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Ischemic Time Associated with Activation of Rejection-Related Immune Responses

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Abstract : Ischemia/reperfusion injury (I/RI) is the major cause of acute renal failure and delayed graft function (DGF) unavoidable in renal transplantation. Enormous studies on ischemia damage playing a role in activating graft rejection factors, such as T cells or macrophages, are being reported. Present study was performed to determine whether ischemia time would play an important role in activating rejection-related factors or not in rat models of I/RI. Male Sprague-Dawley rats were submitted to 30, 45, and 60 minutes of warm renal ischemia with nephrectomy or control animals underwent sham operation (unilateral nephrectomy). Renal function and survival rates were evaluated on day 0, 1, 2, 3, 5 and 7. Immunofluorescence staining of dendritic cells (DCs), natural killer (NK) cells, macrophages, B cells, CD4+ and CD8+ T cells were measured on day 1 and 7 after renal I/RI. Survival rates dropped below 50% after day 3 in 45 minutes ischemia. Histologic analysis of ischemic kidneys revealed a significant loss of tubular architecture and infiltration of inflammatory cells. DCs, NK cells, macrophages, CD4+ and CD8+ T cells were infiltrated from a day after I/RI depending on ischemia time. Antigen presenting cells (DCs, NK cells or macrophages) and even T cells were infiltrated 24 hours post-I/RI, which is at the time of acute tubular necrosis. During the regeneration phase, not only these cells increased but B cells also appeared in more than 45 minutes ischemia. The numbers of the innate and the adaptive immune cells increased depending on ischemia as well as reperfusion time. These changes of infiltrating cells resulting from each I/RI model show that ischemic time plays a role in activating rejection related immune factors and have consequences on progression of renal disease in transplanted and native kidneys.

Key Words **:** Renal ischemia/reperfusion injury, antigen presenting cells, rejection, inflammation, renal transplantation.

Introduction

Renal ischemia/reperfusion injury (I/RI) not only is the major cause of acute renal failure (ARF) but also leads to delayed graft function (DGF) of renal allografts, increased acute rejection, and enhanced chronic allograft nephropathy (18). Renal injury after ischemia appears to be a consequence of tissue hypoxia from interrupted blood supply but also from the process of reperfusion, leading to an active inflammatory response, which contributes to the resultant tissue injury (17,22).

Following transplantation, antigen independent stimuli, including ischemia, reperfusion, surgical injury, and systemic stress, may contribute to the initiation of an inflammatory response (10). Several inflammatory mediators have been identified in the pathogenesis of renal I/RI, including leukocyte adhesion and subsequent infiltration in inflamed sites, activation of complement/membrane attack complex, and up-regulation of cytokines/chemokines (1-2).

I/RI initiated inflammatory response provokes an increased level of acute host immunological reactivity (18). The rapid appearance within kidney tissue of the innate immune system components, such as neutrophils and macrophages, following I/RI provides clear support for an antigen-independent mechanism of tissue injury (14).

Dendritic cells (DCs) are the major professional antigen-presenting cells of the immune system. Transplantation of a syngeneic renal graft increased DC accumulation in the kidney (13). Activated trafficking of DCs following I/RI by showing their capacity to migrate to the kidney draining lymph node, leading to antigen presentation and activation of naive T cells were demonstrated (8). Recent studies have identified T lymphocytes as important mediators in renal I/RI (4,15) as well as I/RI of the liver (24) and the lung (20). T cell activation has been demonstrated to occur totally inde-

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pendently of antigen (16). B cells may also participate in I/RI. Partial protection from renal I/RI in T cell or B cell deficient mice have conferred that these cells mediate renal I/RI (5,15). Genes coding for histocompatibility and antigen-presenting factors, calcineurin and mammalian target of rapamycin pathway-associated proteins identified alloimmunity genes affected by I/RI and at 36 hours after I/RI the up-regulated expression was demonstrated in 30 and 60 minutes of ischemia (9).

In I/RI without the presence of an alloantigen, such as in native kidney I/RI, one could invoke the unveiling of a self-antigen presented by an antigen presenting cell (APC) that was normally not exposed to the immune system, but somehow expressed during tissue injury (16). Therefore, ischemia and duration-related immune changes not only contribute to additional kidney damage but also affect renal function and mortality after the initial insult. This eventually decreases the graft survival rates following renal transplantation due to increased rejection responses.

In this study, we investigated whether ischemia time would play an important role in activating rejection related factors or not in rat models of I/RI. Analyzing immune modulation in moderate or severe ischemia at early and later stages of I/RI would provide us clues for targeting the immune initiation factors to control rejection or DGF in I/RI organ transplantation.

Materials and Methods

Ischemia-Reperfusion Animal Model

All animals were treated according to the regulations for the Care and Use of Laboratory Animals in Kangwon National University. I/R was induced in anesthetized (ketamine, 100 mg/kg intraperitoneal injection) 8 week-old male Sprague-Dawley (SD), rats weighing 220~250 g. After a mid-abdominal incision, the left renal vascular pedicle was bluntly dissected and clamped with atraumatic clamp for 30 (n = 12), 45 (n = 38) or 60 (n = 44) minutes. Right-side nephrectomy was carried out and the incisions were temporarily closed during ischemia. During ischemia, animals were kept on a heating pad to maintain rectal temperature of $37 \pm 0.5^{o}C$. Directly after visual confirmation of kidney reperfusion, the wounds were sutured, and the animals were allowed to recover with free access to food and water. Sham operated animals (n = 12) underwent similar surgical procedures of right nephrectomy without the ligation of left renal pedicle.

Renal Function

For evaluation of functional recovery, tail bleeds were collected from rats before surgery and at 1, 2, 3, 5 and 7 days after reperfusion. Blood samples were tested for serum creatinine levels (mg/dl) using Spotchem EZ (SP-4430, ARKRAY INC., Japanease) chemistry analyzer.

Histology

The rats were sacrificed at day 1 and 7 after I/RI. Kidney samples were collected for immunofluorescene, hematoxylin and eosin (H&E) staining.

Immunofluorescence staining

Activation of rejection related immune cells we studied are DCs, CD4, CD8 T cells and B cells. Natural killer (NK) cells and macrophages were selected to demonstrate the expression of inflammatory changes induced by I/RI. Frozen kidney tissue sections (5 um) were air dried and permeabilized with Triton X-100 (Sigma, USA). After blocking with 1% BSA in PBS (Sigma, USA) for 30-minutes, sections were incubated with primary antibodies diluted in 1% BSA in PBS overnight at $4^{o}C$. Slides were washed in PBS and incubated with secondary fluorochrome-conjugated antibodies for 2 hours. The following monoclonal antibodies (AbD Serotec, Oxford, UK) were used in 1 : 100 dilutions: NK cell (mouse anti rat CD161), CD4+T cell (mouse anti rat CD4 domain) and DC (mouse anti rat OX-62). Macrophage (ED1, mouse anti rat CD68) and CD8+T cell (mouse anti rat CD8 alpha) were used in 1 : 200 dilutions. B cell (mouse anti rat CD45RA) was used in 1 : 400 dilution. Goat anti mouse IgG:FITC (rat adsorbed) was used for secondary antibody. The entire kidney section was viewed and cells were counted in the area of the cortex and corticomedullary junction using confocal laser microscope (Olympus DP50). Total numbers of cells were quantified in a blinded fashion and arbitrarily graded by two people.

Statistical analysis

Data are expressed as mean values ± SD. Comparison between groups was performed by Dunnet's test. Kaplan-Meier analysis was used to analyze the survival rate. $P < 0.05$ was considered significant. SAS 9.0 was used.

Results

All ischemia groups showed increase in serum creatinine levels at day 1. The peak level was reached at day 1 in 30-minutes, day 2 in 45-minutes, and day 3 in 60-minutes ischemia group. The renal function of 30-minutes ischemia group returned to normal level at day 2 whereas in 45- and 60-minutes ischemia groups it did not recover until day 7. Two ischemic groups (45- and 60-minutes) showed significant difference in their serum creatinine level compared to sham groups (Fig 1-A).

Survival rates began to drop after two days after I/RI in severe ischemia groups. Steep decrease was followed in 60-minutes ischemia group reaching down to 4% at 4 days after I/RI and no survival left after one week. In 45-minutes ischemia group, 41% animals remained until day 7 since day 4. All the sham animals and 30-minutes ischemia group animals survived during the whole observation period (Fig 1-B).

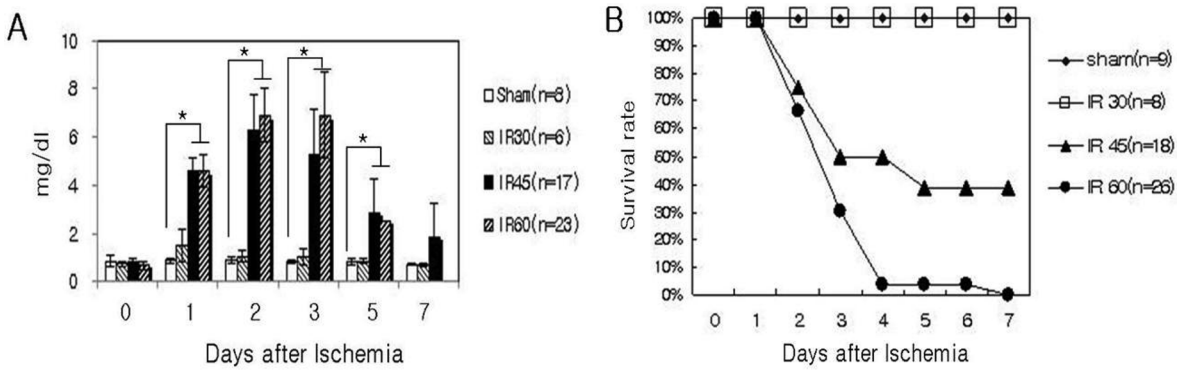

Fig 1. Serum creatinine levels (A) and survival rates (B) from sham and I/R groups. Results are shown as mean values ± SD. *$P < 0.05$ versus sham at the same time.

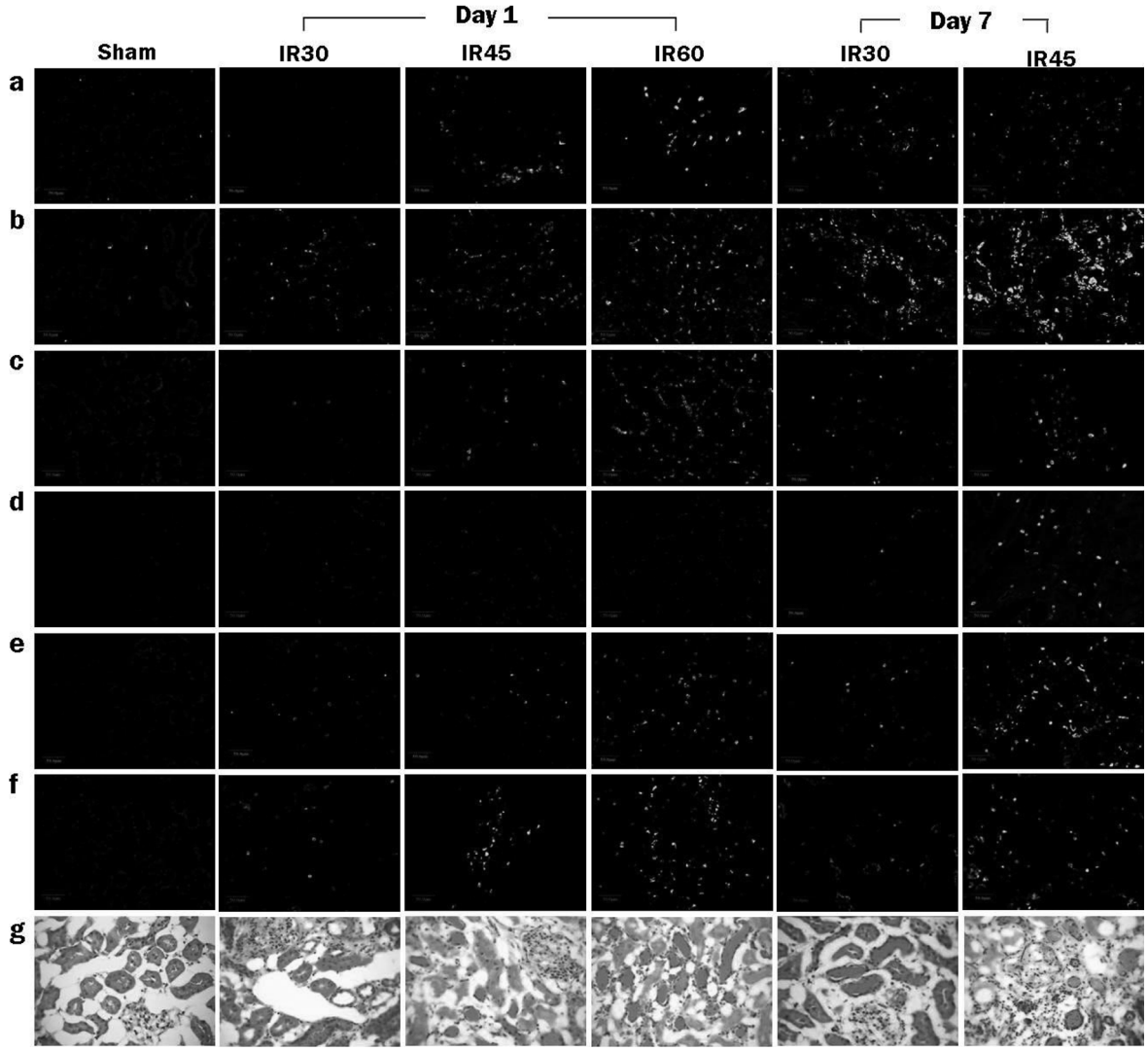

Fig 2. Infiltration of Cells. a. Natural Killer cells (CD161), b. Macrophages (CD68), c. Dendritic cells (OX62), d. B cells (CD45RA), e. CD4 T cells (CD4 domain), f. CD8 T cells (CD8α) at day 1 and day 7 after I/RI by immunofluorescence staining. histological assessment of tubular injury at day 1 and day 7 after I/RI by H&E staining. DCs, NK cells, macrophages, CD4+ and CD8+ T cells were infiltrated from a day after I/RI depending on ischemia time. Macrophages were especially expressed abundantly compared with other cells and in sham animals. B cells were shown only at 7 days after I/RI and not on day 1.

Table 1. Table 1. Grading of infiltrating cells. On day 1 after I/RI: sham (n=3), 30 (n=5), 45 (n=4) and 60 minutes (n=5) of ischemia. On day 7: sham (n=3), 30 (n=3) and 45 minutes (n=5) of ischemia.

	Day 1				Day 7		
	Sham	30	45	60	Sham	30	45
NK cell	−	−	+	+	−	++	++
Macrophage	+−	++	++	++	+−	+++	++++
Dendritic cell	−	+−	+	++	−	+	+
B cell	−	−	−	−	−	+−	+
CD4+ T cell	−	+	+	++	−	+	++
CD8+ T cell	−	+−	++	++	−	+	++

(−) Negative, (+−) Trace(1-20 cells), (+) Moderate(20-50 cells), (++) Strong (50-200 cells), (+++) Very strong (200 -500 cells), (++++) Intense (500 ↑cells). Grading was done arbitrarily observing whole section of ×20 and ×40 slides by two people.

H&E staining of kidney sections at day 1 and 7 after renal I/RI showed infiltration of inflammatory cells and a significant loss of tubular architecture. Destruction of tubule cells and loss of brush borders increased in relation to duration of ischemia and reperfusion. (Fig 2)

Immunofluorescence staining of kidney slices expressed all the cells we intended to observe after I/RI. Arbitrarily graded cell expressions were as table 1. DCs, NK cells, macrophages, CD4+ and CD8+ T cells were infiltrated from a day after I/RI depending on ischemia time. Macrophages were especially expressed abundantly compared with other cells and in sham animals. B cells were shown only at 7 days after I/RI and not on day 1 (Fig 2 and Table 1).

Discussions

The present study demonstrates that prolonged ischemia promotes immune cell expression either at a day or seven days after reperfusion using immunohistochemistry. The serum creatinine level of moderate ischemia (30-minutes ischemia group) reaches its peak and return to normal level faster than the two other severe ischemia groups. The mean serum creatinine level of 60-minutes ischemia group is higher than 45-minutes ischemia group at day 2 and 3 however, there was no significant difference. Thus the degree of renal damage due to ischemia time could not be distinguished by serum creatinine level alone. This can be explained with the survival rates. The serum creatinine level of animals who recovered from I/RI decreases whereas it kept on increasing until those who could not recover from ongoing reperfusion injury die. The survival rates began to drop at day 2 and 3 also. Generally, renal function and survival rates are steady 4 days after I/RI. However, in 60-minutes ischemia group the damage was impossible to recover and all animals in this group died at 7 days after I/RI. The histological assessment of renal tubule destruction at day 1 and 7 support that even the serum creatinine level is returned to the normal level, the kidneys still need more time for repair of the damaged structures. The duration of ischemia time gave loss of tubular architecture more significantly whereas the duration of reperfusion injury infiltrated more inflammatory cells.

Macrophages and NK cells were constituted a major component of the innate immune system acting in nonspecific as well as specific defense (19). Macrophages, especially, were expressed in relatively large numbers. This is thought to be of the fact that macrophages may promote early injury responses after I/RI (11). Our results showed trafficking of macrophages into kidneys of both sham-operated and I/RI rats. Thus laparatomy and surgical trauma induce infiltration of macrophages into rat kidneys which caused nonspecific inflammatory response. However, this data is somehow not consistent with established data showing that laparotomy causes a systemic inflammation response syndrome that can involve distant organs and that laparotomy alone leads to lymphocyte changes (12).

Main function of DCs is to process antigen material and present it on the surface to other cells of the immune system, thus to function as antigen-presenting cells (19). Exposure time to ischemia affected the expression of DCs the most sensitively suggesting that DCs might function as antigen-presenting cells specifically associated with ischemia time. DCs showed clearly distinguished increase in cell numbers among three ischemia groups at a day after reperfusion. At 7 days after reperfusion DCs were slightly increased in 30 and 45 min I/RI groups. It is reported that accumulation of DCs in liver and kidney was observed within 1h and peaked at 24 h after I/RI (23) but DCs infiltrated more at 7 days than at 1 day after I/RI in our data. This difference in results can be explained to the fact that Zhou et al. used 60 min I/RI model and observed until 24 hours after reperfusion, whereas the I/RI models we observed until 7 days after reperfusion is 30 and 45 min I/RI groups. Due to the fact that our 60 min I/RI group did not survive to 7 days after reperfusion, we only have the results for 24 hours after reperfusion. We, however, assume that 60 min I/RI group would show an increase in DCs expression at 7 days after reperfusion as the other I/RI groups did.

DCs are components of the innate immune system that then transfer information to the adaptive immune system, the T cells, and trigger naive T cell response (3,11 and 16). Increased numbers of DCs expressing a more mature phenotype were identified in ischemic kidney in the early phase (within 24 h) after I/RI while these activated DCs with a strong capacity to induce T cell proliferation migrated from the kidney into the renal lymph node 1 day after I/RI (8). This could mean that, since we did not distinguish between immature and mature DCs, by the time we began detecting immune cells, T cell proliferation has already been induced by mature DCs or even if immature DCs consisted the popu-

lation of DCs they are still contributing to the expansion and differentiation of regulatory T cells. This might be the reason why DCs and T cells are both expressed in day 1 and day 7 after I/RI. In severe renal I/RI T cells were reported to be prominent after 1 week (21). T cells were also expressed in larger numbers at 7 days than day 1 after reperfusion.

CD8+ cytotoxic T cells are thought to mediate acute rejection associated with MHC I molecules whereas CD4+ T cells are involved in a chronic rejection of MHC II molecules (7). Our results did not show much differences in number of two types of T cells except the fact that in severe I/RI CD8+T cells were expressed more in the early stage (day 1).

Interestingly, B cells started to be observed at the late repair phase. Expression of B cells is a sign of humeral immune rejection, which suggest that I/RI can provoke antigen independent immune response. According to our results of day 7, ischemia time also affected the expression of B cells. As previously mentioned, B cell deficient mice were shown to be relatively protected from renal I/RI (5). Transfer of serum from wild-type mice restored the injury, suggesting a role of a soluble mediator such as antibody. However, RAG-KO mice deficient in both B and T cells were not protected from I/RI (6). In the future, the pathophysiology of B cells should be investigated to establish its role in I/RI and in transplanted organ, along with rejection related immune modulators acting as an antigen.

For more accurate quantification of present results, molecular level study is needed. The amplified immune reaction in ischemic kidney might be mediated by cytokine-dependent pathway (3). Future study might be needed to analyze cytokines (e.g. IFN-γ, TFN-α, IL-1β and IL-6) for demonstrating the amplified immune reaction. In future study, to investigate whether I/RI could act as antigen triggering immune rejection, we need to explore the expression of the T cell costimulatory molecules and the macrophage markers along with antigen presenting cells.

In conclusions, expression of immune cells, especially antigen presenting cells, at seven days after I/RI proves that I/RI evoke immune responses. Regardless of reperfusion time, increase in cell numbers in severe ischemia groups over moderate ischemia group shows that ischemic time affects the degree of immune responses. As cells or factors that cause tissue injury in the inflammatory response associated with ischemia may be critical for the termination of inflammation and for tissue repair at later stages, these changes of infiltrating cells resulting from each I/RI model may support that ischemia time plays an important role in activating rejection-related immune factors.

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허혈 시간과 거부반응 관련 면역반응

남현숙 · 최진영 · 김윤태 · 강경선* · 권혁무 · 홍종해 · 김두 · 한태욱 · 문태영* · 김지희*** · 조병렬*[2] · 우흥명[1]**

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요 약 : 허혈/재관류 손상은 급성신부전의 주요 원인이며 이식된 신장의 기능지연을 유발하여 거부반응의 발생을 증가시킨다. 본 연구에서는 쥐의 허혈/재관류 손상모델에서 허혈시간에 따른 면역세포의 변화를 평가하였다. 8주령 수컷 SD rat의 좌신을 각각 30, 45, 60분간 허혈/재관류 동안에 우신을 적출 하였고, 대조군은 우신만 적출하였다. 신장기능은 0, 1, 2, 3, 5, 7일에 각각 평가하였으며, 허혈/재관류 후 1일과 7일에 신장조직을 채취하여 면역형광염색 (DCs, NK cells, macrophages, B cells, CD4+ and CD8+ T cells)과 H&E 염색을 실시하였다. 허혈 시간이 증가할수록 신장기능이 감소되었으나, 신장 세뇨관괴사와 염증세포의 침윤이 증가하였다. 허혈/재관류 후 신장조직에서 DCs, NK cells, macrophages, CD4+ T cells, CD8+ T cells의 침윤 증가와 재관류 후 7일째 B cells의 침윤이 관찰되었다. 면역세포는 허혈시간 뿐 아니라 재관류 시간이 증가에 따라 뚜렷하게 관찰되었다. 이 결과는 허혈시간이 증가됨에 따라 면역반응이 증가될 수 있으며, 허혈재관류 손상이 비항원성 면역반응을 유도할 수도 있다는 것을 의미한다.

주요어 : 신장 허혈/재관류 손상, 항원제시 세포, 거부반응, 염증, 신장 이식.