CHAPTER 2

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

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ABSTRACT

Ischemia/reperfusion injury (IRI) has a major impact on graft survival after transplantation. Renal proximal tubular epithelial cells (PTEC) located at the cortico-medullary 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 classical pathway activation, supported by studies in C1q-depleted serum and the use of blocking antibodies to C1q. The activation of the classical 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.

INTRODUCTION

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 (1;2). 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 post ischemic injury (3).

The complement system is a complex cascade of proteins that can be activated by three different pathways (4). Each pathway is activated by a different set

of pattern recognition molecules. The classical 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 Mannan-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 classical 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 (5). All pathways converge at the level of C3 and further downstream activation leads to formation of the membrane attack complex C5b-9 (MAC) (4).

Complement activation is a key feature of renal IRI, as has been demonstrated both in the clinical setting as well as in experimental models (6). 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 (7), an crucial constituent of the AP, or mice treated with anti-factor B antibodies (8) show reduced injury, whereas C4-deficient mice were shown to be as susceptible to renal IRI as wildtype mice (9). 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 (11). 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 (12).

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 (13-15). The role of these antibodies in renal IRI is controversial (16;17).

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 cortico-medullary proximal tubular epithelial cell (PTEC) as an important target (9;18). Renal IRI is associated with a reduction in membrane-bound complement regulators on PTEC (18). Moreover PTEC interact with properdin (19), which can serve as a focus for AP activation (5).

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 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.

METHODS

Cell culture

Immortalized human renal proximal tubular epithelial cells (HK-2, kindly provided by M. Ryan, University College Dublin, Ireland)(20) were grown in serum-free DMEM/HAM-F12 (Bio-Whittaker, Walkersville, US) 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), tri-iodothyronine (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 pre-transplant biopsies or from kidneys not suitable for transplantation and cultured as described earlier (21).

Immortalized mouse renal proximal tubular epithelial cells (IM-PTEC, kindly provided by Dr. G. Stokman, Gorlaeus Laboratories Leiden, Netherlands) were derived from a single proximal tubular epithelial cell of an Immorto mouse (Charles River, Maastricht, The Netherlands) based on the double expression of aquaporin-4 and CD10/neprilysin. Immorto mice express a temperature sensitive, interferon gamma dependent variant of the SV40 large T antigen. Cells are grown under permissive conditions at 33°C 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), tri-iodothyronine (40 ng/ml), prostaglandin E1 (5 ng/ml), hydrocortisone (36 ng/ml, all purchased from Sigma), mouse IFN-gamma (10 ng/ml; R&D systems, Uithoorn, The Netherlands) and maintained for at least one week under restrictive conditions at 37°C in the absence of IFN-gamma to ensure re-differentiation.

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% O_2 and 5% CO_2) or hypoxia (5% $C0_2$ and 95% N_2) for 48 hours in a humidified modular incubator chamber (Billups-Rothenberg, Del Mar, US). Since it is known that cultured PTEC are resistant to hypoxia in high glucose levels (22), 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 5% or 30% pooled human serum (NHS) or mouse serum (NMS) respectively, diluted in DMEM/HAM-F12 for 1h at 37 °C. As a control, cells were reoxygenated in medium without serum. Finally cells were washed in PBS, harvested with non-enzymatic dissociation buffer (Sigma) and resuspended in FACS-buffer (1% BSA, 0.02 % sodium azide and 2,5 mM CaCl2 in PBS). Necrosis was induced by incubating fresh HK-2 cells and IM-PTEC at 56°C for 30 minutes, after which the cells were incubated with 5% NHS or 5% NMS for 1h at 37 °C.

Analysis of complement activation pathways

In order to elucidate which pathways of complement are involved in C-activation by hypoxic cells, several complement inhibitors were evaluated. EDTA, at a final concentration of 5 mM, was used to inhibit all pathways of C-activation. MgEGTA, at 5 mM, was used to block both the CP and LP. To further assess the role of the LP, D-Mannose (200 mM; Sigma) or increasing doses of mAb 3E7 (anti-MBL mAb kindly provided by Dr. T. Fujita, Fukushima, 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 Prof. 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 (23). 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 phosphatemonoester phosphorylcholine (Sigma), an antigen for natural IgM. Phosphorylcholine (20mM) was first preincubated with 5% NHS for 15 min at room temperature and next incubated with hypoxic HK-2 cells for 1h at 37 °C. Binding of serum IgM and deposition of C3, C4 and C5-b9 on normoxic and hypoxic human PTEC was studied using 5% NHS from eleven 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 non-enzymatic 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, 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 Prof. C. E. Hack,), human C3d (Quidel, San Diego, USA), human C4d (Quidel), human C5b-9 (mAb AE11, kindly provided by Dr T. E. Mollnes, 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 Ig (DAKO, Glostrup, Denmark,). Deposition of C3 on mouse PTEC was detected using a rabbit polyclonal antibody anti-mouse C3 (in house generated) (24), followed by RPE-conjugated polyclonal goat anti-rabbit Ig (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, 1µg/ml, Molecular Probes, Leiden, the Netherlands) and Annexin V-FITC (25 µg/ml, VPS Diagnostics, Hoeven, the Netherlands) was used for exclusion of apoptotic and necrotic cells.

Assessment of functional lectin pathway activity by ELISA

Functional activity of the LP was assessed using mannan-coated plates as previously described (25). Shortly, NHS preincubated with L- or D-Mannose (Sigma) was incubated for 1 hour 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 OD 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 normal human serum (NHS) from healthy donors and pooled mouse serum from C57BL/6 (NMS) and RAG-/- mice was 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 (26) 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

Complement activation by hypoxic HK-2 cells and primary human PTEC

In order 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

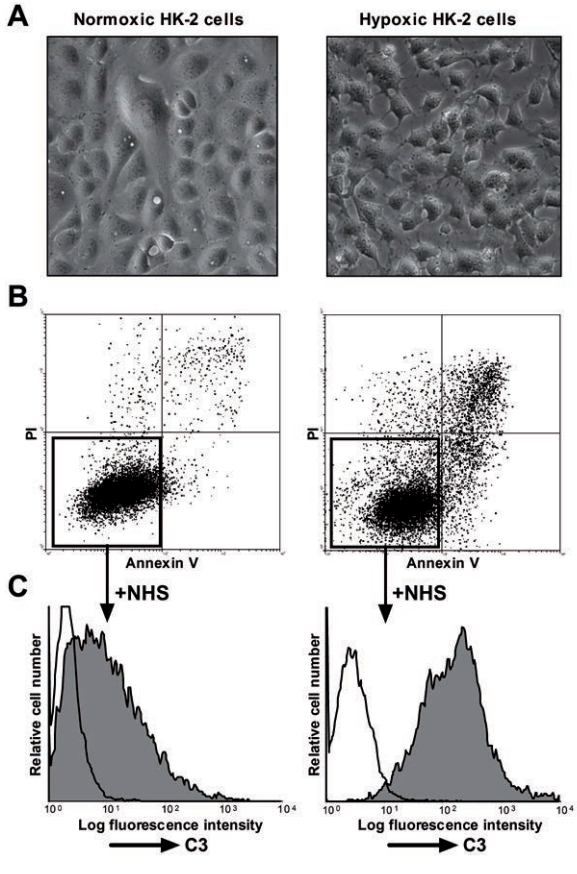

Figure 1. Induction of hypoxic stress. HK-2 cells were subjected to normoxic or hypoxic conditions for 48 hours. Photographs (20x magnification) showing normoxic (a; left) and hypoxic cells HK-2 cells which are stressed, rounded and have lost their tight junctions with neighboring cells (a; right). Cells were stained with PI and Annexin V and apoptotic/ necrotic cells were excluded from further analyses (b). C3 deposition was determined by FACS after 1 hour reoxygenation of cells in 30% NHS (c).

with Propidium iodide and Annexin-V staining (Fig. 1B). In all cases, apoptotic and necrotic cells were excluded from the analysis.
Reoxygenation of the cells in 30% normal human serum (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 (19).
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 C-fragments (Fig. 2D, 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. 2A-E).

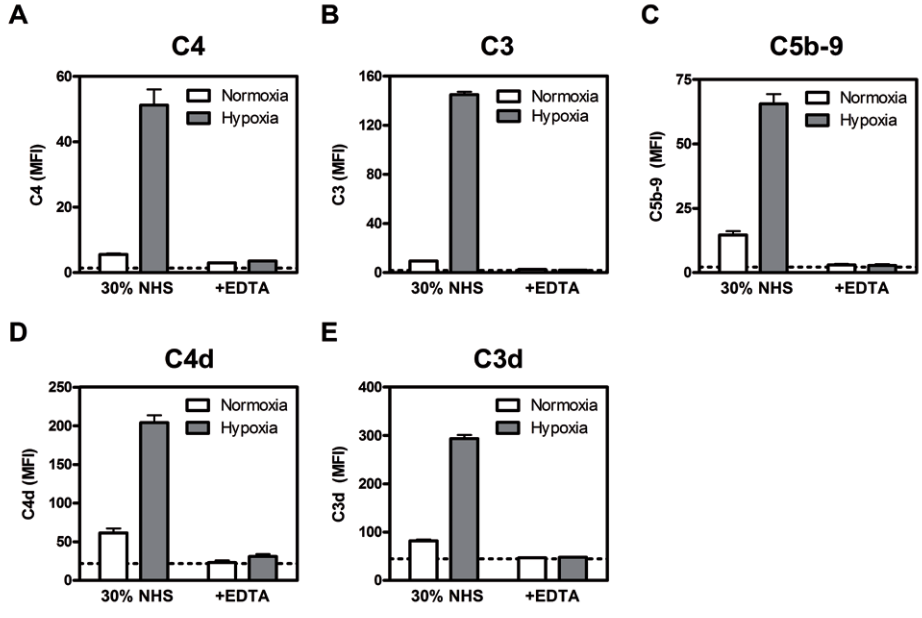

Figure 2. Complement activation by hypoxic HK-2 cells. Normoxic (white bar) and hypoxic (grey bar) HK-2 cells were reoxygenated in 30% NHS and stained for C4 (a), C3 (b), C5b-9 (c), C4d (d) and C3d (e) deposition. As a control complement activation was blocked using EDTA. Dotted lines represent background staining. Results given are the mean (+SD) MFI of triplicate cultures and are representative of 3 independent experiments.

In addition to the HK-2 cell line, also primary human PTEC derived from different donors (n=5) were subjected to 48 hours of hypoxia followed by 1 hour reoxygenation in 5% serum (Fig. 3A, B). While normoxic PTEC induced low-grade C-activation, hypoxia/reoxygenation of these cells resulted in a significant

increase of 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 complement activation was observed when comparing 5 or 30% serum as a source of complement (data not shown), therefore 5% serum was used for further experiments.

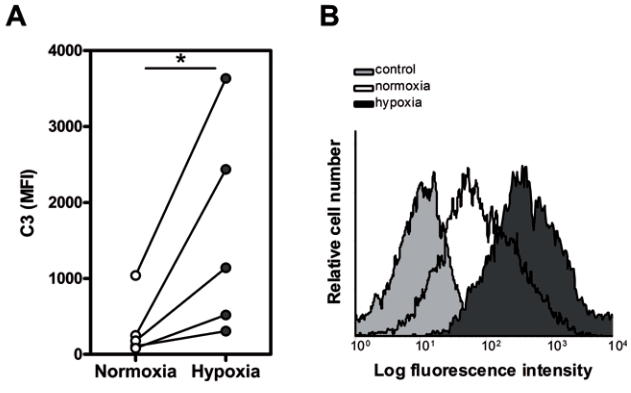

Figure 3. Complement activation by hypoxic primary PTEC. Primary human PTEC (n=5) were subjected to normoxic or hypoxic conditions for 48 hours and reoxygenated in 5% serum for 1 hour. C3 deposition was determined by flow cytometry (a,b). White dots represent normoxic and dark grey dots hypoxic conditions.

A prominent role for alternative pathway 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 (7;8). Therefore, similar as with the human PTEC, mouse PTEC were cultured under hypoxic conditions for 48 hours and reoxygenated in normal mouse serum (NMS) in the 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 follow 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.

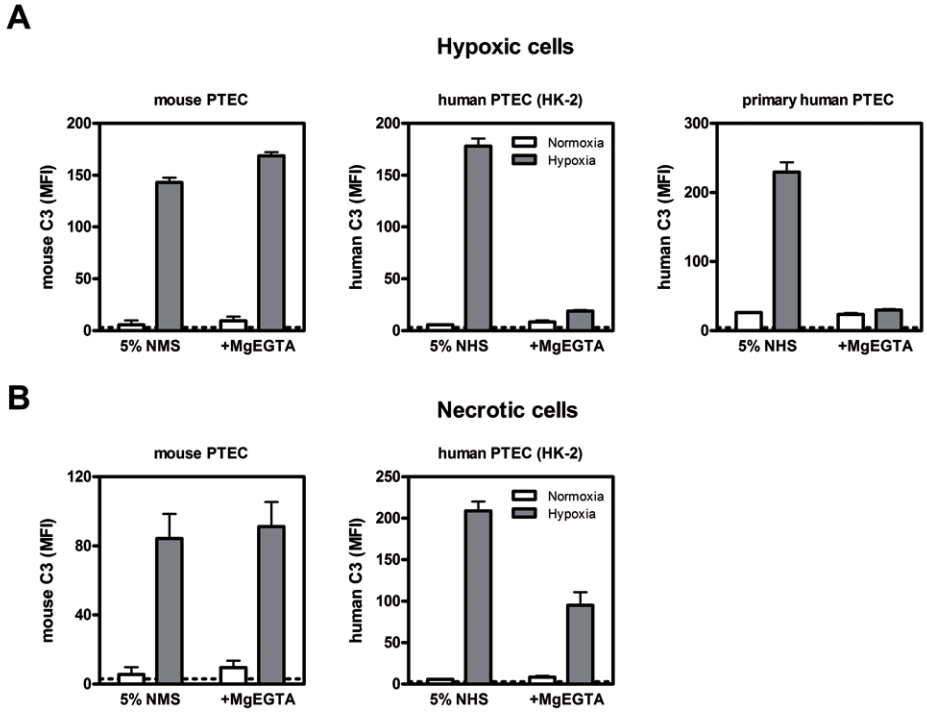

Figure 4. In contrast to mouse, hypoxic human PTEC hardly activate the alternative pathway. (a) To study the contribution of the alternative pathway human (HK-2) and mouse PTEC (IM-PTEC) were rendered hypoxic (a) or necrotic (b) and incubated with 5% NHS or NMS respectively, in the presence or absence of MgEGTA, to block classical and lectin pathway activation. C3 deposition was determined using flow cytometry. White bars represent normoxic and grey bars hypoxic conditions. Results given are the mean (+ SD) MFI of triplicate cultures and are representative of 3 independent experiments.

No contribution of lectin pathway 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 (10-12). 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. 5B). 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 classical pathway activation

The studies above indicate that hypoxic human PTEC exclusively activate the CP in human serum. To obtain more detailed insight in the mechanisms of CP activation by hypoxic human PTEC, we assessed the effect of inhibition of the CP using 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

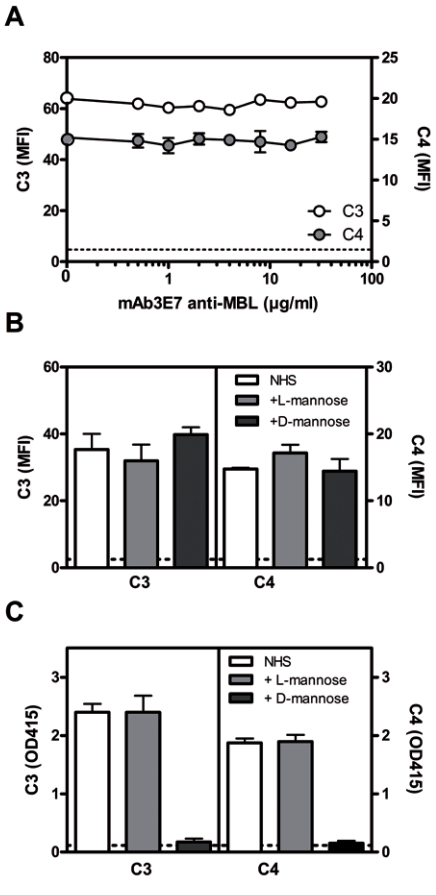

Figure 5. No involvement of lectin pathway of complement. The lectin pathway was studied by (a) incubating hypoxic HK-2 cells with 5% NHS with an increasing dose of blocking antibodies to MBL (mAb 3E7). White dots represent C3 and grey dots C4 deposition. Besides, hypoxic HK-2 cells (b) or mannan coated plates (c) were incubated with NHS in the presence or absence of 200 mM L-mannose (light grey bar) or D-mannose (dark grey bar) after which C3 and C4 deposition was determined. White bars represent the condition with NHS only. Results given are the mean (+ SD) MFI of triplicate cultures and are representative of 3 independent experiments.

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 normal mouse serum.

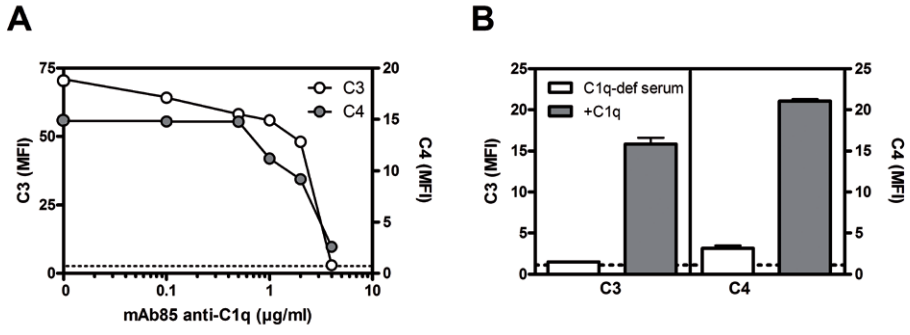

Figure 6. Prominent role for the classical pathway of complement. To study the contribution of the classical pathway, hypoxic HK-2 cells were incubated with 5% NHS with an increasing dose of blocking antibodies to C1q (mAb 85), after which C3 (white dots) and C4 (grey dots) deposition was determined. (a) Hypoxic HK-2 cells were incubated with 5% C1q-deficient serum (white bars), or this serum was reconstituted with purified C1q (100 µg/ml)(grey bar) (b). Results given are the mean (+ SD) MFI of triplicate cultures and are representative of 3 independent experiments.

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 (27;28). 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 neo-epitopes exposed on hypoxic cells and subsequently function as a focus of CP activation by hypoxic human PTEC.

Correlation between IgM levels and complement 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 to normoxic cells (Fig. 8A-C). To determine whether there was an association between IgM binding and classical C-activation we assessed in parallel the binding of IgM and the deposition of C3, C4 and C5b-9 on hypoxic PTEC (Fig. 8D-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.

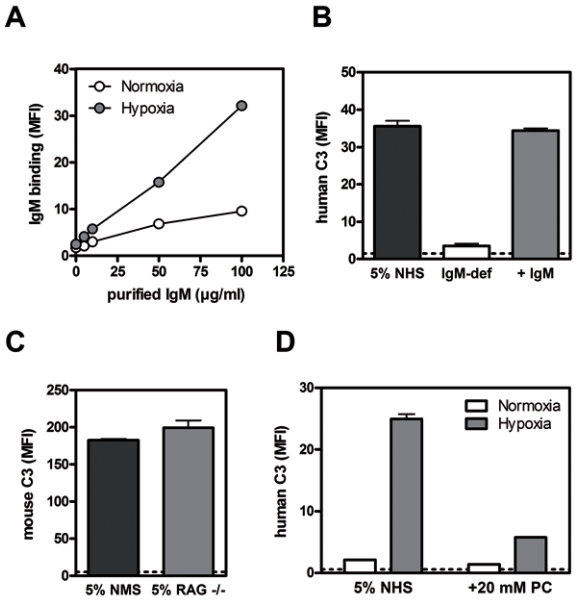

Figure 7. In contrast to mouse PTEC, complement activation by hypoxic human PTEC is IgM-dependent. To establish if complement activation was dependent on bound immunoglobulins, the binding of purified IgM (a) to normoxic and hypoxic HK-2 cells was assessed. To study functional consequences of IgM binding, 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). Results given are the mean (+ 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). Results given are the mean (+ SD) MFI of triplicate cultures and are representative of 2 independent experiments.

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. Complement 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. Complement 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 (29), which is partially mediated through binding of properdin (19). Nevertheless, in our model C-activation was markedly increased

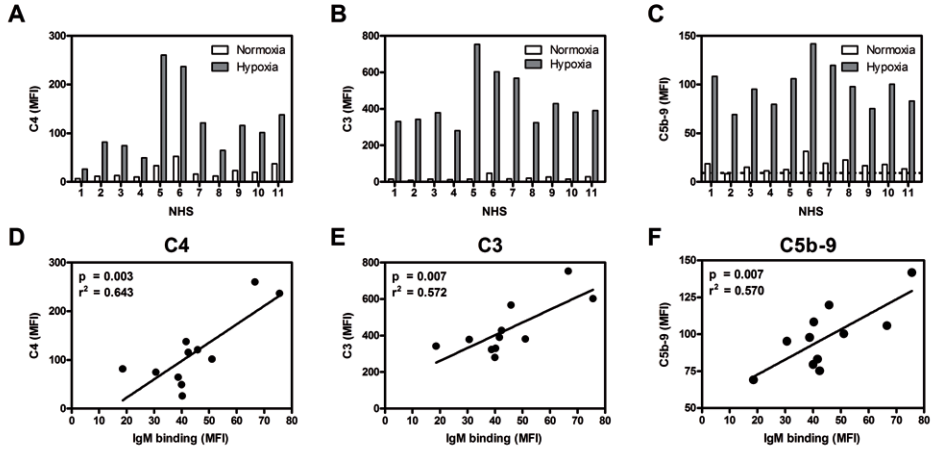

Figure 8. Hypoxia induces IgM-mediated complement activation. Normoxic (white bar) or hypoxic (grey bar) HK-2 cells were incubated with 5% serum derived from eleven different healthy volunteers, and C4 (a), C3 (b) and C5b-9 (c) deposition was determined. Simultaneously, binding of IgM was measured by 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.

as a result of hypoxia/reoxygenation compared to 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 deposited 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 (11), and also renal deposition of MBL has been demonstrated (10). Moreover, we have previously demonstrated that renal allograft recipients with low MBL levels show a better graft survival (30). 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 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 (31;32), 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 were 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 (27;33). 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(34). 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 (13), heart (14), intestine (35) and limb (28) occurs through binding of natural IgM. However, in renal IRI in mice it has been shown that immunoglobulins do not play a role (17). These findings are in line with reports showing that C4-deficient mice were not protected against renal IRI (9), so it appears that in mice IRI can proceed independently of C4 and immunoglobulins. To confirm this in our model we used mouse PTEC which were rendered hypoxic and were incubated with normal mouse serum supplemented with MgEGTA to block CP and LP activation. Additionally we also used serum from RAG -/- mice which lack immunoglobulins. Indeed, C-activation still proceeded in the absence of immunoglobulins or an active CP and LP, indicating that the AP plays an important role in mice. These data are in contrast to hypoxic human PTEC which primarily activate the CP via IgM but in agreement with *in vivo* studies in mice (7;8). Also in a pig model, it was recently shown that the CP and LP of complement were involved in renal IRI (12). Importantly, these authors showed reduced ischemic injury when pigs were treated with C1INH, a specific inhibitor of both the CP and LP (36;37). Interestingly, this study (12) also demonstrated that these two pathways were activated in renal transplant recipients suffering from delayed

graft function (DGF). The co-localization of C4d with both C1q and MBL in graft biopsies obtained from these patients indicated that both these pathways were activated on peritubular capillaries, within the interstitium, and on the glomerular endothelium.

The potential difference in the mechanisms of C-activation by mouse and human cells, as presented in the current manuscript, has important implications for the interpretation of experimental data obtained in mice. To successfully develop therapeutic interventions targeted towards C-activation, it is essential to establish the validity of murine data relative to what takes place in the human situation. Because this study is limited to an *in vitro* model, further studies are needed to delineate the role of natural IgM and complement in the human situation following renal I/R.

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.

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