Anti-Inflammatory and Renal Protective Actions of Stanniocalcin-1 in a Model of Anti-Glomerular Basement Membrane Glomerulonephritis

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We have previously shown that stanniocalcin-1 (STC1) inhibits the transendothelial migration of macrophages and T cells, suppresses superoxide generation in macrophages, and attenuates macrophage responses to chemoattractants. To study the effects of STC1 on inflammation, in this study we induced a macrophage- and T-cell-mediated model of anti-glomerular basement membrane disease in STC1 transgenic mice, which display elevated serum STC1 levels and preferentially express STC1 in both endothelial cells and macrophages. We examined the following parameters both at baseline and after anti-glomerular basement membrane antibody treatment: blood pressure; C_{3a} levels; urine output; proteinuria; blood urea nitrogen; and kidney C₃ deposition, fibrosis, histological changes, cytokine expression, and number of T cells and macrophages. Compared with wild-type mice, after anti-glomerular basement membrane treatment STC1 transgenic mice exhibited: i) diminished infiltration of inflammatory macrophages in the glomeruli; ii) marked reduction in crescent formation and sclerotic glomeruli; iii) decreased interstitial fibrosis; iv) preservation of kidney function and lower blood pressure; v) diminished C₃ deposition in the glomeruli; and vi) reduced expression of macrophage inhibitory protein-2 and transforming growth factor-β2 in the kidney. Compared with baseline,

wild-type mice, but not STC1 transgenic mice, had higher proteinuria and a marked reduction in urine output. STC1 had minimal effects, however, on both T-cell number in the glomeruli and interstitium and on cytokine expression characteristic of either TH1 or TH2 activation. These data suggest that STC1 is a potent anti-inflammatory and renal protective protein. (Am J Pathol 2009, 174:1368–1378; DOI: 10.2353/ajpath.2009.080476)

Stanniocalcin-1 (STC1) is a 25-kDa homodimeric glycoprotein hormone involved in calcium regulation in bony fish,¹ in which elevation of serum calcium triggers the release of STC1 from the corpuscles of Stannius,² organs associated with the kidneys.³ On circulation in the gill and intestine, STC1 inhibits calcium influx from the aquatic environment to the blood to maintain stable concentrations of calcium in the blood.⁴ Mammalian STC1 mRNA is ubiquitously expressed, and the highest levels of STC1 expression are found in the ovary, kidney, prostate, and thyroid.^{5–7} It was previously suggested that STC1 protein does not circulate in the blood of mammals⁸ except during pregnancy and lactation⁹; however, recent data suggest that mammalian STC1 is blood-borne, attached to a soluble protein.¹⁰ The cellular distribution of STC1 mRNA and protein in mammalian organs is not always parallel. In the kidney for example, *in situ* hybridization revealed restricted expression of STC1 mRNA in the cortical and medullary collecting ducts, whereas the protein is detected along the entire nephron.^{11,12} Similarly, the distribution of STC1 mRNA does not parallel the distribution of the protein in cellular elements of the ovary and pregnant uterus.¹³ Thus, STC1 is produced and secreted

Supported by the O'Brien Kidney Center (grants P50 DK64233-01, DK062828, and R01 DK077857), the National Institutes of Health (fellowship training grant DK62703-04 to Y.H.L.), and the Hong Kong Research Grant Council (RGC CERG HKU 7592/06M).

Accepted for publication December 16, 2008.

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by one cell type yet is sequestered by, and functions in, neighboring cells,^{13,14} consistent with paracrine/auto-crine action. The significance of blood-borne STC1 remains unclear.

Unlike the well-defined role for STC1 in regulating serum calcium in fish, little is known about the function of mammalian STC1. Initial studies suggest that STC1 may have a role in wound healing,¹⁵ cellular metabolism,¹⁶ angiogenesis,¹⁷ steroidogenesis,¹⁸ muscle and bone development,^{19,20} phosphate uptake in the kidney and gut,^{21,22} and cancer biology.²³ Thus, through the evolutionary process from fish to mammals, STC1 appears to have acquired new roles and functions in the various organs in which it is expressed.

Previous data from our laboratory suggest that STC1 suppresses superoxide generation in macrophages through induction of mitochondrial uncoupling protein-2, diminishing macrophage function (Y. Wang, unpublished data) and attenuating the response of macrophages to chemoattractants.²⁴ STC1 is normally expressed on the apical surface of endothelial cells in kidney arterioles, venules, and glomerular capillaries.²⁵ It maintains the expression of tight junction proteins in a tumor necrosis factor (TNF)- α -treated endothelial monolayer and blocks TNF- α -induced increase in endothelial permeability.²⁶ Consistent with these data, we have shown STC1 attenuates transendothelial migration of macrophages and T cells.²⁵ We hypothesized that through suppression of macrophage function and inhibition of transendothelial migration of leukocytes, STC1 may provide potent anti-inflammatory action. To test this hypothesis, in this study we applied the anti-glomerular basement membrane (GBM) glomerulonephritis (GN) disease model to STC1 transgenic (Tg) mice, which exhibit elevated serum levels of STC1.²⁷ Notably, these mice also exhibit preferential expression of the transgene in endothelial cells and macrophages.

Experimental Anti-GBM GN is a model of rapidly progressive GN, and is characterized by proteinuria, macrophage and T-cell infiltration, glomerular crescent formation, and Th1 antibody and cytokine responses. Macrophages and T cells play a critical role in the pathogenesis of anti-GBM GN, and their number correlates with the percentage of crescentic glomeruli.^{28–34} Consistent with our hypothesis, STC1 transgenic mice show diminished number of inflammatory/exudative macrophages within the glomeruli and renal protection from anti-GBM GN.

Materials and Methods

Sheep anti-mouse GBM antibody was a gift from Dr. Hui Lan (University of Hong Kong, Hong Kong, People's Republic of China). Polyclonal rabbit anti-STC1 antibodies were a gift from Dr. Gert Flik (Radboud University, Nijmegen, Netherlands).³⁵ Antibodies for mouse pan-macrophage marker (CD68) were purchased from AbD Serotec (Raleigh, NC). Actin antibodies were purchased from Chemicon (Temecula, CA). ICAM-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

MCP-1 antibodies were previously described.³⁶ Antibodies for mouse macrophage and dendritic cell marker (F4/80) and T lymphocytes (CD3) were purchased from BD Pharmingen (San Diego, CA). Fluorescein isothiocyanate (FITC)-tagged monoclonal antibodies for sheep IgG were purchased from R&D Systems (Minneapolis, MN). FITC-tagged antibodies for mouse C3 were purchased from Cedarlane Laboratories (Burlington, Canada). FITC-tagged antibodies for mouse IgG were purchased from Sigma (St. Louis, MO). Anti-mouse IgG, IgG₃, IgG₁, IgG_{2a}, IgG_{2b} antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). Rat anti-mouse C_{3a} was purchased from BD Pharmingen. ECL plus reagent and horseradish peroxidase-linked anti-mouse IgG were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). All other chemicals and reagents were purchased from Sigma.

Anti-GBM GN Model

Transgenic overexpression of STC1 is driven by the mouse metallothionein I minimal promoter over C57B/6CBA genetic background, and displays strong preferential expression of STC1 transgene mRNA in the liver, heart, and brain, and low in numerous other tissues including the kidneys.²⁷ However, our data show STC1 is preferentially expressed in macrophages and endothelial cells, where it is best appreciated in the glomerular capillaries. STC1 is detectable in the serum of wild-type (WT) mice, and as expected serum levels of STC1 in Tg mice were significantly higher (see data and Varghese et al²⁷). Twenty-five-gram, 1-year-old male mice were used. They were placed on *ad libitum* food and water intake throughout the experiment. Accelerated anti-GBM GN was induced in Tg and WT mice of similar genetic background by subcutaneous priming with 1 mg/mouse of normal sheep IgG in Freund's complete adjuvant, followed 7 days later by an intravenous injection of 0.2 mg/g of sheep anti-mouse GBM antibody. Mice were euthanized 10 days after anti-GBM antibody injection. Studies were approved by an institutional review committee.

Blood Pressure Measurement and Renal Function Assessment

Systolic blood pressure was recorded by tail plethysmography using the BP2000 blood pressure analysis system (Visitech Systems, Inc., Apex, NC) in conscious mice at baseline and before euthanasia. Urinary protein concentrations were determined by the Bradford method, adapted to a microtiter plate assay. Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA) was added to the diluted urine samples, and the absorbance at 595-nm wavelength was read on an ELX800 microplate reader (Bio-Tek Instruments, Winooski, VT). The protein concentrations were calculated by reference to bovine serum albumin standards (Sigma).

Serum Urea Measurement

Serum samples were treated with urease (US Biochemical Corp., Cleveland, OH) and the resultant ammonia was reacted with O-phthaldialdehyde/2-mercaptoethanol reagent (Sigma) in phosphate buffer (pH 7.4) for 30 minutes. Urea was measured as fluorescence (excitation at 405 nm and emission at 455 nm).

Measurement of Serum C_{3a} and Mouse Anti-Sheep IgG Isotypes

Blood samples were taken from the tail vein at time 0, 7 days after priming with sheep IgG, and at completion of the experiment (day 14). Sera were isolated and kept at -80°C until use. Serum C_{3a} and mouse anti-sheep IgG isotypes were measured using enzyme-linked immunosorbent assay. For quantitation of mouse anti-sheep IgG, we measured relative OD of sera at 1:3000 dilutions.

Elution of Antibodies from the Kidneys

Kidneys were perfused with saline and stored at -80°C until used. The cortical portions of the kidneys were dissected out by slicing with a razor blade, mixed with cold phosphate-buffered saline (PBS) (pH 7.4), and homogenized in a 5-ml Dounce homogenizer. This homogenate was spun at $400 \times g$ for 7 minutes at 4°C . The pellet was washed with cold PBS seven times and recovered by centrifugation. Elution of the antibodies from the pellet was performed as previously described.³⁷ First, the sediment was suspended in 0.2 mol/L glycine buffer, pH 2.5 (5 parts buffer:1 part sediment, v/v), and incubated at room temperature with constant shaking for 2 hours.³⁷ Then, the mixture was spun at $10,000 \times g$ for 30 minutes at 4°C . The supernatant was recovered and immediately brought to pH 7.0 with 0.1 N NaOH, followed by dialysis against PBS (several changes). The amount of IgG in the eluate was measured using enzyme-linked immunosorbent assay.

Morphometric Analysis

Tissue sections were evaluated by a kidney pathologist who was unfamiliar with the experimental protocol. Interstitial volume was determined using a point-counting technique on trichrome-stained sections. The interstitial volume was expressed as the percentage of grid points of a 1-cm² graded ocular grid, which lay within the interstitial area, viewed at $\times 20$ magnification. Five to ten random fields were used for morphometry. Crescent formation was counted from more than 100 glomeruli for each mouse and expressed as the percent of positive glomeruli of the total number examined. Total macrophages (CD68⁺), resident macrophages/dendritic cells (F4/80⁺), and T cells (CD3⁺) infiltrating the glomeruli and interstitium were counted, and the results were expressed as total cell number per glomerulus, or 10 interstitial grids (1-cm² graded ocular grids viewed at $\times 20$ magnification), respectively. The extent of glomerular sclerosis was expressed as percent of periodic acid-Schiff-

positively stained area per whole glomerular area. Each area was measured by tracking the glomerular tuft aided by computer manipulation using Mac Scope version 6.02 (Mitani Shoji Co., Ltd., Fukui, Japan). The extent of interstitial fibrosis was determined by Masson's trichrome, and is based on survey of the whole area of the cortex in the individual kidney sections and expressed as percentage of the field using Mac Scope version 6.02.

Immunohistochemistry

Ten days after injection of anti-GBM antibody, kidney tissue was fixed in 10% formaldehyde followed by dehydration in graded alcohols and embedded in paraffin blocks using standard techniques. Five- μm sections were cut, dried, and rehydrated. Labeling was performed using polyclonal rabbit anti-STC1 (1:1000 dilution)³⁵ or monoclonal rat anti-mouse F4/80 antigen antibodies (1:100 dilution), and detection was performed using peroxidase enzyme-based detection system (Vector Laboratories, Burlingame, CA). Control for labeling was performed in the presence of nonimmune IgG, and showed no staining. Staining with anti-CD68 (1:100 dilution), anti-CD3 (1:50 dilution), FITC-tagged anti-sheep IgG (1:80 dilution), and FITC-tagged anti-mouse IgG (1:50 dilution) was performed using frozen sections (5 μm in thickness), applying standard techniques. Photomicrographs were taken using a Labophot-2 Nikon (Tokyo, Japan) microscope with a MagnaFire Olympus (Tokyo, Japan) digital camera.

Western Blotting

Kidney tissue was homogenized using a Polytron, for 30 seconds, in a modified RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mmol/L ethylenediaminetetraacetic acid, containing 1 mmol/L phenylmethyl sulfonyl fluoride and 1 $\mu\text{g}/\text{ml}$ leupeptin) and centrifuged for 10 minutes at 1400 rpm at 4°C to remove cell debris. Fifty μg of total kidney lysates or sera were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and incubated with antibodies against: actin (1:10000 dilution), STC1 (1:1000 dilution), ICAM-1 (1:1000 dilution), or MCP-1 (1:1000 dilution). After washing with TBST buffer (20 mmol/L Tris, pH 7.6, 137 mmol/L NaCl, 0.1% Tween-20), the membrane was incubated with horseradish peroxidase-conjugated anti-goat IgG. The bound antibodies were visualized using chemiluminescence.

RNase Protection Assay

Riboprobes for GATA-3, interleukin (IL)-6, IL-10, IL-12, IL-18, interferon- γ , MCP-1, MIF, macrophage inhibitory protein (MIP)-2, RANTES, transforming growth factor (TGF)- β , TNF- α , TCA-3, GAPDH, and the ribosomal protein L-32 were generated by polymerase chain reaction using cDNA templates. Total RNA was isolated from whole kidneys of WT and STC1 Tg mice 10 days after anti-GBM Ab injection, using RNazol (Tel-Test, Friendswood, TX). Three μg of total RNA from each sample were

Figure 1. Inhibition of anti-GBM GN in STC1 Tg mice. **A:** Periodic acid-Schiff- and Masson's trichrome-stained kidney tissue. Anti-GBM Ab injection to WT mice produced severe crescentic GN, characterized by extra- and intracapillary mononuclear infiltration, hyaline cast formation (H), glomerulosclerosis, and interstitial fibrosis (see trichrome staining). STC1 Tg mice display reduced inflammation, crescent formation, and fibrosis after anti-GBM. **Arrow** points to a crescent. G, Glomeruli; H, hyaline casts in dilated tubules. **B:** STC1 Tg mice display fewer crescents, fewer glomerular endocapillary lesions, fewer sclerotic glomeruli, and less expansion in the tubulointerstitial compartment.

used in RNase protection assay using the Torrey Pines Biolabs kit (Houston, TX) as previously described.²⁴ Phosphorimage quantitation was performed using the PhosphorImager SI scanning instrument and ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

Data were expressed as mean $\pm$ SEM. Statistical significance was determined by unpaired *t*-test. A *P* value less than 0.05 was considered statistically significant.

Results

Inhibition of Anti-GBM GN in STC1 Tg Mice

In the following experiment, we sought to determine the impact of transgenic overexpression of STC1 on inflammation, proteinuria, kidney function, and blood pressure in a mouse model of anti-GBM GN. As shown in Figure 1A, after the administration of anti-GBM Ab, WT mice developed severe crescentic GN, associated with intra-

and extracapillary mononuclear cell infiltration, tubular dilatation, intratubular hyaline cast formation, glomerulosclerosis, and interstitial fibrosis. On the other hand, compared with WT mice, STC1 Tg mice treated with anti-GBM Ab displayed 92% fewer sclerotic glomeruli, 73% fewer crescents, 75% fewer glomerular endocapillary lesions, and 82% less expansion in the tubulointerstitial compartment (Figure 1B). Compared with baseline, WT mice demonstrated fourfold increase in blood urea nitrogen (BUN), 50% reduction in urine output, doubling of proteinuria after anti-GBM; whereas, STC1 Tg mice displayed no significant differences in BUN, urine output, or proteinuria (Figure 2); moreover, STC1 Tg mice displayed lower blood pressure after anti-GBM compared with WT Tg mice, consistent with renal protection by STC1 (Figure 2). Control studies indicated equal deposition of sheep anti-mouse-GBM IgG on the GBM in both WT and Tg mice (Figure 3A), and in agreement with a recent report, we also detect STC1 in the serum of WT mice, albeit at low levels compared with STC1 Tg mice (Figure 3B). Important for our hypothesis, Tg mice exhibit preferential expression of STC1 transgene in macrophages (Figure 3E) and endothelial cells, where it is best

Figure 2. Inhibition of crescentic GN in STC1 Tg mice. WT mice had fourfold rise in BUN, 50% reduction in urine output, and more proteinuria; there were no significant changes in BUN, urine output, or proteinuria in STC1 Tg mice. Although baseline blood pressure was similar in WT and STC1 Tg mice, it was higher in WT mice after anti-GBM GN, consistent with renal protection by STC1.

appreciated in the glomerular capillaries (Figure 4). Of interest, some injured tubules (based on morphology) in WT mice displayed increased expression of STC1, the significance of which remains to be determined. Otherwise, the expression (Western blot on whole kidney lysate; Figure 3, C and D) and distribution of STC1 protein (determined by immunohistochemistry; Figure 4) were similar in the kidneys of WT and STC1 Tg mice. It should be emphasized that overexpression of STC1 in macrophages and endothelial cells is not discernible on Western blot, using whole kidney lysate, likely because of the small contribution to total kidney STC1 (Figure 3, C and D).

STC1 Tg Mice Exhibit Decreased Infiltration of the Glomeruli with Inflammatory Macrophages after Anti-GBM GN

We hypothesized that transgenic overexpression of STC1 in the endothelium and macrophages will diminish leukocyte infiltration into the glomeruli/kidney, in the context of

Figure 3. **A:** Equal binding of sheep IgG to GBM in WT and STC1 Tg mice. Ten days after anti-GBM Ab injection, frozen sections of kidney tissue were labeled with FITC-tagged anti-sheep IgG Ab. Equal binding of sheep anti-mouse GBM antibody to the GBM in both WT and STC1 Tg mice is illustrated (arrows point to glomeruli). **B:** STC1 levels in the serum. Western blot demonstrates higher levels of STC1 protein in the serum of STC1 Tg mice compared with WT mice. **C** and **D:** STC1 levels in the kidney. Western blot and bar graph demonstrate equal expression of STC1 in the kidneys (representing whole kidney lysates) of WT and STC1 Tg mice after anti-GBM. **E:** STC1 expression in macrophages. Dual staining for STC1 (red) and F4/80 (brown, a marker of macrophages and dendritic cells) demonstrates high-level expression of STC1 in kidney macrophages/dendritic cells of STC1 Tg mice compared with WT mice.

anti-GBM GN. Hence, we examined the number of T cells ($CD3^+$) and macrophages [total macrophage count ($CD68^+$), versus $F4/80^+$ cells (representing resident macrophages and dendritic cells³⁸)] in the kidney/glomeruli, 10 days after anti-GBM Ab injection, a time point that correlates with peak infiltration of macrophages and T cells in experimental mouse anti-GBM GN.^{39,40}

After anti-GBM Ab injection, $F4/80^+$ cells (resident macrophages and dendritic cells) increased fivefold in the interstitium and periglomerular region of WT kidneys, but only twofold in the corresponding regions of STC1 Tg mice kidneys (Figure 5 and Table 1). Because the number of $F4/80^+$ cells (macrophages/dendritic cells) in the

Figure 4. Expression of STC1 in the endothelium. Immunohistochemistry studies reveal high levels of STC1 expression (brown) in the endothelium of STC1 Tg mice (best appreciated in the glomerular capillaries), compared with WT mice. Also, expression of STC1 in injured tubules of WT mice after anti-GBM GN. G, Glomeruli; T, tubule.

interstitium was identical to the number of CD68⁺ macrophages (Table 1), we conclude that most, if not all interstitial macrophages in both WT and STC1 Tg mice were resident. The lower number of resident macrophages in the interstitium of STC1 Tg mice compared with WT is consistent with reduced interstitial injury in STC1 Tg mice and less need for repair. F4/80⁺ macrophages/dendritic cells were nearly absent from the glomeruli of both WT and STC1 Tg mice at baseline, and their number increased minimally, but equally after anti-GBM GN (Figure 5 and Table 1). After anti-GBM GN, CD68⁺ macrophages were abundant in the glomeruli of WT mice, but were nearly absent from the glomeruli of STC1 Tg mice. Based on the paucity of F4/80⁺ cells (macrophages/dendritic cells) in the glomeruli (in both WT and STC1 Tg), we conclude that most macrophages infiltrating the glomeruli of WT mice after anti-GBM GN were of the inflammatory/exudative variety, and these were absent in STC1 Tg mice. As shown in Figure 5 and Table 1, T cells were predominantly interstitial and increased almost to the same degree after anti-GBM GN in both WT and STC1 Tg mice (3.7-fold versus 2.7-fold, respectively). In the glomeruli, T cells were not detected at baseline and increased minimally but equally after anti-GBM GN in both WT and STC1 Tg mice.

Our data suggest that in a mouse model of anti-GBM GN, inflammatory/exudative macrophages predominate in the glomeruli, whereas resident macrophages are absent, consequently, tipping the balance toward glomerular injury and less repair. Our data also suggest that circulating STC1 and/or transgenic expression of STC1 in macrophages and/or endothelium diminish the number of

inflammatory/exudative macrophages within the glomeruli during anti-GBM GN, protecting from kidney injury.

Decreased Expression of MIP-2 and TGF- β 2 in the Kidney of STC1 Tg Mice after Anti-GBM GN

Anti-GBM GN is associated with increased expression of several cytokines/lymphokines including IL-1 β , TNF- α , TGF- β , MIF, MIP2, and MCP-1.^{41–47} We performed RPA on RNA representing whole kidney tissue from WT and STC1 Tg mice after anti-GBM GN and this revealed no significant changes in mRNA expression of T-cell-related cytokines (TCA-3, IL-18, IL-6, and RANTES), and more importantly, genes characteristic of TH1-mediated T-cell responses (IL-12, and interferon- γ) or TH2-mediated responses (IL-10, GATA-3) (Figure 6, A–C). On the other hand, MIP-2 and TGF- β 2 were lower in STC1 Tg mice. The expression of MCP-1 mRNA was slightly lower in STC1 Tg mice, and hence, we determined protein levels using Western blotting and found similar MCP-1 protein levels in the kidneys of WT and STC1 Tg mice. Additionally, we found no difference in the level of Intercellular adhesion molecule-1 (ICAM-1) protein (Figure 6, D and E). These data are consistent with diminished activation of inflammatory macrophages, and as a result decreased signaling to fibrosis. Our data also suggest that STC1 has no significant effect on T-cell-mediated immunity in the context of anti-GBM GN, at least at the 10-day time point.

Decreased Deposition of Mouse C₃ in the Glomeruli of STC1 Tg Mice after Anti-GBM GN

The inflammatory injury in the acute phase of experimental anti-GBM GN is initiated by binding of the heterologous antibody to the GBM and is complement-dependent.⁴⁸ The autologous phase of the disease is mediated by the immune response against the heterologous antibody affixed to the GBM and represents a delayed hypersensitivity reaction measurable as mouse C₃ and IgG deposition in the glomeruli.⁴⁹ Hence, we studied the deposition of mouse C₃ and IgG in the glomeruli of WT and STC1 Tg mice 10 days after the administration of sheep anti-mouse GBM Ab. Although the deposition of mouse IgG in the glomeruli was similar in WT and STC1 Tg mice (Figure 7A), we found little mouse C₃ in the glomeruli of STC1 Tg mice (Figure 7A). For better assessment of mouse antibody response, we measured mouse anti-sheep IgG isotypes in the serum and performed elution studies of mouse IgG from the kidneys after anti-GBM GN, and found similar levels (Figure 7, B and C). Moreover, because we observed little C₃ deposition in the glomeruli of STC1 Tg mice after anti-GBM GN, we determined serum concentrations of C_{3a}, an index of activated complement (through the classic and alternate pathways), and found equivalent C_{3a} levels at baseline, after priming with sheep IgG and after anti-GBM GN (Figure 7D), suggesting that renal protection by STC1 is not mediated through inhibition of complement activation. Local C3 deposition and synthesis have been shown to

Figure 5. STC1 Tg mice exhibit decreased infiltration of macrophages into the glomeruli after anti-GBM GN. In a model of mouse anti-GBM GN, F4/80⁺ cells (resident macrophages and dendritic cells) were absent from the glomeruli in both WT and STC1 Tg mice. Macrophage infiltration into the glomeruli was observed only in WT mice, and the macrophages were predominantly F4/80⁺/CD68⁺ (inflammatory/exudative). T cells (CD3) were predominantly interstitial and increased to a similar degree in WT and STC1 Tg mice after anti-GBM GN. Image resolution was dictated by available antibodies. Staining for F4/80 was performed on paraformaldehyde-fixed sections, whereas staining for CD68 and CD3 was performed on frozen sections. G, Glomeruli.

correlate with local inflammation and cytokine release.^{50–52} Hence, our data suggest that despite comparable antibody response in the autologous phase of anti-GBM GN (Figure 7B), C₃ deposition in the glomeruli of STC1 Tg mice is diminished, consistent with reduced

macrophage-mediated inflammation. Furthermore, the comparable expression of markers characteristic of TH1-mediated (IL-12, and interferon- γ) or TH2-mediated (IL-10, GATA-3) T-cell responses within the kidney after anti-GBM, suggests that STC1 does not affect T-cell

Table 1. Macrophage (F4/80⁺, CD68⁺) and T-Cell (CD3) Count in the Glomeruli and Tubulointerstitium (TIN) of Wild-Type (WT) and STC1 Transgenic Mice. F4/80 is a Marker of Resident Macrophages and Dendritic Cells; CD68 is a Pan-Macrophage Marker.

	No. of F4/80 ⁺ cells per glomerulus section	No. of CD68 ⁺ cells per glomerulus section	No. of CD3 cells per glomerulus section	No. of F4/80 ⁺ cells per 10 grids in TIN	No. of CD68 ⁺ cells per 10 grids in TIN	No. of CD3 cells per 10 grids in TIN
WT control (n = 10)	0.1 ± 0.1	0	0.1 ± 0.1	17.0 ± 4.0	22.5 ± 0.5	14.0 ± 1.0
STC1 Tg control (n = 7)	0	0	0.03 ± 0.03	20.0 ± 1.5	19.0 ± 2.0	14.7 ± 1.5
WT anti-GBM (n = 7)	4.1 ± 0.3	13.0 ± 1.8	2.7 ± 0.9	110.9 ± 10.3	111.3 ± 15.9	53.4 ± 4.5
STC1 Tg anti-GBM (n = 6)	2.5 ± 0.5	3.0 ± 0.7	3.5 ± 0.3	38.8 ± 6.5	39.3 ± 4.0	39.8 ± 4.9

activation in the context of anti-GBM GN, at least, not at the 10-day time point after anti-GBM GN (Figure 7B).

Discussion

Our cumulative data suggest that stanniocalcin is a critical naturally occurring anti-inflammatory protein.^{24,25} It acts through a number of novel mechanisms that affect endothelial and macrophage function, when combined these effects produce potent inhibition of inflammation. First, we found STC1 decreases intracellular calcium in macrophages,²⁴ and hence, is expected to diminish cell mobility, cell migration, and the response to antigenic stimuli (all involve changes in intracellular calcium signal, acting as a second messenger). Indeed, we found STC1 diminishes the mobility of macrophages and their response to chemoattractants.²⁴ The effects of STC1 are not limited to one chemokine because it blocks the migration of macrophages in response to different chemokines/cytokines.²⁴ Second, in addition to decreasing intracellular calcium, STC1 suppresses macrophage function by decreasing superoxide generation through a novel mechanism that involves up-regulation of mitochondrial uncoupling protein-2 (manuscript submitted for publication). Third, the anti-inflammatory action of STC1 is mediated in part through its effects on the endothelium. The endothelium

plays a critical role in the migration of macrophages from the circulation to the injured tissue. Exposure of endothelial cells to cytokines (TNF- α or IL-1 β for example; produced by inflammatory cells and injured tissue) induces two key changes that facilitate leukocyte migration: opening of the tight junctions between neighboring endothelial cells, allowing inflammatory cells to traverse the endothelial barrier,⁵³ and synthesis and presentation of adhesion molecules at the cell surface, which tether circulating leukocytes to the endothelium. These changes favor the migration of inflammatory cells through the newly formed openings in the endothelial tight junctions.^{53–56} STC1 blocks the effects of cytokines (TNF- α or IL-1 β) on the endothelium, preserving endothelial seal in cytokine-treated endothelial cells.²⁶ Consistent with these data, we showed STC1 dose dependently decreases the migration of macrophages and T cells across cytokine-treated endothelial monolayer.²⁵ The *in vivo* equivalent of our tissue culture results would be: exposure of endothelial cells to STC1 (circulating, or locally produced by the endothelium) would preserve endothelial tight junctions and inhibit extravasation of macromolecules and inflammatory cells to the injured tissue; and exposure of macrophages to STC1 would decrease their mobility and function. To validate our hypothesis, we examined the outcome of anti-GBM GN (mediated by T cells and mac-

Figure 6. STC1 Tg mice exhibit decreased expression of MIP-2 and TGF- β . **A–C:** Densities of respective bands corresponding to RNase protection assay were normalized to L32, and data represent the means and SE of at least six independent determinations. ****** $P < 0.01$. **D** and **E:** Western blots and bar graph show MCP-1 and ICAM-1 protein expression in the kidneys of WT and STC1 Tg mice after anti-GBM GN.

Figure 7. A: Decreased deposition of mouse C₃ but not IgG in the glomeruli of STC1 Tg mice after anti-GBM GN. FITC-labeled anti-mouse C₃ or IgG antibodies were used to determine the deposition of mouse C₃ and IgG in the glomeruli of WT and STC1 Tg mice 10 days after the administration of anti-GBM Ab. **Top:** Representative images for C₃. **Bottom:** Representative images for mouse IgG. G, Glomerulus. **B–D:** Equivalent complement activity, anti-sheep IgG isotypes in the sera, and mouse IgG levels in the kidneys of WT and STC1 Tg mice. Bar graphs depict eluted mouse IgG from the kidneys (B), mouse anti-sheep IgG isotypes in the serum at baseline and after priming with sheep IgG (C), and serum C_{3a} levels at baseline, after priming with sheep IgG and after anti-GBM Ab administration (D). Data represent the means ± SEM of six independent determinations.

rophages) using a Tg mouse, which exhibits high circulating STC1 protein and preferential expression of the transgene in macrophages and endothelium.

Injection of anti-GBM Ab into WT mice produced severe renal failure, associated with a fourfold rise in BUN, 50% reduction in urine output, and increased proteinuria. On the other hand, and consistent with our hypothesis,

administration of anti-GBM Ab to STC1 Tg mice produced no significant change in BUN, urine output, or proteinuria, indicating protection from the injurious effects of anti-GBM Ab. This was confirmed on review of kidney histology in STC1 Tg mice, which showed no significant changes in the number of sclerotic glomeruli, the number of crescentic lesions, and the degree of interstitial expansion and fibrosis after anti-GBM GN. Acute GN is frequently associated with an increase in blood pressure, which results from the loss of functional parenchyma, the decline in urine output, fluid retention, and activation of humoral factors.^{57,58} Importantly, blood pressure was higher in WT mice after anti-GBM GN compared with STC1 Tg mice. Transgenic overexpression of STC1 has not been shown to affect blood pressure in mice without renal disease.²⁷ Hence, the lower blood pressure we observed in STC1 Tg mice after anti-GBM is consistent with renal protection.

A detailed analysis of tissue macrophages and lymphocytes provided additional insights into mechanisms of renal protection by STC1. Macrophages and T cells within tissue are heterogeneous. Resident macrophages play an important role in tissue repair and are characterized as F4/80^{high}, CX3CR1^{high}, GR-1^{low}, CCR2[−], CD62L[−], and MP20^{−59–61}; they are self-regenerative and/or may originate from circulating progenitors.^{62,63} On the other hand, inflammatory/exudative macrophages are short-lived, do not proliferate, produce inflammatory cytokines, and contribute to tissue injury; they are characterized as F4/80^{low}, CX3CR1^{low}, MP20^{high}, GR-1^{intermediate}, CCR2⁺, CD62L⁺.^{59–61,64} Both populations carry the pan-macrophage marker, CD68. Similarly, the nature of the T-cell response (TH1 versus TH2) determines cell-mediated immunity and the course and severity of autoimmune anti-glomerular basement membrane disease.³¹ Remarkably, the predominant macrophage population in the glomeruli of WT mice after anti-GBM GN was exudative/inflammatory (CD68⁺/F4/80[−]) with almost complete absence of F4/80⁺ cells (resident macrophages/dendritic cells). In contrast, there was no significant change in the number of macrophages in the glomeruli of STC1 Tg mice compared with baseline. This finding is critical and is consistent with our hypothesis; that is, overexpression of STC1 in the endothelium blocks the action of cytokines on endothelial tight junctions and preserves endothelial integrity, preventing macrophage migration across the blood vessels. Thus, attenuation of MIP-2 and TGF- β expression in the kidney and the lower deposition of C3 in the glomeruli are consistent with inhibition of macrophage-dependent kidney injury in STC1 Tg mice.

T cells were predominantly interstitial and increased almost to the same degree in WT and STC1 Tg mice after anti-GBM GN, suggesting that STC1 has no significant effect on T-cell infiltration, at least at the 10-day time point. Additionally, we found no difference in the expression of cytokines characteristic of TH1- or TH1-mediated T-cell responses, suggesting that STC1 does not affect T-cell activation. A recent report suggested that macrophage depletion in the course of anti-GBM GN prevents proteinuria and glomerular macrophage infiltration, but not the accumulation of CD4⁺ or CD8⁺ T cells, indicating

that macrophages are common effectors for both CD4 and CD8 T-cell-dependent injury³⁴ and that macrophage depletion decreases the recruitment of T cells to the injured kidney. Thus, our data are consistent with these observations and specifically implicate exudative/inflammatory macrophages in mediating kidney injury in the course of anti-GBM GN. Although the number of interstitial macrophages was lower in STC1 Tg mice after anti-GBM GN, compared with WT, interstitial macrophages were almost entirely of the resident variety and not inflammatory/exudative, and the smaller number of interstitial resident macrophages in STC1 Tg after anti-GBM GN may be a reflection of lesser injury, and hence, lesser need for reparative macrophages. Alternatively, STC1 may have direct effect to decrease interstitial macrophage proliferation or recruitment.

Paciga and McCudden and their colleagues^{16,18,65} reported the existence of STC1-binding protein/receptor in multiple tissues. They used a stanniocalcin-alkaline phosphatase fusion protein in binding assays and concluded there are high-affinity (0.25 to 0.8 nmol/L), saturable and displaceable binding sites for STC1.^{16,18,65} Besides impairing macrophage function, it is unknown if STC1 affects the function of other cells involved in inflammation. In conclusion, STC1 effectively blocks inflammation in the kidney in the context of anti-GBM GN; and this function could be developed into novel methods to combat inflammation.

Acknowledgment

We thank Dr. Ping Zhang for technical assistance.

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