Alternative pathway complement activation induces proinflammatory activity in human proximal tubular epithelial cells

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Abstract

Background. Proximal tubular epithelial cells express a surface C3-convertase activity which induces C fixation and insertion of the C5b-9 membrane attack complex (MAC) into the cell plasma membrane. The physiological consequences of this phenomenon are unknown.

Methods. The effect of C fixation on the production of inflammatory mediators by human proximal tubular epithelial cells in culture was explored.

Results. Proximal tubular epithelial cells incubated with a sublytic amount of normal human serum as a source of C, but not with heat-inactivated human serum, showed a time-dependent calcium influx and a concomitant release of ¹⁴C-arachidonic acid (¹⁴C-AA). Eicosanoid synthesis following the arachidonic acid mobilization was studied as prostaglandin E₂ release. Mg²⁺/EGTA, which did not prevent C activation by the C3-convertase, and p-bromodiphenacyl bromide, a phospholipase A₂-inhibitor, inhibited mobilization of ¹⁴C-AA. These results suggest the activation of an extracellular Ca²⁺-dependent, phospholipase A₂. Complement fixation was associated with the synthesis of proinflammatory cytokines such as IL-6 and TNF-α. Experiments with C6-deficient sera indicated that the release of ¹⁴C-AA and the production of cytokines were dependent on the insertion of the terminal components of complement in the plasma membrane. Indeed, the reconstitution of normal haemolytic activity of C6-deficient sera with purified C6 restored also the release of ¹⁴C-AA and the production of cytokines.

Conclusions. In vitro complement activation on the proximal tubular cell surface triggers the generation of proinflammatory mediators, which may potentially contribute to the pathogenesis of tubulointerstitial injury.

Key words: complement; cytokine; membrane attack complex; proximal tubule; tubular injury

Introduction

The development of tubular interstitial injury is a common finding in the progression of several forms of glomerulonephritis. The importance of this phenomenon is underlined by its correlation with renal functional impairment [1–3]. Nevertheless, the mechanisms responsible for the spread of tissue damage from the glomerular to the tubular compartment are still unclear. The well-documented correlation between the tubular interstitial damage and the severity of proteinuria suggests that proteins abnormally filtered through the glomerular capillary wall can potentially exert toxicity on tubular cells, thereby triggering a tubular interstitial inflammatory process [1–3]. Among the plasma proteins escaping into the urine in nephrotic syndrome, complement fractions are potential candidates for causing tubular injury. In fact, histological studies have shown that the alternative pathway of complement can be directly activated by the brush border of proximal tubules of normal rat [4] or human kidney [5]. The presence of a potent C3-convertase activity on the surface of proximal tubular cells was confirmed in a recent in vitro study on cultured human proximal tubular epithelial cells (PTEC), which demonstrated that the activation of the alternative pathway of complement leads to fixation of the C5b-9 MAC neoantigen on their surface with consequent C5b-9-mediated cytotoxicity [6].

The tubular interstitial lesions observed in progressive glomerulonephritis are characterized by the interstitial infiltrate of mononuclear cells and accumulation of extracellular matrix within the interstitial space. In non-immunological experimental disease characterized by severe proteinuria, the deposition of complement in the brush-border of proximal tubules is accompanied by early monocyte/macrophage transmigration from the circulation into the tubulointerstitial space with subsequent mononuclear cell infiltration and fibroblast proliferation and differentiation [7].

Although several hypotheses were proposed, the mechanisms leading to interstitial inflammatory cell accumulation are still unclear.
In the present study we provide evidence that activation of the alternative pathway of complement on the surface of cultured PTEC leads to the arachidonic acid mobilization with production of autacoids and to the synthesis of proinflammatory cytokines.

Subjects and methods

Proximal tubule epithelial cell preparation and characterization

Surgical specimens of kidneys were used as the source for establishing cultures. The fibrous capsule was removed and portions of tissue were dissected from the outer cortex, minced, and forced through a graded series of meshes to remove interstitial fragments and glomeruli. PTEC were pelleted, resuspended, and plated in RPMI 1640 supplemented with 17% heat-inactivated fetal calf serum (Sigma), 50 U/ml penicillin, and 50 mg/ml streptomycin. Endotoxin was not detectable in either preparation when tested by limulus assay. PTEC were characterized, as previously described [4], on the basis of: (a) minimal staining for desmin and negative staining for factor VIII-VWF; (b) strong staining for cytokeratin and actin; (c) staining of 90–96% of cells for alkaline phosphatase as determined by the naphthal AS-MX method; and (d) increased cAMP production after stimulation with 100 nM PTH but not 1 mM ADH.

Experiments were performed on confluent monolayers at the 4th–5th passage in culture to avoid monocyte contamination. The results obtained from PTEC cultured from four different donors were comparable. Each individual experiment was performed in triplicate.

Source of complement

For in vitro experiments the following sera were used as sources of complement: (a) normal human serum pooled from 10 healthy donors; in some experiments sera were complement-inactivated by heating at 56°C for 1 h (HIHS); (b) C6-deficient human serum (C6-def HS) purchased from Sigma Chemical Co. The CH₄₅ levels in sera were determined by haemolytic activity. Restoration of the normal haemolytic activity was obtained after addition of 50 mg/ml human purified C6 (Sigma) to C6-def HS. Formation of C5b-9 neoantigen on PTEC cell surface was detected by immunofluorescence (Figure 3), induced a significant increase in 45Ca⁺⁺ influx (Figure 2). with the stimuli. The reaction was started by adding 2 mM CaCl₂, 0.5 mCi ⁴⁵Ca⁺⁺ and the different agonists. Termination was achieved by adding 2.5 mM EGTA and 5 mM ruthenium red (final concentration) on ice to remove cell-surface-bound Ca⁺⁺. After washing twice with balanced-salt solution, the pellet was solubilized with formic acid and the radioactivity was measured in the presence of 4 ml Instagel.

Measurement of arachidonic acid release

The release of ¹⁴C-arachidonic acid (¹⁴C-AA) from cellular lipids, mainly phospholipids, was measured using the method described by Hirata et al. [9]: 5 × 10⁵ cells were incubated in 2 ml with 0.15 mCi ¹⁴C-AA (55.5 Ci/mol) at 37°C for 1 h. The cells were washed twice with modified Grey’s buffer and incubated in 2 ml of the same buffer. The release was measured after treatment with different stimuli at 37°C.

Measurement of prostaglandin E₂ and cytokines

The release of prostaglandin E₂ (PGE₂) by PTEC incubated with NHS or HIHS was studied using an enzyme immunoassay system (BIOTRAK, Amersham Life Science, Amersham UK). The production of TNF-α and IL-6 was evaluated by ELISA (Quantikine, R&D Systems, Minneapolis MN).

Results

Generation of inflammatory mediators subsequent to the activation of the alternative pathway of complement on the PTEC surface, was studied using a concentration of NHS that was found sublytic in preliminary experiment [6]. PTEC incubated with 5% NHS but not with HIHS showed a time-dependent influx of ⁴⁵Ca⁺⁺ (Figure 1a) which temporally correlated with the release of ¹⁴C-AA (Figure 1b) in the supernatants.

P-bromodiphenacyl bromide, an inhibitor of PLA₂, [10], prevented the mobilization of ⁴⁵Ca⁺⁺ (Figure 2a) that chelates calcium from extracellular medium. Mg²⁺/EGTA-HS did not interfere with the fixation of complement by alternative pathway on PTEC surface [6].

¹⁴C-AA release was dependent on formation of C5b-9 MAC on PTEC plasma membrane. In fact, C6 Def HS, which is unable to form MAC as assessed by immunofluorescence (Figure 3), induced a significant inhibition of ¹⁴C-AA release (Figure 4). Reconstitution of C6 Def HS with purified human C6 restored the ¹⁴C-AA release. The production of autacoids following the AA release was assessed by measuring the release of PGE₂; NHS but not HIHS stimulated a sustained production of PGE₂ by PTEC (Figure 5).

Measurements of cytokines into cell-free supernatants indicate that NHS, but not HIHS, stimulates the production of TNF-α (Figure 6a) and IL-6 (Figure 6b). The dependency of cytokine synthesis on the fixation of terminal components of complement was shown by using C6-def HS and by restoring the ability to form MAC after addition of purified C6 (Figure 7a,b).

Discussion

The existence of a potent C3-convertase activity on the surface of PTEC has been well established by
several studies performed on tissue sections [4,5] and on primary cultures [6,11–13] The physiological function of this enzymatic activity is unclear but is probably related to the cleavage of other substrates since complement components are normally not filtered into the urine unless in the presence of gross alteration of glomerular permeability.

Indeed, studies on patients with non-selective proteinuria demonstrated the occurrence of tubular C3 deposits which positively correlated to the urinary complement excretion [14]. Moreover, C3 excreted in the urine of proteinuric patients, was shown to be capable of undergoing activation [14]. Recently, the urinary excretion of C5b-9 MAC neoantigen and its deposition in tubular epithelium was detected in proteinuric nephropathies either in the presence or absence of MAC glomerular deposits [15,16]. This suggests that complement, once filtered through the glomerular barrier, may be activated by the brush border of proximal tubules. The increased urinary excretion of C3 and MAC may reflect this phenomenon. Deposition of C3 and C5b-9 along the luminal border of proximal tubular cells was found in aminonucleoside nephrosis unless in the presence of gross alteration of glomerular permeability.

Fig. 1. (a) $^{45}$Ca uptake and (b) $^{14}$C-arachidonic acid release from PTEC incubated at 37°C for various periods of time without any addition or with addition of 5% normal human serum (NHS) or heat inactivated human serum (HIHS).
increased glomerular permeability occurring in proteinuric conditions may favour an increased urinary excretion of C5b-9, which is normally detectable within the circulation. Recently it has been shown that in human renal infarction activation of complement and insertion of terminal components of complement may occur within the proximal tubules when complement components enter in the intratubular urinary space of ischaemic tubules [19]. It has been suggested that the activation of complement may depend either on the
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presence of a C3 convertase in the brush border of proximal tubules or on the release of proteases in the ischaemic tissue [19]. Tubulointerstitial deposits of complement have been also related to an interstitial activation of complement by ammonia [20] and to a cytokine-inducible synthesis of C3 by PTEC [21].

Whether the fixation of complement on the surface of PTEC is a confined phenomenon or else contributes to the pathogenesis of the tubular interstitial injury by triggering generation of inflammatory mediators is unknown. The present work supports the hypothesis that complement activation on the surface of tubular cells may generate mediators that can be instrumental in the induction of tubulointerstitial injury. It is well known that the insertion of C5b-9 generated by an antigen-antibody reaction on the surface of various cell type has a stimulatory action [22–24]. However, the results of the present study indicate that PTEC, in the absence of an antibody-mediated mechanism are per se capable of activating complement and fixing C5b-9 on their surface as results of the contact of complement component with a C3-convertase present on PTEC surface. Therefore, we investigated whether the complement activation on PTEC surface could stimulate generation of inflammatory mediators. Our attention was focused on the release of AA, which is the precursor of potent autacoids, and of TNF-α and IL-6, cytokines which mediate several vascular and cellular events of inflammation. TNF-α, in particular, is a homotrimeric polypeptide with a wide range of biological activities [25]. This cytokine was shown to be involved in several steps of the inflammatory process such as vascular permeability alteration, leukocyte-endothelium adhesion, fibroblast activation and extracellular matrix formation, either by acting directly or through the synthesis of several secondary mediators.

IL-6 was shown to share with TNF several inflammatory properties as well as to possess specific regulatory role in growth and differentiation of different target cell types [26].

The mobilization of AA observed in the present study was related to an influx of extracellular Ca

$^{2+}$ and to the activation of PLA

$^{2}$, since this phenomenon is inhibited by Mg$^{2+}$/EGTA and by p-bromophenacyl bromide, an inhibitor of PLA

$^{2}$. Both the release of AA and the synthesis of cytokines occurred as consequences of complement activation and fixation of MAC on PTEC surface. Indeed, when PTEC were incubated with C6-def HS, which is unable to fix the terminal component of complement and form MAC on the cell surface, no significant release of AA or production of cytokines was observed. The ability to fix the terminal components of complement and to induce AA release and cytokine production was restored by purified C6. The present experiments cannot precisely indicate which of the terminal components of complement is primarily involved in cell stimulation, since besides C5b-9 also C5b-7 and C5b-8 may display biological activity [27]. Moreover, we observed a complement-induced release of PGE

2. PGE

2, a metabolite of cyclooxygenase-dependent metabolic pathway of AA, may either amplify or inhibit the inflammatory phenomena [28]. Indeed it has been reported that PGE

2 in association of complement-derived bioactive peptides significantly enhances vascular permeability at the site of inflammation [28]. On the other hand, PGE

2 inhibits the activation and the recruitment of inflammatory cells [28]. In the present study we evaluated the PGE

2 as a marker of production of AA-derived bioactive metabolites. We cannot exclude that other cyclooxygenase or lipoxigenase metabolites, not considered in this study but potentially relevant for the modulation of inflammatory reaction, are produced during complement activation on PTEC.

The vulnerability of PTEC to complement injury may also reflect the absence or scarcity on their brush border of complement regulatory proteins such as CD55 and CD46, that inhibit the complement activation cascade at C3 convertase level, and CD59, that inhibits the formation of MAC. Morphological studies have shown that complement regulatory proteins on the surface of PTEC are mainly restricted to the basolateral site [29].

The results of the present study suggest that complement activation on the cell surface and insertion of the terminal components of complement may trigger the generation of proinflammatory mediators from PTEC in vitro.

One can speculate that in patients with complementuria due to non-selective proteinuria, PTEC may directly participate in the activation of complement cascade at the luminal site of proximal tubules, leading
to generation of mediators that may contribute to the pathogenesis of tubulointerstitial injury.

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