Endothelial-to-mesenchymal transition and renal fibrosis in ischaemia/reperfusion injury are mediated by complement anaphylatoxins and Akt pathway

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ABSTRACT

Background. Increasing evidence demonstrates a phenotypic plasticity of endothelial cells (ECs). Endothelial-to-mesenchymal transition (EndMT) contributes to the development of tissue fibrosis. However, the pathogenic factors and signalling pathways regulating this process in ischaemia/reperfusion (I/R) injury are still poorly understood.

Methods. We investigated the possible role of complement in the induction of this endothelial dysfunction in a swine model of renal I/R injury by using recombinant C1 inhibitor in vivo.

Results. Here, we showed that I/R injury reduced the density of renal peritubular capillaries and induced tissue fibrosis with
The activation of ECs occurring in I/R [10, 11] leads to an endothelium and chemokines, with the subsequent enhancement of increased expression of cell adhesion molecules, such as ICAM-associated with significant morbidity during transplantation [6]. Renal I/R injury is associated with significant morbidity during transplantation [7], and it is considered an acute inflammatory disease [8, 9]. The activation of ECs occurring in I/R [10, 11] leads to an increased expression of cell adhesion molecules, such as ICAM-1, and chemokines, with the subsequent enhancement of endothelium–leucocyte interactions [12]. As a final result, the number of micro-vessels in renal tissue declines after I/R injury; this process has been described as ‘vascular rarefaction’ and has been associated with the down-regulation of angiogenic factors and the up-regulation of angiogenesis inhibitors [11]. The vascular rarefaction is thought to be linked with the development of EC swelling [11], narrowing of the vessel lumen and chronic hypoxia [13], with subsequent development of tubular injury and tubulo-interstitial fibrosis.

During the reperfusion phase, ECs are the primary target of different pro-inflammatory agents, including the complement system, which plays a critical role in the pathogenesis of I/R [14, 15]. Complement orchestrates immunological and inflammatory processes, contributing to various immune and inflammatory diseases [16]. Among the signalling pathways that regulate inflammation and cell survival, the Akt pathway might play a pivotal role in I/R injury [17] and vascular homeostasis [18].

To date, little is known about the role of EndMT in renal I/R injury and about the pathogenic factors regulating vascular rarefaction at renal level. The aim of our study was to investigate the possible role of complement in the induction of EndMT and renal fibrosis in I/R injury.

**MATERIALS AND METHODS**

**Renal I/R injury model**

After approval by the ethical committee of the Ministry of Health, ten 4-month-old female Large White pigs weighing 20 kg underwent experimental surgery under general anaesthesia [19]. The pigs underwent open surgical procedure. The renal arteries and vein were isolated, and a vessel loop was positioned around the renal artery with a right-angle clamp. A renal biopsy was performed before ischaemia (T0). Then the ischaemic phase was induced pulling on the vessel loop. Five minutes before the end of the ischaemia time, recombinant C1-INH (500 U/kg) was injected in the ear vein of five animals; in another group of five animals, an equal volume of vehicle was infused at the same time point (control group). After 30 min of ischaemia, multiple biopsies were then performed at 15, 30 and 60 min during reperfusion; animals were sacrificed 24 h after the surgical procedure.

**Cell isolation and culture**

Immortalized human umbilical vein ECs line (EA.hy926) obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) were grown to confluence in DMEM high-glucose medium supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 2 mM l-glutamine (Sigma Aldrich). Confluent cells were stimulated with C3a (CalbioChem), C5a (Vincibocheo) and/or Akt inhibitor IV (Akt-inhIV,Calbiochem) for the indicated time period. Akt-inh IV is a cell-permeable benzimidazole compound that inhibits specifically Akt phosphorylation/activation. ECs were processed by immunostaining or lysed for protein extraction.

**Confocal laser scanning microscopy**

Paraffin-embedded swine kidney sections and cultured ECs were stained or double stained for CD31, FSP-1, α-SMA, von Willebrand Factor (vWF), pAktSer473 (Santa Cruz Biotechnologies, Santa Cruz, CA), C4d (AbD Serotec, MorphoSys, UK) and monoclonal anti-EC antibodies (Hy clad Biotechnology), recognized as a swine isoform of human vWF. C4d staining was conducted on paraffin-embedded swine kidney sections as previously described [19]. Renal biopsies were de-paraffinized and underwent epitope unmasking through three microwave (750 W) cycles of 5 min in citrate buffer (pH = 6). For each experiment, 10^5 cells were placed on a cover slip and fixed in 3.7% paraformaldehyde. The slides were incubated with the appropriate blocking solution, primary antibodies (anti-CD31 1:20, anti-FSP-1 1:50, anti-α-SMA 1:100, anti-vWF 1:40, anti-
pAkt\textsuperscript{Ser473} 1:100, anti-C4d 1:50) and the appropriate secondary antibodies (Alexa Flour 488 and 555 goat anti-rabbit; AlexaFlour 555 and 488 anti-mouse, Molecular Probes, Eugene, OR, USA). All sections were counterstained with TO-PRO-3 (Molecular Probes). All the antibodies cross-react with pig tissue. Negative controls were prepared with irrelevant antibody. Specific fluorescence was acquired using the confocal microscope Leica TCS SP2 (Leica, Wetzlar, Germany). Fluorescence levels were quantified using Adobe Photoshop software and expressed as area fraction (%). The number of CD31\textsuperscript{/}/FSP-1\textsuperscript{+} and CD31\textsuperscript{/}/α-SMA\textsuperscript{+} cells was measured in at least 10 high-power (×630) fields/sections by two independent observers blinded to the origin of the slides. The final counts were the mean of the two measures. In no case, inter-observer variability was higher than 20%.

**Immunohisto- and cyto-chemistry analysis**

Renal fibrosis was assessed by Masson’s trichrome staining. For immunohistochemical staining, paraffin-embedded sections of swine renal biopsies or cultured EC first incubated with \( \text{H}_2\text{O}_2 \) (3%) were exposed to protein block solution (Dako, Glostrup, Denmark) and then with the primary antibody (rabbit anti-human C1-INH, kindly provided by Prof. M.R. Daha, University of Leiden, Leiden, the Netherlands, recognizing both the endogenous and the recombinant form [20] and diluted 1:400) or with Caspase-3 (diluted 1:20). The immune-complexes were detected by the Peroxidase/DAB Dako Real EnVision Detection System, according to manufacturer’s instructions (Dako). The reaction was visualized by a brown precipitate, counterstained with Mayer’s haematoxylin (blue) and mounted with glycerol (DakoCytomation, Carpintera, CA, USA). Specific endothelial C1-INH immunostaining was quantified using Adobe Photoshop software and expressed as positive pixel/total pixel.

**Western blotting**

The western blotting for Akt was performed as previously described [21]. To detect α-SMA protein expression, we used an anti-α-SMA monocolonal antibody (Santa Cruz Biotechnol; 1:5000 dilution in TBS). Bands were quantified using Image J 1.34 Software.

**Immunophenotypic analysis of EC by flow cytometry**

Phycoerythrin (PE)-conjugated anti-CD31, anti-VE-cadherin (Biologend) and anti-N-cadherin (Biologend) antibodies were used. After surface incubation for 15 min at 4°C, cells were washed with the FACs buffer (phosphate-buffered saline pH 7.2, 0.2% bovine serum albumin and 0.02% sodium azide). Intracellular staining for unconjugated-FSP-1 (Abcam) was preceded by fixation and permeabilization with IntraPrep\textsuperscript{TM} kit (Instrumentation Laboratory) and incubation for 25 min at 4°C. Cells were then washed and labelled with secondary Antibody AlexaFluor 488 (Molecular Probes) for 25 min at 4°C. Finally, cells were washed twice and data were acquired using a FC500 (Beckmann Coulter) flow cytometer and analysed using Kaluza software. The area of positivity was determined using an isotype-matched mAb; a total of 10\textsuperscript{4} events for each sample were acquired.

**Statistical analysis**

Data were expressed as mean ± standard deviation (SD). Difference between groups was analysed by \( t \)-test analysis. A \( P \)-value of <0.05 was considered statistically significant.

**RESULTS**

**Complement inhibition prevented vascular rarefaction and fibrosis in renal I/R injury**

To characterize renal ECs, we first used an antibody (Ab) directed against CD31 (Figure 1a–c). Compared with basal condition (Figure 1a), I/R injury induced a significant reduction in CD31 expression at 24 h indicating the occurrence of vascular rarefaction (Figure 1b). The reduction in ECs density at 24 h was particularly evident within the peritubular capillaries. Considering the activation of classical and lectin pathways on renal ECs [19], we investigated whether complement inhibition might affect vascular damage in our model. Treatment with C1-INH preserved ECs density preventing the occurrence of vascular rarefaction (Figure 1c and d).

When then we investigated α-SMA expression (Figure 1e–g) as markers of activated myofibroblast, as expected, the presence of α-SMA in normal kidney was limited to the wall of small and medium renal arteries (Figure 1e). Interestingly, we found the presence of this marker with a similar distribution to the peritubular capillaries network (Figure 1f). Treated animals had a significant reduction in interstitial α-SMA expression (Figure 1g and h).

We then stained the kidney sections using Masson’s trichrome staining in normal pig kidney before the induction of I/R damage. As expected, we found very limited presence of collagen deposition in the interstitial space and at glomerular level (Figure 1i). Twenty-four hours from I/R injury, we observed a strong increase in collagen deposition in tubulo-interstitial and in the glomerular area (Figure 1j). Pigs treated with C1-INH showed limited collagen deposition maintain renal fibrosis at basal level (Figure 1k and l). These data indicated that C1-INH preserved the loss of renal vessels (Figure 1d) and prevented the acute development of tubulo-interstitial fibrosis (Figure 1h and l).

**Vascular rarefaction is associated with EndMT and complement activation**

The significant reduction in renal vessels led us to hypothesize the possible occurrence of ECs apoptosis. Therefore, we performed an immunohistochemical staining for Caspase-3. As expected [19], apoptotic tubular cells increased dramatically after 60 min and 24 h from I/R injury (Figure 2a and b). On the contrary, we found the absence of apoptosis in ECs and vessels (arrows, Figure 2a and b). ECs were characterized by alterations both in large vessels and in capillaries. Masson’s trichrome (Figure 2c and d) showed clear morphological changes in ECs, appearing rounded and detached from the basal lamina after 24 h from reperfusion (Figure 2c and d). C1-INH treatment preserved ECs physiological conformation and position, strictly tight to the basal lamina of the vessels.

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Interestingly, ECs expressed clear signs of EndMT as indicated by interstitial CD31/FSP-1 co-expression (Figure 2e) and CD31/α-SMA co-localization at capillary level (Figure 2g). The number of transitioning ECs was significantly reduced in treated pigs (Figure 2f and h; quantification Figure 2i and j).

**Figure 1:** Complement inhibition prevented vascular rarefaction and fibrosis in renal I/R injury. In a swine model of I/R injury, CD31 expression was widely present within peritubular capillaries before ischaemia (A), but significantly reduced after 24 h of reperfusion (B). Infusion of C1-INH completely reversed these effects (C and D). No α-SMA+ cells could be observed within the interstitial space in basal conditions (E). I/R induced a dramatic increase of these cells at 24 h (F) with a pattern resembling the peritubular capillaries network. Interstitial α-SMA expression was totally abrogated by C1-INH treatment (G and H). Masson’s trichrome staining highlighted a strong presence of collagen deposition at tubulo-interstitial level after 24 h of reperfusion in renal tissue of control pigs (J) respect to basal condition (I). C1-INH was able to abrogate collagen deposition in treated pigs (K and L). The quantitative analyses of CD31 (D), α-SMA (H) and Masson’s trichrome (L) stainings were performed as described in the section ‘Materials and Methods’ and expressed as mean ± SD (*P = 0.03, **P = 0.04) of at least three independent pigs for each group. Original magnification: ×63. To-pro-3 was used to counterstain nuclei (blue).

**C3a-induced EndMT in vitro**

Complement anaphylatoxins are considered to play an important role in vascular damage [22]. Therefore, we investigated whether C3a could induce phenotypic alterations in ECs in vitro. Compared with basal condition (Figure 3a), C3a induced significant down-regulation in the expression of the
constitutive marker vWF (Figure 3b and c). At the same time, C3a-activated ECs significantly increased α-SMA protein expression, as shown both by confocal microscopy (Figure 3d and e) and by western blotting (Figure 3f). These data indicated that C3a was able to drive the EndMT process in vitro.

**Role of Akt pathway in C3a and C5a-mediated EndMT**

Recent evidence showed that the Akt pathway might regulate EC differentiation [23]. We investigated whether anaphylatoxins could affect Akt activation in ECs. As shown in Figure 4a, C3a induced a progressive and time-dependent phosphorylation of AktSer473 that was statistically significant at 15 min when compared with basal level. We then exposed ECs to C3a in the presence of Akt-inh IV (5 µM) (Figure 4c). Interestingly, Akt inhibition hampered EndMT with significant reduction of C3a-induced α-SMA expression, as indicated both by western blotting (Figure 4b) and by confocal microscopy (Figure 4d). Moreover, we performed a double-staining for CD31 and FSP-1, and analysed ECs by flow cytometry. As shown in Figure 4d, control ECs expressed CD31 (95 ± 3.2%) and low levels of FSP-1 (2 ± 0.7%). When ECs were exposed to C3a significantly reduced CD31 expression (51 ± 5.0%) while increasing FSP-1 levels (15 ± 2.0%). Interestingly, Akt inhibition abrogated the C3a-induced EndMT by restoring EC phenotype (Figure 4d). We also analysed the effects of the other anaphylatoxin C5a (Figure 4e). After 24 h of C5a stimulation, we observed a significant reduction of the endothelial VE-cadherin and an increase of fibroblast N-cadherin expressions with respect to basal condition. This indicated that C5a was also capable of altering the ECs phenotype by inducing EndMT in 24 h. Interestingly, C5a-induced EndMT was also abrogated by inhibition of Akt.

**Inhibition of complement activation led to abrogation of Akt signalling in vivo**

We then analysed the activation of the Akt pathway in our model of I/R injury. Before ischaemia (Figure 5a), renal tissue did not present C4d deposits and phosphorylation of AktSer473. I/R injury induced a deposition of C4d, accompanied by Akt phosphorylation (Figure 5b). By using an antibody directed against vWF (Figure 4c and d), we found that Akt activation occurred also at the EC level (arrows, Figure 5c). Interestingly, C1-INH blocked the effects of I/R injury on pAktSer473, maintaining the activation of this pathway as in basal condition (Figure 5d). Quantification of the overall pAktSer473 signal by confocal microscopy demonstrated a significant inhibition in treated pigs compared with control animals (Figure 5e).

**Localization of C1-INH on damaged EC**

In order to characterize the localization and distribution of recombinant C1-INH in ischaemic kidney, we analysed renal tissues from control and treated pigs by using an antibody capable to recognize both native and recombinant C1-INH (Figure 6a and b). The infusion of the recombinant protein led to C1-INH localization within glomerular and peritubular capillaries after 30 min from reperfusion (Figure 6a). On the contrary, control pigs did not show any binding of native C1-INH during the reperfusion phase (Figure 6a). These data led us to hypothesize that the recombinant C1-INH might recognize damaged ECs. Therefore, we compared the binding of recombinant C1-INH on cultured ECs (Figure 6c and d) in normal condition or in the presence of cellular stress (Figure 6e and f).
C1-INH did not show significant binding on healthy ECs (Figure 6d). On the contrary, we observed an increased cellular binding of C1-INH on ECs that were activated by H$_2$O$_2$ (Figure 6f).

**DISCUSSION**

In this study, we demonstrated a critical role for complement in the acute induction of the EndMT process via the Akt pathway. In our model, vascular rarefaction occurring in renal I/R injury was associated with significant alterations in EC phenotype, accompanied by the development of tubulo-interstitial fibrosis. This process was driven *in vitro* and *in vivo* by the activation of the Akt pathway and finally hampered by inhibition of the complement system.

I/R injury plays a role in the pathogenesis of delayed graft function [7], a condition associated with an increased incidence of acute rejection episodes and worse long-term graft survival. Our data suggest that I/R injury might be pivotal in the acute induction of renal fibrosis, leading to early chronic allograft dysfunction in transplanted kidney [6, 7]. Although renal fibrosis is considered to be mainly caused by resident fibroblasts, recent studies showed that activated fibroblasts could emerge from trans-differentiation of resident renal cells such as ECs [24]. This process, named EndMT [3], could be considered an extreme form of EC plasticity [4]. EndMT is involved in the progression of renal fibrosis in chronic kidney disease [25]. During EndMT, ECs lose specific endothelial markers and gain myofibroblast markers such as FSP-1 and α-SMA [2, 4]. Alterations in renal ECs might lead to progressive hypoxia of renal tubular epithelial cells with alteration in prostaglandin synthesis and generation of reactive oxygen species [10]. In addition, the renal vascular system lacks comparable regenerative potential [10, 26]. In response to ischaemic damage, renal vascular cells modify F-actin expression and the distribution of vWF on the cellular surface [27]; ultra-structural changes occur in renal EC and smooth muscle cells, suggesting that maintaining endothelial integrity is critical to the recovery of graft function [27]. In accordance with our data, ECs modify their phenotype after renal I/R injury showing CD31$^+$ with FSP-1$^+$ and α-SMA$^+$ expression [26]. When we quantified the CD31$^+$/α-SMA$^+$ ECs, we found that ~20–30% of the total α-SMA$^+$ cells emerging after I/R damage were also CD31$^+$ (Figure 2g), suggesting a different origin for the other activated myofibroblasts. In accordance, EndMT occurred in about 10% of the emerging fibroblast during renal fibrosis [25], thereby contributing to the development of renal fibrosis [13].

To date, little is known about the pathogenic factors regulating EndMT at the renal level. The activation of complement plays a major role in mediating tissue damage [16, 28]. Interfering with complement activation significantly reduces the damage associated with I/R injury [12, 19]. In our swine
**FIGURE 4:** C3a- and C5a-induced EndMT is mediated by Akt activation. Western blotting analysis showed that Akt^{Ser473} phosphorylation in cultured ECs was induced by C3a $10^{-7}$M in a time-dependent manner (A, *P = 0.02). Pre-incubation with a specific Akt inhibitor (Akt-inh, 5 µM) significantly reduced the C3a-induced α-SMA protein expression, as shown both by western blotting (B, *P = 0.03) and confocal microscopy (C). All data represent the mean ± SD of three independent experiments. Flow cytometry analysis of ECs showed the phenotypic changes induced by 24 h activation with C3a (D) and C5a (E, black line) respect to basal condition (D and E, grey dotted line). Using Akt-inh, ECs maintained their phenotype even in presence of C3a (d) and C5a (e, black dotted line). Results are representative of three independent experiments.
model, we detected C4d deposits primarily on peritubular and glomerular capillaries [19]. Therefore, ischaemic ECs might represent complement-activating surfaces by the exposure of auto-antigens [29]. To characterize EC activation in vitro and in vivo, we used CD31 and vWF as endothelial markers to follow the EndMT process. Interestingly, IL-1β and TGF-β could induce selective chromatin modifications in EC, repressing vWF and promoting COL1A2 genes expression, therefore suggesting a key role of EndMT in intestinal fibrosis during inflammatory bowel disease [30]. Moreover, the fact that C3a and C5a induced the secretion of vWF from EC in vivo [31] might be in line with the reduced surface expression during EndMT in vitro (Figure 3a and b). The involvement of complement in vascular pathology has also been demonstrated in different models such as myocardial infarction, arterial aneurysms and atherosclerotic lesions [22]. Therefore, it might be possible to hypothesize that complement might induce EndMT in other organs promoting tissue fibrosis [13, 16, 32].

**Figure 5:** Akt activation induced by I/R is inhibited by C1-INH. Renal cells showed limited activation of Akt pathway before I/R injury (A). Tubulo-interstitial C4d deposits were associated with Akt phosphorylation (purple) after 30 min from I/R injury (B). Renal EC (vWF, green staining) showed Akt phosphorylation (purple) after 30 min from reperfusion (C). C1-INH treatment led to an abrogation of Akt phosphorylation in renal EC (D). Total pAkt [Ser473] levels were quantified as described in the ‘Materials and Methods’ section and expressed as mean ± SD of at least three independent animals for each group (E) (*P = 0.03 versus T0, **P = 0.02 versus T30, T60 ctr). Nuclei were stained with TO-PRO-3 (blue). Original magnification: ×63.
In our study, we investigated the involvement of complement in mediating EC activation by using a recombinant form of C1-INH, a potent inhibitor of proteases of the classical and lectin complement pathways (C1r, C1s and MASP2) [33]. In addition, there are some data demonstrating that C1-INH might affect the activation of the alternative complement pathway [34], as we demonstrated by reducing Factor B deposition [19]. Animal studies showed that C1-INH could protect liver, intestine, heart and brain tissue from I/R damage [33, 35–37]. In addition, C1-INH has anti-inflammatory activities that are independent from complement activation [38, 39]. C1-INH can bind to ECs by adhesion molecules such as P and E-selectins [40], by interfering with endothelial–leucocyte interaction during inflammation. Interestingly, here we demonstrated that recombinant C1-INH localized preferentially at EC level, while the endogenous form was not present. Our findings are in line with the study of Gesuete et al. in ischaemic brain injury [35] showing that recombinant C1-INH preferentially localized to cerebral vessels without signs of endogenous C1-INH binding. This characteristic might be particularly relevant since complement components recognize ischaemic ECs during I/R, with the subsequent local release of anaphylatoxins and amplification of inflammatory process [22]. It might be possible to hypothesize that the recombinant protein acquired a different pattern of glycosylation which might explain the increased binding to EC surface. However, further studies are needed to characterize the C1-INH specific binding sites on ischaemic EC and the possible existence of a signalling pathway associated with C1-INH binding.

In addition, while preserving ischaemic kidney from tubular damage and recruitment of inflammatory cells [19], we found that C1-INH blocked Akt activation on EC in vivo. Akt pathway is particularly involved in inflammation, and could play a central role in I/R injury [17, 41, 42]. We demonstrated that renal tubular epithelial cells are the main cell type involved in the activation of Akt pathway [17]. Interestingly, we found a strong activation of Akt in ECs after I/R injury that seems to be mediated in vivo and in vitro by complement. Moreover, Akt is involved in EC trans-differentiation within the endocardium [23]. Our data demonstrate that C3a activates Akt phosphorylation in vitro and the specific inhibition of Akt signalling abrogated EndMT. This effect was also reproducible in presence of the potent anaphylatoxins C5a, preserving the expression of endothelial markers. We can then hypothesize that interfering with complement C3a and C5a at EC level might prevent EndMT and vascular rarefaction at the level of different organs, thereby improving cell viability and metabolic activity of ECs.

In conclusion, our data demonstrate for the first time that the process of EndMT and vascular rarefaction at renal level is activated by I/R injury through priming of the complement system and subsequent activation of the Akt pathway leading to renal fibrosis. Considering the significant contribution of vascular compartment in the development of chronic allograft dysfunction, we hypothesize that therapeutic inhibition of

**FIGURE 6:** Recombinant C1-INH specifically binds activated EC. Recombinant C1-INH localized at the level of glomerular and peritubular renal capillaries (A) in ischaemic kidneys. Control animals did not present staining for endogenous C1-INH in ischaemic kidney (B). When ECs were cultured in control medium (C), co-incubation with C1-INH did not induce any binding to the cells (D). On the other hand, in the presence of H2O2-induced cellular activation (E), C1-INH presented a significant binding on damaged ECs (F).
these systems may be essential to prevent vascular damage and tissue fibrosis in transplanted kidney.

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**CONFLICT OF INTEREST STATEMENT**

B. O. and E.v.A. are employed in Pharming Group.

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