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LABORATORY INVESTIGATION

Localization of clusterin in the epimembranous deposits of passive Heymann nephritis

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Localization of clusterin in the epimembranous deposits of passive Heymann nephritis. The membrane attack complex of complement (MAC) plays an important role in the mediation of proteinuria in experimental membranous nephropathy induced by Heymann antiserum. SP-40,40 is a recently described serum protein which appears to inhibit the formation of cytolytic MAC in a manner analogous to S protein/vitronectin. SP-40,40 is homologous to proteins originally isolated from rat and ram seminal fluid (sulfated glycoprotein 2 and clusterin, respectively). By current convention, these proteins are considered clusterin homologues. The objective of this study was to examine the participation of rat clusterin in passive Heymann nephritis. Using an antibody to rat clusterin as an immunofluorescent probe, clusterin deposits were demonstrated along the glomerular capillary wall in an identical pattern to rat C3 and C5b-9. Decomplementation using cobra venom factor prevented proteinuria and intraglomerular MAC formation. The epimembranous clusterin were not detected in the complement-depleted animals. The role of clusterin in the mediation of glomerular injury remains unknown, but it is probably related to in situ formation of the terminal complement cascade where it may play a regulatory role.

The membrane attack complex (MAC) of complement participates in the mediation of certain types of glomerular injury. Its role has been most convincingly demonstrated in the experimental model of passive Heymann nephritis (PHN) [1–6]. Immunofluorescence studies of human renal biopsies have also confirmed the presence of MAC both in association with glomerular immune deposits and in regions of glomerular sclerosis [reviewed in 2].

The MAC consists of the terminal complement components C5b, C6, C7, C8, and C9 and it may be generated in one of two ways [7]. The C5b-7 complex, which is amphiphilic, may insert into the lipid bilayer of the cell membrane, followed by the addition of C8 and multiple C9 molecules to form the cytolytically active C5b-9(m). Alternatively, S protein/vitronectin may bind to C5b-7 resulting in the formation of a hydrophilic, nonlytic SC5b-9 complex which remains in the fluid phase. S protein/vitronectin thus plays an important role as an inhibitor of C5b-9(m) formation and may function in vivo to regulate end-organ injury.

Recently another protein was identified within the human SC5b-9 complex and shown to be an inhibitor of the hemolytic activity of complement in vitro. SP-40,40 [8, 9], or complement cytolysis inhibitor (CLI) [10], is a serum alpha-globulin of 80 kD molecular mass which consists of two distinct, disulfide-linked, polypeptide chains each of 40 kD. The entire molecule is the product of a single gene [9]. This original work by Murphy and his colleagues [8, 9] has been confirmed by others [10]. Based on amino acid sequence analysis, the rat homologue is sulfated glycoprotein 2 (SGP-2) [11]. Rat SGP-2 in turn has high homology with ram clusterin [12, 13]. SGP-2 was first identified in the supernatant of primary cultures of rat Sertoli cells [14], and clusterin was first identified in ram rete testis fluid [15] from which it was isolated [16]. These reproductive tract proteins are also present in plasma. The role of clusterin in the complement cascade has been inferred by homology but has not yet been proven in vivo [9, 17]. Still under active investigation, this group of homologous proteins, referred to hereafter as clusterin by recent concensus [17], may inhibit the cytolytic activity of MAC by binding to C5b6 [8, 9, 18], or by combining with the nascent C5b-7 complex [10, 19], to inhibit the insertion of C5b-7 into cell membranes. Although the biological role of clusterin in vivo is unknown, the demonstration of SP-40,40 in human renal biopsies in a pattern similar to MAC and S protein [8, 20], lends to speculation that it may play an important regulatory role.

The purpose of this study was to examine the participation of clusterin in a MAC-dependent experimental model of glomerular injury, PHN.

Methods

Animals

Female Lewis rats weighing 100 to 120 g were purchased from Charles River Breeding Laboratories (Wilmington, Massachussetts, USA). They were fed standard rat chow and given free access to water.

Induction of passive Heymann nephritis

PHN was induced with sheep immune serum raised against rat brush border antigens (Heymann antiserum, provided by Dr. B. Noble, State University of New York at Buffalo, Buffalo, New York, USA) [21]. Each animal was given a single intraperitoneal injection of 11.1 mg sheep IgG/100 g body weight. On day 6, the rats were housed individually in metabolic cages to obtain 24-hour collections of urine. Urinary albumin excretion rates were quantitated by radial immunodiffusion, as described in a previous study [22]. The animals were

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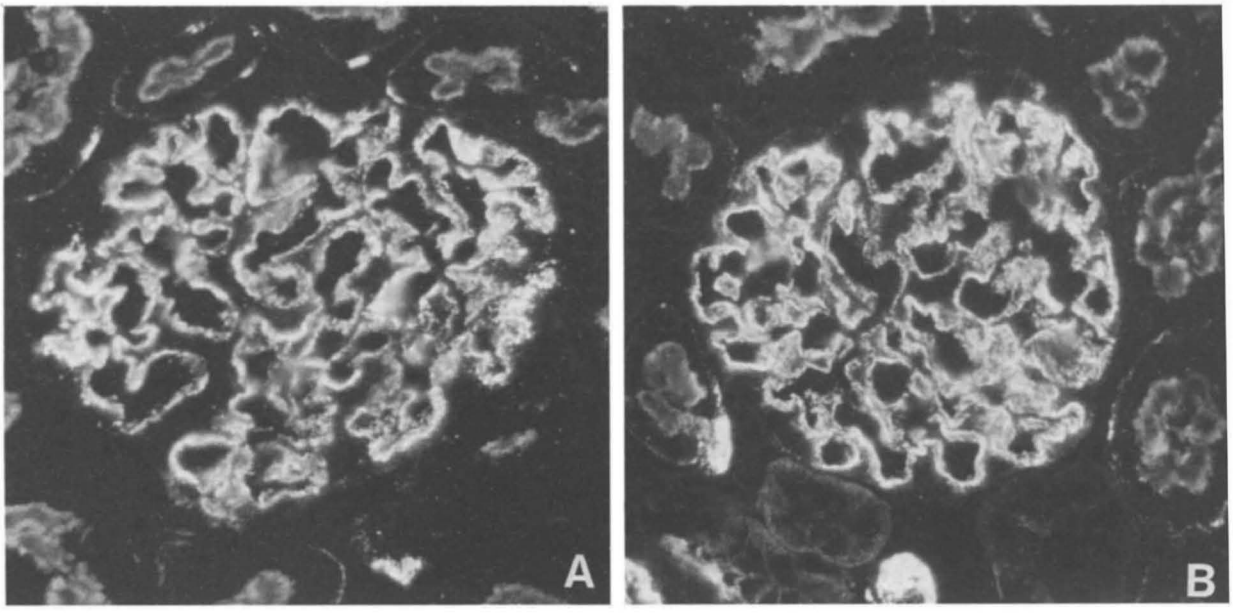

Fig. 1. *Immunofluorescence photomicrographs illustrating the glomerular deposition of sheep anti-rat brush border antibody (IgG).* The pattern and intensity of straining was similar in complement-sufficient (**A**) and decomplemented animals (**B**) 7 days after administration of the antiserum (A × 505; B × 570).

sacrificed on day 7 by complete exsanguination under general anaesthesia with enflurane and nitrous oxide. The kidneys were harvested immediately. Pieces of renal cortex were snap-frozen in 2-methylbutane, precooled in liquid nitrogen and stored at −70°C.

Decomplementation

One group of rats ($N = 6$) was depleted of complement using cobra venom factor (Cordis Laboratories, Miami, Florida, USA). Complement depletion was induced the day prior to the administration of Heymann antiserum by the i.p. injection of CVF (300 U/kg) in three equal doses over 24 hours. Complement depletion was maintained by single daily doses of CVF, 125 U/kg on days 0, 1, 2 and 150 U/kg on days 3 to 6. Results were compared to a group of complement-replete rats ($N = 5$). Complement depletion was verified by completely negative staining of glomeruli for rat C3 and by a 81 to 91% reduction in serum total hemolytic complement (CH50) levels compared to levels obtained using pooled normal rat serum. The effect of decomplementation on the renal distribution of clusterin in normal rats was evaluated using the same CVF protocol. The kidneys of normal rats were examined following 24 hours ($N = 2$) and 3 days ($N = 2$) of complement depletion. CH50 levels in this group of rats were reduced by 97 to 99% of baseline values measured prior to CVF administration. The CH50 assay was performed according to the method of Kabat and Mayer [23]. For this assay, sheep erythrocytes were obtained from Woodlyn Laboratories, Guelph, Ontario, Canada, and rabbit anti-sheep hemolysin from Gibco Laboratories, Burlington, Ontario, Canada.

Immunofluorescence studies

Sections of renal cortex (3 μ thick) were acetone-fixed and stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat C3, and FITC-conjugated rabbit anti-sheep IgG (Organon Teknika, Cappel Division, West Chester, Pennsylvania, USA). By indirect immunofluorescence, sections were stained for rat C5b-9 using a murine monoclonal anti-rat C5b-9 antibody [24] (provided by Dr W. Couser, University of Washington, Seattle, Washington, USA), followed by FITC-conjugated goat anti-mouse IgG $F(ab')_2$ (Tago, Inc., Burlingham, California, USA). Deposition of rat clusterin was assessed by indirect immunofluorescence staining using a rabbit antiserum (provided by Dr. M. Griswold, Washington State University, Pullman, Washington, USA) which was originally published as anti-rat dimeric acidic glycoprotein (DAG-protein) antibody [25], followed by FITC-conjugated goat anti-rabbit IgG (Organon Teknika). DAG-protein was initially isolated from primary cultures of rat Sertoli cells and has been renamed sulfated glycoprotein 2 (SGP-2) [11]. It is the rat homologue of clusterin [12, 13]. The specificity of anti-DAG antiserum was demonstrated in the original publication [25] both by immunoprecipitation and Western blot analysis using supernatants of cultured rat Sertoli cells. Specific reactivity with the two polypeptide monomers of DAG was demonstrated. The original paper also reported a minor cross reactivity with Band 4 protein, but this minor band was subsequently shown to be due to reactivity with the precursor SGP-2 molecule and not Band 4 protein [11]. The FITC-conjugated antisera used in the indirect immunofluorescence studies were preabsorbed with normal rat plasma and shown to be non-reactive with control kidney sections.

Results

Decomplementation prevented the development of albuminuria following the injection of Heymann antiserum. The mean urinary albumin excretion rate on day 6 was 58.5 ± 18.1 mg/100 g body weight for the animals with an intact complement cascade compared to a mean of 0.8 ± 0.3 mg/100 g body weight

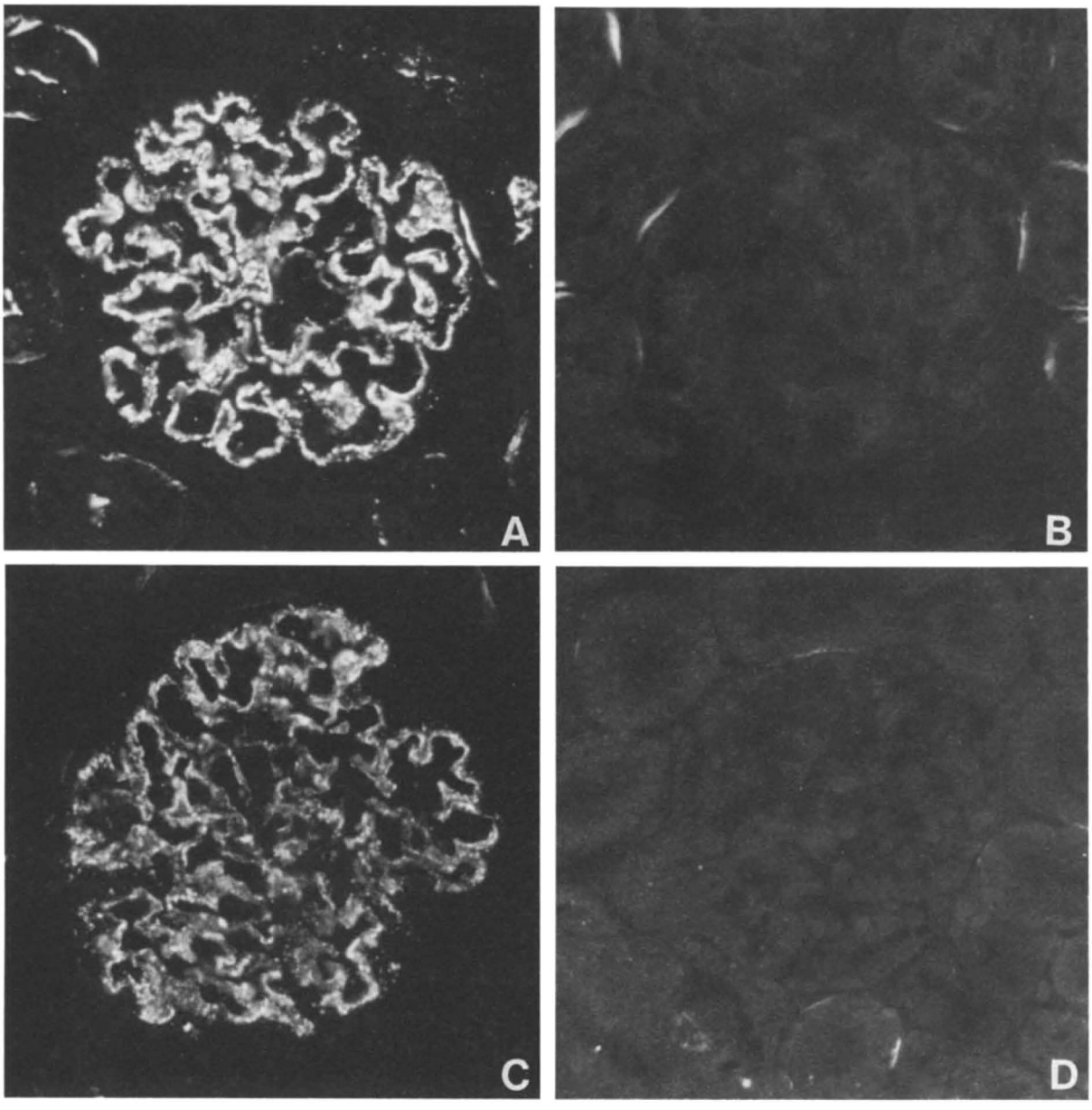

Fig. 2. *Immunofluorescence photomicrographs illustrating the glomerular deposition of complement proteins in animals with passive Heymann nephritis (day 7).* In normal animals, bright granular deposits of rat C3 (**A**) and rat C5b-9 (**C**) were present along the glomerular capillary wall. CVF treatment depleted serum complement and completely inhibited the glomerular deposition of C3 (**B**) and activation of the terminal complement cascade, C5b-9 (D) (A × 500; B × 495; C × 525; D × 490).

in the CVF-treated animals ($P < 0.001$, Student's *t*-test for independent means).

The pattern and intensity of glomerular staining for sheep IgG was similar in both groups (Fig. 1). There were diffuse granular deposits (3 to 4^+ intensity) along the glomerular capillary walls. In the complement-sufficient animals, rat C3 and rat C5b-9 were present in a similar pattern (3^+ intensity) (Fig. 2). In the CVF-treated animals, glomerular staining for rat C3 and rat C5b-9 was completely negative (Fig. 2). In normal rats, clusterin was present in a faint linear pattern along the epithelial aspect of the glomerular capillary wall reflecting focally into mesangial regions (Fig. 3). This pattern was also observed in normal rats depleted of complement by CVF. Focal discontinuous deposits were also present along some tubular basement membranes, Bowman's capsule and the adventitia of arterioles. This pattern was preserved in both experimental groups, although the intensity of staining was significantly reduced in the CVF-treated group. In the experimental animals with an intact complement cascade, clusterin was also present in a granular pattern (1 to 2^+ intensity) along the glomerular basement membrane, similar in distribution to the sheep IgG, rat C3 and rat C5b-9. The pattern of staining for clusterin in the CVF-

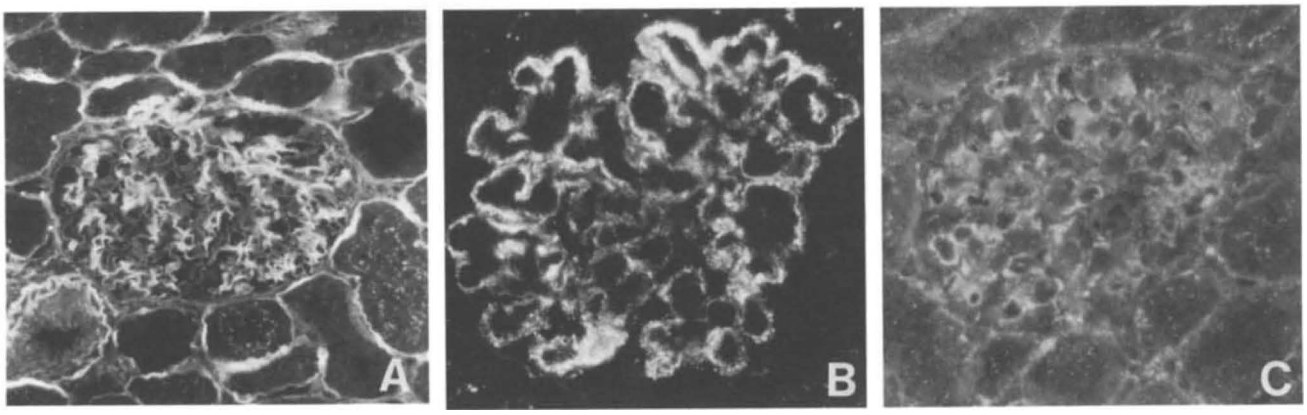

Fig. 3. *Immunofluorescence photomicrographs illustrating the presence of rat clusterin in passive Heymann nephritis, using anti-SGP-2 antiserum.* In normal rats (**A**), faint linear deposits of clusterin are present predominantly along the epithelial aspect of the glomerular capillary wall reflecting into mesangial regions and in a discontinuous pattern along some tubular basement membranes, Bowman's capsule, and the adventitia of arterioles. In rats with PHN (day 7) (**B**), clusterin is also present in the epimembranous immune deposits. Complement-depletion with CVF prevented the interaction of clusterin with the glomerular immune deposits (**C**) (A ,24 × 250; B × 320; C × 370).

treated rats was similar to normal rats. No granular deposits were present.

Discussion

Clusterin is present in the epimembranous immune deposits of experimental membranous nephropathy in association with rat C5b-9. As a result of systemic complement depletion, MAC formation is prevented and deposits of clusterin are no longer present. The demonstration of clusterin in association with subepithelial immune deposits in rat kidney raises several interesting questions about the mechanism of MAC formation within the glomerulus. Similar issues were discussed following the identification of S protein in this location [26–29] and more recently by the identification of SP-40,40 in the SC5b-9 components both in glomerular immune complexes and in areas of glomerulosclerosis in human renal biopsies [20]. It is unlikely that these proteins are deposited by entrapment of SC5b-9/clusterin complexes preformed in the circulation since the complex is extremely large and unable to migrate across the glomerular capillary wall [7, 24]. Left with the option that the terminal complement cascade is activated in situ within injured glomeruli, the presence of clusterin suggests that it may be functioning locally as a regulator of cytolytic MAC formation.

The actual mechanism by which MAC induces proteinuria has not been delineated. Although ultrastructural studies demonstrate the association of MAC with extracellular immune deposits, MAC has also been identified along membranous structures within the mesangium suggestive of cellular debris [5, 30]. In vitro studies demonstrate that Heymann nephritis-inducing antibodies require complement to induce a cytopathic effect on glomerular epithelial cells [31, 32]. Furthermore, C5b-9 can directly activate cultured glomerular epithelial [33–35] and mesangial cells [36, 37]. Thus it remains unclear whether all of the MAC present in the subepithelial immune deposits was initially inserted into the cell membrane or whether other mechanisms enable the biologically active C5b-9(m) to interact with the immune complexes following cellular shedding. Although SC5b-9 has no known pathogenic function, it is still possible that its association with the extracellular immune deposits may not be biologically inert in the kidney [38]. The role of clusterin in the generation of the terminal complement complex in situ is unknown. Based on results from in vitro studies, the predominant role of clusterin may be an inhibitory one through the formation of the cytolytically inactive SC5b-9/clusterin complex. However, just as vitronectin may interact with the biologically active C5b-9(m) during complex formation [39], clusterin appears to do the same thing [20].

Clusterin and S protein/vitronectin seem to be functionally similar proteins. In addition to participating in the formation of an inactive terminal complement complex, both are involved in cellular adhesion. Vitronectin contains the Arg-Gly-Asp(RGD) adhesion sequence and its receptor is a member of the integrin family of membrane receptors involved in cell-cell and cell-matrix interactions [40]. Ram clusterin induces aggregation of several cell types [15, 41] and human clusterin also elicits aggregation of erythrocytes [17]. The clusterin receptor involved in these cellular interactions has not yet been identified. Both clusterin and vitronectin are anionic alpha globulins of approximately 80 kD molecular mass. However, they are immunologically and structurally distinct [8–10]. Unlike clusterin, which is a disulfide-linked heterodimer, vitronectin is a single polypeptide chain. Amino acid sequence analysis demonstrates that they are different proteins. The role of clusterin as an adhesion-promoting molecule may also be important in the genesis of renal injury. However, our study suggests that interaction with the terminal complement cascade is necessary for its recruitment to the site of immune complex formation.

Finally, it appears that the family of clusterin-related glycoproteins may be larger than initially appreciated. Glycoprotein III isolated from bovine adrenal chromaffin granules shows a 72% level of identity with human SP-40,40 and its encoding RNA is also expressed in other organs including the kidney [42]. Glycoprotein III has no cell-aggregating activity, but it is present in both a soluble and membrane-bound form in chromaffin granules and is released in response to acetylcholine.

The present study demonstrates the presence of clusterin in the subepithelial immune deposits of PHN during the process of in situ complement activation. The role of clusterin in the

mediation of renal injury remains unknown but we speculate that it is likely to have an important function.

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