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ORIGINAL ARTICLE

Effect of rapamycin on renal ischemia-reperfusion injury in mice

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Summary

The aim of this study was to determine the effect of rapamycin on renal ischemia-reperfusion injury (IRI) in mice. Renal IRI was induced in male BALB/c mice by clamping both renal pedicles for 45 min. The mice were treated with either vehicle or rapamycin (2 mg/kg/day) by oral gavage, starting 1 day before the IRI and continued daily till killing. The mice were killed on days 1, 3 and 7 after the operation. The severity of the renal IRI was assessed by serum creatinine levels and renal histology. Proliferation of renal tubular cells was quantified by immunohistochemical staining for proliferating cell nuclear antigen (PCNA). One day after the IRI, the serum creatinine levels of rapamycin-treated mice were significantly higher than those of the vehicle-treated mice. Kidney sections from rapamycin-treated mice showed more marked tubular damage and significantly lower number of PCNA-positive cells. The number of PCNA-positive cells in the rapamycin-treated mice remained significantly lower on day 3 after the IRI. By day 7 after the IRI, the serum creatinine levels, renal histology and positive PCNA staining in the kidney sections became similar between the two treatment groups. We conclude that in this murine model of renal IRI, rapamycin treatment aggravates renal IRI during the first 3 days after the insult. This effect might be mediated, at least partly, through inhibition of renal tubular cell proliferation.

Introduction

Ischemia-reperfusion injury (IRI) during renal transplantation is an important cause of early allograft dysfunction and is associated with increased incidence of acute rejection and decreased long-term allograft survival [1,2]. Avoidance or minimization of renal IRI might therefore improve the long-term outcomes of renal transplantation.

The effect of different immunosuppressive drugs on IRI after renal transplantation varies. Cyclosporine, for instance, has been shown to impair the recovery of renal allograft from delayed graft function during the early post-transplant period [3]. Many renal transplant centers have therefore adopted an immunosuppressive protocol that either reduce or delay the introduction of cyclosporine in patients with delayed graft function. Mycophenolate mofetil, on the other hand, has been demonstrated to attenuate IRI in animal model of renal transplantation [4].

Rapamycin is a potent immunosuppressive drug, which acts by inhibiting the proliferation and clonal expansion of interleukin-2-stimulated T cells through the inhibition of a 70-kDa S6 protein kinase, a kinase necessary for cell cycle progression [5,6]. Rapamycin has been used, in combination with cyclosporine and steroids, to prevent acute rejection in renal transplant recipients [7,8]. Earlier studies indicate that rapamycin is by itself non-nephrotoxic [6]. However, in rat models of renal IRI, it has been shown that rapamycin treatment impaired the recovery of the kidneys from acute renal failure [9,10]. Several recent clinical studies have also reported that rapamycin might delay the recovery of the renal allograft from delayed graft function after cadaveric renal transplantation [11,12].

The aim of this study was to further investigate, using a murine model, the effect of rapamycin on renal IRI with special emphasis on its effect on renal tubular cell regeneration.

Materials and methods

Mice

Male BALB/c mice, aged 8–10 weeks, were obtained from the Laboratory Animal Unit of the University of Hong Kong. The mice were maintained in the animal colony of the Laboratory Animal Unit under standard conditions with free access to water and chow. All animal protocols were reviewed and approved by the Committee on Use of Live Animals for Teaching and Research of the University of Hong Kong.

Induction of renal ischemia-reperfusion injury

The mice were anesthetized by intraperitoneal injection of a mixture of midazolam and fentanyl citrate. The left and right renal pedicles were identified through a midline incision and occluded with a microvascular clamp for 45 min. The microvascular clamps were then removed and the kidneys were allowed to reperfuse. Afterwards, the abdomen was closed with continuous sutures in two layers. The peritoneal cavity was filled with warm normal saline before closure. Sham-operated mice underwent a simple laparotomy under identical conditions and served as the operation control. The mice were killed on days 1, 2 and 7 after the IRI operation and the kidneys were harvested for histologic and immunohistochemical analysis. Each experimental group consisted of eight to 10 mice.

Drug administration

Rapamycin was obtained as a gift from Wyeth (HK) Ltd (Hong Kong SAR, People's Republic of China). Rapamycin (2 mg/kg/day) was administered to the mice by oral gavage starting 1 day prior to the induction of IRI and continued daily till killing. The control mice were given vehicle alone on the same schedule. The rapamycin treatment was commenced 1 day prior to the induction of IRI so as to ensure that adequate level of the drug would be present in the mouse's body at the time of the IRI.

Renal function

Blood was collected from the mice by cardiac puncture at the time of killing. Serum creatinine levels were measured by the modified Jaffe's method on a Beckman CX-5 analyzer (Beckman Instruments, Inc., Fullerton, CA, USA).

Renal histology

Kidney tissue fragments obtained at the time of killing were fixed in buffered-formalin and embedded in paraffin. Four micrometer sections were stained with hematoxylin and eosin. The kidney sections were coded and then examined by a single pathologist who was blinded to the treatment groups.

Immunohistochemical staining for proliferating cell nuclear antigen

Paraffin sections were cut at 4-μm-thickness. After dewaxing, antigen retrieval was performed by microwave irradiation for 20 min in 0.01 M sodium citrate buffer (pH 6.0). The sections were then stained with monoclonal antibody against the proliferating cell nuclear antigen (PCNA; Sigma-Aldrich Inc., St Louis, MO, USA). Antibody binding was detected using the avidin-biotin peroxidase method. Endogenous peroxidase was blocked by treatment of the sections with 3% H_2O_2. The sections were stained with the Dako EnVision System (DAKO, Glostrup, Denmark). Negative controls were used in which the primary antibody was omitted. A positive staining result was registered when the nucleus of a cell is entirely filled by the brown color. The outer medulla of the immunohistochemically stained sections were examined (×40 magnification). The number of PCNA-positive cells per high power field was counted.

Statistical analysis

Data were expressed as mean ± SD. Statistical differences between the control and the experimental groups were analyzed using Kruskal–Wallis one-way ANOVA. A P-value of <0.05 was considered significant.

Results

Renal function

The effect of rapamycin treatment on serum creatinine levels in mice subjected to renal IRI is illustrated in Fig. 1. In sham-operated mice, the serum creatinine levels of both vehicle- and rapamycin-treated mice were similar (13.6 ± 1.8 μM vs. 13.9 ± 2.4 μM, $P = 0.64$), indicating that rapamycin treatment did not affect kidney function under normal circumstances. The IRI operation induced a significant increase in serum creatinine levels in both treatment groups on day 1 (19.8 ± 6.3 μM vs. 13.6 ± 1.8 μM, $P = 0.02$ in the vehicle-treated group and 26.8 ± 3.2 μM vs. 13.9 ± 2.4 μM, $P < 0.001$ in the rapamycin-treated group). Moreover, the serum creatinine levels of the rapamycin-treated mice were significantly

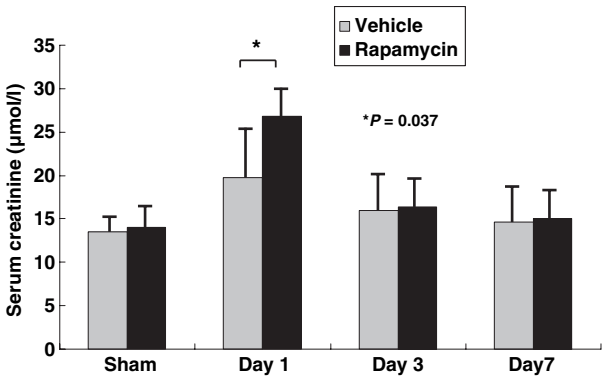

Figure 1 Effect of rapamycin on serum creatinine levels after renal ischemia-reperfusion injury (IRI) in mice. The serum creatinine levels of rapamycin-treated mice were significantly higher than those of the vehicle-treated mice 1 day after the ischemic insult. By days 3 and 7 after the renal IRI, the serum creatinine levels of both treatment groups had improved to near baseline values.

higher than those of the vehicle-treated mice (26.8 ± 3.2 μM vs. 19.8 ± 6.3 μM, $P = 0.037$). By days 3 and 7 after the IRI operation, the serum creatinine levels in the vehicle- and the rapamycin-treated groups had improved to near baseline values and no statistically significant difference could be detected between them (16.4 ± 4.0 μM vs. 16.4 ± 2.4 μM, $P = 0.72$ on day 3 and 14.6 ± 3.0 μM vs. 15.0 ± 2.1 μM, $P = 0.47$ on day 7).

Renal histology

The histology of the kidney sections from the sham-operated mice treated with either vehicle or rapamycin was normal on days 1, 3 and 7 after the operation (data not shown). In mice subjected to renal IRI and treated with vehicle, focal vacuolation of the renal tubular cells was seen on day 1 after the operation (Fig. 2a). In mice subjected to renal IRI and treated with rapamycin, more generalized swelling and vacuolation of the renal tubular cells was observed (Fig. 2b), reflecting more severe renal tubular cell injury. By days 3 and 7 after the IRI, the histologic changes of acute tubular injury in both the vehicle- and the rapamycin-treated mice had largely resolved.

Immunohistochemical staining for PCNA

The effect of rapamycin treatment on PCNA staining in mouse kidneys after IRI is illustrated in Fig. 3. In sham-operated mice treated with either vehicle or rapamycin, there were very few PCNA-positive nuclei per microscopic field (1.5 ± 0.7 vs. 1.4 ± 0.6, P = NS). On the first day after the IRI, the number of PCNA-positive nuclei per microscopic field in the vehicle-treated mice was significantly higher than that of the sham-operated mice

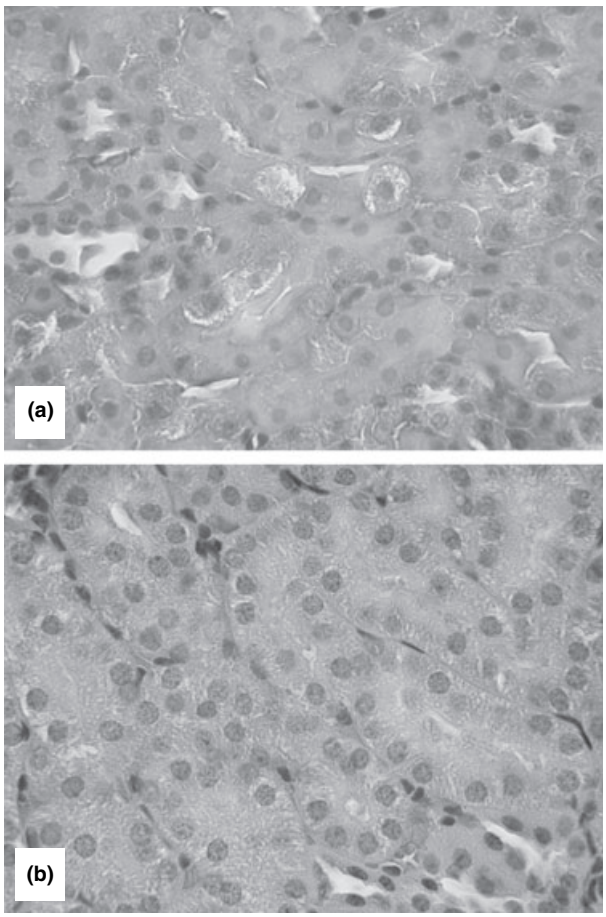

Figure 2 Histopathologic changes in the kidneys of mice subjected to renal ischemia-reperfusion injury (IRI) and treated with vehicle or rapamycin. Representative photomicrographs taken 1 day after the ischemic insult. Rapamycin-treated mice showed more generalized swelling and vacuolation of the renal tubular cells than vehicle-treated mice: (a) vehicle-treated; (b) rapamycin-treated.

(13.5 ± 4.9 vs. 1.5 ± 0.7, $P < 0.005$). In contrast, the number of PCNA-positive nuclei per microscopic field in the rapamycin-treated mice remained at a very low level similar to that of the sham-operated mice. The difference in the number of PCNA-positive nuclei per microscopic field between the vehicle- and the rapamycin-treated mice was statistically significant (13.5 ± 4.9 vs. 1.6 ± 07, $P < 0.005$). Representative photomicrographs showing positive PCNA staining in the vehicle- and rapamycin-treated mice 1 day after the ischemic insult is illustrated in Fig. 4. On day 3 after the renal IRI, the number of PCNA-positive nuclei per microscopic field in the vehicle-treated mice remained significantly higher than that of the rapamycin-treated mice (12.5 ± 7 vs. 1.1 ± 1.1, $P = 0.01$). By day 7 after the renal IRI operation, the number of PCNA-positive nuclei per microscopic field in the rapamycin-treated mice had increased substantially to a

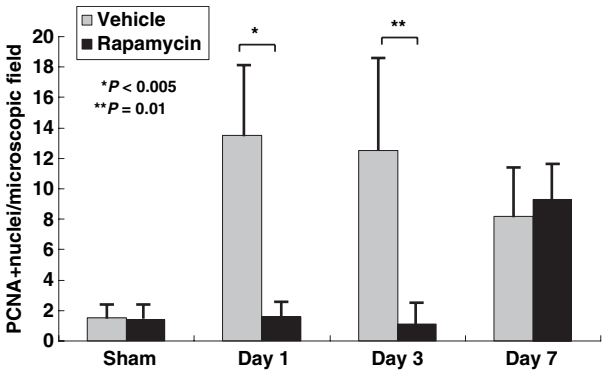

Figure 3 Effect of rapamycin on the expression of proliferating cell nuclear antigen (PCNA) in kidney tissue of mice subjected to renal ischemia-reperfusion injury (IRI). The number of PCNA-positive nuclei per microscopic field in the vehicle-treated mice was significantly higher than that of the rapamycin-treated mice on days 1 and 3 after the renal IRI. By day 7 after the renal IRI, the number of PCNA-positive cells did not differ significantly between the two treatment groups.

level similar to that of the vehicle-treated mice (9.3 ± 2.5 vs. 8.2 ± 3.1, $P = 0.87$).

Discussion

To the best of our knowledge, this is the first report to use a murine model to study the effect of rapamycin on renal IRI. Our results show that rapamycin impairs renal function during the first day and retards the proliferative response of the renal tubular cells during the first 3 days after the ischemic insult.

The results of our study suggest that rapamycin aggravates renal IRI during the first few days after the insult in mice. Our findings are in line with previous studies using rat models of renal IRI in which it was shown that rapamycin treatment impaired the recovery from acute ischemic renal failure [9] and delayed the recovery from IRI after kidney transplantation [10]. Several recent clinical studies have also reported that rapamycin treatment is associated with delayed recovery of the renal allograft in patients with delayed graft function after cadaveric renal transplantation [11,12]. In view of the potential adverse effect of rapamycin on the kidney function after IRI, avoiding the use of rapamycin during the first few days post-transplant should be considered in patients with delayed graft function.

The exact mechanism underlying the adverse effect of rapamycin on renal IRI has not been completely elucidated. Proliferation of the surviving renal tubular epithelial cells to replace the irreversibly injured tubular epithelial cells after the kidney is subjected to IRI serves as an important mechanism of repair [13]. It has been demonstrated that rapamycin inhibits growth factor-induced

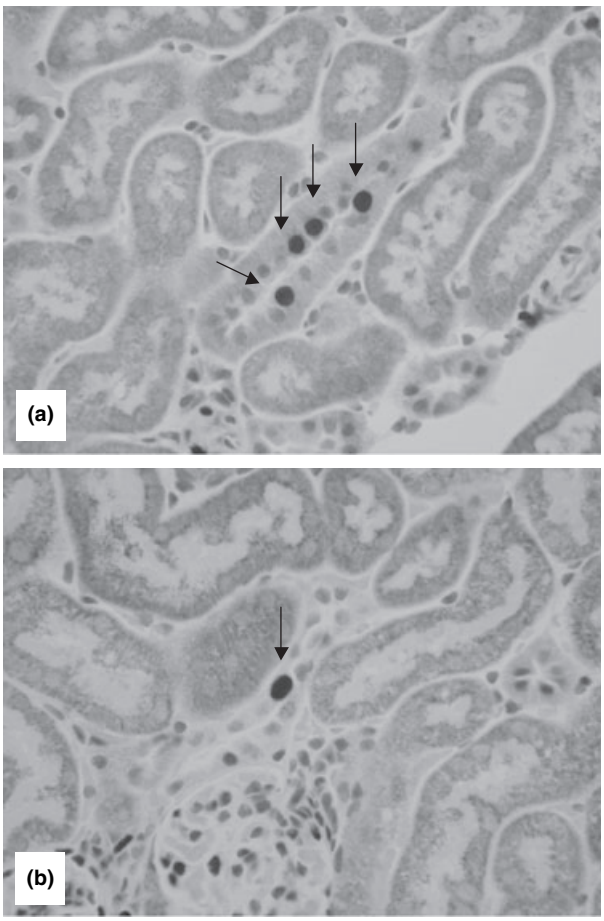

Figure 4 Immunohistochemical demonstration of proliferating cell nuclear antigen (PCNA)-positive cells in kidney sections from mice subjected to renal ischemia-reperfusion injury (IRI) and treated with either vehicle or rapamycin. Representative light microscopic fields of kidney sections obtained 1 day after the ischemic insult: (a) vehicle-treated; (b) rapamycin-treated. The PCNA-positive nuclei were shown with arrows. PCNA-positive nuclei can be abundantly seen in the kidney sections of vehicle-treated mice. In contrast, very few PCNA-positive nuclei could be seen in the kidney sections of rapamycin-treated mice.

proliferation of cultured mouse proximal tubular cells [9]. This finding raises the possibility that rapamycin might impair the recovery of the kidney after acute ischemic injury by inhibiting the regeneration of the renal tubular cells. *In vivo* studies in rats have also shown that rapamycin treatment markedly reduced the proliferative response of the renal tubular cells and significantly impaired renal recovery after IRI [9]. The results of our murine model of renal IRI lends further support to the postulation that rapamycin might aggravate renal IRI by inhibiting the proliferative response of the injured renal tubular epithelial cells.

Other possible mechanisms by which rapamycin aggravates renal IRI include induction of apoptosis of renal

tubular cells and increased intragraft expression of profibrotic cytokines. It has been demonstrated that the number of apoptotic renal tubular cells in rats treated with rapamycin and subjected to renal IRI was substantially higher than that of the vehicle-treated rats [9]. In a rat model of renal isograft, rapamycn treatment was associated with elevated serum creatinine levels and increased intragraft expression of transforming growth factor-β during the early post-transplant period [14].

In our study, rapamycin treatment only caused a transient increase in serum creatinine and worsening of the acute tubular injury on the first day after the ischemic insult. This observation is probably related to the moderate degree of injury to the renal tubular cells in our model as the duration of ischemia was limited to 45 min. In the rat model of renal IRI, however, rapamycin treatment resulted in impairment of renal function for at least 4 days after the injury [9,10]. This apparent discrepancy could be accounted for by the fact that our model of renal IRI is less severe than the rat model in which contralateral nephrectomy was performed prior to clamping of the renal artery. The ischemic injury to the single functioning kidney in the rat model probably resulted in more profound renal tubular injury.

It should also be noted that our murine model of renal IRI is an autologous one and therefore the observed detrimental effects of rapamycin are only transient, in contrast to the allogeneic model, where these effects could have been aggravated by allogeneic innate immune effector mechanisms.

Another interesting observation in our study is that rapamycin treatment only suppressed the proliferation of renal tubular cells during the first 3 days after the IRI. Despite the fact that the mice were treated continuously with rapamycin after the IRI, the amount of PCNA-positive cells in the rapamycin-treated mice increased to a level similar to that of the vehicle-treated mice by day 7 after the ischemic insult. This observation suggests that a rapamycin-insensitive pathway for renal tubular proliferation might become activated after IRI, which allows regenerative proliferation of the renal tubular cells to occur despite inhibition of the mammalian Target of Rapamycin (mTOR) pathway by rapamycin.

Although evidence are accumulating to indicate that rapamycin impairs the recovery of the renal allograft from IRI in the early post-transplant period, the long-term impact of rapamycin treatment on allograft function after IRI is less certain. When rats were subjected to renal IRI and treated with vehicle, rapamycin or cyclosporine, it was found that the glomerular filtration rate measured 7 days after the IRI was similar between the vehicle- and the rapamycin-treated rats but was significantly lower in the cyclosporine-treated rats [15]. Moreover, hypertensive rats which had been subjected to renal IRI and treated with rapamycin for 16 weeks showed reduced proteinuria and glomerulosclerosis, suggesting that rapamycin might ameliorate progressive renal damage after IRI in hypertensive rats [16]. In a recent report, renal transplant patients who had been treated with corticosteroids, low-dose cyclosporine and rapamycin, when compared to patients treated with corticosteroids, full-dose cyclosporine and mycophenolate mofetil, had delayed recovery from delayed graft function but did not have worse graft function at 1 year [17].

We conclude that rapamycin causes transient aggravation of renal IRI in mice. This adverse effect of rapamycin might be mediated, at least partly, through the inhibition of renal tubular cell proliferation. The inhibitory effect of rapamycin on renal tubular cell proliferation is not persistent despite continued administration of the drug. Further studies are warranted to define the long-term significance of rapamycin treatment on kidney function after IRI.

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