Podocyte Bridges between the Tuft and Bowman’s Capsule: An Early Event in Experimental Crescentic Glomerulonephritis

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Abstract. Although experimental crescentic glomerulonephritis starts with an endocapillary inflammation, the crescents themselves seem to originate from the proliferation of parietal epithelial cells (PEC). In this study, an attempt was made to disclose a link between the two processes by a morphologic analysis of early stages of the disease. Mice were immunized with rabbit IgG in complete Freund’s adjuvant on day −6. At day 0, they received an intravenous injection of a rabbit anti–glomerular basement membrane serum. On days 3, 6, and 10, the kidneys were fixed by vascular perfusion for examination by light and electron microscopy. On day 3, morphologic alterations affected mainly the endocapillary compartment; most podocytes appeared to be intact. On day 6, alterations of podocytes were widespread, including foot process effacement and prominent microvillus transformation, and some crescents were found. On day 10, crescents were found in 40% of glomeruli. The most surprising finding was podocytes that adhered to both the glomerular basement membrane and the parietal basement membrane, thus forming bridges between the tuft and Bowman’s capsule. Those podocyte bridges were sparse on day 3 but were regularly encountered on days 6 and 10 in glomeruli without crescents and also as a component of crescents. They were interposed between PEC and later between the cells of a crescent without formation of junctional connection with these cells. It is proposed that the spreading of podocytes on the parietal basement membrane represents a lesion of the parietal epithelium and that this process initiates the proliferation of PEC to form a crescent.

A cellular crescent consists of a multilayered accumulation of cells that limit a diseased glomerulus in the place of the simple epithelium of Bowman’s capsule. Concepts about the cellular composition of crescents have evolved along with the available techniques. Three periods may be identified, of which the two first have been reviewed by Jennette and Hipp (1). In early studies, it was postulated on the basis of structural studies that crescents originated from the proliferation of epithelial cells. In a second period, as antibodies against leukocytes became broadly available, many studies demonstrated the presence of macrophages in crescents, and this cell type was for many years the focus of interest. In a third period, it became possible to identify parietal epithelial cells (PEC) by use of antibodies against cytokeratin (1–4) or other antigens (5,6). It then became evident that, in most patients, PEC are the predominant cell type in crescents. The fact that, in some patients, PEC represented only a small proportion of cells in crescents does not necessarily reflect a heterogeneous origin of crescents. Rather, the cellular composition changes with the evolution of the disease. Indeed, Boucher and colleagues (5) found a predominance of PEC in crescents in patients who showed an intact parietal basement membrane (PBM). In contrast, macrophages were the major cell population of crescents in patients in which ruptures of the PBM were detected, which possibly represents a later stage of disease (5). In contrast to clinical studies, animal models of anti–glomerular basement membrane (GBM) glomerulonephritis offer the possibility to analyze the first days of crescent formation. In rats, crescents consisted of epithelial cells as long as the PBM was intact (7). Rupture of the PBM initiated a fibrocellular organization of crescents, with a high incidence of macrophages (7). In rabbits, macrophages were rare in crescents, in spite of their abundance in the endocapillary compartment (8). In mice, the model used in this study, crescents were composed of PEC, whereas macrophages were lacking (6). Increased proliferation of parietal epithelial cells has been measured in anti-GBM glomerulonephritis in the three species (8–11).

The events that trigger the proliferation of PEC in crescentic glomerulonephritis remain obscure. PEC are not in direct contact with the endocapillary compartment in which the inflammatory process is initiated. Furthermore, it is not known how the simple squamous parietal epithelium acquires the specific organization of a cellular crescent. Clearly, that process implicates more than just cell proliferation. Therefore, in this study, we focused on the morphologic alterations of the parietal epithelium in the early stages of crescent formation. To this...
aim, we used a murine model of crescentic glomerulonephritis in which the disease is induced by intravenous injection of an anti-GBM serum in preimmunized mice. The pathology relies on a cellular immune response, with virtually no role of autologous antibodies (12,13). We observed that formation of podocyte bridges between the GBM and the PBM is an early event in this model of crescentic glomerulonephritis.

Materials and Methods

Animals

Female C57BL/6 mice were obtained, specified pathogen-free, and kept in sterilized filter-top cages during the experimental period. Manipulation of the animals was carried out on a sterile bench. The experimental protocol was approved by the Cantonal Veterinary Office of Zurich.

Induction of Anti-GBM Glomerulonephritis

Female mice, 13 wk old, were immunized by subcutaneous injection of 0.2 mg of rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.2 ml of a 1:1 emulsion with complete Freund’s adjuvant (Sigma, St. Louis, MO). Six days later (day 0), glomerulonephritis was induced by intravenous injection of 0.4 ml of a 1:10 dilution of rabbit anti-mouse GBM serum (14). Urinary protein concentration was evaluated daily by use of dipsticks (Albusix; Miles, West Haven, CT).

Fixation and Tissue Processing

Mice were anesthetized with 17 mg/kg body weight xylazine hydrochloride and 50 mg/kg body weight ketamine hydrochloride, intraperitoneally. Kidneys were fixed by vascular perfusion via the abdominal aorta. The fixative consisted of 3% paraformaldehyde (PFA) and 0.05% picric acid. It was dissolved in a 3:2 mixture of 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mOsm with sucrose) and 10% hydroxyethyl starch in saline (HAES; Fresenius AG, Germany). After 5 min of fixation in situ, the kidneys were removed and cut into coronal slices. Some slices were embedded in paraffin. The remaining tissue was immersed for at least 24 h in the 3% PFA fixative solution, to which 0.5% glutaraldehyde was added. Thereafter, the tissue was postfixed in 1% OsO4 and embedded in epoxy resin.

Light Microscopy and Transmission Electron Microscopy

Light microscopic investigations were carried out on series of 100 sections of 1-μm thickness, cut from epoxy resin-embedded tissue and stained with azure II–methyamine blue. Additionally, series of 16 sections of 5-μm thickness were cut from paraffin-embedded tissue and stained with silver-metheneamine for examination of the basement membranes. For transmission electron microscopy (TEM), ultrathin sections from epoxy resin-embedded tissue were contrasted with uranyl acetate and lead citrate.

Immunofluorescence Microscopy

Collagen type IV was detected using a rabbit polyclonal antibody (Progen Biotec, Heidelberg, Germany) on 1 μm cryosections. The second antibody was goat anti-rabbit Cy3 (Rockland, Gilbertsville, PA). For fibrin detection a goat anti-mouse fibrinogen antibody was used (Nordic Immunologicals, Tilburg, The Netherlands), followed by donkey anti-goat FITC (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania).

Immunohistochemistry

Paraffin sections of 3-μm thickness were rehydrated in alcohol series and finally in phosphate-buffered saline (PBS). For detection of macrophages, we used rat anti-mouse antibodies: anti-MHC class II (clone M5/114,15,2) and anti-F4/80 (clone CI:A3-1). Podocytes were identified with rabbit polyclonal antibodies against WT-1, CD2AP (both Santa Cruz Biotechnology, Santa Cruz, CA), α3 integrin (Chemicon International Inc., Temecula, CA), synaptopodin, and podocin (both gifts from Dr. P. Mundel, Albert Einstein College of Medicine, New York; a manuscript concerning the characterization of podocin, by Schwarz K. et al., has been submitted for publication). Before the rabbit antibodies were used, the sections were submitted to 5 × 5 min microwaving at 600 W. Detection was carried out with Vectastain ABC kits (Vector Laboratories, Burlingame, CA) with the use of peroxidase as label and diaminobenzidine as substrate.

Results

As found previously in this model (13,14), proteinuria started on day 2 after injection of the anti-GBM serum. On day 3, many glomerular capillary loops displayed marked alterations in the endocapillary compartment. On day 6, a broad array of histologic alterations, ranging from almost normal histology to fully developed cellular crescents, were seen among glomeruli of the same kidney. On day 10 after the application of the serum, profound alterations were seen in virtually all glomeruli, and about 40% of them exhibited crescents.

Histopathology of the Glomerular Tuft

A vivid endocapillary inflammation was encountered in many glomeruli on day 3, but, at that time, the majority of capillary loops still appeared healthy. On day 6 endocapillary injuries were more widespread, and on day 10 only few loops displayed a normal structure. Qualitatively however, morphologic alterations of the tuft remained similar from day 3 to day 10.

Because of widespread mesangiolysis, mesangial spaces were dramatically expanded. Subendothelial spaces created by detachment of endothelium from the GBM contained variable amounts of a proteinaceous material (hyaline), cell processes of mesangial cells (which frequently had lost contact with the GBM), and inflammatory cells (Figures 1 through 3). In TEM, sparse filaments of fibrin could be detected amid of hyaline material; conspicuous accumulation of fibrin was found in only few capillary loops in TEM as well as by immunofluorescence (not shown). Adhering mononuclear cells and neutrophils were seen in the capillary lumina (Figures 1 and 2). Although the GBM was often attenuated over extended areas, we encountered sites of complete dissolution in only two instances. Some glomeruli displayed an endocapillary necrosis of most or even all loops. The most striking alteration of podocytes was the formation of villi and clublike protrusions of the luminal cell domain (microvillous transformation) (Figures 1, 3, and 4). At day 10, the vast majority of podocytes were affected. Simplification of the foot process pattern up to foot process effacement was also widespread (Figures 1 through 3); the remaining interdigitating podocyte cell processes were connected either through slit membranes or, more frequently, by tight junction-
like structures. In addition, podocytes frequently contained large intracytoplasmic vacuoles; some microcysts were also observed. In heavily damaged loops, circumscribed detachment of podocytes from the GBM also occurred. The subepithelial space created by detachment appeared to be empty or it contained basement membrane–like material (Figure 1). Of note, the continuity of the podocyte layer along the GBM was preserved even in the most heavily damaged glomeruli.

**Cell Bridges between the Tuft and Bowman’s Capsule in Glomeruli without Crescents**

In the 1-μm section series, we traced glomeruli with respect to alterations that might represent early stages of crescent formation. Thereby, we observed, as the earliest structural change, already found on day 3, cellular bridges between the tuft and Bowman’s capsule. These bridges consisted of one or a few epithelial cells that were attached to both the GBM and the PBM (Figures 2 and 3). From their structure, most notably from the presence of foot processes at the tuft side, these bridging cells appeared to be podocytes. At the capsular side, their cell processes, displaying manifold elaboration, were interposed between PEC and attached to the PBM (Figure 2). Cell-to-cell contacts were rarely found between these bridging cells and PEC; in most cases gaps were seen between them, which indicates the absence of junctional belts (Figure 2). The processes of the bridging podocytes often covered large areas of the PBM (Figure 2). Frequently, they extended between parietal cells and the PBM (Figure 4A).

The bridging cells were often assembled in groups (Figure 4E), enclosing extracellular spaces that were filled with basement membrane–like material (Figure 3), which showed immunoreactivity for collagen type IV (Figure 5). Because these spaces began at the GBM and extended to the PBM, they might represent extracellular routes for diffusion of substances from the capillaries to the PBM.

By means of immunocytochemistry, we could show that the cells forming the bridges between the tuft and Bowman’s capsule were positive for WT1 (Figure 6A), integrin α3 (Figure 6B), synaptopodin (Figure 6C), podocin (Figure 6D), and CD2AP (Figure 6E), as were the podocytes of the tuft; parietal cells were negative for these markers.

In several glomeruli that presented podocyte bridges, we encountered cells within the parietal epithelium that contained mitotic figures (Figure 7). Mitotic figures in PEC are extremely rare in healthy kidneys.

![Figure 1. Injured capillary loops (day 10) (transmission electron microscopy [TEM]). In the two loops on the right, the endothelium has detached from the glomerular basement membrane (GBM); an inflammatory cell is visible in the subendothelial space. The third loop is necrotic and filled with inflammatory cells (∗). Flattened foot processes of podocytes show densely packed microfilaments (†), and the apical cell portions display microvilloous transformation (→). C, capillary lumen; PE, parietal epithelium.](image-url)
Structure of Crescents

Crescents were generally separated from the interstitium by a continuous PBM. At day 10, a rupture in the PBM was found in only 5 of 136 crescentic glomeruli examined on 5-μm-thick serial sections stained with silver-methenamine. In our hundreds of TEM pictures, we encountered in only one instance a rupture of the PBM.

The morphology of most cells in the crescents was compat-
ible with an epithelial origin; however, junctional complexes could not be detected. Crescents often contained accumulations of cell debris, generally in the vicinity of the PBM. Fibrin was not detected in crescents, neither by TEM nor by immunofluorescence. The cells were separated from each other by abundant basement membrane–like material that mostly was in continuity with the PBM (Figure 8) and showed immunoreactivity for collagen type IV (not shown). Because the cells of the innermost layer of a crescent were also not connected by junctions, the intercellular spaces of a crescent communicated with Bowman’s space (Figure 8).

Immunostaining with anti-MHC class II and F4/80 antibodies revealed that macrophages represented only a small fraction of cells in a crescent. Many crescents showed no immunoreactivity at all (Figure 6F). Likewise, immunoreactive cells were rare in Bowman’s space. It must be stressed that immunoreactive cells in the extracapillary compartment are not necessarily macrophages, given that podocytes in injured glomeruli may express macrophage markers (15). In contrast to bridging cells and podocytes on the tuft, the vast majority of cells within a crescent were immunonegative for WT-1, synaptopodin, CD2AP, α3 integrin (not shown), and podocin (Figure 6D), which indicates that they were not podocytes.

Quantification of Cellular Bridges

Serial sections across complete glomeruli were carried out, to obtain insights in the frequency of cellular connections between the tuft and Bowman’s capsule. Fifty-nine glomeruli were followed in three series of semithin (1 μm) sections, each series derived from a different animal killed at day 10. The
results (Table 1) show that cellular bridges are common before crescents have developed and are a consistent attribute of later stages, when crescents are present. Twenty-eight glomeruli in section series from two control mice did not reveal any connection between the tuft and Bowman’s capsule.

Periglomerular Interstitial Response

Glomeruli with an apparently intact parietal epithelium, i.e., glomeruli without tuft adhesion or crescent, generally displayed no or little periglomerular inflammation. In contrast, crescents were associated with abundant periglomerular inflammatory cells (Figures 6F and 8). There was often a striking spatial association between a crescent and an infiltrate (Figure 6F). Focal inflammatory reactions were also often seen in the periglomerular interstitium in the vicinity of podocyte bridges. Infiltrates comprised macrophages (Figure 6F), T cells (CD3+, not shown), and few neutrophils. In infiltrated areas, curtain-like cytoplasmic processes of fibroblasts covered the lesion from outside. The crescent and the periglomerular reaction were usually separated from each other by an intact PBM; as described above, disruptions of the PBM were rare.
Discussion

This study dealt with the early stages of crescent formation in a murine model of crescentic glomerulonephritis. The organization of crescents was similar to that reported for other models of the crescentic glomerulonephritis in mice (6,16,17), as well as for early stages of the crescentic glomerulonephritis in humans (1,5,18,19). Most of the cells in early crescents are likely of epithelial origin, derived from proliferation of PEC (2,3,8–11,20). They are embedded in a basement membrane–like material without being joined to neighboring cells by junctions. As was shown previously in experimental models, crescentic glomerulonephritis starts as an endocapillary inflammation (8,9,14,21). In this study, the inflammation was vividly flourishing 3 d after the initiation of the disease. Crescents were encountered after 6 d, and after 10 d they had developed in about 40% of glomeruli. It is an open question how these two processes, endocapillary inflammation and crescent formation, are related to each other.

The essential new finding of this study was that podocytes frequently form bridges between the tuft and Bowman’s capsule.
Podocyte Bridges in Glomerulonephritis


The effects of manipulating the activities of proteins of the proinflammatory cytokine tumor necrosis factor (TNF) increases the paracellular permeability of epithelia and decreases the response to any challenge), podocytes exhibit (which is the most common change seen in podocytes in crescents) in anti-GBM glomerulonephritis in mice (14). It is not likely that disruption of junctional complexes occurs spontaneously in inflamed glomeruli, given that we never found bare areas of PBM.

The rupture of junctions in the parietal epithelium at the site of intrusion by podocytes might play a central role in the proliferation of PEC and the organization of the crescent. Molecular links between junctional complexes and the pattern of growth in epithelia have been recently disclosed. Oncogene Raf-1 induces the loss of both tight junctions and contact inhibition of growth in a parotid epithelial cell line. Introduction of exogenous occludin in Raf-1–activated cells not only restores the tight junctions in monolayers but also suppresses growth in soft agar (29). Functional inhibition of the E-cadherin/catenin complex suppresses contact inhibition of motility in a mammary carcinoma cell line (30). In Drosophila ovary epithelium, a tight molecular link has been disclosed among junctional belts, cell polarity, and cell proliferation (31). PEC adjacent to podocytes rupturing into the parietal epithelium are in a situation similar to cells at the margin of the lesion of a monolayer in in vitro models of wound healing. Their edge that faces the lesion has lost junctional contacts. This induces the formation of lamellipodia and a crawling behavior (32). The decreased stability of microtubules at the noncontacting edges probably contributes to this phenomenon (33). The cells behind the first row also participate in the generation of force for closure of the defect (32), and they are induced to proliferate (34). In crescentic glomerulonephritis, restoration of the integrity of the parietal epithelium cannot be achieved, because processes of podocytes are interposed between PEC. The combined stimulation of proliferation and migration may, instead, end in crescent formation.

Proteins required for the adhesion to basal membranes are normally localized in the basolateral membrane domain (35). Thus, the adherence of podocytes with part of their luminal membrane to the PBM quite obviously reflects a loss of cell polarity. The positive staining of those cells with α3 integrin alongside the PBM clearly shows the erroneous sorting of a basolateral membrane protein to a portion of the former luminal membrane. The loss of cell polarity may also explain the secretion of extracellular matrix material by apical membrane domains of podocytes; such material is consistently encountered in the niches between bridging podocytes. Moreover, the fact that bridging podocytes do not establish junctions to neighboring PEC suggests that parietal cells at those sites have also lost polarity.

A spatial association between a crescent and a periglomerular inflammation has been observed previously (36), and it was confirmed in this study. This suggests that inflammatory mediators from inside the glomerulus have diffused across the PBM, triggering the interstitial response. The open intercellular spaces between bridging podocytes and PEC provide continuous diffusional pathways from Bowman’s capsule to the periglomerular interstitium. In addition, matrix spaces between bridging podocytes, starting at the GBM and extending into the extracellular spaces within a crescent, might represent a direct diffusion pathway from the endocapillary compartment to the glomerular interstitium.

Figure 7. Mitotic parietal epithelial cell (day 10) (light microscopy). The mitotic figure is adjacent to a bridge that contains a total of four bridging podocytes as identified in the series of semithin sections.
coagulation system in experimental anti-GBM glomerulonephritis have been considered an evidence for a stimulatory role of fibrin in the formation of crescents (37,38). However, because those proteins can promote inflammation independently of fibrin deposition (39,40), the role of fibrin itself in glomerulonephritis has still to be defined. In animal models (8,9,37,41,42) and in human crescentic glomerulonephritis (18,43), necrotizing glomerular lesions, together with breaks in the GBM and exocapillary fibrin deposits, have been observed early in the disease course. In contrast, in this study and a previous study of murine crescentic glomerulonephritis (6), fibrin was found neither in Bowman’s space nor in crescents but was confined to the endocapillary compartment. This discrepancy may be due to the fact that, in this model, the disease advanced slowly (proteinuria did not start before day 2), with a low incidence of GBM breaks and an absence of loop necrosis; thus, the conditions for a leakage of fibrinogen/fibrin into Bowman’s space were simply not established. Nevertheless, crescents did develop in these mice, which suggests that fibrin deposition in Bowman’s space is not necessarily a prerequisite for their development. In this respect, the rather slow and mild evolution of the disease with preservation of the glomerular architecture in early stages of crescent formation provides an opportunity to dissociate the relevance of the various exocapillary factors associated with crescent formation.

In two previous studies on anti-GBM glomerulonephritis in mice, macrophages could not be identified in crescents (6,36).

Table 1. Frequency of cellular connections of the tuft with either BC or a crescent

<table>
<thead>
<tr>
<th>Glomeruli (n = 59)</th>
<th>Frequency (%)</th>
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<tbody>
<tr>
<td>With crescent</td>
<td></td>
</tr>
<tr>
<td>with connection between tuft and crescent</td>
<td>22 (100)</td>
</tr>
<tr>
<td>with cell bridge between tuft and BC outside crescent</td>
<td>20 (90)</td>
</tr>
<tr>
<td>Without crescent</td>
<td></td>
</tr>
<tr>
<td>with cell bridge between tuft and BC</td>
<td>37 (62.7)</td>
</tr>
<tr>
<td>without cell bridge</td>
<td>19 (51.4)</td>
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“Three series of 100 sections (1-μm epon sections), from the three animals killed at day 10, have been evaluated. These series contained 59 complete glomeruli. In addition, a large number of cell bridges between the tuft and Bowman’s capsule (BC) or a crescent were encountered by light and transmission electron microscopy in glomeruli of all experimental animals.

coagulation system in experimental anti-GBM glomerulonephritis have been considered an evidence for a stimulatory role of fibrin in the formation of crescents (37,38). However, because those proteins can promote inflammation independently

Figure 8. Ultrastructure of crescentic glomeruli (day 10) (TEM). (a) Three adhesions of the tuft to the crescent are visible (circles). Aside from the adhesions, the urinary space is open, although very narrow. There is a conspicuous interstitial inflammation all around the glomerulus. (b) A podocyte is partly embedded in the crescent and thus mediates a tuft adhesion. The cells in the crescents are not linked by junctions. The extracellular matrix consists of sheaths of basement membrane–like material (*). It communicates with Bowman’s space (†) and with the PBM (‡). a, afferent arteriole; Po, podocyte.
In this study, the incidence of cells labeled with F4/80 or anti–MHC class II was very low in most crescents and in Bowman’s space. Also, we found by TEM that cells with a morphology suggestive of a macrophage in crescents were rare. Thus, accumulation of macrophages in Bowman’s space does not appear to be at the origin of crescents in this model. Because macrophages infiltrated the tuft and the periglomerular interstitium, the GBM and the PBM appear to efficiently

Figure 9. Development of a crescent shown in schematic drawings. In (a through d), the right half of the glomerulus shows the morphology of a healthy glomerulus; the left half shows pathological alterations observed in anti-GBM glomerulonephritis. Only alterations shown in a were found on day 3. All alterations shown in Panels a through d could be found on days 6 and 10. (a) Inflammatory cells (violet) infiltrate the tuft. The endothelium (purple) in two capillary loops has detached from the GBM. A cell process of a podocyte has intruded between PEC and established a bridge between the GBM and the PBM (basement membranes appear as black lines). A diffusion pathway exists from Bowman’s space toward the periglomerular interstitium, where an interstitial infiltration (green) composed of fibroblasts and inflammatory cells starts to develop. (b) The podocyte bridge involves two cells. The periglomerular inflammation is conspicuous. A mitotic figure is seen in a PEC. (c) A continuous network of basement membrane–like extracellular matrix (yellow) fills the space between bridging podocytes, between bridging podocytes and PEC, and between processes of PEC that have lost contact the PBM. The rupture of junctions between foot processes opens a matrix route from the endocapillary compartment toward the periglomerular interstitium. (d) The lesion displays the characteristic aspect of an early crescent.
limit the access of inflammatory cells to Bowman’s space. Actually, breaks in either of the two basement membranes were rare in this study. It is likely that high incidences of macrophages in crescents, as often reported in human crescentic glomerulonephritis, reflects the rupture of basement membranes in advanced stages of the disease (5). This may apply for fibrin as well.

It is obvious that the proposed role of podocytes in crescent formation is, to some extent, the reverse of what is seen in the formation of tuft-to-capsule adhesion in classic focal segmental glomerulosclerosis (44,45). In focal segmental glomerulosclerosis, the degeneration and finally the loss of podocytes represent the decisive event that permits the affixation of parietal cells to a naked site of GBM, whereas, in crescentic glomerulonephritis, the podocytes seem to be in an hyperactive state, which allows them to break through the parietal epithelium and to attach to the parietal basement membrane. There is no indication of a loss of podocytes in early stages of crescentic glomerulonephritis.

This study has uncovered a novel type of lesion in crescentic glomerulonephritis. In response to an endocapillary inflammation, podocytes appear to undergo specific cellular changes that cause them to break through the parietal epithelium and to attach to the PBM. This represents a potentially important pathogenic step in the development of a crescent.

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