C5a Receptors in Renal Transplantation
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DOI:
10.1016/j.jim.2015.02.010
10.1016/j.molimm.2012.08.013

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Novel Insights in Localization and Expression Levels of C5aR and C5L2 under Native and Post-Transplant Conditions in the Kidney

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Molecular Immunology 2013;53: 237-245
ABSTRACT

Aims
The complement system, and especially C5a, plays an important role in the pathophysiology of renal diseases and post-transplant renal injury. The two receptors for C5a are C5a receptor (C5aR) and C5a-like-receptor-2 (C5L2). Only renal C5aR expression has been reported, although exact localization and alterations in expression after transplantation are unknown.

Materials and Results
Renal C5aR and C5L2 expression and localization were analyzed immunohistochemically. C5aR and C5L2 expression was analyzed in human kidney biopsies obtained from living donors and patients suffering from acute tubular necrosis, acute cellular and vascular rejection or IF/TA.

C5aR was expressed in the thick ascending limb of Henle’s loop and first part of the distal convoluted tubule (DCT). Under inflammatory conditions, C5aR was de novo expressed in proximal tubuli. C5L2 was expressed in the kidney and localized to DCT1, DCT2 and connecting tubule. Persistent distal tubular expression of both receptors was demonstrated after renal transplantation.

Conclusions
This study shows distinct renal expression patterns for C5aR and C5L2. Our findings suggest a functional role for renal C5L2 rather than being a C5a decoy receptor. Future studies focusing on renal C5a-C5aR interaction should take differential C5aR and C5L2 expression into account, alongside abundant C5aR expression on infiltrating cells.
INTRODUCTION

The complement system plays an important role in different inflammatory conditions in the kidney, such as glomerulonephritis (Abe et al., 2001, Quigg, 2003), dense deposit disease (Pickering and Cook, 2011), atypical hemolytic uremic syndrome (Loirat and Fremeaux-Bacchi, 2011), lupus nephritis (Walport, 2002) and in transplanted kidneys (Pratt et al., 2002, Sacks et al., 2003, Serinsoz et al., 2005). The complement system can be activated via three different pathways: the classical, alternative and lectin pathway (Walport, 2001). Initiation of each of the pathways leads to activation of C3 and C5, and subsequent formation of the membrane attack complex. Inherent to complement activation is generation of anaphylatoxins C3a and C5a, of which C5a has the most potent chemokinetic and pro-inflammatory properties. Recent studies have shown that C5a is a key component in the development of renal injury in different renal pathologies (Abe et al., 2001, Bao et al., 2005, De Vries et al., 2003a, De Vries et al., 2003b, Gueler et al., 2008, Kose et al., 2010, Walport, 2002).

There are two known receptors for C5a, namely C5a receptor (C5aR) (Gerard and Gerard, 1991) and C5a-like receptor 2 (C5L2) (Ohno et al., 2000). Both receptors were initially identified on immune cells and are abundantly expressed on neutrophils. Stimulation of C5aR expressed on neutrophils by C5a initiates multiple inflammatory responses, such as chemotaxis and release of cytokines and reactive oxygen species (Lee et al., 2008, Monk et al., 2007, Rabiet et al., 2007). Expression of C5aR has been found in non-immune tissue as well, including kidney, brain, liver and lung tissue (Haviland et al., 1995). Expression of renal C5aR has been investigated under diverse inflammatory conditions, and has been reported on both proximal and distal tubuli (Abe et al., 2001, Fayyazi et al., 2000, Gueler et al., 2008, Zahedi et al., 2000). However, the exact localization of renal C5aR expression under native conditions remains to be elucidated.

On immune cells, C5L2 is expressed alongside C5aR and is thought to serve as a decoy receptor for C5a, thereby counterbalancing C5aR initiated responses (Bamberg et al., 2010, Gao et al., 2005, Lee et al., 2008, Monk et al., 2007). As has been demonstrated for C5aR, expression of C5L2 has been found in non-immune tissue as well (Ohno et al., 2000, Okinaga et al., 2003). Similar to co-expression of C5aR and C5L2 on immune cells, protein expression of C5L2 alongside C5aR has been reported on astrocytes (Rabiet et al., 2007). However, C5L2 protein expression in the kidney has not been reported so far.

Currently, inhibitors of C5 and C5aR are used clinically in treatment of several renal diseases, for example in atypical hemolytic uremic syndrome (aHUS). In animal models, inhibition of C5 or C5aR during renal ischemia-reperfusion injury (Arumugam et al., 2003, De Vries et al., 2003a, De Vries et al., 2003b, Zheng et al., 2008) and renal transplantation (Gueler
et al., 2008, Lewis et al., 2008, Li et al., 2010) has been shown to be beneficial as well. Clinical trials using C5 inhibitors in renal transplantation for treatment of humoral rejection are currently under investigation (Stegall et al., 2011). These recent developments emphasize the importance to identify the presence and exact localization of C5aR and C5L2 expression in the kidney under native conditions and after renal transplantation.

The aim of the present study was to investigate the expression and localization of C5aR and C5L2 in the kidney under native and inflammatory conditions in a renal transplant setting. Expression of renal C5aR and C5L2 was investigated in living kidney donor biopsies and in biopsies obtained under different conditions after transplantation, namely acute tubular necrosis (ATN), acute cellular and vascular rejection and interstitial fibrosis and tubular atrophy (IF/TA).

**MATERIALS AND METHODS**

2.1 Immunohistochemistry

Frozen sections (4 µm) from discarded kidneys from brain-dead donors were fixed in acetone for 10 min at room temperature (RT). Endogenous peroxidases were blocked with 0.09% H$_2$O$_2$ in phosphate buffered saline (PBS) for 30 min at RT. Paraffin embedded tissue sections (4 µm) from unaffected areas of kidneys following surgical renal tumor excision were deparaffinized and antigen retrieval was performed using 0.1M Tris/HCl buffer pH 9. Endogenous peroxidases were blocked with 0.3% H$_2$O$_2$ in PBS for 30 min at RT. Sections were incubated with primary monoclonal antibody to human C5aR, clone S5/1, directed against aa15-21 of the extracellular N-terminal region (Hycult, Uden, The Netherlands, (Oppermann et al., 1993)), monoclonal antibody to human C5aR, clone W17/1, directed against the extracellular N-terminal region (Hycult), polyclonal antibody to human C5aR, N9-29, directed against N-terminal aa9-29 (provided by M.R. Daha from Leiden University Medical Center, Leiden, The Netherlands), polyclonal antibody to C5L2 (Hycult) for 1 h at RT. As primary antibodies controls, PBS and isotype controls were performed (Dako, Glostrup, Denmark). Sections were incubated with appropriate horseradish peroxidase-conjugated secondary and tertiary antibodies (Dako). Antibodies were diluted in PBS with 1% bovine serum albumin (Sanquin, Amsterdam, The Netherlands) and, if necessary, 1% normal human serum (Sigma-Aldrich, St. Louis, MO, USA). The reaction was developed by addition of 3-amino-9-ethylcarbazole (AEC) and 0.03% H$_2$O$_2$. Sections were counterstained with Mayer’s haematoxylin solution (Merck, Darmstadt, Germany) or Periodic acid-Schiff (PAS) and embedded in Kaiser’s glycerine gelatine (Merck).

In further analysis of exact renal C5aR and C5L2 localization and expression of these receptors in renal biopsies, monoclonal antibody to C5aR S5/1 and polyclonal antibody to
C5L2 were used. The specificity of both antibodies was verified by western blotting.

2.2 Immunofluorescence

Frozen and paraffin-embedded sections were treated as described in 2.1. For C5aR and C5L2 localizations studies, monoclonal antibody to human C5aR, clone S5/1 and polyclonal antibody to human C5L2 were double stained with primary antibodies directed to specific markers for different regions in the distal nephron, i.e. polyclonal antibody to human uromodulin (Abcam, Cambridge, UK), polyclonal antibody to parvalbumin (Abcam) and monoclonal antibody to calbindin-D-28K (Sigma-Aldrich). Primary antibody controls for double stainings were performed using PBS controls. Sections were incubated with appropriate horseradish peroxidase-conjugated and FITC-conjugated secondary and tertiary antibodies. Antibodies were diluted in PBS with 1% bovine serum albumin (Sanquin) and, if necessary, 1% normal human serum (Sigma-Aldrich). Binding of primary antibodies detected by horseradish peroxidase-conjugated secondary and tertiary antibodies were visualized using TSA Tetramethylrhodamine System (PerkinElmer LAS, Boston, USA). Sections were counterstained and embedded using Vectashield with DAPI (Vector Laboratories, Burlingame, Canada).

2.3 Kidney biopsies

Renal C5aR and C5L2 expression was analyzed in frozen sections (2 µm) from human kidney biopsies that were diagnosed with acute rejection grades Banff Ia, Banff Ib, Banff IIA, Banff IIB, IF/TA (renal biopsies with at least 30% IF/TA) and ATN (all groups n=6). As healthy controls, we used biopsies from living kidney donors obtained at donation (before retrieval with kidney in situ and before clamping arterial and venous circulation of the kidney, n=6). Biopsies were taken using a 16-gauge needle (Acecut®, TSK Laboratory, Japan). In addition, frozen sections from unaffected areas of kidneys following surgical tumor excision (4 µm, n=6) were included. Sections were stained with monoclonal antibody to human C5aR, clone S5/1 and polyclonal antibody to human C5L2, as described in 2.1. C5aR and C5L2 expression were analyzed by two individual observers.

RESULTS

3.1 Renal C5aR expression

Expression of C5aR in the kidney was examined using two different monoclonal antibodies, S5/1 and W17/1, and one polyclonal antibody, N9-29 (Fig 1). Both in frozen and paraffin-embedded sections of renal tissue, S5/1 stained granulocytes (Fig 1A). In addition, S5/1 revealed C5aR expression by distal tubuli both in frozen (Fig 1B) and paraffin-embedded sections (Fig 1D). The C5aR expression was localized at the basolateral side of these tubuli. W17/1 stained distal tubuli on frozen sections as well (Fig 1C), but this antibody was not
applicable for staining paraffin sections. Using N9-29 (Fig 1E), morphological similar distal
tubuli were stained in paraffin sections, when compared to S5/1 (Fig 1D). Besides strong
expression in distal tubuli, S5/1 occasionally showed weaker basolateral C5aR expression
in proximal tubuli. Specificity of C5aR antibodies was confirmed by PBS and appropriate
isotype controls (data not shown).

3.2 Renal C5aR localization
To characterize the precise localization of C5aR expression by distal tubuli, detailed
morphology of C5aR positive tubules was examined on paraffin embedded sections and
double stainings with multiple distal tubular markers were performed (Fig 2). We found that
C5aR is mainly expressed in the thick ascending limb of Henle’s loop (TAL) and to a lesser
extent in the first part of the distal convoluted tubule (DCT1). First, morphological examination of paraffin embedded sections suggested that TALs and DCTs are expressing C5aR (Fig 2A). In addition, the majority of C5aR positive tubuli showed colocalization with uromodulin, a marker for TAL (Fig 2B). Double staining of C5aR and parvalbumin localized C5aR to DCT1 as well (Fig 2C). However, the intensity of C5aR staining in parvalbumin positive tubuli was lower than in parvalbumin negative tubuli. C5aR did not show any colocalization with calbindin (data not shown), which marks the second part of the distal convoluted tubule (DCT2) and the connecting tubule (CNT).

3.3 Renal C5L2 expression
To better understand the potential role of C5a on renal epithelial cells, renal expression of C5L2 was analyzed as well (Fig 3). With the antibodies we used, tubular C5L2 expression appeared to be less intense than tubular C5aR expression. Similar to C5aR, we observed C5L2 expression in distal tubuli at the basal side of epithelial cells (Fig 3A). A PAS counterstaining demonstrated that C5L2 expression was present within the tubular basement membrane of distal tubuli (Fig 3B). Specificity of the primary antibody directed to C5L2 was verified by PBS and isotype controls, and by western blotting (data not shown).
3.4 Renal C5L2 localization

To study possible co-localization of C5aR and C5L2, the type of distal tubuli expressing C5L2 was investigated in detail, using the same markers used for localizing renal C5aR. C5L2 did not show any colocalization with TAL-marker uromodulin (data not shown). Serial sections stained with C5L2 and parvalbumin (antibodies both of rabbit origin) indicated expression of C5L2 in DCT1 (Fig 3C and 3D respectively). In addition, colocalization of C5L2 with calbindin was found, implicating expression of C5L2 in DCT2 and CNT (Fig 3E).

Double staining of C5aR and C5L2 revealed the expression of these two receptor to be almost
Figure 4: C5aR expression in human kidney biopsies from living kidney donors and transplanted kidneys.
Representative pictures from C5aR stained frozen sections of biopsies obtained from (A) living donor kidney, (C) ATN, (D) Banff Ia, (E) Banff Ib, (F) Banff IIa, (G) Banff IIb, (H) IF/TA, (B) Frozen section from an unaffected part of a kidney following surgical tumor excision (RCC). (I) C5aR positive distal tubuli are located apart from C5aR positive leukocyte influx. (J) Representative picture of weaker C5aR expression in proximal tubuli (PT) alongside strong C5aR expression in TAL in paraffin embedded tissue. Magnifications A-I 200x, J 400x.a
Figure 5: C5L2 expression in human kidney biopsies from living kidney donors and transplanted kidneys. Representative pictures from C5L2 stained frozen sections of biopsies obtained from (A) living donor kidney, (C) ATN, (D) Banff Ia, (E) Banff Ib, (F) Banff IIa, (G) Banff IIb, (H) IF/TA. (B) Frozen section from an unaffected part of a kidney following surgical tumor excision (RCC). Arrow heads indicate positive tubuli. (I) Perivascular C5L2 positive leukocyte influx observed in acute rejection. Magnifications 200x.
mutual exclusive (Fig 3F). When positive for both C5aR and C5L2, the tubuli showed weak expression for both of the receptors, when compared to tubuli which were single positive for one of the receptors (Fig 3F, inset).

3.5 Renal C5aR expression in kidney transplant biopsies
To gain insight in renal C5aR and C5L2 expression before and after transplantation, expression of these two receptors was analyzed in renal biopsies obtained from living kidney donors and in post-transplant renal biopsies diagnosed with ATN, acute cellular or vascular rejection or IF/TA (Fig 4 and Fig 5). The biopsies from living kidney donors were obtained at time of donation before clamping of the arterial and venous circulation. In addition, expression of the receptors was analyzed in renal tissue obtained from macroscopically unaffected parts of kidneys following surgical tumor excision (RCC).

The intensity of renal C5aR expression in distal tubuli was comparable between biopsies obtained from living donors (Fig 4A), and all post-transplant biopsies, i.e. ATN, acute rejection and IF/TA (Fig 4C-4H). No C5aR expression was found in proximal tubuli in biopsies from living donors. In contrast, in several post-transplant biopsies, weak C5aR expression was observed at the basolateral side of proximal tubuli, alongside stronger C5aR expression in

Figure 6: Schematic representation of the localization of C5aR and C5L2 expression and tubular markers in the nephron. Linear representation of a nephron with a glomerulus, the proximal tubule (prox), the loop of Henle (loop), the thick ascending limb of Henle’s loop (TAL), the first and second part of the distal convoluted tubule (DCT1, DCT2), the connecting tubule (CNT) and the collecting duct. Renal C5aR is expressed in the TAL and fades out along the DCT1, while C5L2 expression arises in DCT1 and is stronger expressed in the DCT2 and CNT. Uromodulin is a marker for TAL, parvalbumin is a DCT1-marker and calbindin marks the DCT2 and CNT.
distal tubuli (Fig 4J). Notably, the intensity of C5aR expression in distal tubuli was increased in RCC (Fig 4B), compared to biopsies from living donors (Fig 4A) or post-transplant biopsies (Fig 4C-4H). Additionally, C5aR expression was observed in proximal tubuli in RCC.

Besides C5aR expression on tubuli, C5aR positive infiltrating leukocytes were detected in the interstitium of all post-transplant biopsies. Interestingly, loss of tubular C5aR expression was observed in areas with abundant C5aR positive infiltrating leukocytes. On the contrary, in areas with less C5aR positive leukocytes, tubular C5aR expression was more intense (Fig 4I).

3.6 Renal C5L2 expression in kidney transplant biopsies

Renal C5L2 expression levels in distal tubuli were comparable between living kidney donor biopsies (Fig 5A) and post-transplant biopsies obtained from patients with ATN and all stages of acute rejection (Fig 5C-5G). However, almost no C5L2 expression was observed in kidney biopsies obtained from patients with IF/TA (Fig 5H). In addition, clearly increased distal tubular expression of C5L2 was observed in RCC (Fig 5B), when compared to biopsies from living donors (Fig 5A) and all post-transplant biopsies (Fig 5C-H). Expression of C5L2 by proximal tubuli was not observed in any of the tissues.

Besides C5L2 expression on distal tubuli, infiltrating C5L2 positive leukocytes were observed in post-transplant biopsies. The majority of interstitial infiltration in post-transplant biopsies was negative for C5L2, but positive for C5aR. On the contrary, perivascular leukocyte infiltration was found to be C5L2 positive, but C5aR negative, particularly in acute rejection biopsies (Fig 5I). This phenomenon was only sporadically observed in ATN and IF/TA biopsies.

DISCUSSION

The important role of anaphylatoxin C5a generated by complement activation in the pathophysiology of renal disease and post-transplant renal injury is well established. Detailed knowledge on the expression pattern and function of receptors for C5a in the kidney should be acquired to continue with clinical application of intervention studies. There are two known receptors for C5a, namely C5aR and C5L2. C5aR expression on tubular cells within the kidney has been reported, although the exact localization of this receptor is unknown. The present study shows that renal C5aR is predominantly expressed in TAL and DCT1. In addition, to our knowledge, we are the first to show that also C5L2 is expressed on renal epithelial cells. However, in contrast to C5aR, renal C5L2 expression was localized in DCT1, DCT2 and CNT. Subsequently, persistent expression of both receptors on distal epithelial cells was demonstrated after renal transplantation.

Expression of C5aR in human renal tissue has been reported in both proximal and distal
Renal Localization of C5a Receptors

Chapter 2

However, the type of distal tubuli expressing C5aR has not been further specified. Notably, tubular expression of C5aR has even been suggested to be debatable (Kiafard et al., 2007). Therefore, we first confirmed renal C5aR expression by using three different antibodies. It was found that all three antibodies predominantly stained distal tubuli, and using different tubular markers, the distal tubuli expressing C5aR were identified as being TAL and DCT1. In line with previous reports, we also observed C5aR expression in proximal tubuli of post-transplant biopsies and in macroscopically unaffected parts of kidneys following surgical tumor excision (Abe et al., 2001, Fayyazi et al., 2000, Gueler et al., 2008, Zahedi et al., 2000). In addition to previous studies, C5aR expression was also analyzed in renal tissue from healthy individuals. Our study clearly shows that C5aR expression in biopsies from healthy kidney donors, obtained before organ retrieval with the kidney still in situ, is only found on distal tubuli. Therefore, we propose that, under native conditions, C5aR is only expressed on distal tubuli, but can be induced in proximal tubuli under inflammatory conditions.

Considering C5aR induction on proximal tubuli, interleukin-6 (IL-6) may be a potential candidate responsible for inducing C5aR under inflammatory conditions. IL-6 has been shown to induce C5aR expression in rat hepatocytes (Koleva et al., 2002). In addition, in a model of septic rats, anti-IL-6 treatment reduced C5aR expression in renal tissue (Riedemann et al., 2003). Moreover, IL-6 may be responsible for proximal C5aR induction in unaffected parts of kidneys following surgical tumor excision as well, since RCC is frequently associated with paraneoplastic symptoms, of which some can be attributed to IL-6 production by the tumor itself (Blay et al., 1997, Tsukamoto et al., 1992).

To speculate about a possible role of C5aR on tubular cells, it is essential to know more about renal expression of C5L2. To our knowledge, we are the first to demonstrate C5L2 expression in the kidney. Although C5aR is expressed in TAL and DCT1, renal C5L2 expression was confined to DCT1, DCT2 and CNT. Double staining revealed that C5aR and C5L2 are almost mutually exclusive expressed. DCT1 was positive for both C5aR and C5L2, but showed weak expression for both receptors, compared to tubuli which were single positive for one of the receptors. Our data suggest that C5aR is expressed in TAL and fades out along DCT1, while C5L2 expression arises in DCT1 and is stronger expressed in DCT2 and CNT (Fig 6). Although C5aR and C5L2 are both expressed in the distal nephron, it is remarkable that these two receptors are not expressed on the same cell type. Expression of these two receptors on the same cell type would be coherent with the hypothesis that C5L2 serves as a decoy receptor for C5a. Indeed, simultaneous expression of C5aR and C5L2 has been reported on both immune and non-immune cells (Rabiet et al., 2007). Possibly, C5L2 might have cellular functions of its own rather than only being a decoy receptor. Further investigation is required.
to elucidate the underlying reasons for the separated expression of C5aR and C5L2 on different cell types and the functions of C5aR and C5L2 in the distal nephron.

To get more insight into the regulation of C5aR and C5L2 expression, we examined the expression of both receptors after transplantation. Human post-transplant kidney biopsies from patients diagnosed with ATN, acute cellular or vascular rejection or IF/TA were analyzed for C5aR and C5L2 expression. Using this unique material, we were able to study expression levels in healthy renal tissue and different inflammatory circumstances after transplantation. The intensity of C5aR expression in distal tubuli remained comparable in living donor biopsies and post-transplant biopsies. In addition, C5aR was found to be abundantly expressed on infiltrating cells. Interestingly however, distal tubular C5aR expression was found to be lost in areas with severe leukocyte infiltration. It is known that binding of C5a to C5aR leads to internalization of the receptor (Bamberg et al., 2010, Okinaga et al., 2003, Rabiet et al., 2008). Therefore, it is possible that excessive generation of C5a leads to internalization of tubular C5aR and tubular loss of C5aR. We envision that internalization, down regulation or shedding of C5aR could prevent overstimulation of C5aR on distal tubuli, thereby dampening renal inflammation.

C5L2 expression in living donor biopsies and ATN and acute rejection biopsies remained at the same level. However, C5L2 expression was almost absent in biopsies with IF/TA, suggesting that cellular C5L2 expression is inhibited or that C5L2 is shedded from the tubular cell membrane under influence of the microenvironment during IF/TA. Renal fibrosis is one of the histological hallmarks of IF/TA, and C5aR has been described to promote renal fibrosis (Boor et al., 2007). Since C5L2 is thought to serve as a decoy receptor for C5a, thereby counterbalancing C5aR initiated responses, tubular loss of C5L2 in IF/TA might be involved in development of fibrosis observed in IF/TA.

In conclusion, this study shows that C5aR and C5L2 are both expressed in the kidney, but that the receptors are located in a different region of the distal nephron. In addition, we showed that C5aR, but not C5L2, is expressed in proximal tubuli under inflammatory conditions. In post-transplant human kidney biopsies persistent C5aR and C5L2 expression in distal tubuli was found. Our findings imply that for further studies on the role of C5aR and C5L2 in the pathophysiology of renal diseases, differential expression of the receptors during inflammation and expression at different parts of the nephron should be taken into consideration.

ACKNOWLEDGMENTS

We thank Anita Meter-Arkema for excellent technical assistance.
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