Autoantibodies to hLAMP-2 in ANCA-Negative Pauci-Immune Focal Necrotizing GN

Andrea Peschel,* Neil Basu,† Alexandra Benharkou,* Ricarda Brandes,* Markus Brown,* Régis Dieckmann,* Andrew J. Rees,* and Renate Kain*

*Clinical Institute of Pathology, Medical University of Vienna, Austria; and †School of Medicine and Dentistry, University of Aberdeen, Aberdeen, United Kingdom

ABSTRACT
Pauci-immune focal necrotizing GN (piFNGN) is usually associated with ANCA that are thought to be pathogenic. However, 10%–15% of patients are ANCA negative and the cause of their injury is unknown. We previously reported a high frequency of autoantibodies to human lysosome-associated membrane protein-2 (hLAMP-2) in ANCA-associated piFNGN, and have now investigated whether the same is true in ANCA-negative patients. Of 11 patients, 8 (73%) had anti–hLAMP-2 antibodies detected by ELISA and confirmed by immunoblotting and indirect immunofluorescence. The autoantibodies from all 8 patients bound to native LAMP-2 purified from human glomeruli and recombinant hLAMP-2 expressed in ldlD cells, both with molecular masses of 110 kD. However, in contrast to anti–LAMP-2 antibodies from ANCA-positive patients, these antibodies from ANCA-negative patients failed to bind the more complexly glycosylated native neutrophil hLAMP-2 (190 kD). Treatment with the deglycosylating enzyme, endo-β-galactosidase, reduced the mass of neutrophil hLAMP-2 to 110 kD and enabled autoantibody binding. Similarly, pre-treating neutrophils with endo-β-galactosidase or neuraminidase converted ANCA assay results from negative to positive. Finally, IgG from LAMP-2-positive ANCA-negative patients bound specifically to normal human kidney sections and to human glomerular endothelial cells in culture. In conclusion, in patients with ANCA-negative piFNGN, we have identified autoantibodies to hLAMP-2 that bind native glomerular but not neutrophil hLAMP-2, suggesting a role in pathogenesis.

LAMP-2 is a heavily glycosylated membrane protein that traffics from the cell surface to lysosomes, where it is most abundant and is critical for cellular homeostasis and responses to stress. We originally discovered autoantibodies to human LAMP-2 (hLAMP-2) as part of a systematic search for autoantibodies to glomerular membrane proteins in piFNGN and have reported their high prevalence in piFNGN. We consistently find that >80% of patients presenting with piFNGN in European cohorts have circulating autoantibodies to hLAMP-2 that rapidly became undetectable after immunosuppressive treatment. Although another group reported a lower overall incidence, the frequency of anti-hLAMP-2 antibodies at presentation in their cohort was still highly significantly increased, with a frequency 10-fold higher than healthy controls.

RESULTS

There were a few individuals with ANCA-negative piFNGN in our previous cohorts who had autoantibodies to hLAMP-2 detected by ELISA and confirmed by Western blotting and indirect immunofluorescence assays. This was unexpected because LAMP-2 is expressed in neutrophils (Figure 1A) and patients’ autoantibodies almost invariably recognize peptide epitopes that remain accessible after glycosylation. Accordingly, anti–hLAMP-2 antibodies would be expected to have positive fluorescence ANCA assays even when antibodies to MPO and PR3 are absent. In an attempt to explain the apparent paradox, we identified all of the ANCA-negative patients with piFNGN treated by us and re-analyzed sera taken at the time they first presented with biopsy-proven active disease.

We identified 11 patients who had ANCA-negative results at presentation with piFNGN and without detectable antibodies to MPO or PR3 whose subsequent assays remained consistently negative (Figure 1B): two of these patients were included in our previously reported series. The clinical characteristics and results of ANCA, anti-MPO, anti-PR3, and anti-hLAMP-2 assays of these 11 ANCA-negative patients are shown in Table 1. One of these patients who had previously tested positive for ANCA and antibodies to MPO and PR3 had become negative for all tests after immunosuppressive treatment (Table 1).

Figure 1. LAMP-2 in human neutrophils and characteristics of ANCA-negative patients. (A) LAMP-2 is found in compartments that partially overlap with PR3 and MPO in human PMNs. (B) Clinical characteristics and results of ANCA, anti-MPO, anti-PR3, and anti-hLAMP-2 assays of 11 ANCA-negative patients with (n=8) or without (n=3) evidence of autoantibodies to hLAMP-2 with isolated renal disease (K) or systemic disease (L, lung; S, skin; J, joint). Sera from ANCA-negative patients react with hLAMP-2. (C) IgG in sera from LAMP-2–positive/ANCA-negative patients does not give positive ANCA reactivity using commercially available PMN cytospin preparations by indirect immunofluorescence. Original magnification, ×630 in A; ×400 in C. IIF, indirect immunofluorescence.
reported cohorts and nine were not (Table 1). All of the patients presented with deteriorating renal function and typical features of pIFNGN. Renal biopsies confirmed the diagnosis and presence of active injury with focal necrosis and crescents affecting at least 50% of glomeruli. The morphologic appearances were indistinguishable from ANCA-positive patients in our cohorts but injury was apparently confined to the kidney in 9 of 11 patients (82%), suggesting more limited disease. On retesting, all 11 patients had negative indirect immunofluorescence ANCA assays (Figure 1C) as well as ELISA results for antibodies to MPO and PR3. This confirms they were true ANCA-negative patients.

When tested for antibodies to hLAMP-2, 9 of 11 patients (82%) had positive results in our standard ELISA with bacterially expressed unglycosylated recombinant human LAMP-2 as a substrate, whereas two were negative (Figure 2A). The positive ELISA results were confirmed in eight of these by Western blots (Figure 2B) and indirect immunofluorescence on IdId cells stably transfected to express glycosylated human LAMP-2 targeted to the plasma membrane (IdId/hLAMP-2H; Figure 2C). Thus, positive results in three independent validated assays unequivocally established that 8 of 11 (73%) ANCA-negative patients had autoantibodies to hLAMP-2 with titers indistinguishable from those of ANCA-positive patients.18,19 Anti–LAMP-2 antibodies were not detected in the remaining three patients.

ELISA and Western blotting showed that the autoantibodies from ANCA-negative patients bind to the protein backbone of hLAMP-2, whereas indirect immunofluorescence confirmed that the epitopes recognized remained accessible in glycosylated recombinant hLAMP-2 expressed in IdID cells but were cryptic in native neutrophil hLAMP-2 (Figure 1C). hLAMP-2 is heavily glycosylated with 19 potential N-linked glycosylation sites and a cluster of O-linked glycosylation sites at the hinge region between the two homologous halves of the extracellular domain.23 Carbohydrate contributions between a half and three quarters of the molecular mass of hLAMP-2, depending on the complexity of glycosylation that varies with cell type and activation status. Neutrophil hLAMP-2 is particularly

| Table 1. Effect of removal of sialic acid residues and/or removal of polylactosamines on ANCA staining pattern |
|---|---|---|---|---|
| Patienta | Sex | Age (yr) | IIF | MPO/PR3 |
| | | | ANCA C| GEnC (U/ml) |
| ANCA-negative/hLAMP-2 | autoantibody positive | | | |
| A | F | 60 | − | + | − | + |
| B | F | 65 | − | NA | − | + |
| C | F | 33 | − | + | − | + |
| D (39) | M | 33 | − | + | − | + |
| E | F | 22 | − | − | − | + |
| F | F | 85 | − | − | − | + |
| G | F | 70 | − | NA | − | + |
| H (70) | F | 17 | − | + | − | + |
| ANCA-negative/hLAMP-2 | autoantibody negative | | | |
| I | M | 29 | − | NA | − | − |
| J | F | 73 | − | + | − | − |
| K | M | 59 | − | − | − | − |
| ANCA-positive/hLAMP-2 | autoantibody negative controls | | | |
| L | M | 78 | P | NA | MPO 22 | − |
| M (57) | F | 57 | C | NA | PR3 20 | − |
| N (51) | M | 58 | C | − | PR3 50.55 | − |
| O (9) | F | 76 | C | PR3 360 | − |
| P | M | 45 | C | − | PR3 39.8 | − |

All 11 ANCA-negative patients (patients A–K) had negative results in standard, commercially available indirect immunofluorescence assays; however, patient sera with antibodies to hLAMP-2 exhibited binding to GEnC by IIF using either frozen sections of human kidney or immortalized glomerular endothelial cells (n=8, A–H). Using sera from those eight patients for IIF, removal of either N-glycans (n=1, E) or both, removal of sialic acid and polylactosamines (n=7, A–D, F–H) from normal human granulocytes, enhanced fluorescence intensity and revealed either a membrane bound staining (n=2) and/or an (atypical) cANCA pattern (n=7). The remaining three patients did not exhibit hLAMP-2 antibodies by ELISA and treatment of granulocytes did not alter their ANCA negativity by IIF assays (patients I–K). The ANCA staining pattern of five patients with either cANCA/anti-PR3 antibodies (n=4) or pANCA/anti-MPO antibodies (n=1) was not altered by carbohydrate removal (patients L–P), nor did treatment with the enzymes change negative results from sera of healthy controls (n=3). IIF, indirect immunofluorescence; F, female; −, negative; +, positive; membrane +++, membrane accentuated staining; C, cytoplasmic ANCA; NA, not available; M, male; atypical, atypical staining pattern; no difference, no difference in staining pattern to untreated cells; P, perinuclear ANCA.

aNumbers in parentheses next to the patients’ assignment (A–P) refer to patients in Supplemental Table 3 published previously by Kain et al. (18).
heavily glycosylated and carries complex polylactosamine carbohydrate moieties, presumptively to protect it from degradation by neutrophil proteases.24 By contrast, native hLAMP-2 from glomeruli is less complexly glycosylated and has a molecular mass of 110 kD, which is identical to recombinant hLAMP-2 expressed in Chinese hamster ovary cells or the mutant ldlD cell line we use for indirect immunofluorescence assays.18,19

To test the influence of glycosylation on autoantibody binding, we purified LAMP-2 separately from membrane-enriched fractions of lysates of human glomeruli and neutrophils by two-stage affinity purification using a rabbit polyclonal anti–hLAMP-2 and a mouse mAb (H4B4), respectively, coupled to protein A Sepharose columns. The affinity-purified glomerular and neutrophil hLAMP-2 had the expected molecular masses of 110 and 190 kD, respectively, reflecting the complexity of their glycosylation.18,19 Autoantibodies from the eight LAMP-2 positive patients bound glomerular hLAMP-2 but failed to bind to 190-kD neutrophil hLAMP-2 (Figure 3A). However, enzymatic treatment with endo-β-galactosidase, which removes polylactosamines, reduced the molecular mass of the neutrophil hLAMP-2 to 110 kD, similar to that of glomerular hLAMP-2,23 and facilitated autoantibody binding. By contrast, anti–hLAMP-2 antibodies from ANCA-positive patients usually bind fully glycosylated native neutrophil hLAMP-2.21 Interestingly, the specific polyclonal anti–LAMP-2 antibody recognized a second weak band of 110 kD in some neutrophil LAMP-2 preparations (Figure 3, A and C). This probably represents incompletely glycosylated hLAMP-2 isolated from immature neutrophils as has previously been described.23 This band was present in LAMP-2 preparations used to test sera from two patients (H and F): IgG from their sera reacted with it but not with the dominant 190 kD species (Figure 3, A and C). IgG from the three ANCA-negative patients without detectable anti–LAMP-2 antibodies failed to bind any of the LAMP-2 preparations (Figure 2C, Supplemental Figures 1A and 2).

The Western blots suggest that the anti–LAMP-2 antibodies in ANCA-negative
patients fail to bind to fixed neutrophils because the epitopes they recognized are occluded by carbohydrate moieties. We tested this by pretreating the human neutrophils with deglycosylating enzymes before performing the ANCA assay. Pretreatment with neuraminidase to remove sialic acid residues enabled the patients’ autoantibodies to bind neutrophil LAMP-2 (Supplemental Figure 1B) and resulted in positive indirect immunofluorescence for ANCA in eight relevant ANCA-negative patients, whereas pretreatment with endo-β-galactosidase to remove polylactosamine side chains rescued binding in 7 patients (Figure 3B, Table 1). Again, the sera from the three ANCA-negative patients without anti-hLAMP-2 antibodies failed to bind neutrophils even after treatment with deglycosylating enzymes (Supplemental Figure 1C). Binding of antibodies to MPO and PR3 in neutrophils was unaffected by treatment with either deglycosylating enzyme (Supplemental Figure 1C) using sera from ANCA-positive patients without detectable autoantibodies to hLAMP-2 (Supplemental Figure 2, A and B). Thus, anti-LAMP-2 antibodies in ANCA-negative patients do not bind fully glycosylated LAMP-2 in mature human neutrophils.

The autoantibodies in sera from the ANCA-negative patients bound native human glomerular LAMP-2 in Western blots; thus, we used indirect immunofluorescence to test whether they also bind LAMP-2 in glomeruli of tissue sections of normal human kidney. Anti–LAMP-2–positive and anti–LAMP-2–negative sera were incubated with sections of unfixed frozen normal human tissue that had been preincubated with high salt (1.8% NaCl) citrate buffer to remove nonspecifically bound IgG and binding visualized with an affinity-purified sheep–anti-human IgG antibody. Sera were available from 8 of 11 ANCA-negative patients (6 positive for antibodies to LAMP-2 and 2 negative—there was insufficient serum to test patients B, G, and I). IgG from all six LAMP-2–positive patients tested bound specifically to glomeruli with a predominantly

Figure 3. Autoantibodies in ANCA-negative patients bind to native human glomerular but not fully glycosylated neutrophil LAMP-2. (A and C) Patients’ autoantibodies bind to affinity-purified native human glomerular hLAMP-2, whereas immunopurified, fully glycosylated 109 kD hLAMP-2 from neutrophils is not recognized. However, in some patients (F and H), reactivity with an incompletely glycosylated form of hLAMP-2 at 110 kD is observed (A and C, right). (B) Enzymatic deglycosylation of PMN preparations with neuraminidase and endo-β-galactosidase (endo-βGal) treatment before indirect immunofluorescence abolishes staining with CD15s (SialylLex) and Tomato lectin staining, respectively. IgG from patients’ sera containing anti–LAMP-2 antibodies binds only to neutrophils after enzymatic deglycosylation, whereas binding of a mAb to hLAMP-2 is not influenced by enzymatic treatment. The control serum from a healthy control remains negative in all conditions. Original magnification, ×400.
endothelial pattern (Figure 4A, Supplemental Figure 2C) similar to what we previously described for ANCA-positive patients with coexisting anti–LAMP-2 antibodies. We confirmed that autoantibodies in all six sera bound the glomerular endothelium using conditionally immortalized human glomerular endothelial cells (GEnCs), kindly provided by Dr. Simon Satchell and Professor Peter Mathieson (Bristol, UK) (Figure 4A). By contrast, IgG from the two ANCA-negative LAMP-2-negative patients did not bind to glomeruli, nor did IgG from two LAMP-2-negative/ANCA-positive patients used as controls (Supplemental Figure 2C).

Finally, we used inhibition with purified soluble human glomerular LAMP-2 to confirm the specificity of autoantibody binding to human glomeruli and GEnCs. First we defined the optimal concentration of soluble LAMP-2 in a dose response curve to inhibit IgG binding from the serum of one patient with high anti–LAMP-2 antibody concentration (patient A). Soluble glomerular hLAMP-2 inhibited binding in a dose-dependent fashion both of the patient’s autoantibodies and of the monoclonal anti–LAMP-2 antibody. The results were similar using either normal human kidney sections or ldlD cells stably expressing hLAMP-2 (Figure 4, B and C). Complete inhibition of binding was achieved with a hLAMP-2 concentration of 4.4 ng/μl. Accordingly, double this concentration was used for inhibition experiments with the remaining five sera samples and this abrogated IgG binding in every case (Supplemental Figure 2C).

DISCUSSION

The results, summarized in Table 1, clearly identify anti–hLAMP-2 antibodies in patients with biopsy-proven active piFNGN in a situation in which canonical ANCA cannot be detected and thus are highly unlikely to be the cause of
injury. This mirrors the situation in two
experimental models in rats: (1) WKY
rats immunized with the hLAMP-2 mo-
lecular mimic, FimH,18 who develop au-
toantibodies to rat LAMP-2 associated
with piFNGN without anti-MPO or
PR3 antibodies; and (2) env-pX rats
that spontaneously develop small vessel
vasculitis together with high titers of au-
toantibodies to LAMP-2 antibodies
again without antibodies specific for
MPO or PR3.26 Thus, clinically and in
rodent models, the presence of autoan-
tibodies to hLAMP-2 is correlated with
active vasculitis even in the absence of
antibodies to MPO and PR3. Notably,
the converse occurs in patients with AAV
in remission when antibodies to LAMP-2
are rare despite the continued presence
of autoantibodies to MPO or PR3,4,27,28
further strengthening the case that autoanti-
tibodies to LAMP-2 are pathogenic.

In conclusion, we have identified
injury-associated autoantibodies to
hLAMP-2 that bind glomerular but not
neutrophil LAMP-2 in a group of ANCA-
negative patients. This suggests that
autoantibody binding to hLAMP-2 in the
glomerulus is sufficient to cause injury in
these patients and that binding to neu-
traphils is dispensable. This has implica-
tions for understanding the pathogenesis
of AAV more generally. It also has im-
portant implications for the develop-
ment of anti–LAMP-2 assays. There are
currently no clinical-grade assays for
measuring hLAMP-2, necessitating our
use of three independent assays. We and
others are working intensively to develop
assays suitable for general use; however,
the results reported here caution against
the use of heavily glycosylated hLAMP-2
preparations as assay substrates,22 such as
those expressed in human embryonic
kidney 293 cells, because of the risk
that pathogenetically relevant epitopes
will be occluded and that autoantibodies
to them will go undetected.

CONCISE METHODS

Patients and Controls
Sera from 11 ANCA-negative patients (8
hLAMP-2 positive and 3 hLAMP-2 negative)
and 5 ANCA-positive (hLAMP-2 negative)
control sera were identified from two inde-
pendent series of patients presenting with
FNGN in the context of small vessel vasculitis
from Vienna and Aberdeen. The patients’
details are summarized in Figure 1A and
Supplemental Figure 1. Two of these pa-
tients were included in our previous study
and nine were not.18 Sera from three healthy
volunteers served as controls in all experi-
ments.

Permission to use patients’ sera for auto-
antibody testing was granted by the relevant
ethics committees of the Medical University
of Vienna and the University of Aberdeen.

Standard ANCA and Autoantibody
Assays
Serial dilutions of all sera were routinely
tested for ANCA (The Binding Site), as
previously described.18,19,21 One or more se-
rum samples from each patient was tested for
ANCA specificity using commercially avail-
able ELISA systems (PR3/MPO Combikit,
ORGenTeC, or VARELISA, Phadia).

Generation of Recombinant and
Purification Mutant hLAMP-2 for
Western Blotting and ELISA and
Indirect Immunofluorescence Assay
for Antibodies to hLAMP-2

Methods and assays to test for the presence
of autoantibodies to hLAMP-2 in patients’
sera using Escherichia coli expressed
hLAMP-2 extracellular domain as GST fu-
sion protein for ELISA and Western blot
and IdID cells stably expressing hLAMP-2
on the cell surface for indirect immuno-
fluorescence were previously described in
detail.18,19

Indirect Immunofluorescence on
Sections of Human Kidney,
Immortalized GEnCs, and
Polymorphonuclear Neutrophil
Granulocytes

Reactivity with structures of human kidney
was tested on 4-μm unfixed frozen sections
of a normal human kidney that had proved
unsuitable for transplantation. Kidney sec-
tions were treated with 100 mM sodium
citrate buffer (pH 6.6) with 1.8% NaCl to
remove nonspecifically bound IgG before
indirect immunofluorescence with patient
sera and antibodies specific for hLAMP-2.

Reactivity of patients’ IgG with GEnCs,
maintained in the presence of and without
the addition of TNF-α, and preparations of
normal human granulocytes was assayed by
indirect immunofluorescence using com-
mercially available cytopep preparations of
polymorphonuclear neutrophilic granulo-
cytes (PMNs) (Inova). To test the influence
of glycosylation, neutrophils were treated
with PNGaseF (P0704S; BioLabs), neur-
aminidase (sialidase, P07205; BioLabs), and
endo-β-galactosidase (Escherichia freundii,
100455; Seikagaku Corporation) in their ap-
propriate buffers and used for indirect im-
munofluorescence with hLAMP-2–positive
and hLAMP-2–negative sera from 11
ANCA/MPO/PR3-negative patients. As con-
trasts, we used hLAMP-2–negative sera from
five patients with ANCA-associated FNGN
(one with pANCA/MPO and four with
cANCA/PR3 antibodies) and sera from three
healthy volunteers. Pictures were taken in
each experiment at fixed exposure times dete-
rmined by a positive control antibody to
hLAMP-2 (H4B4) and ranged between 400–
600 milliseconds.

Purification of Native hLAMP-2,
Western Blotting, and Inhibition
Experiments

Native hLAMP-2 was purified from human
glomeruli and PMNs. In a two-stage pro-
cedure, membrane protein-enriched frac-
tions (TX 114 fraction)21 of glomerular and
PMN cell lysates were passed sequentially
through two protein A Sepharose columns,
respectively, containing immobilized poly-
clonal and monoclonal (H4B4) antibodies
specific for hLAMP-2. Bound hLAMP-2
protein was recovered by acidic elution
and separated by SDS-PAGE with subse-
quent transfer onto polyvinylidene fluo-
ride or nitrocellulose membranes and
used for Western blot analysis to determine
the capacity of patients’ autoantibodies to
bind to unglycosylated and glycosylated
forms of hLAMP-2. Increasing concentra-
tions of immunopurified glomerular
hLAMP-2 (0.044, 0.44, 4.4, and 8.4 ng/μl)
were used to inhibit binding of patients’
sera or H4B4, a mAb to hLAMP-2, to
hLAMP-2 in sections of normal human
kidney, immortalized GEnCs, or IdID cells
stably expressing hLAMP-2 on the cells
surface.
Antibodies and Reagents

The following primary antibodies were used: monoclonal mouse anti–HLAMP-2, clone H4B4 (Developmental Studies Hybridoma Bank, University of Iowa), and monoclonal mouse anti–HLAMP-2 (clone CD3) and rabbit anti–HLAMP-2 (932B) (kindly provided by Prof. Minoru Fukuda of Burnham Institute, La Jolla, CA). Secondary antibodies used in indirect immunofluorescence were Alexa Fluor 488–conjugated F(ab’)2 fragment of goat anti-mouse IgG (H+L) and goat anti-rabbit IgG (H+L) (Invitrogen) and FITC-conjugated sheep Ig to human IgG (INOVA Diagnostics).

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DISCLOSURES

None.

REFERENCES


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CORRECTION