P2Y1 Gene Deficiency Protects from Renal Disease Progression and Capillary Rarefaction during Passive Crescentic Glomerulonephritis

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The metabotropic receptor P2Y1 is necessary for full ADP-induced platelet activation and is localized on various intrinsic renal cell types, including mesangial cells, podocytes, and endothelial cells. To date, nothing is known about the role of the P2Y1 receptor during inflammatory renal disease. The role of the P2Y1 receptor was investigated using 22 P2Y1 gene–deficient (−/−) and 27 wild-type (wt) mice during the time course of passive crescentic nephrotic glomerulonephritis. Six P2Y1−/− and six wt mice served as undiseased controls. Renal tissues were harvested on days 1, 10, and 28 after disease induction. No renal phenotype was found in P2Y1−/− versus wt mice. In contrast, during crescentic glomerulonephritis, approximately 30% of all wt mice died, whereas all P2Y1−/− mice survived. Renal function as assessed by creatinine clearance measurements, glomerulosclerosis, and tubulointerstitial injury indices as well as glomerular and interstitial matrix expansion were improved significantly in P2Y1−/− compared with wt mice. These changes were preceded by reduced glomerular and peritubular capillary rarefaction indices in P2Y1−/− compared with wt mice. The alteration of the rates of both peritubular apoptosis and endothelial cell proliferation suggests improved capillary preservation in P2Y1−/− mice early in disease (day 10) and an additional enhanced repair reaction in P2Y1−/− mice at the late time point (day 28), whereas injury on day 1 seemed to be equivalent in both groups. It is concluded that loss of P2Y1 receptor function safeguards against capillary loss, fibrosis, and death by renal failure during experimental crescentic glomerulonephritis.


Received May 6, 2006. Accepted November 20, 2006.

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ISSN: 1046-6673/1802-0494
an ideal model to investigate our hypothesis that P2Y1 receptor plays an important role not only in thromboembolism but also in a complex inflammatory disease.

In our model of experimental passive nephrotoxic nephritis, heterologous serum against glomerular extracts is injected in mice, leading to similar changes as seen in human crescentic glomerulonephritis. This model is characterized by an early influx of inflammatory cells, including platelets, with subsequent platelet aggregation and activation, formation of fibrin thrombi, and injury of endothelial and other endogenous renal cells (19). At later time points, kidneys develop crescent formation, glomerulosclerosis, and tubulointerstitial fibrosis (19) with progressive loss of glomerular and peritubular capillaries.

In this study, we investigated the role of the P2Y1 receptor during the course of passive crescentic nephrotoxic nephritis as a complex inflammatory disease model in vivo. We show that survival of nephritic P2Y1 −/− mice is improved markedly and associated with reduced renal capillary loss, glomerulosclerosis, and fibrosis.

Materials and Methods

Animal Model and Experimental Design

Twenty-eight male and female P2Y1 −/− mice and 33 age- and gender-matched C57/b16 wild-type (wt) mice at the age of 26 wk were used in this study. All mice were fed standard mouse chow (Altromin 1324; Spezialfutterwerke, Lage, Germany) and tap water ad libitum.

In the initial experiment, passive nephrotoxic crescentic glomerulonephritis was induced using exactly the antibody described previously (provided by R.J. Johnson and S.J. Shankland [19]) and adjusted to body weight in 16 P2Y1 −/− and 16 wt mice; six P2Y1 −/− and six wt mice served as undiseased controls. Mice were killed on days 10 and 28. Because of the high mortality of nephritic wt mice after the early time point (day 10), five additional wt mice were exposed to nephritis and killed on day 28.

A second experiment was performed to investigate injury and the immune response to the disease-inducing antibody at a very early time point. Disease was induced in six age- and gender-matched P2Y1 −/− and six wt mice. A survival biopsy was performed 20 h after disease induction, and mice were killed on day 28. The day before the mice were killed, a 24-h urine collection was performed in all mice to assess proteinuria and creatinine clearance. On the day of killing, bleeding time was determined in 0.9% (wt/vol) NaCl solution at 37°C after capping of the terminal 5 mm of the mouse tail. Mice were anesthetized by a combination of ketamine (100 mg/kg) and xylazine (5 mg/kg), and blood was collected via puncture of the inferior caval vein. Mice were perfused via the heart with 0.9% (wt/vol) NaCl solution and Rheomacrodex that contained 0.05% novocaine, and the right kidney was harvested for immunohistochemical analysis. Afterward, the mice underwent perfusion fixation with 48 mM Na2HPO4 and 14 mM KH2PO4 that contained 3% paraformaldehyde and 14 mM KH2PO4 that contained 3% glutaraldehyde and the left kidney was harvested for morphometric evaluation (not for the second experiment). Serum and urine samples were stored at −70°C until analysis. All studies were approved by the local ethics committee.

Tissue Processing and Immunohistochemical Staining

Tissue for light microscopy was fixed in methyl Carnoy’s solution or 3% paraformaldehyde, embedded in paraffin, and cut into 3-μm sections for indirect immunoperoxidase staining as described elsewhere (20,21). For immunoperoxidase staining, tissue sections were incubated with the following primary and secondary antibodies as indicated: 19A2, a murine IgM mAb against the proliferating cell nuclear antigen (PCNA) (21), for detecting actively proliferating cells (Chemicon, Temecula, CA); MECA-32, a murine IgGl mAb specific for detecting endothelial cells (gift from R. Hallmann, Lund University, Lund, Sweden) (22); biotinylated lectin from Lycopersicon esculentum (tomato) for staining of glomerular capillaries (Sigma-Aldrich, Munich, Germany) (23); a polyclonal rabbit antibody against the P2Y1 receptor (24); a polyclonal rabbit antiserum against the P2Y1 receptor (25); a biotinylated polyclonal goat antibody to human/bovine collagen IV (Southern Biotechnology Associates, Birmingham, UK); CT-CD8a, a rat anti-mouse CD8α mAb (Caltag, Burlingame, CA); CT-CD4, a rat anti-mouse mAb against CD4 (Caltag), FA-11, a rat anti-mouse mAb against CD68 (Serotec, Duesseldorf, Germany); and a rat anti-mouse glycoprotein Ib antibody for specific staining of platelets (gift from B. Nieswandt, Virchow-Institute, Würzburg, Germany) (26). Negative controls for immunostaining included either deletion of the primary antibody or substitution of the primary antibody with equivalent concentrations of an irrelevant murine or rat mAb or preimmune rabbit IgG. All tissue sections were incubated with primary antibodies overnight at 4°C. Afterward, specific biotinylated secondary antibodies (all by Zymed, San Francisco, CA) were applied followed by peroxidase-conjugated avidin D (Vector Laboratory, Burlingame, CA) and color development with diaminobenzidine with nickel chloride for nuclear staining and otherwise without nickel.

Tissues for immunofluorescent staining were embedded in OCT (Lab-Tek Products, Naperville, IL) and snap-frozen in liquid nitrogen. Tissue sections were incubated overnight with a specific Alexa Fluor 555 donkey anti-sheep antibody (Invitrogen, Karlsruhe, Germany) for evaluation of the binding of the disease-inducing antibody and with an Alexa Fluor 555 donkey anti-mouse antibody (Invitrogen) for evaluation of the autologous immune response via murine IgG. Complement activation was evaluated via specific staining for C3 using a FITC-conjugated antibody (Cappel, ICN Biomedicals, Eschwege, Germany). For the evaluation of the cellular immune response, tissue sections were incubated with specific antibodies for CD4, CD8α, and CD68 overnight followed by an Alexa Fluor 488 donkey anti-rat antibody.

Quantitative Analysis of Immunostaining and Capillary Rarefaction

Glomerulosclerosis was evaluated individually in at least 50 randomly selected glomeruli under ×400 magnification on periodic acid-Schiff (PAS)-stained sections using a semiquantitative scoring system from 0 to 4 (0, normal glomeruli; 1, mesangial thickening of <25%; 2, mesangial proliferation and thickening up to 50%; 3, obliteration of capillaries and diffuse sclerosis up to 75%; and 4, complete capillary obliteration and thrombosis with global sclerosis up to 100%). Values are given as score ± SD. Fibrin deposits were identified on tissue sections that were stained with acid fuchsin orange G, in which fibrin deposits demonstrate an intense orange-red color. Glomerular fibrin deposition was evaluated individually in at least 50 randomly selected glomeruli under ×400 magnification using a semiquantitative scoring system (0, no fibrin deposits; 1, up to 25%; 2, >25% and up to 50%; 3, >50% and up to 75%; and 4, >75% of the gcs contains fibrin deposits.)

Cortical expression of collagen IV and glomerular expression/deno-
sition of collagen IV, fibronectin, IgG, sheep-IgG, and C3 were quantified using computer-assisted image analysis software (MetaVue; Visithron Systems, Puchheim, Germany). At least 20 cortical fields of vision and at least 50 glomeruli per section were analyzed using ×400 magnification. Cortical expression of collagen IV is given as mean ± SD per mm², and fibronectin, IgG, sheep-IgG, and C3 positivity is given as percentage positive area per gcs. IgG is given as an integrated value including the covered area as well as the intensity of the staining. Peritubular capillary loss was examined through a 10 × 10 eyepiece grid using a ×40 objective. The grid covers an area of 0.0625 mm² at this magnification. Each section that contained no MECA-32–positive peritubular capillary was counted. This scoring system thus inversely reflects peritubular capillary rarefaction, whereby low values represent intact capillaries (maximum is 100). Glomerular capillary rarefaction was determined after staining for lectin from Lycopersicon esculentum (tomato). A semiquantitative scoring system from 0 to 3 was used (0, 0 to 25%; 1, 25 to 50%; 2, 50 to 75%; and 3, 75 to 100% loss of glomerular capillaries) (27). For each biopsy, 50 gcs were evaluated in a blinded manner. PCNA-positive as well as CD68⁺ cells were counted in 50 consecutive glomeruli and in 20 cortical fields of vision for assessment of the tubulointerstitium. CD4⁺ and CD8⁺ cells were counted in 20 cortical fields of vision for assessment of the tubulointerstitium and expressed as mean ± SD per mm². Glomerular platelet infiltration was assessed after staining for glycoprotein lb using a semiquantitative scoring system from 0 to 4 (0, no; 1, 1 to 25%; 2, 26 to 50%; 3, 51 to 75%; and 4, 76 to 100% platelet-positive area per glomerulus). For each biopsy, at least 50 gcs were evaluated in a blinded manner.

**Immunohistochemical Double Staining**

For determination of the number of proliferating endothelial cells, double immunostaining for PCNA, a marker of cell proliferation, and MECA-32, an endothelial cell–specific marker, was performed as described previously (29). The controls for this double-staining procedure consist of either omitting or replacing the secondary antibody and of omitting or replacing the primary antibody with an irrelevant mouse mAb. The number of proliferating endothelial cells was evaluated by counting the number of cells that stained for both PCNA (black) and MECA-32 (brown) as PCNA⁺/MECA-32⁺ cells, respectively, and was expressed as a mean ± SD per gcs or per mm² counting 20 areas of each biopsy.

**Terminal Deoxynucleotidyl Transferase—Mediated dUTP-Biotin Nick-End Labeling Assay**

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) assay as described previously (30). The number of TUNEL-positive apoptotic cells was counted in 50 sequentially selected glomeruli and given as the mean number either per gcs or per mm² after counting 20 peritubular areas of each section.

**Morphometric Analysis**

For morphometric analysis, semithin sections (0.5 μm) of the left kidneys were investigated for glomerular geometry and preservation of glomerular capillaries and endogenous glomerular cell types by morphometric and stereologic techniques as described in detail elsewhere (31–33).

**Electron Microscopy**

In several randomly selected mice per group, ultrathin sections of the renal cortex were investigated qualitatively using a Zeiss EM 902 (Zeiss Co., Oberkochen, Germany) at various magnifications.

**Cell Culture**

Mouse mesangial cells were cultured from isolated glomeruli using a standard sieving procedure and characterized by positive staining for α-smooth muscle actin and α8-integrin and the lack of the epithelial marker WT-1, endothelial marker MECA-32, and the macrophage/monocyte marker F4/80. Mesangial cells were grown in DMEM that contained 10% FCS, insulin (5 μg/ml), and penicillin/streptomycin at 37°C and 5% CO₂.

Cultured mouse glomerular endothelial cells (gLEND) provided by H. Schöckelmann, University of Kiel, Kiel, Germany) were immortalized by transfection with polyclona middle-T antigen as described previously (34). Cells were characterized by immunocytochemistry via positive staining for the typical endothelial cell markers MECA-32 and CD31 and the lack of staining for MC makers such as α-smooth muscle actin and α8-integrin, as well as epithelial cell markers such as WT-1 and cytokeratin, and maintained in DMEM that contained 10% FCS, 1 mM pyruvate, penicillin/streptomycin, nonessential amino acids, and 5 μM mercaptoethanol at 37°C and 5% CO₂ (35).

Mouse podocytes (provided by KH Endlich, University of Greifswald, Greifswald, Germany) that were isolated from kidneys of ImmortoMouse mice (Charles River, St. Louis, MO) that carry a temperature-sensitive mutant of the immortalizing SV40 large T antigen under control of the IFN-γ–inducible H-2Kb promoter were grown in RPMI 1640 that contained 10% FCS, penicillin/streptomycin, and γ-IFN at 33°C (36).

**Reverse Transcriptase–PCR of P2Y1 Receptor**

Ten-micrometer-thick frozen sections from renal tissues were microdissected using a Palm Microlaser-System (P.A.L.M. Microlaser Tech., Bernried, Germany) after staining with crystal purple. At least 50 glomeruli and an area of 100,000 μm² of tubulointerstitial tissue were captured in RTL-buffer (Qiagen, Hilden, Germany). Cultured mouse mesangial cells, gLEND, and undifferentiated podocytes as well as cortical renal tissue, microdissected glomeruli, and microdissected tubulointerstitium were lysed in RTL buffer, and RNA was purified using RNeasy micro-columns (Qiagen) with subsequent DNase digestion following the manufacturer’s instructions. Reverse transcription was performed using reverse transcription reagents from ABgene (Hamburg, Germany) following the manufacturer’s instructions.

The primers used were as follows: mP2Y1 forward TGG CGT GGT TGG CGT GTA CCC TCT CAA GTG and reverse ACC GTG CTC GCA AAT TCA TCG TT. The expected size of the product was 410 bp. PCR was performed according to a previously described protocol in which used primers also have been described (37).

**Detection of Anti-Sheep IgG**

Anti-sheep IgG antibodies in sera from mice that were collected 28 d after disease induction were detected by ELISA. A sheep-IgG fraction (90 μg/ml) was coated overnight at 4°C on a 96-well microtiter plate (Maxisorp, Nunc, Denmark) using 50 mM sodium carbonate buffer at pH 8.5. After blocking with 5% skim milk and 1% BSA in PBS for 2 h, sera (diluted 1:100 in PBS) were incubated for an additional 2 h. This and all subsequent incubation steps were conducted at room temperature. Serum from a mouse that was diseased with nephrotic serum nephritis was diluted in a range between 1:12.5 and 1:800 and used as standard. A dilution factor of 1:100 was regarded as 1. After a washing step with PBS that contained 0.01% Tween 20, the plate was incubated for 30 min with peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech Europe, Freiburg, Germany) diluted 1:2000 in PBS that contained 0.01% Tween 20. Nonbound secondary antibody was removed by washing before substrate solution (0.4 mg o-phenylene...
diamine with 0.03% H₂O₂ in 50 nM sodium phosphate at pH 5.5) was added. Substrate reaction was stopped with 2 N H₂SO₄ and analyzed at 492 nm using an ELISA reader (Sunrise; Tecan, Crailsheim, Germany).

Miscellaneous Measurements
Urinary albumin was measured using a mouse-specific sandwich ELISA system (Bethyl Lab, Montgomery, TX). Serum creatinine, urea, and urinary creatinine were measured using an autoanalyzer (Beckman Instruments, Brea, CA).

Statistical Analyses
All values are expressed as means ± SD. Statistical analysis was performed using SPSS 13.0 (SPSS, Munich, Germany). The Kolmogorov-Smirnov test was applied to test for normal distribution followed by a Mann-Whitney U test. Statistical significance was defined at \( P < 0.05 \).

Results
P2Y1 −/− Mice Have No Renal Phenotype
To exclude a relevant renal phenotype and differences in renal function or underlying autologous inflammation in P2Y1 −/− mice, we investigated albuminuria (0.8 ± 0.3 versus 1.2 ± 0.5 μg urinary albumin/μg urinary creatinine) and creatinine clearance (2.1 ± 1.1 versus 1.4 ± 0.01 ml/min; NS) as well as the autologous deposition of IgG (1.2 ± 0.2 versus 1 ± 0.3% positive area per gcs; NS) and C3 (0.1 ± 0.07 versus 0.17 ± 0.17% positive area per gcs; NS) and the presence of platelets (1.62 ± 1.04 versus 2.31 ± 2.19 per gcs; NS) in kidneys of wt and P2Y1 −/− mice. Kidneys of P2Y1 −/− mice demonstrated no difference regarding all of these parameters compared with wt mice. For further investigation of the relevance of P2Y1 receptor function, passive nephrotoxic glomerulonephritis was induced in P2Y1 −/− and wt mice.

Survival and Renal Function Are Improved Significantly in Nephritic P2Y1 −/− Mice
Unexpected, P2Y1 −/− mice demonstrated a markedly improved survival compared with wt controls during the course of nephrotoxic nephritis (Figure 1A). Whereas all P2Y1 −/− mice survived until day 28 after disease induction, approximately 50% of all wt mice died between day 10 and the day of killing. Whereas albuminuria as a parameter of urinary creatinine was not different (on day 10 75 ± 86 versus 41 ± 68 and on day 28 369 ± 169 versus 123 ± 81 μg albumin/μg creatinine versus wt mice), renal function as indicated by creatinine clearance was improved in nephritic P2Y1 −/− compared with surviving wt mice on day 28 (Figure 1B). It seems that the P2Y1 receptor influences mechanisms that are relevant for injury and/or repair during this model of nephrotoxic nephritis.

Nephritic P2Y1 −/− Mice Show Reduced Glomerular and Tubulointerstitial Injury
A hallmark of nephrotoxic nephritis is the development of glomerular crescents as well as the development of progressive glomerular sclerosis and tubulointerstitial fibrosis. Although there was already a trend toward a decrease of all of these parameters of progressive injury on day 10 in P2Y1 −/− mice, crescent formation (Figure 2A), glomerulosclerosis (Figure 2B), and tubulointerstitial fibrosis (Figure 2C) were reduced significantly in P2Y1 −/− compared with wt mice on day 28. Representative examples of the day 28 PAS stainings are shown in Figure 2D for nephritic wt mice and in Figure 2E for P2Y1 −/− mice. Assessment of glomerular fibrin deposition demonstrated reduced fibrin deposition in P2Y1 −/− mice compared with wt mice on day 10 (Figure 2F).

Glomerular and Tubulointerstitial Matrix Expansion Is Reduced in Nephritic P2Y1 −/− Mice
Because progressive renal disease is associated with glomerular and tubulointerstitial matrix expansion, we also assessed glomerular fibronectin and collagen IV as well as tubulointerstitial collagen IV expression by computerized morphometry. Glomerular staining for fibronectin was reduced significantly on day 28 in P2Y1 −/− compared with wt mice (Figure 3, A through C). Glomerular as well as tubulointerstitial collagen IV expression in nephritic P2Y1 −/− mice was decreased markedly on day 10 and on day 28 (Figure 3, D through G).

P2Y1 −/− Mice Demonstrate Reduced Glomerular and Peritubular Capillary Loss during Nephritis
Because renal sclerosis/fibrosis is thought to be linked and potentially caused by capillary loss (14), we sought to investigate peritubular and glomerular capillary rarefaction during nephritis (Figure 4). Glomerular capillary rarefaction as assessed by a semiquantitative scoring system after staining for Lycopersicon esculentum lectin was attenuated markedly in P2Y1 −/− compared with wt mice at any time point (Figure 4A). Peritubular capillary rarefaction as assessed after staining for MECA-32 also was reduced markedly in nephritic P2Y1 −/− mice on day 28 (Figure 4B). On day 10, only a trend toward improvement of peritubular capillary rarefaction was seen in nephritic P2Y1 −/− mice (Figure 4B). Examples of the MECA-32 staining in mice with prominent capillary loss (score 70; Figure 4C) and capillary protection (score 48; Figure 4D) are
demonstrated for the better understanding of the evaluation system. In parallel, by morphometry, the area of the glomerular capillary convolute in P2Y1\(-/-\) mice was reduced on day 10 (3.66 ± 0.53 versus 4.48 ± 0.53 × 10^{-3} mm²; \(P < 0.01\)) but increased significantly on day 28 (5.21 ± 0.28 versus 4.08 ± 0.63 × 10^{-3} mm²; \(P < 0.01\)) compared with wt mice.

In accordance with these findings, electron microscopy of peritubular capillaries on day 28 depicts that P2Y1\(-/-\) mice demonstrate less swelling of endothelial cells (Figure 5, A and B) and a reduced thickness of the endothelial barrier compared

**Figure 2.** Markers of renal disease progression are improved markedly in nephritic P2Y1\(-/-\) mice. Glomerular crescent formation (A), glomerular sclerosis (B), and tubulointerstitial fibrosis (C) were assessed in periodic acid-Schiff (PAS)-stained sections. The percentage of crescents as defined by more than two extracapillary cell layers was counted in 50 randomly selected glomeruli. For glomerular sclerosis, a semiquantitative score from 0 to 4 and for tubulointerstitial fibrosis a score from 0 to 3 was used as described in Materials and Methods. Mean values per glomerular cross-section and per mm² cortex are depicted. (D and E) Representative PAS-stained sections in nephritic wt (D) and P2Y1\(-/-\) mice (E) on day 28. Fibrin deposition was evaluated after acid fuchsin orange G staining using a semiquantitative score from 0 to 4. Data are means ± SD are shown (F). *\(P < 0.05\).

**Figure 3.** P2Y1\(-/-\) mice show a reduction of glomerular and tubulointerstitial matrix accumulation during crescentic nephritis. Glomerular matrix expansion as indicated by fibronectin (A through C) or collagen IV (D through G) immunostaining was assessed using computer-assisted image analysis. In either case, a reduction of matrix accumulation could be demonstrated in nephritic P2Y1\(-/-\) mice. Representative images of the fibronectin staining (brown color) in wt (B) and P2Y1\(-/-\) mice (C) on day 28 are depicted. Glomerular (D) and tubulointerstitial matrix expansion (E) were quantified by collagen IV staining using computer-assisted image analysis of cortical areas and demonstrated differences between P2Y1\(-/-\) and wt mice. F (wt mice) and G (P2Y1\(-/-\) mice) show representative images of the collagen IV staining on day 10. *\(P < 0.05\).
with wt mice (Figure 5, C and D). For investigation of potential mechanisms of the remarkable capillary protection in P2Y1−/− mice, the balance of cellular proliferation and apoptosis was observed during nephritis.

**Figure 5.** Electron microscopy of peritubular capillaries from P2Y1−/− and wt mice on day 28. Magnifications: ×3000 in A and C; ×4400 in B and D.

**Figure 4.** Glomerular and peritubular capillary rarefaction is prevented markedly in nephritic P2Y1−/− mice. Glomerular capillary rarefaction score was assessed in 50 randomly selected glomeruli after staining for tomato lectin using a semiquantitative score from 0 to 3 (A). Peritubular capillary rarefaction (B) was evaluated after staining for MECA-32 using a 10 × 10 grid as overlay covering an area of 0.0625 mm² at ×400 magnification. All fields without a positively stained capillary were counted and given as mean per grid (100), reflecting the lack of peritubular capillaries. Images of MECA-32 staining covered by a grid in day 28 nephritic wt (C) and P2Y1−/− mice (D) are shown to illustrate the evaluation of capillary loss. *P < 0.05.

**Proliferative Response Was Altered in Nephritic P2Y1−/− Mice**

Because the balance of cellular proliferation and apoptosis in response to renal injury plays a widely known role for the repair/progression process, we also investigated the apoptotic and the proliferative response during the course of disease. Glomerular cell proliferation as assessed by PCNA staining was reduced significantly in P2Y1−/− mice on day 10 but demonstrated no differences thereafter (Figure 6A). In parallel, interstitial cell proliferation was decreased on day 10 in P2Y1−/− mice (Figure 6B), whereas no differences occurred on day 28. Neither the rates of apoptotic glomerular nor interstitial cells as assessed by TUNEL staining were significantly different in P2Y1−/− compared with wt mice (Figure 6, C and D).

As a potential mechanism for the prevention of peritubular capillary rarefaction in nephritic P2Y1−/− mice, we examined the proliferative response of peritubular endothelial cells by double staining for MECA-32, an endothelial cell marker, and PCNA, a marker of cellular proliferation. Peritubular endothelial cell proliferation in P2Y1−/− mice was decreased on day 10 but increased on day 28 compared with nephritic wt mice (Figure 6E).

Morphometric analysis further strengthened these findings, because on day 28, the total number of glEND in P2Y1−/− mice was enhanced significantly (113 ± 23 versus 74 ± 15; P <
0.01) compared with wt mice, resulting in an increased total number of glomerular cells in P2Y1−/−/H11002 mice (261 ± 38 versus 202 ± 29; P < 0.01). By contrast, the number of mesangial cells (142 ± 17 versus 123 ± 35; NS) as well as podocytes (104 ± 16 versus 140 ± 75; NS) was unchanged. No difference appeared on day 10.

In general, it has to be considered that all changes in the nephritic P2Y1−/−/H11002 group are compared with a group of nephritic wt mice, in which the high mortality of 50% caused a positive selection process. Therefore, all beneficial changes that were seen in the P2Y1−/−/H11002 mice very likely would have been even greater, if not just the surviving mice would have been considered.

Figure 6. The balance of cellular proliferation and death is modulated in nephritic P2Y1−/−/H11002 compared with wt mice. Proliferating glomerular (A) and peritubular (B) cells were counted using proliferating cell nuclear antigen (PCNA) as a marker for proliferating cells. Apoptotic glomerular (C) and peritubular cells (D) were counted after performance of the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay. Quantification of the proliferative activity of peritubular renal endothelial cells was done after double immunostaining for PCNA (proliferation marker) and MECA-32 (endothelial cell marker; E). (F) Representative picture of this double-staining technique, where PCNA-positive nuclei are shown in black and MECA-32-positive endothelial cells are shown in brown. PCNA-/MECA-32-positive proliferating peritubular endothelial cells are marked by arrows. (*P < 0.05)

High Mortality in wt Mice Is a Consequence of Severe Renal Failure

Almost all wt mice that had died during our study were found in their cages the morning after their death, and neither functional nor histologic examination was possible. However, one nephritic wt mouse was found clinically in very severe condition and consequently killed immediately on day 14. Analysis of blood samples confirmed the uremic status of the mouse by serum creatinine of 0.41 mg/dl (normal value <0.1) and serum urea of 719 mg/dl. Histologic indicators of glomerular and tubulointerstitial injury demonstrated that glomerulosclerosis (3.03) and tubulointerstitial fibrosis (1.2) scores were far above the mean values of wt mice on day 10 (glomerulo-
sclerosis 1.7 ± 0.5; tubulointerstitial fibrosis 0.42 ± 0.31). Fourteen percent of glomeruli showed crescents. In parallel, this mouse demonstrated enhanced glomerular (0.53) and peritubular (17.5) capillary rarefaction scores compared with wt mice on day 10 (glomerular 0.37 ± 0.36; peritubular 12.4 ± 4.9). These changes were paralleled by glomerular (14.1, by fibronectin staining) and tubulointerstitial (13.4, by collagen IV staining) matrix expansion (values of wt mice on day 10: 6.6 ± 4.3 for fibronectin; 10.3 ± 1.5 for collagen IV). This example strongly suggests that the death of nephritic wt mice during this study occurred as a consequence of severe renal failure/uremia as reflected by histologic and functional alterations. It also suggests that the differences that are found between P2Y1−/− and wt groups are underestimated as a result of positive selection in the wt group on day 28 (mortality rate >50%).

Evaluation of the Immune Response after Disease Induction

Because we wanted to differentiate further between effects of the P2Y1 receptor on injury and repair during this disease model, we evaluated the immune response after injury. To detect differences related to the P2Y1 receptor early after disease induction, we performed a separate experiment and investigated mice 20 h after disease induction and on day 28. During this experiment, one wt mouse died on day 20.

Glomerular binding of the disease-inducing sheep anti-rabbit antibody was equivalent in P2Y1−/− and wt mice (Table 1). Whereas 20 h after disease induction complement C3 still was negative in all kidney sections, it was increased on day 28 but equal in both groups (Table 1).

Immunofluorescence staining for mouse IgG demonstrated no differences on days 10 (3.1 ± 1.1 versus 2.6 ± 0.9; NS) and 28 (2.6 ± 0.7 versus 2.7 ± 1; NS) in P2Y1−/− versus wt mice. In addition, we measured serum titers of mouse anti-sheep IgG on day 28 by ELISA, which were equivalent in P2Y1−/− and wt mice (Table 1).

To exclude differences regarding the cellular immune response, we assessed the number of glomerular/tubulointerstitial CD4+, CD8+, and CD68+ cells as well as the platelet infiltration. Whereas no differences occurred regarding CD4+ cells, P2Y1−/− mice demonstrated an increase of CD68+ monocytes/macrophages in the tubulointerstitium 20 h after disease induction (Table 1). This difference did not occur in the glomerular compartment. The number of CD8a+ cells was decreased in P2Y1−/− compared with wt mice on day 28 (Table 1). Glomerular platelet infiltration as well as bleeding times (105 ± 50 versus 95 ± 8 s; NS) were unchanged in P2Y1−/− versus wt mice (Table 1). Histologic evaluation of PAS-stained tissue sections demonstrated no differences 20 h after disease induction.

Localization of the P2Y1 Receptor on Intrinsic Renal Cells and Renal Tissues

Two different polyclonal antibodies against the P2Y1 receptor were evaluated extensively for immunostaining using frozen or paraffin-embedded tissue sections with multiple demasking procedures. Because no reproducible staining results could be obtained, we used reverse transcriptase–PCR to localize P2Y1 receptor RNA in renal tissues and cultured renal cells. Thereby, we detected P2Y1 receptor RNA in extracts of cortical tissue (kidney), laser-microdissected glomeruli (glomeruli), and tubulointerstitium from three different mice as well as in cultured (undifferentiated) podocytes, mesangial cells, and glEND (Figure 7).

Discussion

In this study, we investigated the role of the ADP receptor P2Y1 using P2Y1−/− mice during the course of passive nephrotoxic glomerulonephritis. The P2Y1 receptor has been shown to be present on various renal cells (4,5). Because in healthy P2Y1−/− mice no specific renal phenotype was apparent, we examined the contribution of this nucleotide receptor to renal inflammatory disease by using a crescentic nephritis model with an early influx of inflammatory cells such as monocytes/macrophages, lymphocytes, and platelets with subsequent

Table 1. Evaluation of cellular and autologous immune response in P2Y1−/− and wt micea

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<th>Disease Induction and Inflammatory Response</th>
<th>20 H</th>
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<td>wt P2Y1−/−</td>
<td>25.5 ± 0.8</td>
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<tr>
<td>0.1 ± 0.04</td>
<td>0.11 ± 0.03</td>
<td>19.3 ± 4.2</td>
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<tr>
<td>0.95 ± 0.3</td>
<td>1.1 ± 0.7</td>
<td>7.8 ± 2.3</td>
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<tr>
<td>0.93 ± 0.1</td>
<td>0.99 ± 0.7</td>
<td>3.2 ± 1.4</td>
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<tr>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.01</td>
<td>0.01 ± 0.03</td>
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<tr>
<td>0.9 ± 0.4</td>
<td>1.4 ± 0.7</td>
<td>1.2 ± 0.6</td>
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<td>18.4 ± 4.4</td>
<td>26.9 ± 6.4b</td>
<td>22.5 ± 4.8</td>
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<tr>
<td>ND</td>
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<td>17.5 ± 3.7</td>
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<tr>
<td>ND</td>
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<td>0.93 ± 1.4</td>
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<td>0.99 ± 0.7</td>
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aSheep-IgG and C3 deposition are expressed as percentage per glomerular cross-section (gcs) as assessed after specific staining using computer-assisted image analysis. Platelets were counted after glycoprotein Ib staining in glomeruli. CD4+ and CD8a+ cells were counted in at least 20 fields of vision. CD68+ cells were counted in glomeruli and the tubulointerstitium. All data are given either per gcs or per mm² tissue section. Serum titers of anti-sheep IgG were measured by ELISA as described in Materials and Methods. ND, not determined; P2Y1−/−, P2Y1 gene–deficient; wt, wild-type.

bp < 0.05.
platelet aggregation and activation (19). P2Y1 receptor directly or via platelet activation may contribute to injury of endothelial and other endogenous renal cells, which is followed by crescent formation and glomerulosclerosis as well as tubulointerstitial fibrosis and accompanied with the progressive loss of glomerular and peritubular capillaries (19).

Although we aimed toward a very severe disease model, it was an unexpected finding that approximately 50% of all wt mice died during the late time course (after day 10) of nephrotoxic nephritis, whereas no nephritic P2Y1−/− mice died during the late time course (after day 10) still was an unexpected finding that approximately 50% of all nephritic wt mice died during the late time course (after day 10) of nephrotoxic nephritis. Because we were not able to establish a reliable and reproducible staining for the P2Y1 receptor in our renal tissues, we used PCR and thereby detected P2Y1 receptor RNA that was present in glomeruli and tubulointerstitial microdissected tissues as well as in cultured glEND, mesangial cells, and podocytes.

Previous studies have demonstrated clearly the importance of capillary preservation and repair (14,17,18,41) for the healing of renal disease in many different experimental models. Because we first hypothesized that the P2Y1 receptor might be involved in platelet-endothelial interaction and capillary injury and repair also has been linked closely with renal disease progression, we examined the degree of renal capillary injury and repair in wt and P2Y1−/− nephritic mice. At all time points, P2Y1−/− mice were protected markedly from glomerular and peritubular capillary rarefaction that accompanies progressive renal scarring in this disease model of nephrotoxic nephritis as assessed by immunostaining and morphometric analysis as well as electron microscopy. Because capillary preservation and repair are regulated mainly by the balance of endothelial cell apoptosis and proliferation, we evaluated further whether this balance was altered in P2Y1−/− compared with wt mice. No significant difference in glomerular or peritubular apoptotic activity between both groups could be determined, although a trend toward a decreased apoptotic activity especially regarding the peritubular compartment suggests a reduced injury reaction in P2Y1−/− mice. In contrast, cellular proliferation within glomeruli and interstitium was modulated in P2Y1−/− mice. In the early phase of disease (day 10), the proliferative activity of glomerular and peritubular endothelial cells was reduced significantly, being consistent also with a reduced injury reaction and an increased endothelial cell preservation in P2Y1−/− mice, in which less compensatory proliferation is required compared with wt mice. During the late phase of disease (day 28), the number of proliferating peritubular endothelial cells then was increased even compared with wt mice, indicating improved endothelial repair during this phase of progressive capillary rarefaction. In addition to the P2Y1-dependent regulation of renal endothelial cell proliferation and apoptosis, mechanisms such as vasculogenesis by systemic precursor cells or endothelial cell migration could be P2Y1 dependent and cannot be excluded by our methods (4,42).

Although nothing is known about the role of the P2Y1 receptor in renal disease from in vitro studies, few in vitro data exist regarding its potential function that might apply to the findings that were seen in our renal model. The widely known expression of the P2Y1 receptor on endothelial cells and other renal cells suggests that either direct or indirect modulation of the

**Figure 7.** Detection of P2Y1 receptor RNA in kidney tissue and cultured renal cells. PCR was performed as described in the Materials and Methods section in cultured murine glomerular endothelial cells (glEND), mesangial cells (MC), (undifferentiated) podocytes (Podo), kidney cortex (kidney), laser-microdissected glomeruli, and tubulointerstitium (TI) from three different mice.
endothelial cell phenotype occurs. Although no clear-cut studies are available regarding P2Y1-mediated proliferation and apoptosis in endothelial cells in vitro, studies in glioma C6 (43) and astrocytoma (44) cells suggest P2Y1 receptor–mediated effects that are consistent with our findings in vivo. Nucleotide-mediated cell proliferation in the glioma cell line C6 (43) could be enhanced via P2Y1 blockade, whereas apoptosis of an astrocytoma cell line was stimulated after de novo introduction of the P2Y1 receptor (44). A recently published work also demonstrated a possible link between the activation of AMP-activated protein kinases via P2Y1 receptors (45) and hypoxic conditions that might influence the regulation of hypoxia-induced factor-1 and thereby the induction of genes such as vascular endothelial growth factor (46). In addition, there is evidence for P2Y1 receptor–mediated NO release in endothelial cells (4) and consecutive activation of the NO/cGMP pathway (47). Considering that NO may modulate capillary repair during wound healing differently depending on the environmental context (48), further experimental studies are needed to determine whether any of these findings apply to the situation in renal disease.

Several recent studies also demonstrated a close link between capillary loss and renal matrix expansion/progressive renal disease. Whether any of these findings apply to the situation in renal (48), further experimental studies are needed to determine whether any of these findings apply to the situation in renal disease.

Conclusion
P2Y1 receptor deficiency leads to enhanced survival and preservation of renal kidney histology and function during crescentic nephrotic glomerulonephritis. This marked protection is accompanied by improved renal fibrosis as well as capillary preservation early on and an additional enhanced capillary repair during the later course of disease. The exact mechanisms of our findings have to be clarified by further in vivo and in vitro studies. Nevertheless, pharmacologic targeting of the P2Y1 receptor might be considered as a promising strategy to prevent progressive renal disease and renal capillary injury/loss during inflammatory renal disease.

Acknowledgments
This work was supported by grants IZKF TP A12 and SFB 423 TP B6 (to C.P.M.H.) and IZKF TP B14 (to B.H.). The skilled technical help of U. Goller, S. Weber, A. Braun, T. Christ, A. Luedke, and M. Klewer is gratefully acknowledged. We thank Prof. R.J. Johnson (University of Florida, Gainesville, FL) and Prof. S.J. Shankland (University of Washington, Seattle, WA) for providing the disease-inducing sheep serum and Prof. B. Nieswandt (University of Würzburg, Würzburg, Germany) for the glycoprotein Ib antibody.

Disclosures
None.

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