Extraglomerular Origin of the Mesangial Cell after Injury

A New Role of the Juxtaglomerular Apparatus

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Abstract

We investigated the origin of the glomerular mesangial cell, a smooth muscle–like cell that provides structural support in the glomerulus. Injection of anti–Thy 1 antibody that binds the Thy 1 antigen on rat mesangial cells eliminated (> 95%) the mesangial population at 20–28 h, while Thy 1-positive cells in the juxtaglomerular apparatus (JGA) were sequestered from the circulation and survived. Single pulse labeling with [^{3}H]thymidine at 36 h labeled Thy 1-positive cells in the JGA and hilus. Serial biopsies demonstrated the progressive migration (5–15 μm/d) and proliferation of these mesangial reserve cells until the entire glomerulus was repopulated. The regenerating mesangial population expressed contractile and migratory proteins preferentially at the leading edge of the migratory front. Single as well as multiple pulse labeling with [^{3}H]thymidine confirmed that the entire mesangial cell repopulation originated from only a few mesangial reserve cells. These reserve cells resided in the extraglomerular mesangium in the JGA and were not renin-secreting cells, macrophages, smooth muscle cells, or endothelial cells. These studies document mesangial cell migration in the anti–Thy 1 model of mesangial proliferative glomerulonephritis and provide evidence for a new role for the juxtaglomerular apparatus in the maintenance of the mesangial cell population. (*J. Clin. Invest.* 1997. 100:786–794.) Key words: glomerulonephritis • mesangial cell • migration • juxtaglomerular apparatus

Introduction

It has recently been appreciated that not all glomerular injury progresses to scarring, but that under certain situations spontaneous healing occurs (1, 2). Complete repair of the glomerular architecture can even occur with mesangiolysis, the most severe form of mesangial cell (MC)[1] injury in which a loss of the mesangium with disintegration of the MC matrix results in ballooning of the capillaries and occasional microaneurysms (3, 4). Some of the processes in glomerular repair have been identified and include recovery of a normal cell population by a balance of proliferation and apoptosis, capillary repair by angiogenesis, and the remodeling of the mesangial matrix by a balance of matrix synthesis and degradation (1–7).

A process that has not been studied extensively in the repair process is MC migration. Despite the importance of cell migration in many basic biological processes such as embryonic development, inflammation, and wound healing, only indirect evidence for in vivo MC migration has been provided to date. In the Habu snake venom model, glomerular repair of microaneurysms is associated with the appearance of desmin-positive MC initially around the edge of, and later within, the aneurysms, suggestive of MC migration (8).

In mesangial proliferative glomerulonephritis in the rat induced by a single injection of anti–Thy 1 antibody (the anti–Thy 1 model), severe mesangiolysis is followed by MC proliferation, apoptosis, angiogenesis, and matrix expansion (4–7). Nevertheless, it is completely unknown how these different processes are coordinated to restore the dramatically altered glomerular architecture within a few weeks, and whether this process requires migration of extrinsic or intrinsic glomerular cells. Even in embryonic glomerulogenesis, where cell migration is presumed to be an important part of glomerular development, in vivo cell migration has not been demonstrated directly, and it is still debated whether glomerular endothelial and mesangial cells originated from common or different precursors, and whether these are of extrinsic or intrinsic derivation (9–11). That MC migration does occur in the anti–Thy 1 model is indirectly suggested by the increased expression of plasma membrane cytoskeleton linking the proteins moesin/radixin preferentially in their filopodial cell extensions (12), but direct evidence for migration in vivo is not available.

In the current paper, we provide evidence that repopulation of the mesangium in the anti–Thy 1 model involves migration of cells from the hilar area and, interestingly, from the extraglomerular mesangium in the juxtaglomerular apparatus (JGA). These extraglomerular cells could be shown to be distinct from macrophages and smooth muscle cells, and demonstrate a new role for the JGA in the maintenance of a normal MC population.

Methods

Experimental design. The anti–Thy 1 model is characterized by an initial complement-dependent mesangiolysis followed by a mesangial cell repopulation. To maximize the ability to detect MC migration in the repopulation process, we desired a dose of anti–Thy 1 antibody that would first kill the entire normal resident MC population so that we could focus exclusively on how new cells repopulate the glomerulus. Therefore, dose response studies were performed in which graded doses of 0.1–1.2 ml/100 g of goat antithymocyte antibody

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; EGM, extraglomerular mesangium; GEN, glomerular endothelial cell; JGA, juxtaglomerular apparatus; MC, mesangial cell; PCNA, proliferating cell nuclear antigen; sm, smooth muscle.

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(plasma) were given to establish a disease model characterized by > 95% loss of MC (n = 2–4 per dose). Confirmation of MC loss was provided by immunostaining for Thy 1 and desmin.

A dose of 0.5 ml/100 g or greater of anti–Thy 1 antibody was found to cause a complete loss of MC in glomeruli by 28 h after injection. This dose was then selected to study the subsequent MC repopulation and migration. The in vivo migration assay was developed by single pulse labeling rats with [^{3}H]thymidine at 36 h (n = 4–5 each) after disease induction (with 0.5 ml/100 g anti–Thy 1 antibody). This results in the labeling of cells proliferating within that 1-h period. Biopsies were then performed at 90 min after labeling and at 5 and 7 d. This allowed one to identify the number and location of proliferating cells at the time of the labeling (the 1 1/2 h biopsy) and at two later time points. The labeled cells could further be identified as MC by immunostaining for the Thy 1 antigen. One could then document migration of these labeled MC cells from the hilar area by demonstrating that the labeled cells were located at progressively farther distances from the hilar area over time.

The studies in which the [^{3}H]thymidine pulse was administered at 36 h allowed us to label and localize the proliferating MC at that time point and to observe their migration over time. As will be shown in Results, the labeled cells that were Thy 1 positive (MC) at this time point were restricted to the hilar area. These labeled MC subsequently migrated to the periphery of the glomerular tuft over time. Additional experiments were performed in which the single pulse labeling of [^{3}H]thymidine was administered at other times to separate groups of rats (4, 12, 20, 36, 44, 52, 60, 68, or 76 h, n = 4–5 each) after disease induction with serial biopsies 90 min after labeling and at 5 and 7 d. This allowed us to study/analyze mesangial repopulation starting at various stages after mesangial ablation. By studying the location of proliferating cells labeled at different time points, it was possible to determine if cells repopulated the mesangium in a single wave (each time point shows cells farther from the hilus) or if there is a continuous supply of new cells from the hilar area (new hilar cells would be labeled at each time point).

The possibility existed that some of the repopulation of the mesangium could involve the migration of nonproliferating MC that would not take up [^{3}H]thymidine and would therefore not be detected by our migration assay. However, it was also possible that the entire repopulation of the mesangium might derive from proliferating MC. To determine which possibility was correct, an additional group of animals (n = 4) was administered multiple [^{3}H]thymidine injections every 8 h between 30 and 70 h after anti–Thy 1 injection to label the entire population of proliferating MC over that time period (based on the estimation that the S phase of most proliferating cells is $\sim$ 8 h). This was followed by a single pulse bromodeoxyuridine (BrdU) injection on day 5. This allowed us to examine whether the entire MC population had incorporated [^{3}H]thymidine, and by including the BrdU injection we could also determine if the late proliferating MC (on day 5) were derived from the earlier proliferating MC (between 30 and 70 h).

Identification of the MC precursors in the extraglomerular mesangium, as well as the phenotypic characteristics of the migrating MC population, was determined by single, double, and triple immunostaining combined with autoradiography using various cell-specific and phenotypic markers. The number and distance of labeled MC from the JGA during the process of glomerular repopulation as well as the percentage of extraglomerular MC labeling was assessed (see detailed methods below).

The migration studies described above were performed using a dose of anti–Thy 1 antibody (0.5 ml/100 g) that results in an almost complete loss of the resident MC population. To determine if the mechanisms of MC repopulation and migration are different if the initial injury is modulated, both a lower (0.3 ml/100 g body wt) and a higher (0.7 ml/100 g body wt) dose of anti–Thy 1 antibody was administered to separate groups of rats (n = 3–6) followed by three [^{3}H]thymidine injections every 8 h starting at different time points (between 24 and 40 h) after the anti–Thy 1 injection.

In vivo disease model. Experimental mesangial proliferative nephritis was induced in 190–230-g male Wistar rats (Simonsen Laboratories, Gilroy, CA) by intravenous injection of goat anti–rat thymocyte plasma as previously described (13). [^{3}H]Thymidine (6.7 Ci/mmol, 1 mCi/ml, 120 μCi/100 g body wt; New England Nuclear, Boston, MA) or BrdU injections (10 mg/100 g body wt; Sigma Chemical Co., St. Louis, MO) were given by intraperitoneal injection. Serial survival biopsies (as described in reference 14) were performed at 90 min after the last (or single) [^{3}H]thymidine and/or BrdU injection (between 4 and 76 h after disease induction) and on day 5. Sacrificial biopsies were obtained on day 7.

Renal morphology and immunohistochemistry combined with autoradiography. Renal biopsies were fixed in methyl Carnoy's solution and embedded in paraffin (14). Immunostaining was performed using an indirect immunoperoxidase technique as described previously (14) using the following primary antibodies: the murine IgG_1 mAb OX-7 (Accurate Chemical and Science Corp.,Westbury, NY), a murine IgG_2 mAb against α-smooth muscle (sm) actin (Sigma Chemical Co.), a murine IgG mAb D33 against desmin (Dako Corp., Santa Barbara, CA), a murine IgG_1 mAb against renin (15) (kind gift of M. Laprade, Sanofi Recherche, Montpellier, France), a murine IgG_1 mAb against rat endothelial antigen RECA-1 (16) (kind gift of A. Duijvestijn, University of Limburg, The Netherlands), a murine IgM mAb against proliferating cell nuclear antigen (PCNA) (19A2; Coulter Immunology, Hialeah, FL), a murine IgG_1 mAb, ED-1, to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells (Harlan Bioproducts for Science, Inc., Indianapolis, IN), a rabbit polyclonal antiserum against moesin and radixin (17) (kindly provided by Dr. Furthmayr, Stanford University, Stanford, CA), and a murine IgG mAb against BrdU (Becton Dickinson & Co., Mountain View, CA). For BrdU immunostaining, the standard protocol was modified by pretreatment of the slides with 0.0001 mg pepsin/ml 0.1 N HCl at 37°C for 30 min, followed by 1.5 N HCl at 37°C for 15 min, and 0.1 M Borax buffer at room temperature for 2 × 5 min before applying the primary antibody. For combination with autoradiography ([^{3}H]thymidine detection), immunostained sections were dehydrated, air dried, dipped in NTB 2 emulsion (Eastman Kodak Co., Rochester, NY), stored in the dark at 4°C for 3 wk, developed, and counterstained with methyl green (Sigma Chemical Co.).

Quantitation of migration. Migration of labeled MC was quantified using a computerized microscope–camera monitor system. Migration of MC was measured from the glomerular hilus and could only be measured in glomeruli in which the hilar and extraglomerular mesangium (EGM) regions were present. For each biopsy, at least 20 cross sections of cortical glomeruli with identifiable EGM and hilar areas were examined out of a total of 80–130 glomeruli per biopsy. Cells positive for both [^{3}H]thymidine and Thy 1 (OX-7) were identified and the distance (in micrometers) from these cells to the glomerular hilus was measured. Both absolute distances and the distance corrected for glomerular diameter were determined. The latter measurement corrects for changes in glomerular size that accompanies disease. Migration was documented by showing that the labeled MC were further from the hilar area on serial biopsies. The migratory speed (micrometers per day) was determined by factoring the change in mean distance divided by the time between the biopsies.

Immunohistochemical single, double, or triple staining combined with autoradiography. To determine the [^{3}H]thymidine-labeled cell types (MC, glomerular endothelial cell [GEN], smooth muscle (–like) cells, renin secreting cells, and macrophages) during glomerular repopulation, double (OX-7/α-sm actin, OX-7/BrdU, OX-7/renin, OX-7/ED-1, OX-7/RECA-1), and triple (OX-7/RECA-1/ED-1, OX-7/α-sm actin/BrdU) immunostaining was performed using an indirect immunoperoxidase technique. The first primary antibody was incubated overnight at 4°C, followed sequentially by biotinylated rabbit anti–mouse IgG_{2A} or IgG_1 serum (Zymed Laboratories Inc., South San Francisco, CA), peroxidase-conjugated avidin D (Vector Laboratories Inc., Burlingame, CA), and color development with diaminobenzidine without nickel chloride. Incubation in 3% H_2O_2/metha-

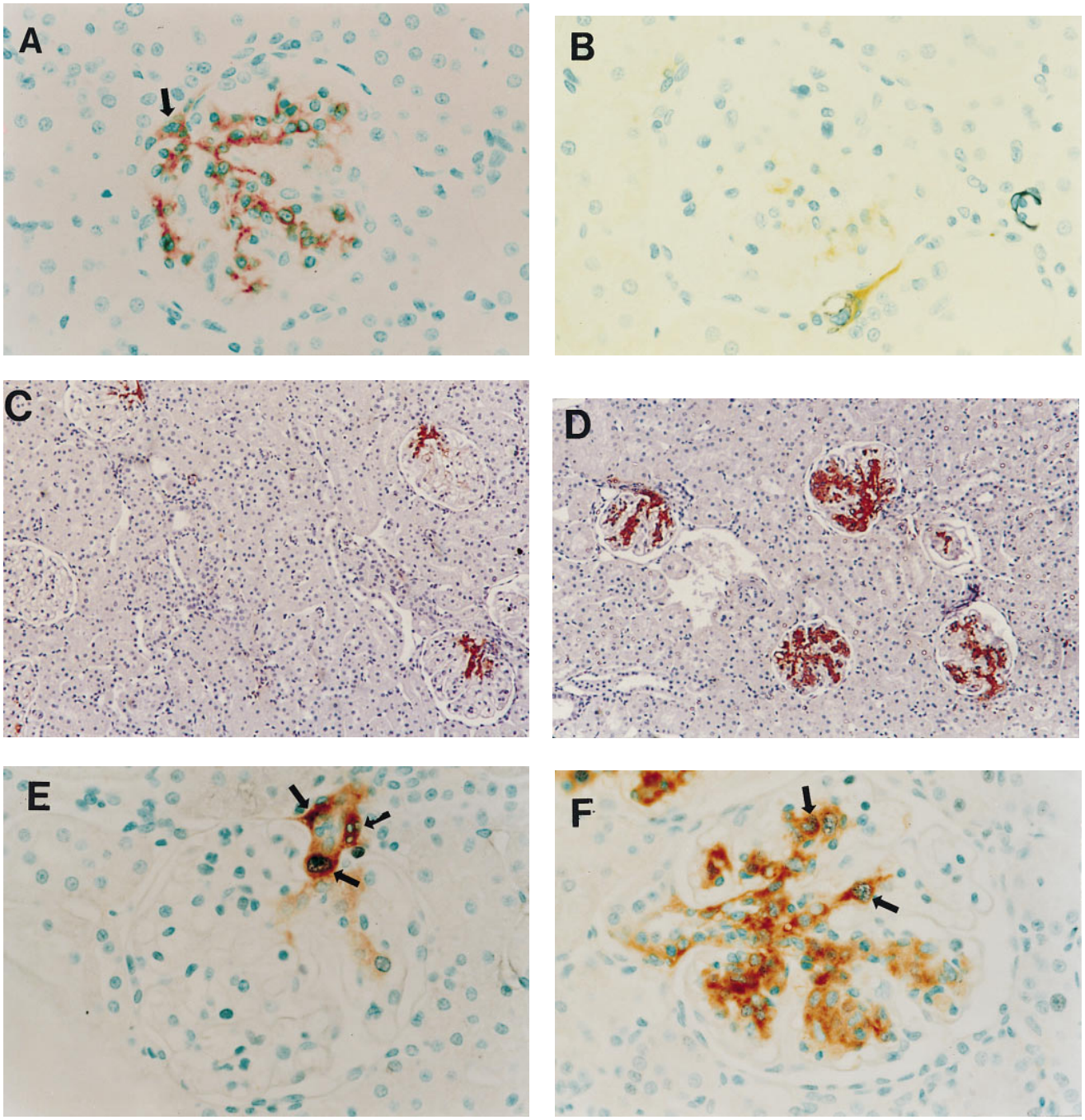

Figure 1. Despite an almost complete loss of MC after anti–Thy 1 antibody injection, repopulation of the mesangium occurs within a few days. (*A*) Immunostaining of the MC population (*brown*) within a normal glomerulus and in the juxtaglomerular apparatus (*arrow*) using the OX-7 monoclonal antibody against the Thy 1 antigen. (*B*) A near complete (> 95%) loss of intraglomerular but not extraglomerular MC (OX-7, *brown*) at 28 h. This loss of MC is replaced by an increasing number of Thy 1-positive MC, starting around day 2 (*C*; OX-7, *brown*) and being almost complete on day 7 of disease (*D*; OX-7, *brown*). (*E*) The early proliferating MC (*arrow*) in the extra-/juxtaglomerular apparatus and the hilar area 36 h after anti–Thy 1 antibody injection using OX-7 staining to identify MC (*brown*) and PCNA staining as a marker for proliferating cells (*black*). (*F*) 6 d after disease induction, MC (OX-7, *brown*) have repopulated the glomerulus and MC proliferation (OX-7, *brown*; PCNA, *grey*) (*arrows*) occurs preferentially at the periphery of the glomerulus.

nol for 20 min prevented any remaining peroxidase activity. For sequential double staining with two murine monoclonal IgG_1 antibodies, an additional blocking step with Fab anti–rabbit and anti–mouse IgG was performed for 1 h. Subsequently, the second primary antibody was applied overnight at 4°C, followed by peroxidase-conjugated rat anti–mouse IgG_1, IgG2A, or IgM antibody and Vector grey (Vector Laboratories Inc.) as the second color reagent. For triple staining, the blocking steps with 3% H_2O_2/methanol and Fab anti–mouse IgG were followed by application of the avidin/biotin blocking reagents (Vector Laboratories Inc.). The third primary antibody was applied overnight at 4°C, followed by biotinylated rabbit or horse anti–mouse IgG_1 or IgG serum (Zymed Laboratories Inc.), alkaline

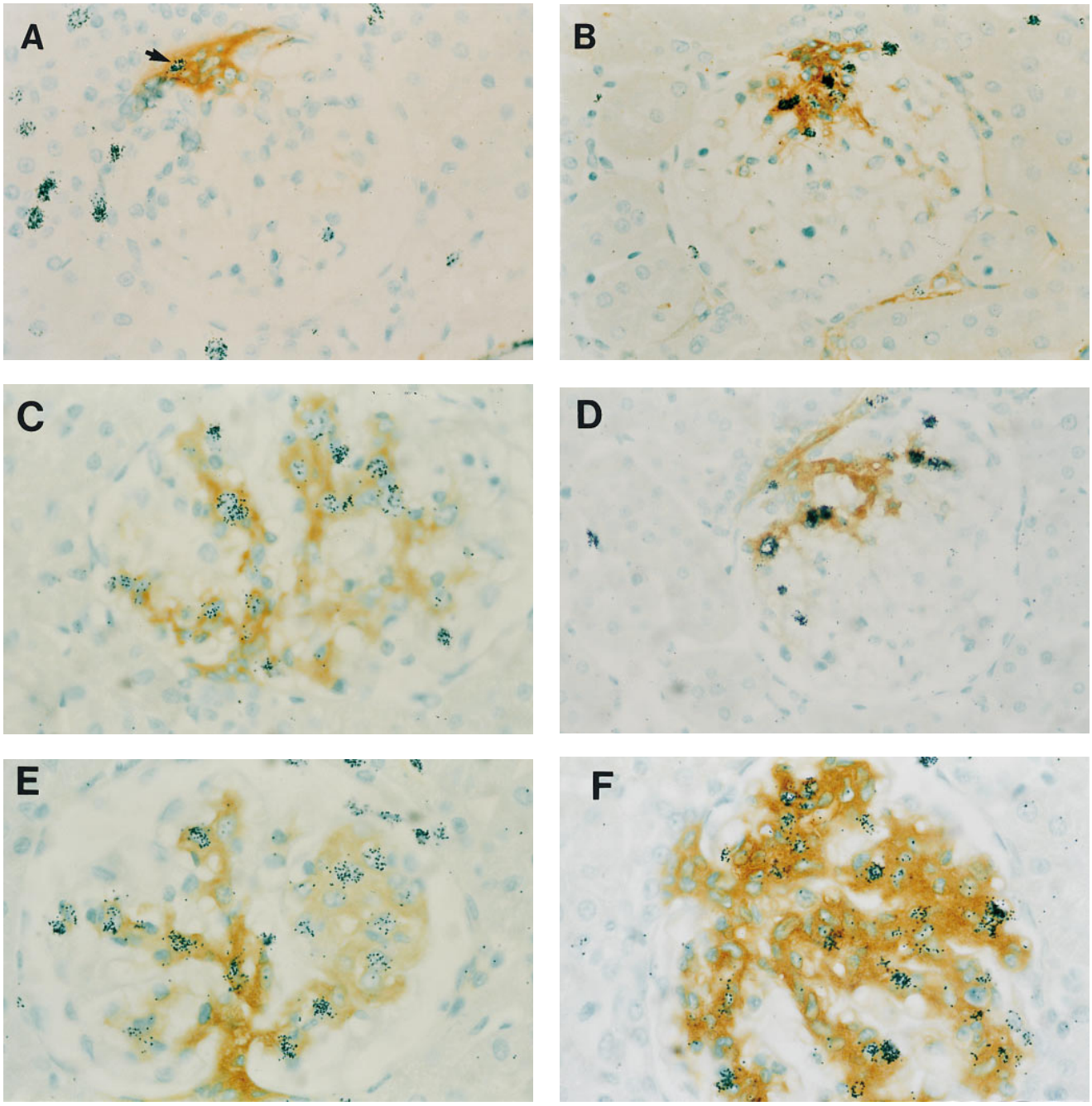

Figure 2. Repopulation of glomeruli in the anti–Thy 1 model occurs through migration of Thy 1-positive 'reserve' cells from the extraglomerular mesangium and hilar area to the periphery of the glomerulus. These pictures show staining for the Thy 1 antigen (OX-7, *brown*) combined with autoradiography for [^{3}H]thymidine incorporation (*grains*). In all pictures, the EGM can be appreciated, in *A*, *B*, and *D* at the top, in *C* and *E* at the bottom, and in *F* at the upper left. (*A*) 36-h [^{3}H]thymidine (*grains*) was present in the nuclei of proliferating extraglomerular MC in the JGA (*arrow*). At this time, only a few [^{3}H]thymidine-labeled MC were positive and they were close to the JGA (*B*). In the same animals on day 5 (after labeling at 36 h), the [^{3}H]thymidine-labeled MC had migrated towards the periphery of the glomerulus while further replicating as evidenced by the increase in labeled MC and a decrease in grain density per cell (*C*). Single pulse labeling with thymidine injections (at 68 h) followed by biopsy 90 min later (*D*) labeled more proliferating MC, which were further away from the hilus compared with the 36-h labeling time point. Biopsies of these latter animals at day 5 (*E*) and on day 7 (*F*) also demonstrated that the labeled MC had migrated further to the periphery of the glomerulus. The almost continuous track of labeled MC from the hilus to the periphery suggests that some MC settle down early after cell division with or without further proliferative cycles while other MC migrate and divide further in the periphery of the glomerulus.

phosphatase–conjugated ABC kit (Vector Laboratories Inc.), and color development with Vector red (Vector Laboratories Inc.) or Histomark Red (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). For some double stainings (OX-7/BrdU for example), the above described step for the third primary antibody (alkaline phosphatase system) was applied for the second primary antibody instead of a second peroxidase system. Controls included omitting either the primary antibodies or replacing them with mouse serum, as well

as omitting either of the secondary antibodies. When autoradiography was combined with staining, the immunostaining was performed first, as previously described (18).

Fluorescent immunohistochemical double staining (as described in reference 12) for the detection of goat anti–Thy 1 antibody (biotinylated rabbit anti–goat antibody; Vector Laboratories Inc.) and MC (OX-7) was performed on 4-μm-thick frozen sections.

Statistical analysis. All values shown are mean±SD. Differences in the distances of labeled cells from the hilus in serial biopsies was determined using the ANOVA test with Bonferroni correction.

Results

Despite an almost complete loss of MC after anti–Thy 1 antibody injection, repopulation of the mesangium occurs within a few days. Injection of goat anti–Thy 1 antibody leads to maximal binding to the Thy 1 antigen on MC within 1 h (19) and to subsequent complement-mediated killing of the MC with the typical features of mesangiolysis on days 1 and 2 after the disease induction (4, 19). Injections with increasing amounts of anti–Thy 1 antibody (0.1–1.2 ml/100 g body wt) led to a progressive increase in the extent and duration of mesangiolysis. At doses of 0.4–0.5 ml/100 g and higher, the normal MC population (∼ 25 Thy 1-positive MC per cross section; Fig. 1 *A*) was almost completely (> 95%) killed by 20–28 h after disease induction, as demonstrated by the loss of intraglomerular Thy 1 staining (Fig. 1 *B*). This true loss of MC could be confirmed by cell counting, by electron microscopy, and by staining with antidesmin antibody, which also detects MC (not shown). From days 2 to 7, the loss of the MC population was replaced by an increasing number of Thy 1-positive MC (Fig. 1, *C* and *D*). This was associated with a progressive decrease in glomeruli that were negative for MC (by OX-7 staining), from 50% at 36 h to 10% at 68 h.

MC repopulation in the anti–Thy 1 model involves migration of Thy 1-positive "reserve cells" from the extraglomerular mesangium and hilar area to the periphery of the glomerulus. By double immunostaining with anti–Thy 1 (OX-7) and PCNA antibodies, the earliest proliferating MC (days 1–3) were localized in the extraglomerular mesangium of the juxtaglomerular apparatus and in the hilar area (Fig. 1 *E*), while proliferating MC later in disease (days 5–7) were frequently localized at the periphery of the glomerulus (Fig. 1 *F*), suggestive of a coordinated movement of MC from the hilus to the glomerular periphery.

To prove that these MC are migrating, a migration assay was developed. The principle is to label cells so they can be tracked within the glomerulus. Because it appeared that the migrating cells were also proliferating, we administered [^{3}H]thymidine as a 'pulse' that would label the proliferating cell population only for the short period (1 h) around the pulse, coupled with immunostaining for Thy 1 antigen to document that the labeled cells were MC in origin. Rats were single pulse labeled with [^{3}H]thymidine at 36 h after anti–Thy 1 antibody injection and were serially biopsied 90 min after labeling and at days 5 and 7. In the 90-min biopsy, the [^{3}H]thymidine- and Thy 1-positive cells were limited to the hilar area and EGM (Fig. 2, *A* and *B*, and Table I). Serial biopsies demonstrated the progressive migration of [^{3}H]thymidine and OX-7-positive MC away from the hilus (Fig. 2 *C*, and Table I). Analysis of the serial biopsies obtained at days 5 and 7 demonstrated that these labeled MC had migrated to the periphery of the glomerulus at a speed of 5–15 μm/d while further replicating as evidenced by an increase in labeled MC number and a decrease in grain number per cell (Table I).

Additional experiments were performed in which the single pulse labeling was done at different times after the anti–Thy 1 injection (between 4 and 76 h). Immunostaining for MC (OX-7 Ab) combined with autoradiography showed that [^{3}H]thymidine injections at 4, 12, or 20 h after disease induction did not lead to any significant [^{3}H]thymidine incorporation by intra- or extraglomerular MC (not shown). Single pulse labeling at earlier times (28–52 h) resulted in the labeling of extra- and intraglomerular MC, which were near the EGM at the 90-min biopsy (Fig. 2 *A*). Single pulse labeling at later time points (60–76 h) resulted in the labeling of more intraglomerular MC that were farther away from the EGM (Fig. 2 *D*, and Table I), whereas fewer extraglomerular MC were labeled compared with earlier times (Table I). [^{3}H]Thymidine uptake by extraglomerular MC started at 28 h after disease induction, peaked at earlier times (36–52 h), and decreased at the late labeling time points (60–76 h). Very few (none) PCNA- and/or BrdU-positive extraglomerular MC could be identified on day 5 (7) of anti–Thy 1 disease. Serial biopsies in the rats pulsed at later times also confirmed the progressive migration of labeled MC to the periphery of the glomerulus (Fig. 2, *D–F*, and Table I).

The late proliferating MC are derived from early proliferating MC. The initial studies suggested that the repopulation of the mesangium involved the migration and proliferation of a

Table I. Absolute Distance of [^{3}H]thymidine-labeled MC from the EGM in Micrometers or Distance after Correcting for Changes in Glomerular Diameter (Measured from the EGM–Hilus Border) as well as Number of Labeled MC and the Percentage of Glomeruli with [^{3}H]thymidine-positive EGM (of Glomeruli with Identifiable EGM) During Anti–Thy Disease

	36-h label			68-h label	
Time of biopsy/time of [^{3}H] thymidine label	37.5 h/36 h	5 d/36 h	69.5 h/68 h	5 d/68 h	7 d/68 h
Distance from EGM (μm)	20.7±10.4	53.7±20.3	38.0±20.1	61.5±24.6	69.6±27.7
Distance from EGM (percent glomerular diameter)	20.0±8.5	57.2±19.1	33.5±13.7	56.3±18.6	64.5±23.3
[^{3}H] thymidine-labeled MC per glomerulus	1.7±0.1	6.8±0.8	3.6±0.5	12.7±2.0	21.8±3.3
Glomerulus with labeled EGM (percent identifiable ECM)	25.2±11.2	ND	18.3±2.1	ND	ND

All the data regarding distance and number of labeled MC on days 5 or 7 are significant ($P < 0.01$) versus the time point of labeling (start, 37.5 or 69.5 h) as well as the comparison of 37.5 vs. 69.5 h at the time of labeling. Data are mean±SD. The SD in regard to the distance measurements reflects the variation on a cellular level and not only on an animal to animal basis.

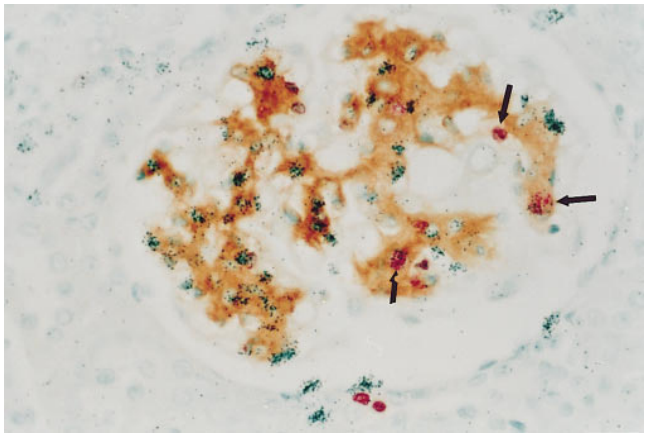

Figure 3. The late proliferating MC are derived from early proliferating MC. Multiple [^{3}H]thymidine injections every 8 h between 30 and 70 h after anti–Thy 1 injection label almost the entire new MC population (OX-7, *brown*) at day 5. BrdU labeling at day 5 demonstrates that most of the proliferating MC (BrdU, *red*) were derived from earlier labeled [^{3}H]thymidine (*grains*) positive MC (OX-7+/BrdU+/[^{3}H]thymidine+, *arrow*).

few MC precursors from the EGM. To determine if the entire MC repopulation could be attributed to this mechanism, multiple [^{3}H]thymidine injections were administered every 8 h to a separate group of rats (based on the average duration of S phase of 8 h). Rats labeled with [^{3}H]thymidine every 8 h between 30 and 70 h after anti–Thy 1 injection resulted in a complete labeling of the MC population on days 5 or 7 in most glomeruli (Fig. 3). In addition, to demonstrate that the MC underwent multiple replications, an additional single BrdU injection was administered on day 5. Proliferating MC at day 5 (BrdU positive) were also positive for the [^{3}H]thymidine label that had been administered 2 d earlier (OX-7+/BrdU+/[^{3}H]thymidine+; Fig. 3).

Consistent with the finding of serial replication of the MC population was the observation in all experiments with multiple [^{3}H]thymidine injections (including experiments with 0.3 and 0.7 ml anti–Thy 1 antibody per 100 g body wt) that the grain density of labeled MC at the first survival biopsy after the last [^{3}H]thymidine injection was higher at the leading edge than of MC in the hilus; while at later biopsies the grain density of peripheral MC was dramatically reduced.

The coordinated repopulation of the mesangium is specific for Thy 1-positive cells. Single, double, or triple immunostaining with cell-specific markers for MC (OX-7), GEN (RECA-1), macrophages (ED-1), renin secreting (renin), or vascular smooth muscle (-like) cells (α-sm actin) combined with autoradiography revealed that this directional migratory flow of cells is specific for Thy 1-positive MC. Macrophages and GEN accounted

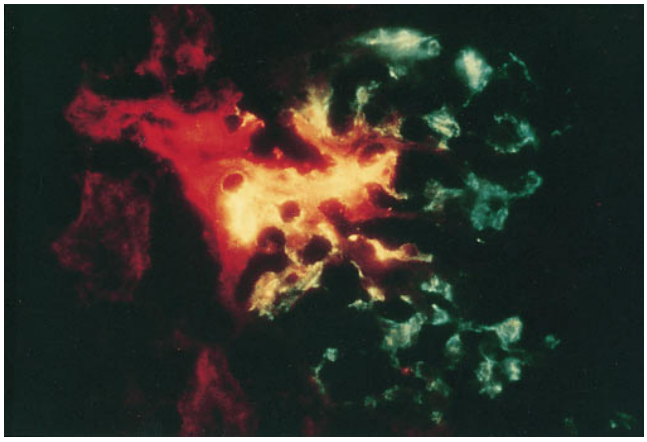

Figure 5. The extraglomerular mesangium is sequestered from the circulation. Double staining (here at 28 h after disease induction) with an anti–goat IgG (FITC, *green*, showing the distribution of the polyclonal goat anti–Thy 1 antibody) and the OX-7 antibody (Texas Red, *red*, marking extra- and intraglomerular MC). At this time, only extraglomerular and hilar MC can be observed (*red*). The disease inducing goat anti–Thy 1 antibody (*green*) is evenly distributed throughout the intraglomerular mesangium, but spares the MC of the juxtaglomerular apparatus (*red*). The yellow staining in the hilar area represents the colocalization of both the injected disease inducing goat anti–Thy 1 antibody and the Thy 1 antigen of the repopulating MC.

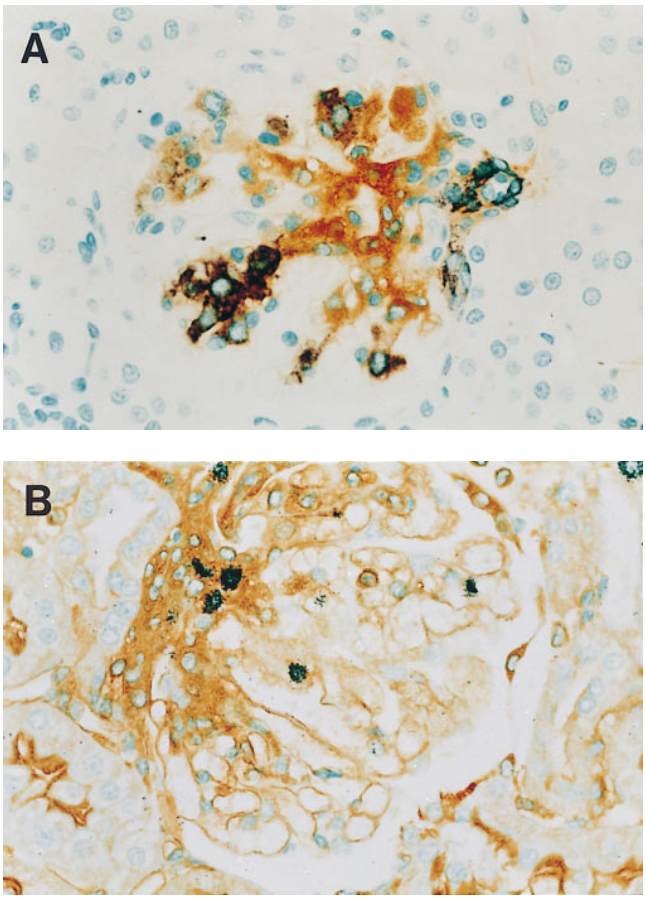

Figure 4. Expression of contractile and migratory proteins (α-sm actin, moesin/radixin) during the process of mesangial repopulation. Double staining for the Thy 1 antigen (MC, *brown*) and α-sm actin (*blue*) in mesangial proliferative nephritis (here at day 4) demonstrates that the cytoskeletal protein α-sm actin is preferentially expressed at the leading edge of the migratory front (*A*). (*B*) The [^{3}H]thymidine labeling (*grains*) of extraglomerular and hilar MC also express moesin/radixin (*brown*). As reported previously (12), moesin/radixin is also expressed by glomerular endothelial cells and parietal glomerular epithelial cells.

for almost all labeled Thy 1-negative cells and were randomly distributed throughout the glomerulus during mesangial repopulation (not shown). [^{3}H]Thymidine labeling of the JGA did not include renin-positive areas and renin staining was usually not seen beyond the hilar region within the glomerulus (not shown). While [^{3}H]thymidine injections between 28- and 44-h labeled Thy 1-positive extra- and intraglomerular MC, the glomeruli did not show any α-sm actin–positive cells at these time points. A few randomly distributed [^{3}H]thymidine- and α-sm actin–positive cells in small and medium sized blood vessels were detected throughout the process of glomerular repopulation with no apparent change in labeling intensity (not shown). Glomerular α-sm actin expression was noted first at 52 h after disease induction in a few Thy 1-positive MC and was preferentially and strongly expressed in MC at the leading edge of the migratory/proliferative front throughout the process of mesangial repopulation (Fig. 4 *A*). Using the cytoskeletal linking proteins moesin/radixin as markers for activated/migratory MC, equivalent kinetics of [^{3}H]thymidine labeling in moesin/radixin-positive extra- and intraglomerular MCs during the process of glomerular remodeling could be observed (Fig. 4 *B*) when compared with the data using OX-7 for identifying MC.

Effect of modulating the degree of mesangiolytic injury. In rats injected with lower doses of anti–Thy 1 antibody, the MC loss was not complete and preferentially involved the periphery of the glomerulus. For example, [^{3}H]thymidine injections in rats with 0.3 ml/100 g anti–Thy 1 antibody demonstrated the same directional flow of proliferating MC from the hilus to the periphery, but the labeled MC started on average from a more peripheral position compared with rats given higher doses of anti–Thy 1 antibody. In these animals, [^{3}H]thymidine uptake by extraglomerular MC was still observed, but fewer glomeruli were involved (positive).

In rats injected with higher doses of anti–Thy 1 antibody, the mesangiolysis was more severe and repopulation of the glomerulus was delayed. In anti–Thy 1 disease induced with 0.7 ml/100 g antibody (for example), the typical wave of migrating MC starting at the hilus and EGM (here more obvious) to the periphery was seen, but occurred in a delayed and slower fashion.

The extraglomerular mesangium is sequestered from the circulation. Double staining (at 4, 12, 20, and 28 h after disease induction) with an anti–goat IgG (visualizing the binding of the goat anti–Thy 1 antibody to MC) and the OX-7 antibody (showing the localization of the extra- and intraglomerular MC) using an indirect immunofluorescent technique demonstrated that the injected anti–Thy 1 antibody did not gain access to the extraglomerular MC of the JGA (Fig. 5). While the anti–Thy 1 antibody appeared equally distributed throughout the intraglomerular mesangium, the MC loss (by OX-7 staining) during mesangiolysis preferentially involved the periphery of the glomerulus (at 4 h) and later involved areas closer to the hilus (12–28 h).

Discussion

The origin of the MC in glomerulogenesis and after injury in glomerulonephritis is unknown. Despite evidence that even severe loss of MC (mesangiolysis) in human and experimental glomerular disease (1–8) can be followed by complete recovery, little is known about the mechanisms responsible for the successful remodeling of the glomerulus. MC migration is likely to be an important part of the coordinated restoration of the glomerular architecture after injury, but has not been shown directly in vivo. This paper describes the first in vivo assay for MC migration, which demonstrated a directional flow of proliferating MC from an extra-/juxtaglomerular site, repopulating the glomerulus in a coordinated fashion after MC injury.

In experimental mesangial proliferative glomerulonephritis in the rat induced by anti–Thy 1 antibody, mesangiolysis (maximal around days 0–2) is followed by MC proliferation (between days 2 and 7), resulting in a transient excess of MC after day 4. It is unknown if this proliferative response originates from randomly surviving intraglomerular MC or from an extraglomerular source. To study the origin and the repopulation of the mesangium after injury, we first sought to eliminate the MC population as completely as possible. Dose–response studies with doses between 0.1 and 1.2 ml anti–Thy 1 antibody/100 g body wt revealed an almost complete loss of the intraglomerular MC staining (OX-7) at 20–28 h after injection of 0.5 ml/100 g or higher. The loss of MC staining was confirmed to be a true loss of MC by cell counting, by electron microscopy, and by staining with antidesmin antibody, which also detects MC.

We then sought to develop a system that would allow us to identify MC early in disease and would allow us to track the migration of these particular MC over time. Double staining for proliferating MC using OX-7 and PCNA antibodies revealed that MC proliferation starts very early after the phase of mesangiolysis. Therefore, [^{3}H]thymidine (or BrdU) seemed to be an excellent marker to track proliferating cells. [^{3}H]Thymidine is quickly absorbed after intraperitoneal injection and is taken up rapidly by proliferating cells during the S phase of the cell cycle and stays with a cell until further replication occurs, which then leads to two equally labeled cells. Its rapid metabolism prevents any further labeling later than 1 h after injection (20). Serial biopsies after single [^{3}H]thymidine injections were used to track the label within the same animals, and combining immunostaining with autoradiography allowed us to identify the specific cell types.

Using this methodology, a directional flow of proliferating MC from the JGA and hilar area towards the periphery of the glomerulus was identified during MC repopulation. The movement of labeled MC was accompanied by further replicative cycles. [^{3}H]Thymidine labeling during MC lysis (at 4, 12, or 20 h) did not result in any significant uptake by MC. Single pulse labeling at 28 h and later, however, identified early proliferating MC at the hilus and JGA, which migrated to the periphery of the glomerulus when examined by serial biopsies at days 5 and 7 (Fig. 2). Whereas single pulse labeling of [^{3}H]thymidine at early time points (28–52 h) after disease induction localized MC close to the hilus at the time of the labeling, later [^{3}H]thymidine pulses (60–76 h) identified more proliferating MC further away from the hilus (Fig. 2, and Table I). At days 5 and 7, these labeled MC had migrated to the periphery of the glomerulus while further replicating, as evidenced by an increase in labeled MC number and decrease in grain number per cell (Fig. 2, and Table I). Measurement of the distance of labeled cells to their apparent origin (border hilus-JGA) demonstrated a consistent movement of MC with an average speed of 10 μm/d throughout the process of repopulation.

The anti–Thy 1 model is a relatively homogenous model in which $>$ 95% of glomeruli are affected. Although the injury is

relatively synchronized, the repopulation of the mesangium appeared to be more variable. Some lobules of glomeruli on day 5 were completely filled with labeled MC out to the periphery, while other lobules were still lacking MC. This pattern is also reflected by the increasing standard deviations of the MC distance measurements over time (Table I). However, when labeled MC were present, they were usually localized in a linear continuity from the periphery to the hilus, suggesting that after replication some MC settle at the site, whereas others continue to migrate to the periphery of the glomerulus.

That the reconstituted MC population was primarily derived from proliferating MC was shown by labeling with multiple [^{3}H]thymidine injections every 8 h between 30 and 70 h, which labeled essentially all of the Thy 1 (OX-7)-positive population (Fig. 3). In the same animals, additional BrdU labeling at day 5 demonstrated that most of the proliferating MC at day 5 were derived from earlier proliferating MC (OX-7+/[^{3}H] thymidine + /BrdU+; Fig. 3) supporting the concept that the MC repopulation involves multiple replication of MC.

The origin of the MC in glomerulogenesis (9–11) and after injury in the adult glomerulus is unknown. For example, it remains unknown whether there is a common precursor cell for GEN and MC (21) that migrates into the immature glomerulus from outside with its origin from primitive pericytes (22) or from metanephric mesenchyme. The transient de novo expression of α-sm actin (a marker for smooth muscle-like cells) in MC during glomerulogenesis (23) and during the proliferative phase in anti–Thy 1 nephritis (14) can be interpreted as the repopulation of the glomerulus with a smooth muscle cell (-like) precursor that finally differentiates into MC or as a transient phenotypical switch of the MC. The results of our current study clearly favor the latter possibility in anti–Thy 1 nephritis. Characterization of the reserve cell of MC repopulation with different cell markers demonstrates that the earliest labeled MC in extra- and intraglomerular positions (between 28 and 44 h) were strictly Thy 1 antigen positive, but α-sm actin and renin negative (data not shown). Therefore, the term 'reserve MC' may characterize these extraglomerular and hilar Thy 1-positive cells most appropriately. Labeled macrophages and glomerular endothelial cells were randomly distributed throughout the glomerulus and did not show a directional movement similar to the Thy 1-positive MC. Expression of α-sm actin by Thy 1-positive MC was seen only after 52 h and was highest in the migratory front during repopulation (Fig. 4 *A*). This finding suggests a role for α-sm actin in movement- or settlement-related (inter-) actions of MC that may well relate to the gradual rebuilding of a new mesangial continuum. Using the migration/activation-related cytoskeletal linking proteins moesin/radixin as MC markers (12), the typical directional flow of migrating MC from the EGM and hilar region could be confirmed (Fig. 4 *B*). Although our study clearly suggests the EGM/hilar region as the source for repopulating MC, our experiments cannot completely exclude the possibility of influx of proliferating Thy 1-positive cells, which migrate from outside the kidney through the hilus into the glomerulus.

How can we explain the pattern of MC repopulation initiating from the JGA and hilus? Helpful insights were provided by the three-dimensional (3-D) reconstruction of the whole mesangium in the rat (24). An ultrastructural 3-D analysis of the normal mesangium in the rat has confirmed a continuity of the mesangium from the vascular pole to the periphery of the glomerulus (24). The reconstructed (3-D) mesangium was divided treelike into 3 lobes and 64 loops. The kinetics and distribution of labeled MC suggests that the MC movement of a few reserve MC from the hilus to the periphery follows this treelike pattern with a coordinated repopulation of lobes/loops of the glomerular mesangium requiring the interplay of settlement, proliferation, and migration (consistent with the above described pattern of the mesangial architecture). This pattern of MC repopulation was also confirmed in animals injected with a lower dose of anti–Thy 1 antibody (0.3 ml/100 g) that does not eliminate the intraglomerular MC completely. It is tempting to hypothesize signaling (or even feedback) mechanisms between the reserve MC and the injured and/or repopulating MC. Interestingly, the kinetics of MC loss in anti–Thy 1 nephritis followed a similar but opposite pattern. MC killing occurred from the glomerular periphery but left the MC within the JGA (and possibly the hilar area) intact. The EGM seemed to be relatively sequestered from the circulation, since the anti–Thy 1 antibody spared this area even at high doses (Fig. 5). However, as some [^{3}H]thymidine labeling of MC in the EGM did occur, presumably there is some exposure of the EGM to circulating substances. The reason why peripheral MC were more susceptible to killing by the anti–Thy 1 antibody is unknown, as the binding of anti–Thy 1 appeared to be evenly distributed within the glomerulus by immunofluorescence (Fig. 5).

The percentage of MC that were derived from the EGM versus the hilar area could not be assessed with certainty, since migration of nonproliferating MC from the EGM to the hilus cannot be detected using this method. Nevertheless, this in vivo migration assay differentiates MC proliferation (assessed by a decrease in grain number and an increase in MC) from MC migration (assessed by the distance of labeled MC from the JGA–hilus border). This in vivo assay may be very helpful in identifying mediators of the migratory response of MC, which may lead to new treatments in MC injury.

In conclusion, we provide the first direct evidence that MC migrate in vivo after glomerular injury. Surprisingly, the repopulation of the mesangium after injury results from the migration of a mesangial-like cell that resides in the JGA. This suggests that the JGA may have the additional function of maintaining the MC population after injury.

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