Natural IgM antibodies are involved in the activation of complement by hypoxic human tubular cells

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van der Pol P, Roos A, Berger SP, Daha MR, van Kooten C. Natural IgM antibodies are involved in the activation of complement by hypoxic human tubular cells. Am J Physiol Renal Physiol 300: F932–F940, 2011. First published February 2, 2011; doi:10.1152/ajprenal.00509.2010.—Ischemia-reperfusion injury (IRI) has a major impact on graft survival after transplantation. Renal proximal tubular epithelial cells (PTEC) located at the corticomedullary zone are relatively susceptible to IRI and have been identified as one of the main targets of complement activation. Studies in mice have shown an important role for the alternative pathway of complement activation in renal IRI. However, it is unclear whether experimental data obtained in mice can be extrapolated to humans. Therefore, we developed an in vitro model to induce hypoxia-reoxygenation in human and mouse PTEC and studied the role of the different pathways of complement activation. Exposure of human PTEC to hypoxia followed by reoxygenation in human serum resulted in extensive complement activation. Inhibition studies using different complement inhibitors revealed no involvement of the alternative or lectin pathway of complement activation by hypoxic human PTEC. In contrast, complement activation by hypoxic murine PTEC was shown to be exclusively dependent on the alternative pathway. Hypoxic human PTEC induced classic pathway activation, supported by studies in C1q-depleted serum and the use of blocking antibodies to C1q. The activation of the classic pathway was mediated by IgM through interaction with modified phosphomonoesters exposed on hypoxic PTEC. Studies with different human sera showed a strong correlation between IgM binding to hypoxic human PTEC and the degree of complement activation. These results demonstrate important species-specific differences in complement activation by hypoxic PTEC and provide clues for directed complement inhibition strategies in the treatment and prevention of IRI in the human kidney.

hypoxia; ischemia; kidney; reperfusion injury

ISCHEMIA-REPERFUSION INJURY (IRI) occurs upon reperfusion of vascularized tissue after an extended period of ischemia. It is an inevitable event in organ transplantation. Clinical and experimental studies have shown that renal IRI has a major impact on short- and long-term graft survival after organ transplantation and accounts for delayed graft recovery and associated morbidity and mortality (21, 23). Restoration of blood flow to ischemic tissue initiates a cascade of inflammatory events, including endothelial dysfunction, neutrophil sequestration, and complement activation (C-activation) which all contribute to postischemic injury (12).

The complement system is a complex cascade of proteins that can be activated by three different pathways (33). Each pathway is activated by a different set of pattern recognition molecules. The classic pathway (CP) is initiated by direct binding of C1q to e.g., apoptotic cells, or by binding to antigen-antibody complexes. The lectin pathway of complement (LP) is activated by interaction of mannose-binding lectin (MBL) or ficolins with sugar moieties. CP and LP activation both lead to deposition of C4 and C2, which result in the generation of the classic C3 convertase that is able to cleave C3. The alternative pathway (AP) is continuously activated at a low level (so-called tickover), does not require C4, and is tightly regulated by membrane-bound and soluble complement regulators. Furthermore, properdin can act as a focal point of AP mediated C-activation upon binding to its ligand (13). All pathways converge at the level of C3, and further downstream activation leads to formation of the membrane attack complex C5b-9 (MAC) (33).

C-activation is a key feature of renal IRI, as has been demonstrated both in the clinical setting as well as in experimental models (37). Moreover, interference with C-activation reduces IRI. Studies in mice have suggested that the AP is predominantly activated in IRI. Mice deficient in factor B (27), a crucial constituent of the AP, or mice treated with anti-factor B antibodies (29), show reduced injury, whereas C4-deficient mice were shown to be as susceptible to renal IRI as wild-type mice (36). Nevertheless, other pathways of C-activation have also been implicated. Deposition of MBL was observed in mouse kidneys after renal IRI (10), and also deficiency of MBL partially protects mice against renal IRI (17). In pigs, different components of the CP and LP were detected after renal ischemia, and therapeutic intervention with C1INH, which interferes with both the LP and CP, was successful (4).

In the heart, muscle, and the intestine, C-activation after ischemia depends on naturally occurring IgM antibodies to intracellular antigens which are externalized upon ischemia (5, 15, 35). The role of these antibodies in renal IRI is controversial (2, 22).

Both endothelial and epithelial cells in the kidney seem to be targets for C-activation following IRI. In the kidney, several studies have implicated the corticomedullary proximal tubular epithelial cell (PTEC) as an important target (28, 36). Renal IRI is associated with a reduction in membrane-bound complement regulators on PTEC (28). Moreover, PTEC interact with properdin (11), which can serve as a focus for AP activation (13).

So far, in human renal IRI the activation pathways by PTEC are still incompletely elucidated. In the present study, we developed an in vitro model to induce hypoxia-reoxygenation and investigated the subsequent effects on C-activation by hypoxic human and mouse PTEC. We specifically focused on the question of which pathways of C-activation are initiated by the PTEC after hypoxic stress. We demonstrate that C-activation by human PTEC as a result of hypoxia-reoxygenation primarily occurs via the CP of complement and is dependent on both IgM antibodies and C1q. In contrast, hypoxia-induced C-activation by mouse PTEC primarily occurs via the AP.
MATERIALS AND METHODS

Cell culture. Immortalized human renal PTEC (HK-2, kindly provided by M. Ryan, University College Dublin, Dublin, Ireland) (26) were grown in serum-free DMEM/HAM-F12 (Bio-Whittaker, Walkersville, MD) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Breda, The Netherlands), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), triiodothyronine (40 ng/ml), epidermal growth factor (10 ng/ml), and hydrocortisone (36 ng/ml, all purchased from Sigma, Zwijndrecht, The Netherlands). Primary human PTEC were isolated from pretransplant biopsies or from kidneys not suitable for transplantation and cultured as described earlier (32).

Immortalized mouse renal PTEC (IM-PTEC, kindly provided by Dr. G. Stokman, Gorlaeus Laboratories, Leiden, The Netherlands) were derived from a single PTEC of an Immorto mouse (Charles River, Maasstricht, The Netherlands) based on the double expression of aquaporin-4 and CD10/nephrilysin. Immorto mice express a temperature-sensitive, IFN-γ-dependent variant of the SV40 large T antigen. Cells are grown under non-restrictive conditions, except in DMEM/HAM-F12 (Bio-Whittaker) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), triiodothyronine (40 ng/ml), prostaglandin E1 (5 ng/ml), hydrocortisone (36 ng/ml, all purchased from Sigma), and mouse IFN-γ (10 ng/ml; R&D Systems, Uithoorn, The Netherlands) and maintained for at least 1 wk under restrictive conditions at 37°C in the absence of IFN-γ to ensure redifferentiation.

Induction of hypoxia-reoxygenation and necrosis. To simulate an ischemic event, cells were grown to confluence in 24-well culture plates at 37°C and subjected to normoxia (21% O2 and 5% CO2) or hypoxia (5% CO2 and 95% N2) for 48 h in a humidified modular incubator chamber (Billups-Rothenberg, Del Mar). Since it is known that cultured PTEC are resistant to hypoxia at high-glucose levels (31), culture medium of the HK-2 cells, primary cultured human PTEC, and mouse IM-PTEC was replaced by identical culture medium, but only containing 2 mM glucose, before induction of hypoxia. To simulate the reperfusion phase, cells were washed and reoxygenated in medium, but only containing 2 mM glucose, before induction of hypoxia. To further assess the role of the LP,D-mannose (200 mM; Sigma) or increasing doses of mannose (0.25, 0.5, 1, 2.5, 5 mM) was used to block both the CP and LP. To further assess the role of the CP, d-mannose (200 mM; Sigma) or increasing doses of mAb 3E7 (anti-MBL mAb kindly provided by Dr. T. Fujita, Fuku- shima, Japan), which both inhibit the binding of MBL to its ligands, were used. To assess the role of the CP, increasing doses of mAb85 (mAb anti-human C1q, kindly provided by Dr. C. E. Hack, Sanquin, Amsterdam, The Netherlands) were used. MAb85 is directed against the globular head regions of C1q and inhibits binding of C1q to activators such as IgM and aggregated IgG (16). To further study CP activation, the binding of IgM (5–100 μg/ml) to normoxic and hypoxic HK-2 cells was assessed. Functional consequences of IgM binding to hypoxic HK-2 cells were studied using 5 or 30% C1q- or IgM-depleted human serum. As a control, the serum was reconstituted with purified C1q (50 μg/ml) or IgM (100 μg/ml), respectively. IgM-dependent C-activation by mouse PTEC was studied by incubating hypoxic mouse IM-PTEC with 5% serum derived from immunoglobulin-deficient RAG−/− mice. Furthermore, the role of IgM in C-activation was studied using the phosphatemoester phosphorylcholine (Sigma), an antigen for natural IgM. Phosphorylcholine (20 mM) was first preincubated with 5% NHS for 15 min at room temperature and next incubated with hypoxic HK-2 cells for 1 h at 37°C. Binding of serum IgM and deposition of C3, C4, and C5b-9 on normoxic and hypoxic human PTEC was studied using 5% NHS (from 11 healthy donors) diluted in serum-free DMEM/HAM-F12 culture medium and incubated on the cells for 1 h at 37°C.

FACS analysis. After incubation with purified IgM or serum as a source of complement, cells were washed in PBS, harvested with nonenzymatic dissociation buffer (Sigma), and resuspended in FACS buffer (1% BSA, 0.02% sodium azide, and 2.5 mM CaCl2 in PBS). Depositions of C3, C4, and C5b-9 and binding of IgM on human PTEC were detected using mouse monoclonal antibodies against human C3 (RFK22, Laboratory of Nephrology, Leiden, The Netherlands); human C4 (C4-4A, anti-C4, kindly provided by Dr. C. E. Hack); human C3d (Quidel, San Diego, CA); human C4d (Quidel); human C5b-9 (mAb AE11, kindly provided by Dr. T. E. Mollems, Nordland Central Hospital, Bodo, Norway); and human IgM (HB57, hybridoma obtained from the American Type Culture Collection, Manassas, VA), respectively, followed by RPE-conjugated polyclonal goat anti-mouse immunoglobulin (DAKO, Glostrup, Denmark). Deposition of C3 on mouse PTEC was detected using a rabbit polyclonal antibody anti-mouse C3 (in house generated) (30), followed by RPE-conjugated polyclonal goat anti-rabbit immunoglobulin (DAKO). All antibody incubations were performed on ice for 30 min. Cell surface staining was assessed using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Propidium iodide (PI, 1 μg/ml, Molecular Probes, Leiden, The Netherlands) and annexin V-FITC (25 μg/ml, V-FITC, H9262, Hoeven, The Netherlands) were used for exclusion of apoptotic and necrotic cells.

Assessment of functional LP activity by ELISA. Functional activity of the LP was assessed using mannan-coated plates as previously described (25). Next, NHS preincubated with l- or d-mannose (Sigma) was incubated for 1 h at 37°C to allow C4 and C3 deposition in the well. The plates were washed and incubated with dig-conjugated C4-4A (anti-human C4) or RFK22 (anti-human C3), followed by HRP-conjugated F(ab’)2 from goat IgG anti-dig (Boehringer Mannheim, Mannheim, Germany). After washing, C3 deposition was quantified with ABTS (Sigma). The optical density at 415 nm was measured using a microtiter plate reader.

Complement and serum reagents. All samples were collected and experiments were performed according to the guidelines of the ethics committee of the Leiden University Medical Center. As a source of complement, pooled NHS from healthy donors and pooled mouse serum from C57BL/6 (NMS) and RAG−/− mice were divided into aliquots and stored at −80°C until used. Human C1q-depleted serum was generated as previously described (25). The C1q-depleted serum showed normal LP and AP activity in hemolytic assays and could be completely restored with purified C1q. Human IgM-depleted serum was generated by immune adsorption using Biogel-coupled anti-human IgM monoclonal antibodies (HB57) at a high salt concentration to prevent C-activation during the procedure and showed normal activity in all three complement pathways. Human C1q (19) and IgM (25) were purified as previously described.

Statistical analysis. Correlation analysis between variables was performed by linear regression, and the significance of differences between groups was calculated by Student’s t-test using GraphPad Prism software (GraphPad Software, San Diego, CA). Differences with P < 0.05 were considered significant.

RESULTS

C-activation by hypoxic HK-2 cells and primary human PTEC. To establish an in vitro model to simulate IRI on PTEC, we cultured HK-2 cells under normoxic or hypoxic conditions. Forty-eight hours of hypoxia were required to induce hypoxic
stress which was accompanied by morphological changes with rounding of cells and loss of tight junctions (Fig. 1A). Despite these changes, the vast majority of both hypoxic and normoxic cell populations (respectively, >75 and >90%) were still viable at this time point, as determined with PI and annexin-V staining (Fig. 1B). In all cases, apoptotic and necrotic cells were excluded from the analysis.

Reoxygenation of the cells in 30% NHS resulted in extensive C-activation by hypoxic HK-2 cells (Fig. 1C). In line with our previous findings, a low level of C-activation was also observed on normoxic cells (11). C-activation by hypoxic HK-2 cells was not restricted to deposition of C3 only (Fig. 2B) but was accompanied by deposition of C4 (Fig. 2A) up to the level of C5b-9 (Fig. 2C). Moreover, using monoclonal antibodies to neoepitopes on C4d and C3d, we demonstrated that the detected C3 and C4 on the cell surface reflects activated complement fragments (Fig. 2, D and E). Furthermore, in all cases, C-activation could be completely blocked using EDTA, showing that the deposition of C4, C3, and C5b-9 was the result of activation of the complement system (Fig. 2, A–E).

In addition to the HK-2 cell line, also primary human PTEC derived from different donors (n = 5) were subjected to 48 h of hypoxia followed by 1-h reoxygenation in 5% serum (Fig. 3, A and B). While normoxic PTEC induced low-grade C-activation, hypoxiareoxygenation of these cells resulted in a significant increase in C-activation and deposition of C3. Interestingly, there was some variance in the degree of C-activation between the five different PTEC (Fig. 3A), indicating that some PTEC were more prone to activate complement after hypoxia-reoxygenation than others. Titration of serum showed that similar C-activation was observed when 5 or 30% serum as a source of complement was compared (data not shown); therefore, 5% serum was used for further experiments.

A prominent role for AP activation by hypoxic mouse, but not human PTEC. Although the activation of complement and deposition of C4 on human PTEC (Fig. 2A) already suggested involvement of the CP or LP, most mouse studies of renal IRI have implicated a dominant role for the AP (27, 29). Therefore, similar to human PTEC, mouse PTEC were cultured under hypoxic conditions for 48 h and reoxygenated in NMS in the

![Fig. 1. Induction of hypoxic stress. HK-2 cells were subjected to normoxic or hypoxic conditions for 48 h. Photographs (×20 magnification) showing normoxic (A; left) and hypoxic HK-2 cells which are stressed, rounded, and have lost their tight junctions with neighboring cells (A; right). Cells were stained with propidium iodide (PI) and annexin V, and apoptotic/necrotic cells were excluded from further analyses (B). C3 deposition was determined by fluorescence-activated cell sorter (FACS) after 1-h reoxygenation of cells in 30% normal human serum (NHS; C).](http://ajprenal.physiology.org/)

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presence or absence of MgEGTA, which blocks both the CP and LP but leaves the AP intact. Indeed, C3 deposition on hypoxic mouse PTEC was not affected by the addition of MgEGTA, indicating that the AP is the predominant pathway activated by hypoxic mouse PTEC (Fig. 4A). In contrast, incubation of hypoxic human HK-2 or hypoxic primary human PTEC with 5% serum in the presence of MgEGTA almost completely prevented deposition of C3 (Fig. 4A), indicating a major involvement of the CP or LP.

Necrotic human cells are known to bind different complement components, leading to activation of the complement system. To study whether C-activation by necrotic human and mouse PTEC also follows different pathways of C-activation, these cells were rendered necrotic and incubated with human or mouse serum, respectively, in the presence or absence of MgEGTA. Indeed, mouse PTEC again exclusively activate the AP as MgEGTA did not have any inhibitory effect (Fig. 4B). Under these conditions human necrotic PTEC now also show some activation of the AP. These data show that hypoxic and necrotic mouse PTEC exclusively activate the AP, while hypoxic human PTEC primarily activate the CP/LP and necrotic human PTEC activate both the AP and CP/LP.

Fig. 2. Complement activation by hypoxic HK-2 cells. Normoxic (open bars) and hypoxic (filled bars) HK-2 cells were reoxygenated in 30% NHS and stained for C4 (A), C3 (B), C5b-9 (C), C4d (D), and C3d (E) deposition. As a control, complement activation was blocked using EDTA. Dotted lines represent background staining. Values are means ± SD mean fluorescence intensity (MFI) of triplicate cultures and are representative of 3 independent experiments.

Fig. 3. Complement activation by hypoxic primary proximal tubule epithelial cells (PTEC). Primary human PTEC (n = 5) were subjected to normoxic or hypoxic conditions for 48 h and reoxygenated in 5% serum for 1 h. C3 deposition was determined by flow cytometry (A and B). *, Normoxic conditions; ○, hypoxic conditions.
No contribution of LP activation by hypoxic human PTEC.

Several studies in mice and humans have shown a role for the LP, which can be activated by MBL, in renal IRI (4, 10, 17). To study the contribution of the LP on C-activation by hypoxic human PTEC, these cells were incubated with NHS in the presence or absence of an inhibiting monoclonal antibody to MBL (Fig. 5A). Inhibition of MBL did not affect C3 or C4 deposition, indicating that the LP is not involved. In line with these findings, blockade of the LP using D-mannose, a ligand for MBL, did not affect C3 or C4 deposition (Fig. 5A). To show that D-mannose is able to inhibit the LP of complement, we applied an ELISA system with mannan-coated plates which specifically activate the LP. C-activation in this assay could be completely blocked using D-mannose, while L-mannose, which does not bind MBL, had no effect (Fig. 5C). Together these data show that inhibition of the LP had no effect on C-activation by hypoxic human PTEC, indicating that there is no involvement of the LP.

IgM binds to hypoxic human PTEC and contributes to CP activation.

The studies above indicate that hypoxic human PTEC exclusively activate the CP in human serum. To obtain more detailed insight into the mechanisms of CP activation by hypoxic human PTEC, these cells were incubated with NHS in the presence or absence of a monoclonal antibody directed against the C1q-globular head domains. Blockade of the CP using this antibody resulted in a dose-dependent inhibition of both C3 and C4 deposition on hypoxic HK-2 cells (Fig. 6A) and primary PTEC (data not shown). Moreover, incubation of hypoxic HK-2 cells in C1q-deficient serum resulted in an almost complete abrogation of C3 and C4 deposition, and reconstitution with purified C1q restored C-activation (Fig. 6B).

The CP can be activated by binding of C1q to different cellular ligands, but also to cell-bound immunoglobulins like IgM. Indeed, we could show a dose-dependent binding of purified IgM to hypoxic human cells (Fig. 7A). Exposure of hypoxic human PTEC to serum depleted of IgM resulted in an almost complete abrogation of C3 deposition, whereas reconstitution of this serum with purified IgM fully restored C-activation. (Fig. 7B), indicating a major role for IgM and subsequent C1q on C-activation by hypoxic human PTEC.

In contrast to human PTEC, incubation of hypoxic mouse PTEC with serum derived from RAG-/- mice, which lack immunoglobulins, still resulted in extensive C-activation (Fig. 7C) to a similar level as NMS.

Recent studies have shown that the binding of IgM to apoptotic cells is mediated for a major part by lyso-phosphatidyl derivatives and that this binding can be blocked using phosphorylcholine (6, 20). Indeed, C-activation by hypoxic human PTEC was blocked significantly by 20 mM phosphorylcholine (Fig. 7D), suggesting that natural IgM antibodies can bind to phospholipid neoepitopes exposed on hypoxic cells and subsequently function as a focus of CP activation by hypoxic human PTEC.

Correlation between IgM levels and C-activation by hypoxic human PTEC.

Eleven sera derived from healthy individuals all induced significant C3, C4, and C5b-9 deposition on hypoxic HK-2 cells, compared with normoxic cells (Fig. 8, A–C). To
determine whether there was an association between IgM binding and classic C-activation, we assessed in parallel the binding of IgM and the deposition of C3, C4, and C5b-9 on hypoxic PTEC (Fig. 8, D–F). We found a significant correlation between IgM binding and C4 ($r^2 = 0.643$), C3 ($r^2 = 0.572$), and C5b-9 deposition ($r^2 = 0.570$), indicating that IgM binding to hypoxic human PTEC plays an important role in activation of the CP of complement after hypoxic stress.

**DISCUSSION**

Several lines of evidence support an important role for the AP of C-activation by proximal tubular cells in in vivo mouse models of renal IRI. However, it is still unclear whether similar pathways of C-activation are involved in the human setting.

Therefore, we studied hypoxia-induced C-activation by human and mouse PTEC. C-activation by hypoxic mouse PTEC was mainly dependent on the AP, compatible with the in vivo observations. In contrast, hypoxic human PTEC primarily activated complement through the CP, with a major involvement of natural IgM antibodies. These results reveal important species-specific differences which might have important implications for complement-directed therapies in renal IRI.

As an in vitro model of renal IRI, both primary human PTEC and HK-2 cells were cultured under hypoxic conditions and reoxygenated in human serum. This resulted in extensive C-activation by the tubular cells and deposition of C4 and C3 up to the level of C5b-9. C-activation could be completely abrogated using EDTA, showing that the deposition of complement factors on the hypoxic cells was the result of activation of the complement system.

In our model, normoxic human PTEC showed some deposition of complement, as described before (3), which is partially mediated through binding of properdin (11). Nevertheless, in our model C-activation was markedly increased as a result of hypoxia-reoxygenation compared with the basal level of C-activation. Furthermore, using MgEGTA, which blocks the CP and LP, C-activation could be completely abrogated to levels of complement deposition on normoxic PTEC, indicating
that the increased deposition of complement on hypoxic cells does not depend on the AP.

Studies with MBL knockout mice have shown a protective effect of MBL deficiency in the setting of renal IRI (17), and also renal deposition of MBL has been demonstrated (10). Moreover, we have previously demonstrated that renal allograft recipients with low MBL levels show better graft survival (1). However, we could not show a role for MBL in our in vitro model using hypoxic human PTEC. Blocking antibodies to MBL or inhibition with D-mannose did not have any effect on C-activation. Although C-activation in the ischemic kidney is largely localized to the tubular epithelium, low-grade C-activation on the endothelium could result in activation of the endothelium and extravasation of serum constituents in the

Fig. 7. In contrast to mouse PTEC, complement activation by hypoxic human PTEC is IgM dependent. To establish whether complement activation was dependent on bound immunoglobulins, the binding of purified IgM (A) to normoxic and hypoxic HK-2 cells was assessed. To study functional consequences of IgM binding, hypoxic HK-2 cells were incubated with 5% IgM-deficient serum. As a control, serum was reconstituted with purified IgM (100 μg/ml; B). IgM-dependent complement activation by mouse PTEC was studied by incubating hypoxic IMPTEC with 5% NMS or RAG −/− serum, after which C3 deposition was determined (C). Values are means ± SD MFI of triplicate cultures and are representative of 3 independent experiments. Dotted lines represent background staining. Hypoxic HK-2 cells were incubated with 5% NHS in the presence of 20 mM phosphorylcholine (PC), and C3 deposition was determined (D). Values are means ± SD MFI of triplicate cultures and are representative of 2 independent experiments.

Fig. 8. Hypoxia induces IgM-mediated complement activation. Normoxic (open bars) or hypoxic (filled bars) HK-2 cells were incubated with 5% serum derived from 11 different healthy volunteers, and C4 (A), C3 (B), and C5b-9 (C) deposition was determined. Simultaneously, binding of IgM was measured by FACS analysis and correlated to the C4 (D), C3 (E) and C5b-9 (F) deposition. P values and correlation coefficients are given in the graphs.
interstitium, which could lead to C-activation on tubular epithelial cells. Therefore, we hypothesize that MBL is mainly involved in LP activation on endothelial cells, as shown before (8, 9), or has effector functions that are still unknown.

Using C1q-depleted serum or blocking antibodies to C1q we could reduce C-activation by hypoxic human PTEC to basal levels, indicating that the complement system is mainly activated via the CP. Interestingly, C-activation by hypoxic cells that were annexin-V and PI positive, and thus excluded from analysis, also occurred via the CP, suggesting that hypoxia-induced apoptotic PTEC expose similar ligands as the hypoxic, but still viable cells.

It is known from several studies that late apoptotic and necrotic cells bind natural IgM antibodies, which will lead to activation of the CP (6, 7). We could also demonstrate such a binding of IgM to hypoxic PTEC. Using IgM-deficient serum, we showed that C-activation by hypoxic cells also occurs via binding of IgM, probably via binding of IgM to phosphorylcholine residues exposed on hypoxic cells, as shown by inhibition of C-activation using phosphorylcholine. Furthermore, we could show a high correlation between IgM binding and C4, C3, and C5b-9 deposition using sera from different donors, suggesting a prominent role for IgM in activation of the CP. It has been proposed that I-PLA2 activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine, leading to binding of natural IgM antibodies and subsequently C-activation (14). It is tempting to speculate that a similar process may be involved on hypoxic human PTEC.

Data from studies in mice indicate that C-activation due to IRI in skeletal muscle (5), heart (15), intestine (34), and limb (20) occurs through binding of natural IgM. However, in renal IRI in mice it has been shown that immunoglobulins do not play a role (22). These findings are in line with reports showing that C4-deficient mice were not protected against renal IRI (36), so it appears that in mice IRI can proceed independently of C4 and immunoglobulin. To confirm this in our model, we used mouse PTEC which were rendered hypoxic and were incubated with NMS supplemented with MgEGTA to block CP (36), so it appears that in mice IRI can proceed independently of C4 and immunoglobulin. Using IgM-deficient serum, we showed that C-activation by hypoxic mouse PTEC primarily occurs via the CP of complement, which is dependent on the binding of IgM. This is in contrast to hypoxic mouse PTEC, which primarily activate the AP of complement. Together, these data provide new clues about the pathways of complement that should be targeted after renal IRI in mice; however, further studies in humans are needed.

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From the results above, we conclude that hypoxia-induced C-activation by human PTEC primarily occurs via the CP of complement, which is dependent on the binding of IgM. This is in contrast to hypoxic mouse PTEC, which primarily activate the AP of complement. Together, these data provide new clues about the pathways of complement that should be targeted after renal IRI in humans; however, further studies in humans are needed.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**REFERENCES**


