				
B1	5	0, 1, 1, 0, 1	0.6	0.55
B2	5	2, 2, 3, 2, 2	2.2	0.45
B3	5	3, 3, 3, 4, 3	3.2	0.45
B4	7	4, 4, 3, 3, 4, 3, 4	3.57	0.53
Allogeneic/fg12 Ab				
C1	4	1, 0, 1, 1	0.75	0.50
C2	4	2, 2, 2, 3	2.25	0.50
C3	5	2, 2, 3, 2, 3	2.4	0.55
Allogeneic/control Ab				
D1	3	2, 2, 3	2.3	0.58
D2	3	3, 4, 3	3.3	0.58
D3	4	3, 4, 3, 4	3.5	0.58

probe was denatured by heating in an 85°C water bath for 5 min, chilled on ice for 1 min, and added to tissue sections for hybridization at 42°C overnight. Posthybridization washing in a series of dilutions of SSC was followed by application of 3% blocking reagent at room temperature for 30 min. After a brief wash in Tris-HCl buffer (pH 7.5), sections were incubated with polyclonal anti-Dig Fab, conjugated to alkaline phosphatase (AP; Boehringer Manheim), and diluted 1/500 in Tris-HCl buffer. Unbound Ab was removed by two 5-min washes with Tris-HCl buffer. A purple reaction product developed when AP substrate, 5-bromo-4-chloro-3-indolyl-phosphate, and NBT, were applied to sections at room temperature for 120 min. Sections were counterstained with methylene green and mounted with Permount for viewing.

Immunohistochemical staining

mfgl2 and hfgl2 prothrombinase in mouse and human rejecting grafts. Immunohistochemical staining for detection of the fgl2 prothrombinase was used to assess fgl2 prothrombinase expression in grafted tissue, where fgl2 mRNA transcripts were evident as previously described (9). Tissues were fixed with 100% methanol at 4°C for 5 min and air dried. They were rehydrated with 0.1 M PBS (pH 7.4) and blocked with 10% normal horse serum in PBS at room temperature for 2 h.

A polyclonal Ab to mfgl2 prothrombinase was produced in rabbits by repeated injections with a 14-aa hydrophilic peptide (CKLQADDHRDPGGN) from exon 1 of the mfgl2 prothrombinase, which had been coupled to keyhole limpet hemocyanin. Ab was purified by affinity columns, and thereafter tissue or cultured cell slices were incubated with Ab (20 μg/ml

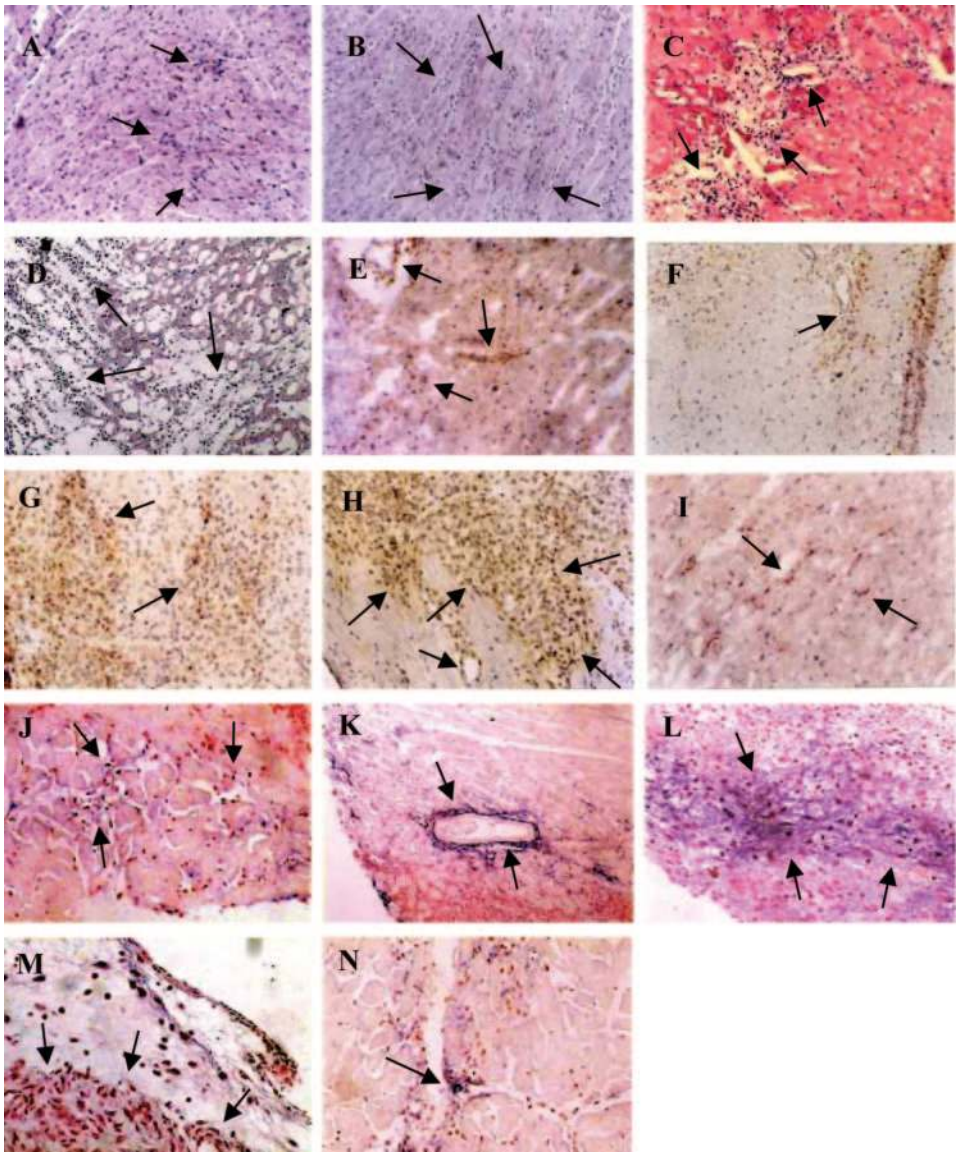

FIGURE 1. mfgl2 expression in mouse heart grafts post-cervical heterotopic heart allotransplantation. *A–D*, Mild (*A*, day 1 posttransplantation; ×100) focal or patchy (*B*, day 3 posttransplantation; ×100) mononuclear inflammation; scattered mononuclear infiltration with myocardium injury (*C*, day 5 posttransplantation; ×100); and severe mononuclear infiltration with confluent myocardium necrosis (*D*, day 7 posttransplantation; ×100). mfgl2 expression was increasingly displayed in the infiltrating mononuclear cells and endothelial cells of the microvascular vessels both by immunohistochemistry (*E–I*) and by in situ hybridization (*J–N*) at day 1 (*E*, in infiltrating mononuclear cells; ×100), day 3 (*F*, in vascular endothelium; *J*, in infiltrating mononuclear cells; ×100), day 5 (*G*, in infiltrating mononuclear cells; *K*, in vascular endothelium; *L*, in infiltrating mononuclear cells; ×100), and day 7 (*H* and *M*, in infiltrating mononuclear cells; ×100) posttransplantation. *I* and *N*, Control, at day 5 posttransplantation in the isogeneic group showing mild focal or patchy mononuclear inflammation (*I*, in infiltrating mononuclear cells; *N*, vascular endothelium; ×100).

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in PBS) at room temperature for 2 h. Subsequently, sections were incubated with immunoperoxidase-conjugated goat IgG (6.7 μg/ml) fraction to rabbit IgG Fc (DakoCytomation) at room temperature for 1 h, following which they were washed five times in PBS with 0.05% Tween 20. Tissue slices were then air dried, mounted with 90% glycerol, and photographed with a Leitz Laborlux fluorescence S microscope.

Fibrin in mouse and human rejecting grafts. For fibrin detection, a rabbit-anti-fibrinogen Ab (DakoCytomation) was used. This reagent is known to react with fibrinogen and fibrin in mouse and human tissues (1, 9). The technique used for detection of fibrin was the standard avidin-biotin complex (ABC) method as previously described (9). The biotinylated secondary Ab was an anti-rabbit IgG linked to peroxidase reacted with 3,3′-diaminobenzidine chromagen, followed by counterstaining with hematoxylin. Serial sections were stained with H&E.

Dual immunohistochemical staining of hfgl2 and fibrin. Dual staining for hfgl2 and fibrin on the same tissue was performed using a Vectastain ABC kit (Vector Laboratories), with second Abs labeled with AP or HRP, respectively.

Immunoperoxidase staining of macrophages and T lymphocytes. A CD68 Ab (DakoCytomation), CD4 Ab, or CD8 Ab was used to detect macrophages (Kupffer cells) or T lymphocytes using the similar methodology described above.

Patient studies

Thirty-one patients who had undergone kidney allotransplantation at Tongji Hospital signed consents for this study. Twenty-one were male, and 10 were female. All patients were diagnosed with acute rejection by histopathologic findings according to the Banff 97 criteria (10). The clinical grade of rejection and time from transplant to rejection are presented in Table III. Percutaneous kidney biopsies were obtained for the purposes of guiding treatment decisions. Histological sections were stained with H&E. A polyclonal Ab against hfgl2 was used to detect the expression of hfgl2 protein by immunohistochemistry as described above. Anti-CD68 (DakoCytomation) and anti-CD8 (DakoCytomation) mAbs were used to detect the presence of macrophages and lymphocytes, respectively, in serial sections to identify the cellular source of hfgl2. Anti-fibrinogen mAb (DakoCytomation) was also used to detect fibrin deposition (8). Dual staining with anti-hfgl2 Ab and anti-fibrinogen Ab was performed to explore evidence for an association between hfgl2 expression and fibrin deposition.

Statistical analysis

Quantitative data were expressed as mean ± SD. Statistical analysis was conducted by using independent *t* test to compare effects of treatment on mean grade of rejection grade. Survival data were measured using a Kaplan-Meier model, and overall strata comparisons were made using log rank (Mantel-Cox) tests. The analysis was conducted on the statistical program for social sciences (SPSS), version 13.0, for Windows. A value of $p < 0.05$ was considered statistically significant.

Results

mfgl2 expression in rejecting grafts post-cervical heterotopic cardiac allotransplantation in mice

Hearts recovered from mice that underwent cervical heterotopic cardiac isogeneic or allogeneic transplantation at predetermined times posttransplantation (days 1, 3, 5, and 7) were examined for the presence of rejection (Table IV; Fig. 1, *A–D*) and expression of mfgl2. Transplantation of isogeneic grafts was used as controls. Expression of mfgl2 was examined at both the mRNA and protein levels by in situ hybridization and immunohistochemistry, respectively. Expression of mfgl2 was first detected at day 1 posttransplant, and expression of mfgl2 increased on days 3, 5, and 7 (Fig. 1, *E–H* and *J–M*). mfgl2 was seen primarily in infiltrating mononuclear cells and endothelial cells of the microvasculature, which correlated with the severity of the histopathological findings in rejecting grafts. There was little or no mfgl2 expression in grafts from the isogeneic group where there were no histological signs of rejection (Fig. 1, *I–N*).

Cellular source of mfgl2 and fibrin deposition in rejecting grafts in mice

By serial section staining, the majority of $CD68^+$, $CD4^+$, and $CD8^+$ cells showed high expression of mfgl2 protein in rejecting grafts (Fig. 2, *A–F*). Fibrin deposition was also detected in the endothelium of microvascular vessels by immunoperoxidase staining by day 5 (Fig. 2, *G* and *H*).

Effects of fgl2 polyclonal Ab on the histopathological improvement in rejecting grafts post-cervical heterotopic cardiac allotransplantation in mice

The pathological grades of the rejecting cardiac grafts from mice treated with or without fgl2 polyclonal Ab based on a modification

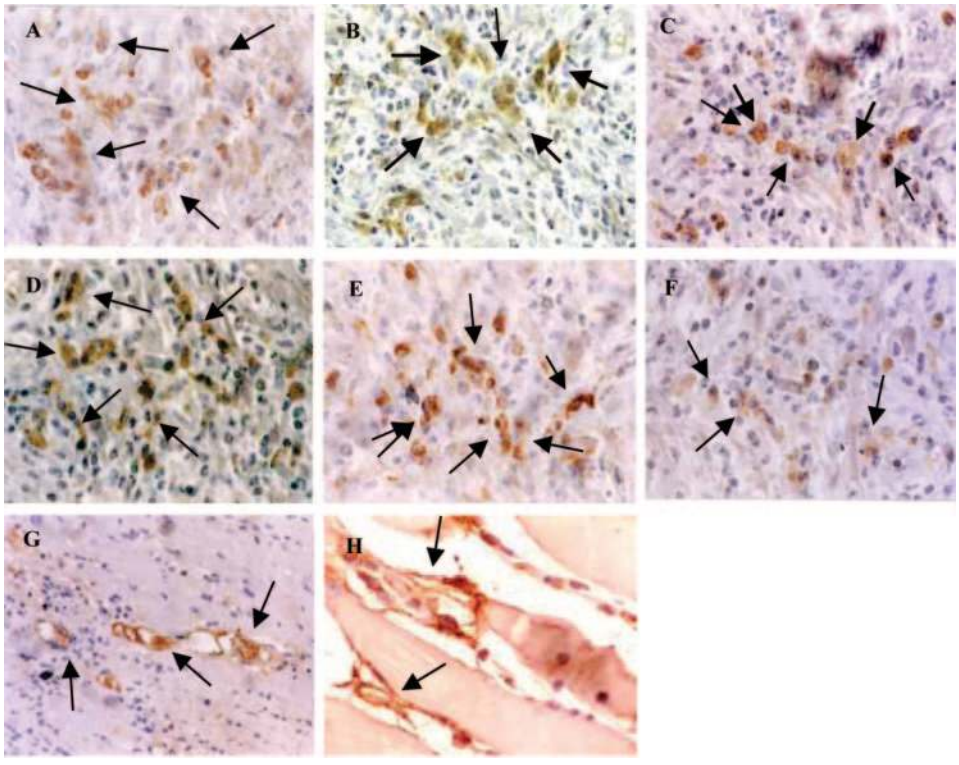

FIGURE 2. Cellular localization of mfgl2 expression and fibrin deposition in rejecting grafts in mice. *A–C* were stained with a fgl2 polyclonal Ab and show fgl2 protein staining in infiltrating mononuclear cells (arrows). *D–F* are serial sections of *A–C* showing that cells costaining for CD68, CD4, and CD8, respectively, strongly express mfgl2; ×200. Fibrin deposition is shown by arrows within the microvascular vessels by immunoperoxidase staining (*G*, ×200; *H*, ×400).

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of the Banff criteria proposed by Billingham et al. (9) (Table II) are summarized in Table IV and Fig. 3. Grade of rejection of fgl2 Ab-treated grafts was statistically reduced when compared with untreated or control Ab-treated allografts on days 3, 5, and 7, whereas control Ab treatment had no effect on rejection grade (Fig. 3*B*). The histological changes within the rejecting cardiac grafts were improved post-fgl2 polyclonal treatment as shown in Fig. 3*A*, *A–C*, compared with that in Fig. 1, *B–D*, or Fig. 3*AD*, in which no Ab treatment or a control Ab was used.

Effect of fgl2 polyclonal Ab on survival of rejecting cardiac grafts post-cervical heterotopic heart allotransplantation in mice

Grafts from mice that had received two injections of 500 μg of fgl2 neutralizing polyclonal Ab had a modest, but statistically significant increase in survival compared with grafts from untreated mice or mice injected with a control polyclonal Ab by log rank (Mantel-Cox) analysis followed by a paired *t* test for individual groups. The mean survival time of Ab-treated mice was with a mean survival time of 9.4 ± 1.1 days compared with a survival time of 7.3 ± 0.5 days of untreated mice; and 7.3 ± 0.6 days of mice receiving control Ab (Fig. 4). Additionally, mice receiving two injections of Fab′ equivalent to 500 μg of polyclonal anti-fgl2 (group E) were protected to the same degree as mice receiving the native anti-fgl2 molecule, with a mean survival of 9.7 + 1.3 days. These data are consistent with the hypothesis that protection occurs through neutralization of fgl2 rather than the involvement of other cells or molecules.

Heart grafts from BALB/cJ mice transplanted into mfgl2-deficient mice were universally rejected on day 6.4 ± 1.2, similar to transplantation of heart grafts from BALB/cJ mice, which were transplanted into $fgl2^{+/+}$ littermate controls. In contrast, heart grafts from fgl2-deficient mice transplanted into BALB/cJ mice had a prolonged survival but were ultimately rejected on day 12.2 ± 1.4 (Table V).

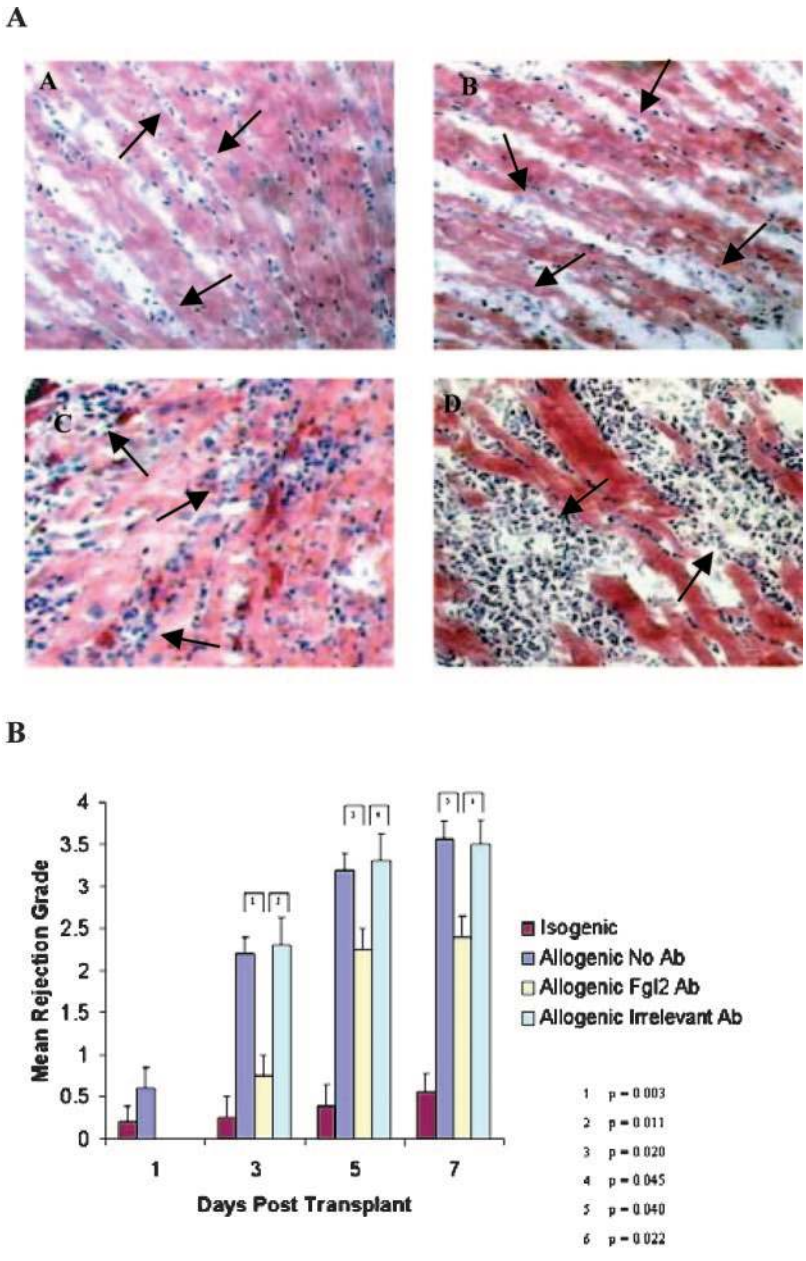

FIGURE 3. Effects of a neutralizing fgl2 polyclonal Ab on the pathology of heart grafts post-cervical heterotopic heart allotransplantation (H&E staining). *A*, Hearts recovered from animals treated with neutralizing anti-fgl2 Ab showed only mild or focal mononuclear inflammation (*A*, *A* and *B*) on days 3 and 5 posttransplantation (×100) and scattered mononuclear cell infiltration with mild myocardial necrosis (*AC*) on day 7 posttransplantation (×100). In contrast, *AD* shows a section of a heart with heavy mononuclear cell infiltrates and severe myocardial necrosis on day 7 posttransplantation from an animal treated with purified rabbit IgG control (×100). *B*, Comparison of rejection grades in groups of mice shown in Tables I and IV, and Fig. 3*A*.

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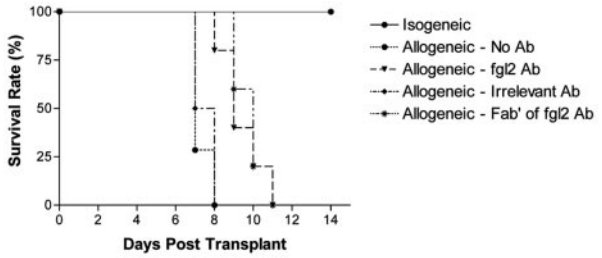
A. Survival Rate



B. Individual Survivals

Groups	Treatment	Survival (days)	Log-Rank p value
A	Isogeneic	14, 14, 14, 14, 14, 14, 14	
B	Allogeneic – No Antibody	7, 7, 8, 7, 7, 7, 8	0.000 [1]
C	Allogeneic–fgl2 Antibody	9, 9, 11, 10, 8	0.014 [2]
D	Allogeneic – Irrelevant Antibody	7, 8, 8, 7	0.498 [3]
E	Allogeneic – Fab' of fgl2 Antibody	11, 10, 10, 9, 9	0.637 [4]

1 Group A vs. Group B
2 Group B vs. Group D
3 Group C vs. Group D
4 Group C vs. Group E

FIGURE 4. Effect of fgl2 polyclonal Ab on the survival of heart grafts post-cervical heterotopic heart allotransplantation. *A*, Survival rate. Four to seven mice in each group received isogeneic (C57BL/6J→C57BL/6J) or allogeneic (BALB/c→C57BL/6J) heart transplants and received no treatment (isogeneic–no treatment, —●—; allogeneic–no treatment, - -●- -), or treatment with 500 μg of fgl2 polyclonal Ab 24 and 72 h posttransplant (allogeneic–fgl2 Ab, –▼–), or treatment with Fab′ from 500 μg of Ab to fgl2 24 and 72 h posttransplantation (allogeneic–Fab′ of fgl2 Ab, –*–) or 500 μg of control rabbit IgG 24 and 72 h posttransplantation (allogeneic–irrelevant Ab, –◆–) as indicated in Table I. *B*, Survival of individual groups. Survival data were measured using a Kaplan-Meier model, and overall strata comparisons were made using log rank (Mantel-Cox) tests.

Immunohistochemical assessment of hfgl2 expression in patients with renal acute allograft rejection

To address the relevance of hfgl2 in human acute allograft rejection, we studied patients with renal acute allograft rejection (Table III). Twenty-one of the patients were male, and 10 were female. The mean time to first rejection posttransplant was 14.9 + 24.1 mo. Grafted renal tissues with histopathological grades IB, IIA, IIB, and III were strongly and uniformly positive for hfgl2 expression, whereas IA lesions had less intense hfgl2 positivity, indicating a close association of hfgl2 expression with the histopathologic rejection and fibrin deposition. Expression of hfgl2 was seen primarily in renal tubule cells, in infiltrating mononuclear cells, and in the endothelium of small renal blood vessels and glomerular capillary wall in close proximity to fibrin deposits (Fig. 5).

By dual-staining immunohistochemistry, $CD68^+$ (macrophages) and $CD8^+$ (T cells) were shown to be the cellular source of hfgl2 (Fig. 6, *A–D*, respectively). Fibrin deposition was seen within the microvascular vessels, glomerular capillary wall and matrix, and the surface of infiltrating mononuclear cells in the blood vessels by immunoperoxidase staining in association with hfgl2 expression (Fig. 6, *E* and *F*).

Table V. *Survival of grafts post cervical heterotopic heart allotransplantation in mfgl2-deficient mice*[a]

Donor	Recipient	Survival (Days)	Mean ± SD
C57BL/6J	BALB/cJ	6, 6, 7, 6, 5	6.0 ± 0.71
$mfgl2^{+/+}$	BALB/cJ	7, 6, 7, 6, 5	6.2 ± 0.84
$mfgl2^{-/-}$	BALB/cJ	12, 14, 11, 12, 12	12.2 ± 1.10
BALB/cJ	$mfgl2^{-/-}$	6, 7, 5, 8, 6	6.4 ± 1.14
BALB/cJ	$mfgl2^{+/+}$	6, 5, 6, 7, 6	6.0 ± 0.71

[a] $mfgl2^{-/-}$ refers to mice on C57BL/6J background in which fgl2 has been deleted by targeted recombination. $mfgl2^{+/+}$ refers to littermate controls of $fgl2^{+/+}$ mice on C57BL/6J background. Female mice were used for all experiments.

Discussion

Fibrin deposition is a common element of many types of immunological reactions including delayed-type hypersensitivity, autoimmune disease, graft rejection, and the response to infectious agents (11, 12). Induction of procoagulant molecules on leukocytes and endothelial cells is thought to play an important role in the pathogenesis of thrombin generation and fibrin deposition at the sites of inflammation. This has been observed in the case of tissue factor, through the widespread constitutive expression of this molecule in subendothelial and perivascular tissues, suggesting that its major function is in initiating coagulation to achieve hemostasis after endothelial cell disruption.

Procoagulants other than tissue factor, which are induced specifically by immune mediators, may also play a critical role in regulating localized fibrin deposition. fgl2, also known as fibroleukin, is a 70-kDa type-2 transmembrane protein, which has been postulated to directly convert prothrombin to thrombin in the absence of factor VII or factor X (13). fgl2 has been shown previously to play a critical role in the pathogenesis of fulminant hepatitis induced by murine hepatitis virus strain 3 (MHV-3) (1, 7). Murine, porcine, and human fgl2 have now been cloned and sequenced, and fgl2 encodes the mouse fibrinogen-like protein (*musfiblp*), a previously described gene isolated from CTLs that shares significant homology to fibrinogen β- and γ-chains (14–16). The murine and human proteins share 78% overall identity, with greater conservation at the C terminus. The C terminus of fgl2 corresponds to a highly conserved region that is found in fibrinogen as well as in other functionally unrelated fibrinogen-related proteins such as tenascin, ficolin, and angiopoietin (17–19).

In the context of innate immune activation, induction of fgl2 in macrophages and endothelial cells has been shown to contribute to the pathogenesis of tissue factor-independent fibrin deposition and organ injury. In support of its role as a coagulant are the observation that neutralizing Abs to mfgl2 prevent both fibrin deposition and death from MHV-3 infection (20). Recent studies have also shown that inhibition of reticuloendothelial cell mfgl2 expression through the use of gene-targeted fgl2-deficient ($fgl2^{-/-}$) mice results in the prevention of MHV-3-induced fibrin deposition, liver injury, and death (7). Furthermore, we have recently reported that murine and human fgl2 prothrombinase/fibroleukin are highly expressed in endothelium, macrophages, and lymphocytes in xenograft rejection (4).

In the current study, elevated expression of mfg12 mRNA and protein was observed on vascular endothelial cells and infiltrating leukocytes, including macrophages, CD4- and CD8-positive T lymphocytes, in rejecting cardiac allografts in a mouse heterotopic cardiac transplant model, in association with deposits of fibrin.

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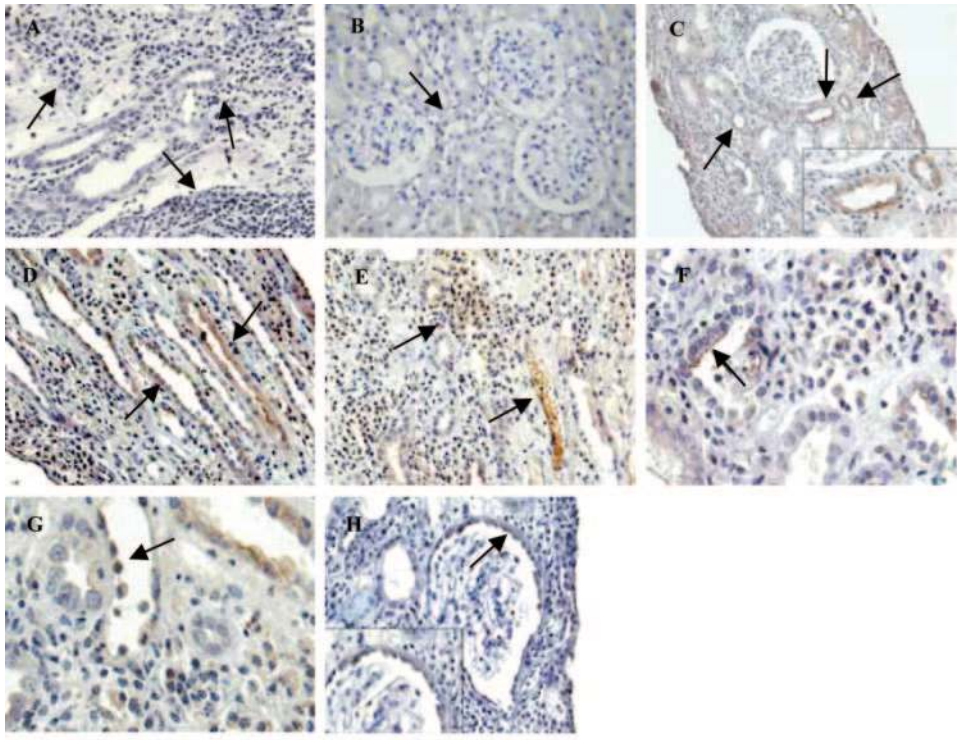

FIGURE 5. hfgl2 expression in patients with renal acute allograft rejection by immunohistochemistry. Kidney biopsy from a patient with renal acute allograft rejection. *A*, A heavy mononuclear cell infiltrate is seen within the stroma and between renal tubular epithelial cells. Vascular endothelialitis is shown with marked hyperplasia, swelling, and abscission (H&E stain; ×400). *B*, hfgl2 is weakly expressed in renal tubules of normal kidney tissue (×200). *C* and *D*, Areas of hfgl2 expression are seen in renal tubular epithelium in the renal cortex (×200, 400) and in the renal medulla (×200). *E* and *F*, Areas of hfgl2 expression are observed in infiltrating phlogistic cells within the kidney stroma (×400) and in the brush border of the renal tubular epithelium (×1000). *G*, hfgl2 expression in leukocytes adhering to the vessel wall (×1000). *H*, hfgl2 expression in cells adhering to the parietal layer of the renal capsule (×400, 1000).

Treatment of mice with a high-titered neutralizing anti-mfgl2 polyclonal Ab reduced the pathological injury and led to modest but statistically significant increase in graft survival. The fact that Fab′ anti-fgl2 was as protective as the native Ab suggests that the protection observed follows from neutralization of mfgl2 and is independent of other (FcR^+) cells/molecules, including complement, in the environment. Furthermore, transplanted hearts from $mfgl2^{-/-}$ mice were largely devoid of fibrin deposition resulting in prolonged graft survival, whereas grafts from wild-type mice transplanted into $mfgl2^{-/-}$ recipients were rejected in a similar tempo, and with similar histopathology, as $fgl2^{+/+}$ littermates. Collectively, these data provide evidence for a role for endothelial cell fgl2 expression in the fibrin deposition associated with allotransplant rejection and suggest that strategies designed to prevent endothelial cell fgl2 expression may prove of benefit to improving allotransplant graft survival. The data are further supported from patient studies in which increased fgl2 and fibrin expression were found mainly in renal tubule cells, infiltrating macrophages, $CD8^+$ T cells, and plasmacytes, as well as endothelial cells in rejecting renal grafts.

Recent studies from Hancock et al. (6) have also examined the relevance of mfgl2 to fibrin deposition in allorejection. Their studies, similar to ours, showed that $mfgl2^{-/-}$ recipient mice rejected

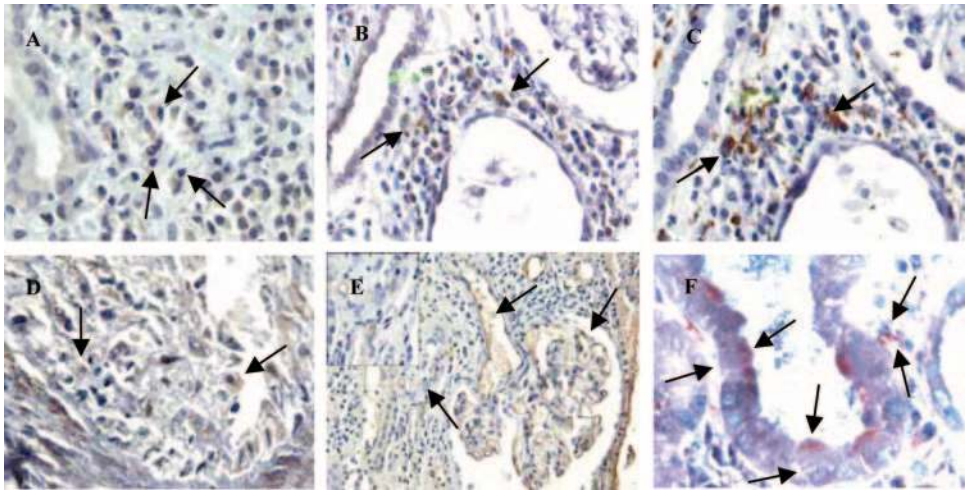

FIGURE 6. Cellular localization of hfgl2 and fibrin deposition in patients with acute renal allograft rejection. *A* and *B* were stained with fgl2 polyclonal Ab and show hfgl2 protein in infiltrating mononuclear cells (×1000). *C* and *D* are serial sections of *B*, respectively, showing dual staining of CD68 and CD8 cells demonstrating that the majority of macrophages and CD8-positive cells highly express hfgl2 (×1000). *E*, Fibrin deposition is shown by arrows within the microvascular vessels, glomerular capillary wall and matrix, and at the surface of the mononuclear cells by immunoperoxidase staining (×1000). *F*, Dual staining for hfgl2 and fibrin within engrafted renal issue: Fibrin (red) deposits can be seen within renal tubules (purple) and around the hfgl2-positive infiltrating cells (purple) in the kidney matrix (×1000).

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donor heart allografts in a fashion analogous to wild-type mice. Collectively, these studies support the hypothesis that (donor) endothelial cell production of mfgl2, rather than an immune-activated infiltrating leukocyte population, accounts for the fibrin deposition in allorejection (6). These data are further supported by our recent studies in xenotransplantation in which fibrin deposition associated with xenograft rejection was largely intravascular rather than associated with infiltrating inflammatory cells (21). Furthermore, xenografts from mfgl2$^{-/-}$ mice transplanted into rats were devoid of thrombosis. These observations collectively suggest that induction of fgl2 on the vascular endothelium accounts for fibrin deposition of allo- and xenotransplant rejection.

It is important to appreciate that fgl2 along with other members of the fibrinogen family of molecules have also been implicated in delivery of signals, which activate various arms of the innate immune system (22). Accordingly, some of the effects mediated by alteration of fgl2 expression might reflect not simply altered thrombin deposition, but also an altered immune activation. As but one example, Chan et al. (23) reported that fgl2 modified expression of costimulatory molecules on developing dendritic cells, with a profound "downstream" effect on cytokine induction following allostimulation. Current studies from our laboratory support the hypothesis that an additional immunomodulatory role for fgl2 does indeed follow binding to a receptor expressed in multiple tissues (R. Liu, manuscript in preparation).

Although not the focus of this paper, the molecular pathways for induction of fgl2 have recently been studied. In murine hepatitis virus infection, nucleocapsid protein induces transcription of fgl2 through the transcription factor hepatic nuclear factor 4α and its cognate receptor (24, 25). In transplantation, fgl2 transcription appears to be regulated by cytokines. Macrophage induction of fgl2 is induced by IFN-γ, whereas preliminary data suggest that fgl2 transcription in endothelial cells occurs in response to TNF-α but not IFN-γ (6). In contrast, induction of tissue factor is NF-κB dependent, and, unlike fgl2, IFN-γ inhibits tissue factor transcription (M. F. Liu, unpublished data).

The importance of fgl2 to allograft rejection is supported first by the observation that expression of fgl2 correlates with rejection (6) and also by the fact that administration of neutralizing Ab to fgl2 diminished the pathological injury and improved graft survival in a similar fashion to that previously reported in murine hepatitis infection (20).

In the development of late interstitial fibrosis seen in cases of chronic rejection, a great deal of attention has been paid to the early fibrin deposition in the matrix in the rejected grafts. Acute rejection is known to be one of the high risk factors for this phenomenon (26, 27). An important observation in this current study in both the experimental mouse model and in humans is the finding of high expression of fgl2 prothrombinase in infiltrating cells within the matrix or interstitial region of the kidney. Interestingly, the expression of fgl2 was in seen in close association with local fibrin deposition, consistent with a role for fgl2 in the pathogenesis of chronic allograft rejection, although further studies are necessary to define a role for fgl2 in chronic rejection.

In summary, these studies in both a murine model and in human renal allograft rejection provide evidence suggestive of a role for fgl2 in the thrombosis associated with acute allograft rejection. The data indicate that endothelial cells rather than leukocyte fgl2 expression accounts for intravascular fibrin deposition. Although not part of this study, data generated by our group and that of Hancock et al. suggest that one mechanism of altered fgl2 transcription is through elaboration of cytokines including IFN-γ and TNF, although additional studies are necessary to determine whether other cytokines are involved in the induction of fgl2. The elaboration of fgl2 during acute allograft rejection and the interstitial fibrin deposition may lead to further injury and development of chronic allograft rejection, but additional studies are required to firmly establish the role of fgl2 in this process, and in particular a potential immunomodulatory role for fgl2 in this process. These studies provide a rationale to target *fgl2* for therapeutic intervention in an attempt to ameliorate both acute and chronic allograft rejection.

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Disclosures

The authors have no financial conflict of interest.

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