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Inflammation and caspase activation in long-term renal ischemia/reperfusion injury and immunosuppression in rats

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Background. We have previously shown the long-term influence of renal ischemia/reperfusion (I/R) injury and immunosuppression on fibrotic genes and apoptosis in a rat model. For the first time, we have now investigated the effects of I/R and immunosuppression on inflammation and caspase activation.

Methods. I/R injury was induced in the right kidney and the left was removed. Cyclosporin (CsA) (10 mg/kg), tacrolimus (0.2 mg/kg), rapamycin (1 mg/kg), or mycophenolate mofetil (MMF) (10 mg/kg) was then administered for 16 weeks. The effects of I/R and immunosuppressants on interstitial inflammation, interleukin (IL)-1β expression, caspase-1 and caspase-3 activation, tubulointerstitial damage, and fibrosis were evaluated.

Results. ED-1+ (a specific rat monocyte/macrophage marker) cells were mainly localized in the tubulointerstitium and periglomerular areas and increased in I/R group compared to controls ($P < 0.01$). This was further increased by CsA, but decreased by tacrolimus, rapamycin, or MMF ($P < 0.05$). The 17 kD active IL-1β remained unchanged, but 35 kD IL-1β precursor was decreased by rapamycin in comparison with I/R group ($P < 0.05$). The 45 kD or 20 kD caspase-1 was increased by I/R or CsA, respectively, and decreased by rapamycin ($P < 0.05$). The 24 kD caspase-3, which proved to be an active caspase-3 subunit, was increased in I/R and CsA groups and deceased by tacrolimus, rapamycin, or MMF ($P < 0.05$), but not 32 kD precursor or 17 kD active caspase-3. The activity data of caspase-1 and caspase-3 exhibited the same trend as Western blotting data. The staining of active caspase-3 was scattered in kidneys, mainly in tubular and interstitial areas, which was consistent with that of ED-1+ cells. There was a strong positive correlation between interstitial inflammation and 24 kD caspase-3 expression or caspase-3 activity ($r = 0.814$ or 0.484), all of which were also closely related with urinary protein ($r = 0.537$, 0.529, or 0.517), serum creatinine ($r = 0.463$, 0.573, or 0.539), tubulointerstitial damage ($r = 0.794$, 0.618, or 0.712) and fibrosis ($r = 0.651$, 0.567, or 0.469), all $P < 0.01$.

Conclusion. This study shows that the mechanisms of long-term I/R injury and immunosuppressants treatment include interstitial inflammation and caspase activation, most clearly demonstrated by the 24 kD active caspase-3.

Key words: long-term renal ischemia/reperfusion injury, immunosuppressants, interstitial inflammation, IL-1β expression and caspase-1 and caspase-3 activation.

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Chronic allograft nephropathy is the principal cause of allograft failure [1, 2]. Many factors are involved in the progression of chronic allograft nephropathy, including immune injury, ischemia/reperfusion (I/R) injury, the inflammatory response, and immunosuppressant drugs [2, 3]. I/R injury is especially important in kidney transplantation, being associated with primary nonfunction, delayed graft function, increased acute rejection, and late allograft dysfunction [4]. The underlying mechanisms of I/R injury have been elucidated, including abnormalities in regional blood flow, endothelial/epithelial cell dysfunction, inflammation, and tubular obstruction [5]. The initial ischemic insult may also lead to the up-regulation of class II major histocompatability complex (MHC) molecules, thus increasing the immunogenicity of the ischemic organ [6]. Although immunity, inflammation, and other factors have tended to be considered and investigated in isolation, it has become evident that these aspects are interdependent and may either coexist or act as distinct entities depending on the time frame and intensity of I/R injury.

The involvement of inflammatory mediators in the course of I/R has been extensively addressed, but the etiology of I/R-induced inflammation remains largely obscure [4]. Many studies suggest that neutrophil recruitment to the site of injury is a central event in the pathogenesis of renal I/R injury [7, 8]. The generation of oxygen free radicals [9] enhances the local expression of adhesion molecules [10, 11] and promotes neutrophil chemotaxis during early reperfusion. The activation of the complement system [12, 13] is also involved in neutrophil

influx and I/R damage. Moreover, it has been revealed that various activated caspases participate in the processing of the proinflammatory cytokines, apart from its well-recognized mission in apoptotic cell death. First, caspase-1 is a widely accepted inflammatory mediator because it is involved in the maturation of interleukin (IL)-1β [14–16] and IL-18 [17, 18], both of which have been implicated in the pathogenesis of renal I/R injury. Caspase-3 activation has been recently linked with inflammation via processing of IL-1β, IL-18, and macrophage inflammatory protein-2 (MIP-2) [19–21]. Caspase-7 is responsible for processing the endothelial monocyte-activating polypeptide II (EMAP-II), which is produced in the early phase of renal I/R injury [4, 22]. Finally, caspase-11, which activates caspase-1 and caspase-3, plays a crucial role in inflammation [23].

Therapeutic strategies for the attenuation of I/R injury by abrogating the inflammatory response have included the use of antibodies against intercellular adhesion molecule (ICAM)-1 [24], tumor necrosis factor (TNF)-α [25] or interferon (INF)-γ [26], the depletion of complement components [10, 27] or neutrophils [28], and inhibition of caspases [29]. However, almost all of these studies were designed to observe the effects on the acute phase of I/R injury. The long-term impact of I/R injury and the modern immunosuppressants used for kidney transplantation on inflammation has been less well studied. The common immunosuppressants used in human kidney transplantation are calcineurin inhibitors, cyclosporine A (CsA) and tacrolimus; a macrolide-triene antibiotic, rapamycin; and a specific inhibitor of inosine monophosphate dehydrogenase, mycophenolate mofetil (MMF). These immunosuppressants have anti-inflammatory effects via different pathways, but their precise mechanisms need to be further explored.

Although a model of renal transplantation developing chronic allograft nephropathy would be the most relevant to the clinical situation, this model, as well as being technically difficult, would require baseline immunosuppression, making it more difficult to assess the relative contributions of each individual immunosuppressant. The I/R rat model, therefore, has been widely used as an alternative substitute that mimics part of crucial changes occurring in chronic allograft nephroapthy. It has previously been shown that tacrolimus and rapamycin, but not CsA, protect against the effects of I/R injury over a 16-week period [30, 31]. Links with fibrotic genes and their effects on extracellular matrix turnover [32] and apoptosis modulating renal tubular cell loss [abstract; Yang B, et al, *J Am Soc Nephrol* 13:96, 2002] have been investigated using this model. In the current study, we have investigated, for the first time, the long-term effects of I/R injury and immunosuppressants on the degree and distribution of inflammatory cells; IL-1 and caspase-1 expression and activity; caspase-3 activation, activity, and localization; tubulointerstitial damage; and fibrosis. In addition, correlations between measures of inflammation, its possible mediators and changes in renal function and histology have been studied.

METHODS

I/R model

The model of renal I/R injury has previously been described [30, 31]. Briefly, groups ($N = 4$ to 6) of male Wistar rats (300 to 350g) were anesthetized with halothane and underwent occlusion of the right renal vascular pedicle for 45 minutes, together with left nephrectomy. This approach has previously been found to be the longest period of renal warm ischemia compatible with survival and induce an appropriate degree of renal injury [33]. After recovery, animals were followed for a period of 16weeks. Rats were housed at constant temperature (20°C) and humidity (45%) on a 12-hour light/dark cycle, fed ad libitum on standard laboratory rat chow, and had free access to tap water. All the experiments were carried out according to the regulations laid down by the Home Office (Animal Scientific Procedure Act 1986, UK).

Drug administration

After the surgical procedure, microemulsion CsA dissolved in pure olive oil (10 mg/kg) (Neoral®) (Novartis Pharmaceuticals, Sussex, UK), tacrolimus powder dissolved in water (0.2 mg/kg) (Prograf®) (Fujisawa Pharmaceuticals, London, UK), rapamycin liquid (1 mg/kg) (Rapammune®) (Wyeth Pharmaceuticals, Berks, UK), MMF (10 mg/kg) (Cellcept®) (Roche Pharmaceuticals, UK), or drug vehicle was administered orally by gavage once daily for 16 weeks. The dosage and administration route of immunosuppressants were chosen based on the previous studies, the clinical doses for humans and their side effects, and our own preliminary data [31, 34–36]. Animals undergoing sham operation (control), laparotomy and vascular pedicle dissection, and unilateral nephrectomy only were included as controls.

Assessment of histologic change and renal function

The sections were stained with picro Sirius Red overnight (0.1% Sirius Red in saturated aqueous picric acid) to detect tubulointerstitial fibrosis, especially collagens I and III [37, 38]. Rapid dehydration was repeated with an initial wash of 0.01 N HCl for 2 minutes, and serial washes of 70% ethanol for 45 seconds followed by 80, 90, and 100% ethanol for 2 minutes. Slides were cleared with two washes of xylene and mounted with DPX mountant. The entire renal cortex of each kidney was semiquantitatively analyzed using a previously validated image analysis system on an Apple Macintosh computer system using the NIH image software (National Institutes of Health, Bethesda, MD, USA) [39].

Hematoxylin and eosin–stained sections of kidney were also semiquantitatively graded for tubulointerstitial damage (tubular dilation or atrophy and interstitial expansion with edema, inflammatory infiltrate or fibrosis) based on a scale of 0 to 3. The normal cortical tubulointerstitium scored 0; mild tubulointerstitial damage affecting up to 25% of an objective field at ×200 magnification scored 1; moderate tubulointerstitial damage affecting 25% to 50% of the field scored 2; and severe tubulointerstitial damage exceeding 50% of the field scored 3. The examiners were blinded to the treatment groups and the randomly selected 12 cortical fields were scored for each animal and the mean score attributed to the animal [40].

Blood obtained at the time of sacrifice was used to measure serum creatinine levels as an indication of renal function. 24-hour urinary protein was determined by the Bradford method as a marker of renal damage. At the end of the 16-week study period, rats were reanesthetized and the right kidney was removed immediately. Renal cortical tissue was fixed in 10% (wt/vol) neutral buffered formalin and paraffin-embedded for histologic and immunohistochemical examination, or snap frozen and stored in liquid nitrogen for total protein analysis.

Evaluation of renal inflammation by ED-1 immunostaining

Renal inflammation was evaluated by ED-1 (a specific rat monocyte/macrophage marker) immunostaining on paraffin-embedded tissues using a Dako ChemMate EnVision™ Detection Kit (Dako, Glostrup, Denmark). Sections were pretreated with 0.125% (wt/vol) trypsin at 37°C for 10 minutes. A monoclonal mouse antirat ED-1 antibody (Serotec Ltd., Oxford, UK) at 1:50 dilution was applied overnight at 4°C. Thereafter, antibody binding was revealed using the peroxidase/diaminobenzidine (DAB) from the Dako kit. Sections were counterstained with hematoxylin and mounted in glycergel (Dako). Negative control sections were incubated with normal mouse IgG at the same protein concentration of primary antibody. Renal inflammation was evaluated by a semiquantitative analysis based on calculating the percentage of ED-1+ cells area fraction (both glomeruli and tubulointerstitium) in 20 fields at ×400 magnification using computer-aided software.

Measurement of tissue IL-1β, caspase-1, caspase-3, or β-actin protein

Ten percent (wt/vol) kidney cortex homogenate was prepared and 20 μg of protein was separated on a 15% (wt/vol) polyacrylamide denaturing gel and electroblotted onto Hybond C membrane (Amersham Life Science, Buckinghamshire, UK) [41]. This was blocked with 5% (wt/vol) milk and probed with a polyclonal rabbit antirat full-length IL-1β (1:1000 dilution) (NIBSC, Potters Bar, UK), caspase-1 (1:500 dilution) (Upstate Biotechnology, Milton Keynes, UK), caspase-3 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody, or a monoclonal mouse anti-β-actin antibody (1:5000; dilution Insight Biotechnology, Middlesex, UK) [42]. The antibody binding was revealed using an antirabbit (caspase-3) or mouse (β-actin) peroxidase conjugate (Dako) or antirabbit (IL-1β and caspase-1) ABC Elite Kit (Vector Laboratories, Peterborough, UK) diluted at 1:1000 and the enhanced chemiluminescence (ECL) detection system (Amersham Life Science). Developed films were semiquantitatively analyzed by scanning volume density using a Bio-Rad GS-690 densitometer and Molecular Analyst version 4 software (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Optical volume density value for IL-1β, caspase-1, or caspase-3 was corrected for loading using β-actin expression. Results were expressed as the percentage of the average control volume density. The size of target protein was determined by comparison with protein molecular weight markers (Bio-Rad Laboratories Ltd.) using the same analysis package [41, 43, 44].

Caspase-1 and caspase-3 activity assays

The activities of caspase-1 and caspase-3 were detected by a modified Fluorometric CaspACE™ Assay System (Promega, Southampton, UK) using 100 μg of protein extracted from renal tissues [41]. This assay is based on the ability of caspase-1 or caspase-3 to cleave the fluorogenic substrate of Ac-YVAD-7-amino-4-methyl coumarin (AMC) or Ac-DEVD-AMC. The specificity of the assay was determined using the caspase-1 inhibitor Ac-YVAD-CHO or caspase-3 inhibitor Ac-DEVD-CHO. The fluorescence of the reaction was monitored at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using an Mx 4000™ Multiplex Quantitative PCR System (Stratagene, Foster City, UK). The fluorescence intensity was calibrated with standard concentrations of AMC and caspase-1 or caspase-3 activity was expressed as picomole AMC liberated per minute per microgram protein at 30°C.

Electroelution

Protein homogenates of the kidney cortex were further analyzed by electroelution using a miniwhole gel eluter system (Bio-Rad), which allows the simultaneous electroelution of multiple protein bands separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) into 14 narrow chambers [45]. Protein (100 μg) from the kidney of rat number 6 in the CsA group, which had the highest caspase-3 activity, was loaded into each well of a 15% SDS-PAGE gel. The proteins in the SDS-PAGE gel was then electroeluted into Tris/boric acid buffer for 20 minutes (the eluter acts as an electrodialyzer removing SDS from protein and leaving them in a nontoxic physiologic buffer). A total of 54 μL

eluates from all chambers were analyzed for caspase-3 activity as described above. Two samples of the original protein homogenate and pure caspase-3 enzyme (Sigma, Dorset, UK) were used as positive controls. Fifteen microliter eluates were again resolved by SDS-PAGE and stained with brilliant blue or processed for subsequent Western blotting with the antibody against full-length caspase-3.

Immunostaining of active caspase-3

The localization of active caspase-3 protein was detected by immunostaining of paraffin-embedded tissues. An affinity-purified rabbit anti-17 kD active caspase-3 antibody (R&D Systems Europe, Ltd., Abingdon, UK) at 1:400 dilution was applied for 1 hour at 37°C. Thereafter, antibody binding was revealed using the peroxidase/DAB from the Dako ChemMate EnVision™ Detection Kit. Sections were counterstained with hematoxylin and mounted in glycergel. Negative control sections were incubated with normal mouse IgG at the same protein concentration of the primary antibody.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The statistical difference was assessed by a signal factor analysis of variance (ANOVA) and the Student *t* test. The correlation between parameters was determined by linear correlation and multiple regression analyses using SPSS software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to be significant [44].

RESULTS

I/R injury, immunosuppressant treatment, and inflammation

The effects of I/R and immunosuppressants on renal inflammation were evaluated by computerised histomorphometry of ED-1 immunostaining. There were very few ED-1+ cells inside glomeruli and interstitium in control kidneys (Fig. 1A). However, ED-1+ cells were dramatically increased in I/R kidneys and localized mainly in the periglomerular, peritubular, expanded interstitial areas (Fig. 1B), and tubular lumens (Fig. 1C).

I/R injury (0.37 ± 0.06%) (Fig. 1E) increased inflammation compared to the controls (sham control 0.07 ± 0.02%) (unilateral nephrectomy 0.08 ± 0.01%) ($P < 0.01$) (Fig. 1D and J). The number of ED-1+ cells in the unilateral nephrectomy group was almost identical to that in the sham control group (Fig. 1J). Therefore, inflammation can be attributed to I/R injury rather than unilateral nephrectomy. CsA (0.63 ± 0.08%) ($P < 0.05$) (Fig. 1F and J) enhanced the infiltration of inflammatory cells into the interstitial area compared to the I/R group. In contrast, inflammatory cell infiltration was reduced by administration of the other immunosuppressants (tacrolimus 0.17 ± 0.04%) (rapamycin 0.102 ± 0.03%) (MMF 0.17 ± 0.02%) ($P < 0.05$ or 0.01) (Fig. 1G to J) when compared to the I/R or CsA group.

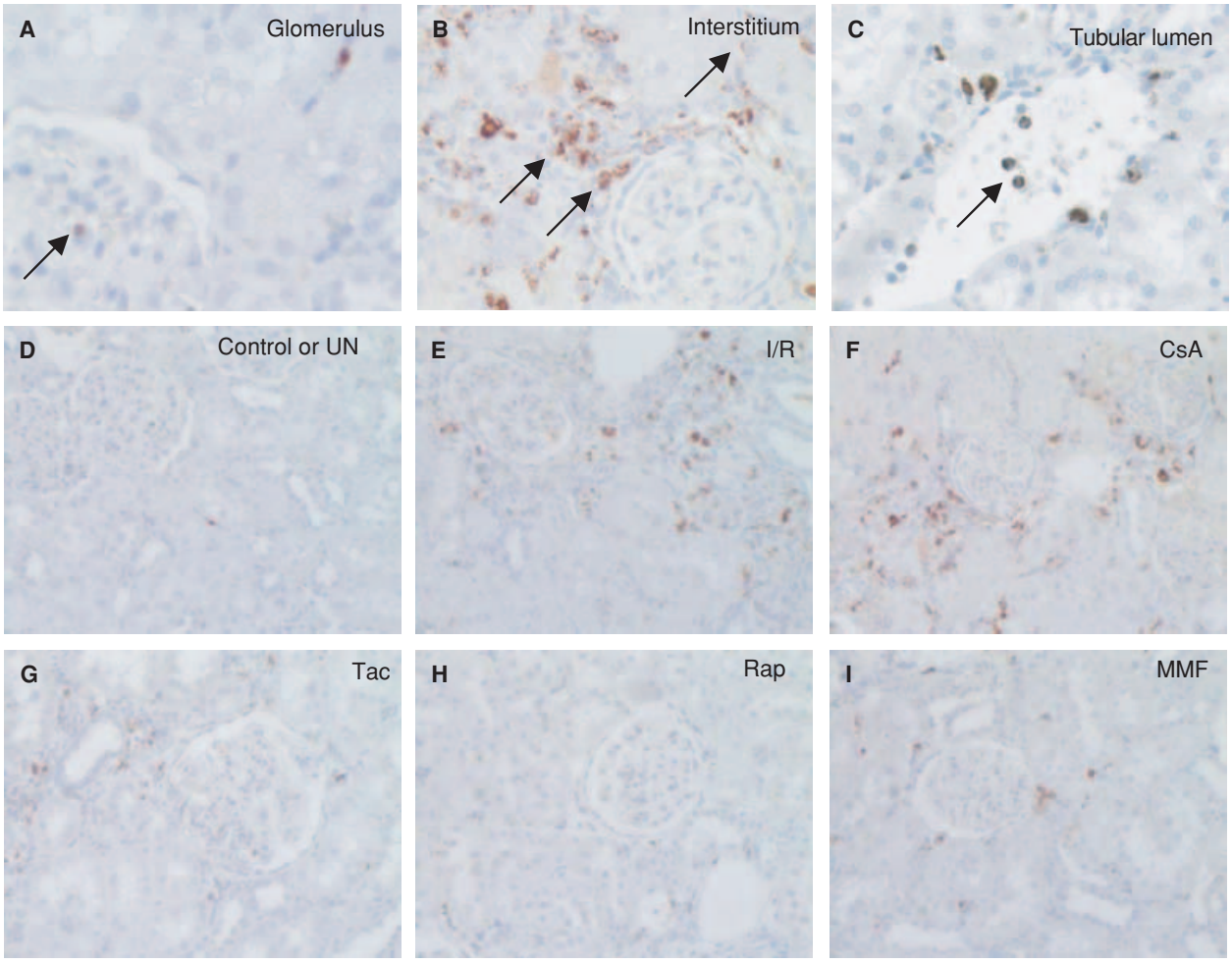
Effect of I/R and immunosuppressants on the protein expression of IL-1β, caspase-1, and caspase-3

The protein expression of IL-1β, caspase-1, and caspase-3 in kidney tissues was determined by Western blotting. The 42 kD β-actin was used to control sample integrity and loading in each lane on the same membrane for detection of IL-1β, caspase-1, or caspase-3 (Figs. 2 to 4). There were no significant differences between the studied groups.

The 35 kD IL-1β precursor was increased in the I/R group (153.5 ± 27.7%) compared to the controls (control 100.0 ± 22.7% and unilateral nephrectomy 96.2 ± 22.8%), but this difference did not reach statistical significance. In comparison with the I/R group, only rapamycin significantly decreased the expression of 35 kD IL-1β (68.5 ± 8.2%) ($P < 0.05$), whereas CsA (135.6 ± 16.0%), tacrolimus (134.7 ± 19.4%), or MMF (101.6 ± 23.3%) had no significant effects (Fig. 2A). Seventeen kD active IL-1β remained statistically unchanged, although I/R (126.7 ± 22.5%) and all four immunosuppressants (CsA 141.7 ± 18.3%; tacrolimus 139.7 ± 19.3%; rapamycin 129.2 ± 16.5%, or MMF 130.0 ± 12.3%) numerically increased its expression when compared to the controls (control 100.0 ± 18.7% and unilateral nephrectomy 98.8 ± 25.6%) (Fig. 2B).

A 45 kD caspase-1 precursor was significantly increased in the I/R group (125.8 ± 4.5%) compared to sham control (100 ± 8.4%) ($P < 0.05$). Rapamycin significantly decreased caspase-1 precursor (83.0 ± 9.6%) ($P < 0.01$), but the other drugs had no such effect (CsA 126.2 ± 4.0%; tacrolimus 113.0 ± 3.2%; or MMF 114.6 ± 9.1%) (Fig. 3A). There was only a marginal difference between the I/R and unilateral nephrectomy group (96.4 ± 17.2) ($P = 0.08$). Although 20 kD active caspase-1 did not change significantly in the I/R group compared to the controls (control 99 ± 16.2% and unilateral nephrectomy 95.2 ± 25.0%), it was significantly increased by CsA (130.4 ± 5.6%) and decreased by rapamycin (66.8 ± 12.1%) (both $P < 0.05$). Tacrolimus (93.8 ± 13.5%) and MMF (86.1 ± 1.6%) had no significant effect on 20 kD active caspase-1 expression (Fig. 3B).

The 32 kD caspase-3 precursor increased in the I/R group (157 ± 13%) compared to controls (control 100 ± 13% and unilateral nephrectomy 95 ± 15%) (both $P < 0.05$) (Fig. 4A). However, compared to the I/R group, the various immunosuppressants did not significantly affect its expression (CsA 191 ± 39%, tacrolimus 206 ± 34%, rapamycin 134 ± 18%, and MMF 188 ± 32%). The 29 kD processing intermediate was also present in the kidneys of all groups (Fig. 4). Interestingly, the 24 kD band, representing active caspase-3, was increased by I/R injury



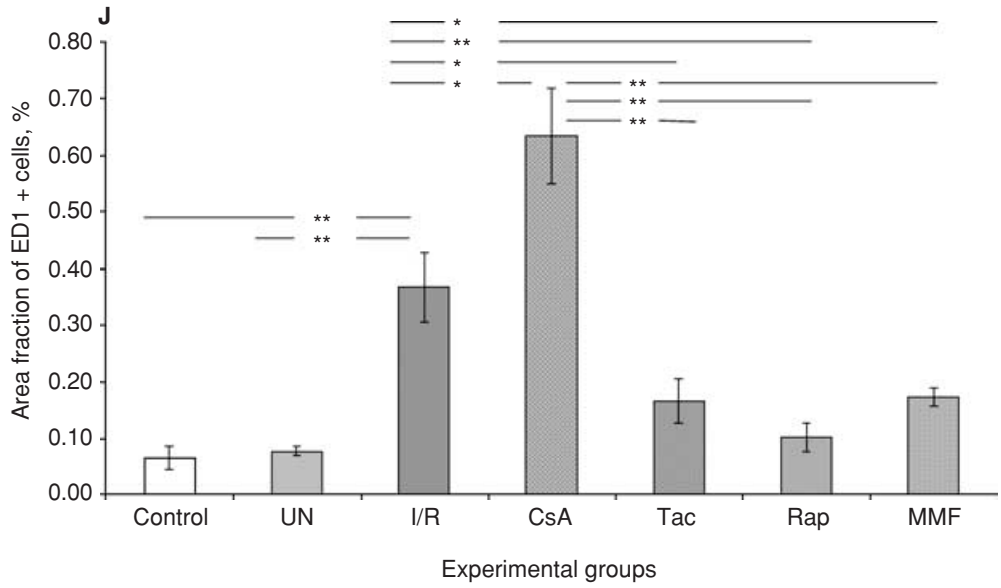


Fig. 1. Endothelin-1 (ED-1+) cells in different compartments of kidneys and staining profiles in various experimental groups. ED-1+ cells were detected in the glomerulus (*A*), peri glomerular areas, peritubular areas, expanded interstitial areas (*B*), and tubular lumen (*C*, arrows) at ×400 magnification. ED-1+ cells were scattered in the controls [control (sham-operated) and unilateral nephrectomy (UN)]; (*D*) and dramatically increased in ischemia/reperfusion (I/R) kidneys (*E*), and were further increased by cyclosporine A (CsA) treatment (*F*), mainly in interstitial areas, but decreased by tacrolimus (Tac) (*G*), rapamycin (Rap) (*H*), and mycophenolate mofetil (MMF) (*I*) treatment at ×200 magnification. Histogram (*J*) shows the ED-1+ staining as a percentage of the whole area in the kidneys of various groups using computer aided semiquantitative analysis. Data represents mean ± SEM ($N = 4$ to 6). $^{*}P < 0.05$; $^{**}P < 0.01$.

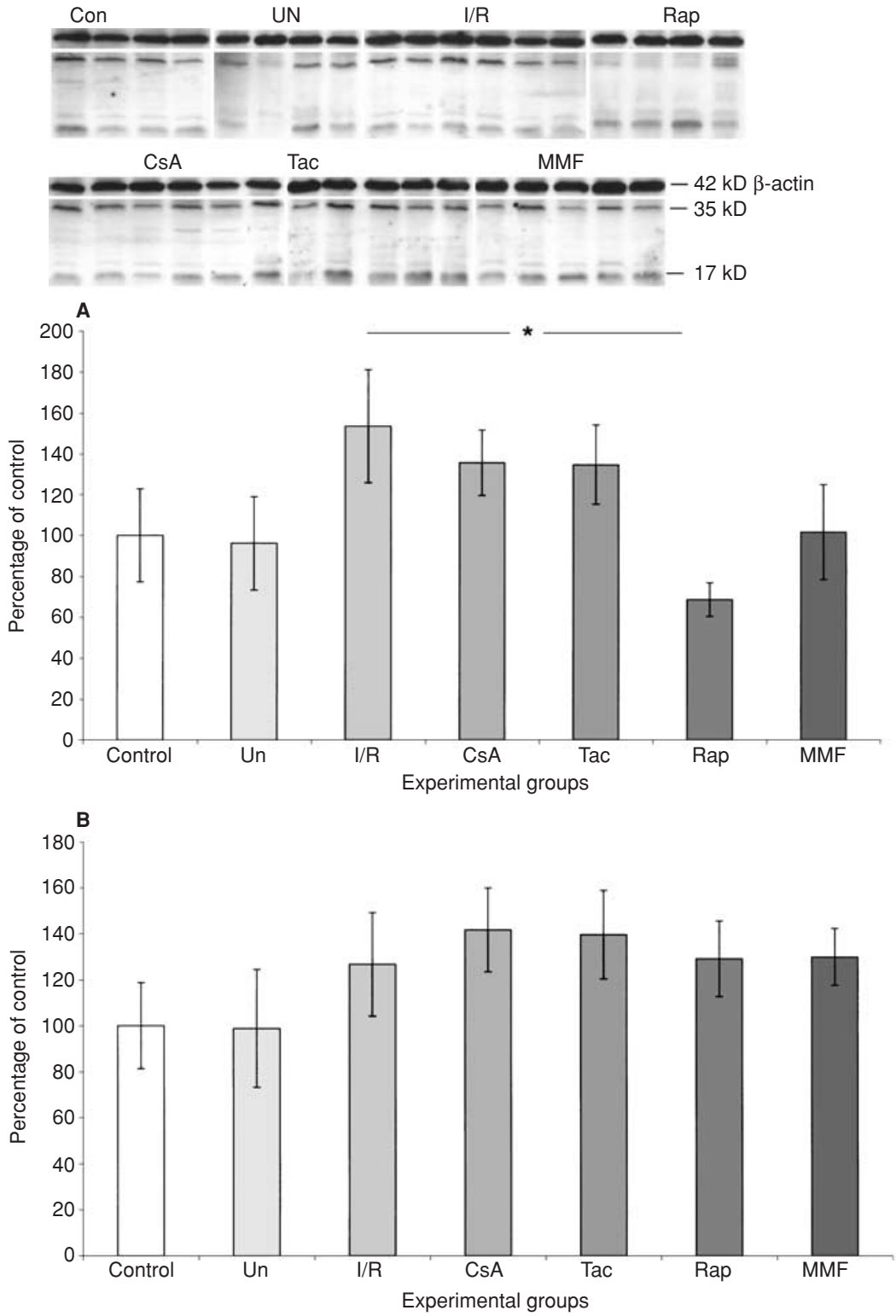


Fig. 2. Expression of interleukin(IL)-1β protein detected by Western blot analysis. Sample immunoblots showed 35 kD precursor and 17 kD active subunit of IL-1β. A 42 kD band of β-actin was detected to show the loading of samples in each lane. Histogram data represent the result of volume density analysis 35 kD (*A*) and 17 kD (*B*) IL-1β using β-actin to correct for loading. Data represent the mean percentage change in volume density compared to the average control value (mean ± SEM) ($N = 4$ to 6). $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations are: control, sham-operated; UN, unilateral nephrectomy; I/R, ischemia/reperfusion; CsA, cyclosporine A; Tac, tacrolimus; Rap, rapamycin; MMF, mycophenolate mofetil.

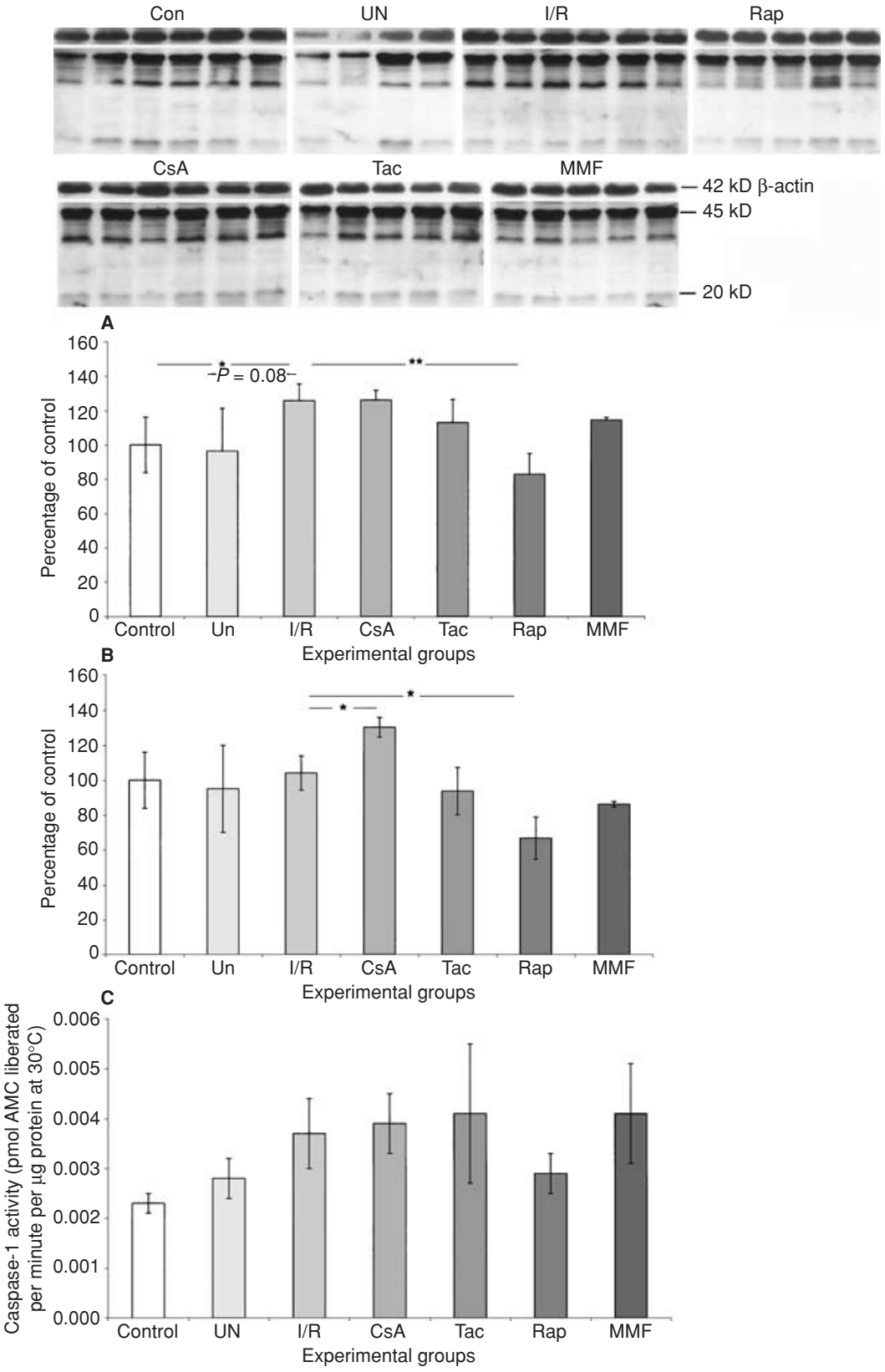


Fig. 3. Caspase-1 Western blots revealed 45 kD precursor and 20 kD active subunit. A 42 kD band of β-actin was detected to show the loading of samples in each lane. Histogram data represent the result of volume density analysis of 45 kD (*A*) and 20 kD (*B*) caspase-1 using β-actin to correct for loading, and the detection of caspase-1 activity (*C*). Data represent the mean percentage change in volume density compared to the average control value (mean ± SEM) ($N = 4$ to 6). $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations are: Control, sham-operated; UN, unilateral nephrectomy; I/R, ischemia/reperfusion; CsA, cyclosporine A; Tac, tacrolimus; Rap, rapamycin; MMF, mycophenolate mofetil.

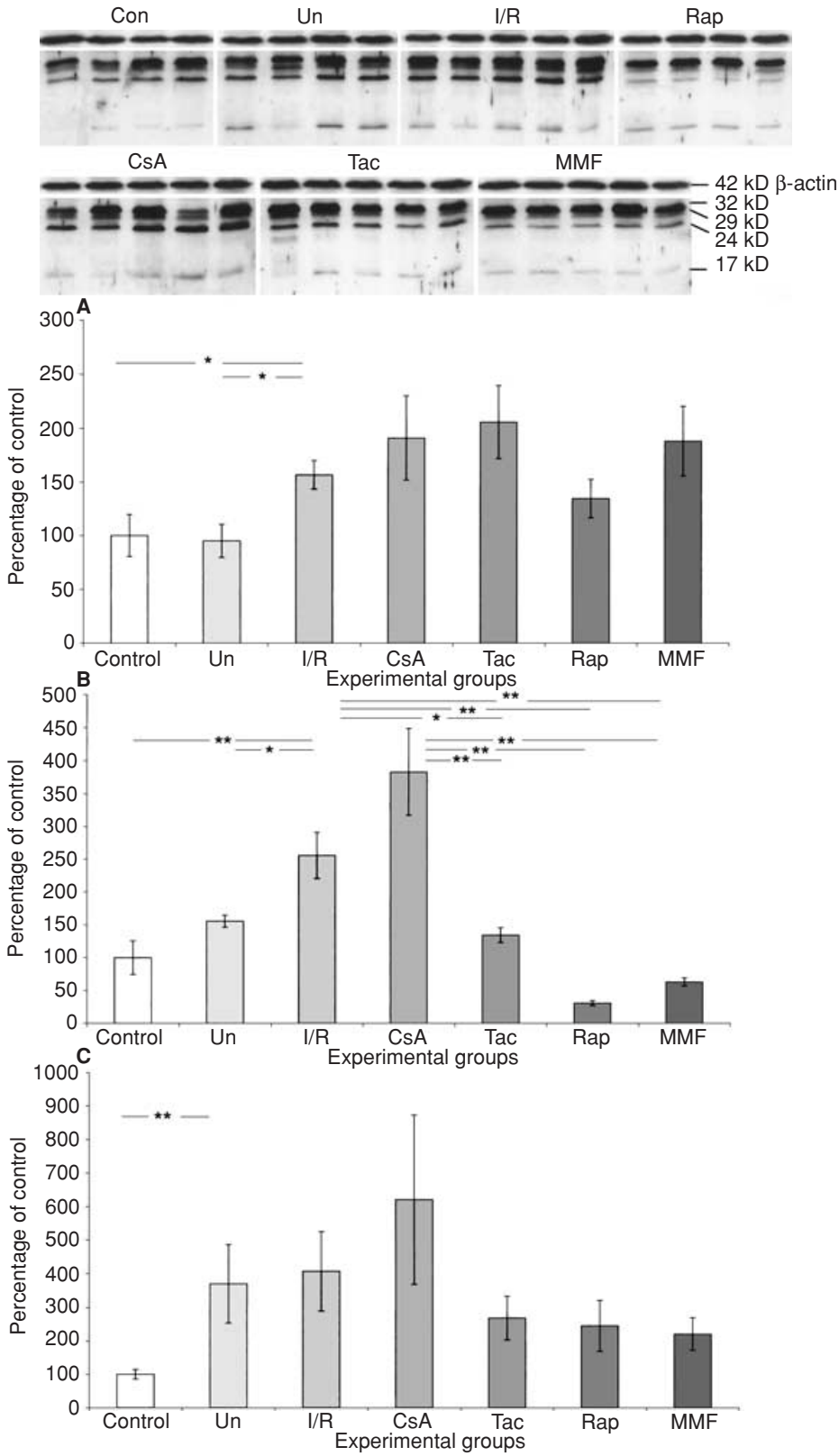


Fig. 4. Expression of caspase-3 protein detected by Western blot analysis. Immunoblots showed a 32 kD band, representing the precursor of caspase-3; a 29 kD band, representing processing intermediate of caspase-3; 17 kD and 24 kD bands, representing the caspase-3 active subunits. The expression of 42 kD β-actin demonstrates the equal loading of samples in each lane. Histogram data represent the result of volume density analysis 32 kD (*A*), 24 kD (*B*), and 17 kD (*C*) caspase-3 using β-actin to correct for loading. Data represent the mean percentage change in volume density compared to the average control value (mean ± SEM) ($N = 4$ to 6). $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations are: control, sham-operated; UN, unilateral nephrectomy; I/R, ischemia/reperfusion; CsA, cyclosporine A; Tac, tacrolimus; Rap, rapamycin; MMF, mycophenolate mofetil.

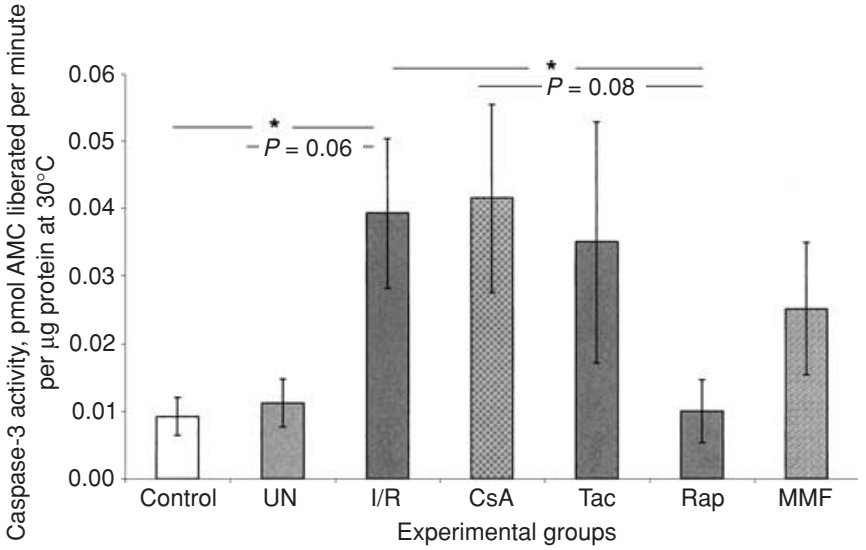


Fig. 5. Caspase-3 activity in kidney tissues assayed by the fluorometric substrate cleavage. This measurement was repeated three times and data represents mean ± SEM (N = 4 to 6). $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations are: control, sham-operated; UN, unilateral nephrectomy; I/R, ischeemia/reperfusion; CsA, cyclosporine A; Tac, tacrolimus; Rap, rapamycin; MMF, mycophenolate mofetil.

(256 ± 35%) compared to the controls (control 100 ± 26%) ($P < 0.01$) (unilateral nephrectomy 155 ± 9%) ($P < 0.05$) (Fig. 6B). CsA led to a further increase (382 ± 66%), but the difference did not reach statistical significance. Tacrolimus (134 ± 11%) ($P < 0.05$), rapamycin (31 ± 4%) ($P < 0.01$), and MMF (63 ± 6%) ($P < 0.01$) significantly decreased the expression of 24 kD caspase-3 compared to the I/R or CsA group (all $P < 0.01$). The 17 kD band, another caspase-3 active subunit, demonstrated similar trends to the 24 kD caspase-3 (I/R 407 ± 118%; CsA 621 ± 252%; tacrolimus 267 ± 65%; rapamycin 244 ± 76%; and MMF 220 ± 48%), but other than an increase in the unilateral nephrectomy group (370 ± 117%) compared to the control (100 ± 15%) ($P < 0.01$), these changes did not reach statistical significance (Fig. 4C).

Effect of I/R and immunosuppressants on caspase-1 and caspase-3 activity

To confirm the expression of caspase-1 and caspase-3 detected by Western blotting, we measured caspase-1 and caspase-3 activity. There was an increased trend in caspase-1 activity in the most of experimental groups over the control except for the rapamycin group, which showed a decrease, but these did not reach statistical significance (control 0.0023 ± 0.0002, unilateral nephrectomy 0.0028 ± 0.0004, I/R 0.0037 ± 0.007, CsA 0.0039 ± 0.0006, tacrolimus 0.0041 ± 0.0014, and rapamycin 0.0029 ± 0.0004) (Fig. 3C). Caspase-3 activity was significantly increased in the I/R kidneys (0.039 ± 0.011) (Fig. 5) compared to the control group (0.009 ± 0.003) ($P < 0.05$), but this increase only reached a marginal statistical significance when compared to the unilateral nephrectomy group (0.011 ± 0.004) ($P = 0.06$). The elevated caspase-3 activity in the I/R kidneys was reduced in the rapamycin-treated group (0.010 ± 0.005) ($P < 0.05$), but did not significantly change in the CsA (0.042 ± 0.014), tacrolimus (0.035 ± 0.018), or MMF (0.025 ± 0.009) treatment group. There was also a marginal difference between rapamycin and CsA treatment ($P = 0.08$). The specific and competitive tetrapeptide inhibitor of caspase-1 Ac-YVAD-CHO or caspase-3 Ac-DEVD-CHO, almost fully inhibited the caspase-1 or 3 activity, demonstrating the specificity of the assay (data not shown).

Electroelution of whole SDS PAGE gels

In order to verify that the 24 kD band illustrated in Western blot is an active caspase-3, proteins resolved by SDS-PAGE were electroeluted into 14 chambers. The eluate from chamber 6 showed the highest caspase-3 activity and the eluate from chamber 10 revealed weaker caspase-3 activity (Fig. 6A). The SDS-PAGE gel loading with eluates from all chambers and staining with brilliant blue revealed the size of the protein in chamber 6 to be of the order of 24 kD. The activity detected in chamber 10 could be due to the 17 kD caspase-3 (Fig. 6B). This was further confirmed by Western blotting using an antibody to full-length caspase-3 (Fig. 6C). These experiments strongly indicate that the 24 kD band is an active caspase-3 protein.

Localization of active caspase-3

The distribution of active caspase-3 protein in kidney sections was revealed by immunostaining. The positive staining was scattered in kidneys, especially in glomerular areas (Fig. 7A). There was more staining in the tubular (Fig. 7B) and interstitial areas (Fig. 7C), and some positively stained cells showing the morphologic changes of apoptosis (such as shrunken cell with condensed nucleus) (Fig. 7B). The positive stained cells were seen in

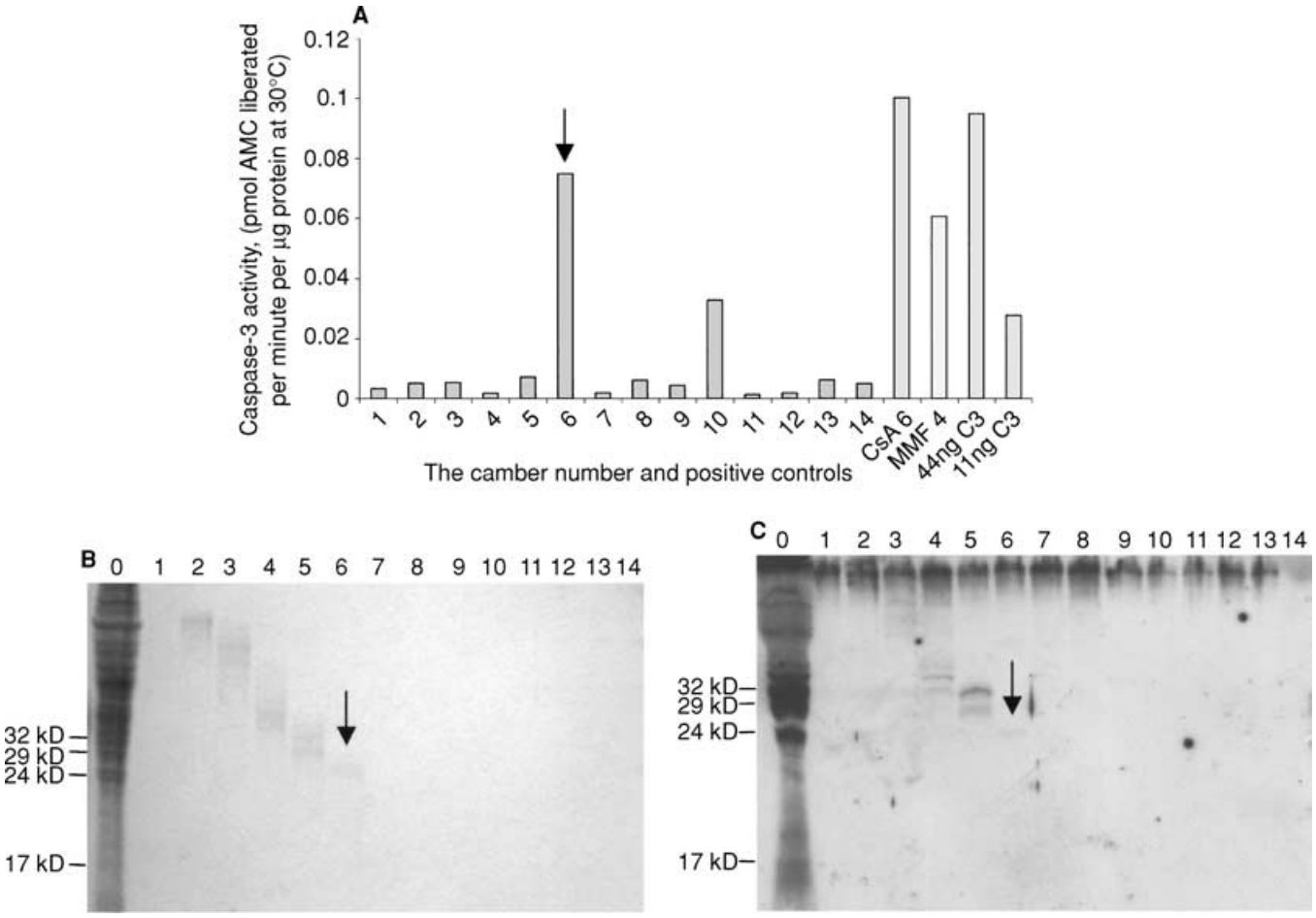


Fig. 6. Caspase-3 activity in electroeluted homogenate samples. Caspase-3 activity was highest in chamber 6 and some weaker activity was detected in chamber 10. 100 µg protein of kidney homogenate from rat number 6 of the cyclosporine A (CsA) group and rat number 4 of the mycophenolate mofetil (MMF) group, and 44 ng or 11 ng protein of pure caspase-3 enzyme were used as positive controls (*A*). Electroeluted samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with brilliant blue. The result showed the size of protein in eluate 6 to be of the order of 24 kD as indicated by an arrow and the protein from eluate 10 to be of the order of 17 kD (*B*). These data were further corroborated by Western blotting using the antibody to whole caspase-3 (*C*).

the tubular lumen as well (Fig. 7D). There was no specific staining in negative control sections.

Renal function and histologic findings

Extracellular matrix deposition in the kidney was studied by Sirius Red staining. The dye molecule intercalates with the tertiary groove in the structure of collagen types I and III and presents a pink color (Fig. 7E) under white light, and is strongly birefringent (Fig. 7F) when observed under polarized light. Sirius Red staining was mainly located in the tubulointerstitium rather than the glomerular interstitium (Fig. 7E and F). As shown in Figure 8A, it was significantly increased in the I/R (1.04 ± 0.12) or CsA group (1.30 ± 0.18) compared to controls (control 0.51 ± 0.04 and unilateral nephrectomy 0.34 ± 0.07) (both $P < 0.01$). Animals treated with tacrolimus (0.61 ± 0.04), rapamycin (0.34 ± 0.08), or MMF (0.82 ± 0.08) had significantly lower staining level than both I/R and CsA-treated animals (all $P < 0.05$).

In the majority of animals, tubulointerstitial damage was presented as tubular dilation and interstitial inflammatory infiltrate and fibrosis. There was no extensive tubular atrophy and the score of tubulointerstitial damage was mainly between mild to moderate (Fig. 8B). The tubulointerstitial damage was significantly increased in the I/R group (0.76 ± 0.15) compared to controls (control 0.04 ± 0.01 and unilateral nephrectomy 0.09 ± 0.02) (both $P < 0.01$). CsA increased the tubulointerstitial damage score when compared to the I/R group, but there was no statistical difference. Rats in the rapamycin (0.18 ± 0.05), tacrolimus (0.53 ± 0.20), or MMF (0.45 ± 0.09) group had less tubulointerstitial damage than the I/R animals, only rapamycin treatment showed statistical significance ($P < 0.01$). However, in comparison with the CsA group, rapamycin or MMF (both $P < 0.01$) significantly decreased

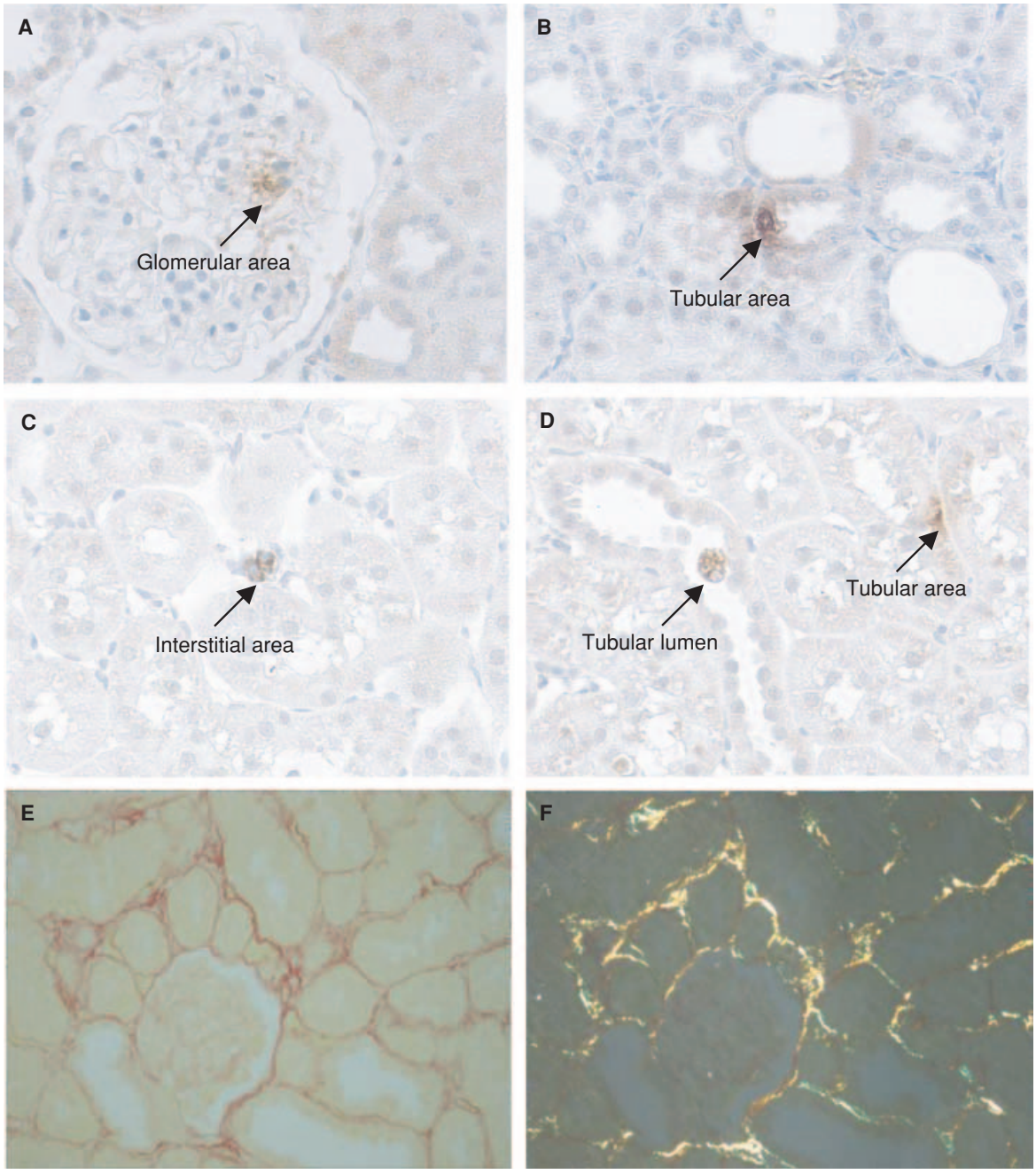


Fig. 7. Localization of active caspase-3 protein and extracellular matrix deposition. Active caspase-3–positive cells were showed in the glomerulus (*A*), tubular area (*B*), expanded interstitial area (*C*), and tubular lumen (*D*, arrows) at ×400 magnification. The positive stained area of Sirius Red staining presented a pink color under white light (*E*) and was strongly birefringent under polarized light (*F*) at ×200 magnification.

the tubulointerstitial damage, but tacrolimus ($P = 0.06$) just reached marginal significance.

As we have previously shown, serum creatinine (μmol/L) (Fig. 9A) was significantly increased in the unilateral nephrectomy group (65 ± 4.4) in contrast to the control (49.8 ± 4.1) ($P < 0.05$). The I/R (88.8 ± 7.0) injury or CsA (88.8 ± 6.8) treatment further raised the level of serum creatinine compared to controls ($P < 0.05$ or 0.01). Animals treated with tacrolimus (61.4 ± 5.5), rapamycin (61.0 ± 2.4), or MMF (64 ± 5.8) had significantly lower serum creatinine level than both I/R and CsA-treated animals (all $P < 0.05$). Urinary protein (mg/mL) (Fig. 9B) excretion was also significantly increased in the I/R group (25.0 ± 5.3) in comparison with controls (control 7.9 ± 1.2 and unilateral nephrectomy 8.0 ± 0.9) ($P < 0.05$). The CsA group demonstrated a higher urinary protein excretion than the I/R group, but this only reached marginal statistical significance (59.1 ± 16.1) ($P = 0.07$). Rats in

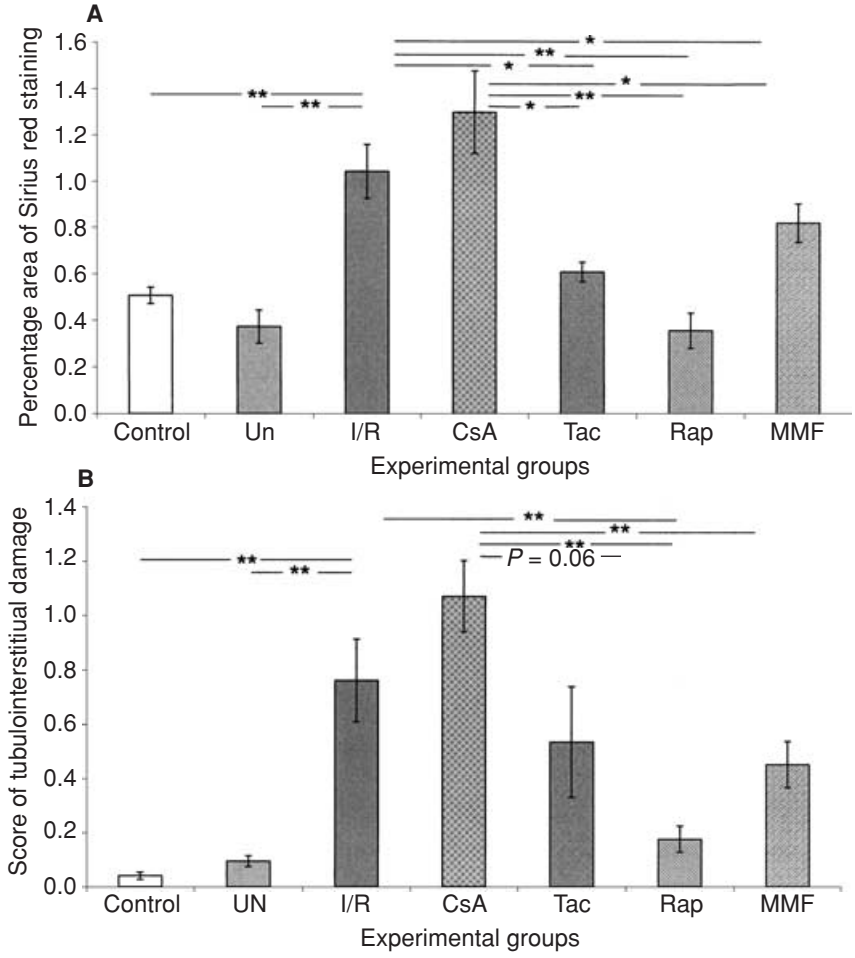


Fig. 8. Changes of Sirius Red staining (*A*) and tubulointerstitial damage (*B*) in different experiment groups. Data are expressed as mean ± SEM ($N = 4$ to 6). $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations are: control, sham-operated; UN, unilateral nephrectomy; I/R, ischemia/reperfusion; CsA cyclosporine A; Tac, tacrolimus; Rap, rapamycin; MMF, mycophenolate mofetil.

the rapamycin group (13.9 ± 6.1) ($P < 0.05$) had significantly less urinary protein excretion than I/R animals, but the lower level in the tacrolimus (16.4 ± 4.2) or MMF (15.8 ± 5.8) group did not reach statistical significance. However, in comparison with the CsA group, tacrolimus and rapamycin ($P < 0.05$), but not MMF ($P = 0.08$), significantly decreased urinary protein excretion.

Correlation between inflammation, apoptosis, caspase-3 protein expression, and renal function

There was a positive correlation between interstitial inflammation and 35 kD IL-1β or 45 kD caspase-1 ($r = 0.406$ and 0.405, respectively) ($P < 0.05$), but not for 17 kD IL-1β or 20 kD caspase-1 (Table 1). A strong positive correlation was revealed between interstitial inflammation and the 24 kD caspase-3 or caspase-3 activity ($r = 0.814$) (Fig. 10A) ($r = 0.484$) (Fig. 10B) ($P < 0.01$), respectively, all of which were also correlated with the change in serum creatinine ($r = 0.463$) (Fig. 10C) ($r = 0.573$) (Fig. 10D) ($r = 0.539$) ($P < 0.01$), respectively, and urinary protein ($r = 0.537$) (Fig. 10C) ($r = 0.529$) (Fig. 9D) ($r = 0.517$) ($P < 0.01$), respectively, and the level of tubulointerstitial damage ($r = 0.794$) (Fig. 10E) ($r = 0.618$) (Fig. 10F) ($r = 0.712$) ($P < 0.01$), respectively, and fibrosis ($r = 0.651$) (Fig. 10E) ($r = 0.567$) (Fig. 10F) ($r = 0.469$) ($P < 0.01$), respectively. To a lesser extent, interstitial inflammation was correlated with 32 kD ($r = 0.457$) ($P < 0.01$) and 17 kD ($r = 0.341$) ($P < 0.05$) caspase-3 protein expression. The expression of 35 kD IL-1β was also linked with 45 kD casapse-1 ($r = 0.466$) ($P < 0.01$) and 24 kD caspase-3 ($r = 0.386$) ($P < 0.05$). Multiple regression analyses showed that caspase-3 activity was more related to 24 kD caspase-3 (standardized β coefficients = 0.435) ($P < 0.05$) compared to 32 kD (standard β coefficients = 0.361) ($P < 0.05$) or 17 kD caspase-3 (standard β coefficients = 0.141) ($P > 0.05$). The 24 kD caspase-3 had the strongest association with the change in inflammation (standard β coefficients = 1.093) ($P < 0.01$) when compared to either the 32 kD (standard β coefficients = 0.183) ($P = 0.074$) or 17 kD caspase-3, 45 kD or 20 kD caspase-1 and 35 kD or 17 kD IL-1β (standard β coefficients = 0.241) ($P = 0.056$).

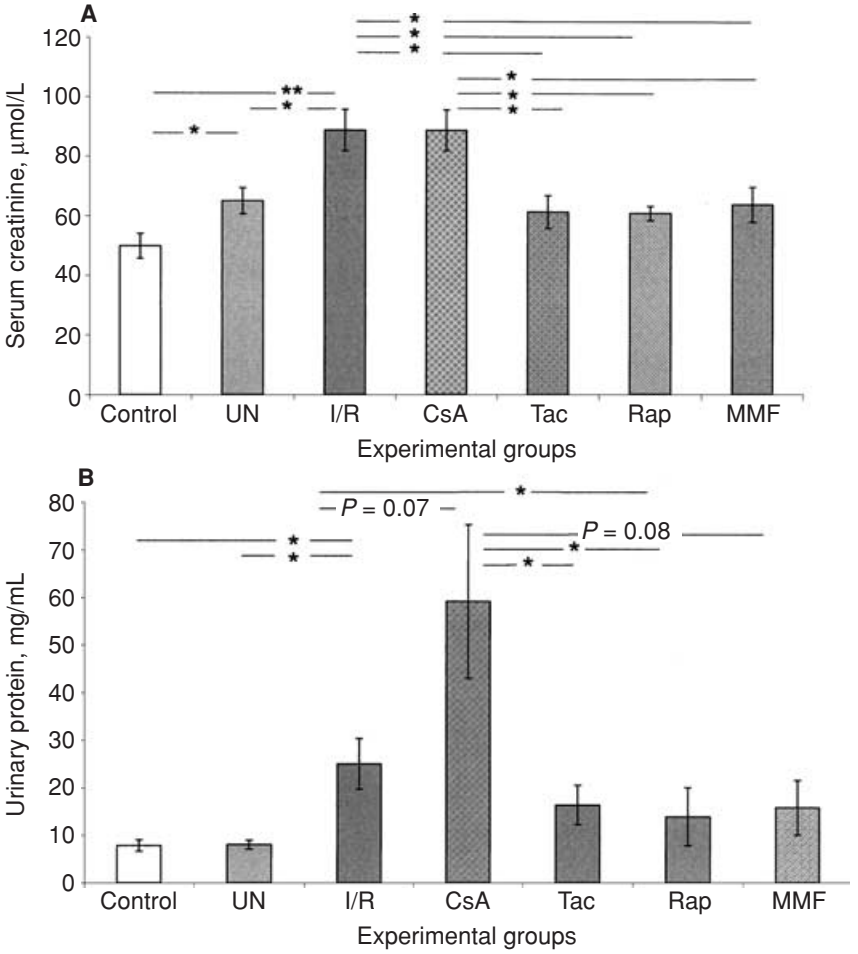


Fig. 9. Changes of serum creatinine (*A*) and urinary protein (*B*) in different experiment groups. Data are expressed as mean ± SEM ($N = 4$ to 6). $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations are: control, sham-operated; UN, unilateral nephrectomy; I/R, ischemia/reperfusion; CsA, cyclosporine A; Tac, tacrolimus; Rap, rapamycin; MMF, mycophenolate mofetil.

DISCUSSION

I/R injury is an important factor influencing both early allograft function and long-term graft survival following kidney transplantation. The inflammation involved in the progression of I/R injury may be mediated by a variety of inflammatory mediators, neutrophil infiltration, caspase activation, and the occurrence of apoptosis [4, 29]. We have previously reported that the immunosuppressants tacrolimus and rapamycin protect against the long-term effects of I/R injury in a rat model and have elucidated the mechanisms underlying the deposition of extracellular matrix [30–32] and the deletion of renal cells by apoptosis [abstract; Yang B, et al, *J Am Soc Nephrol* 13:96, 2002]. However, the precise mechanisms of I/R injury are far from clear and the effects of immunosuppressants on renal I/R injury have not been fully defined. The consequent events after I/R involve not only an early but also a late inflammatory response (lasting several weeks), which is of particular relevance to chronic allograft reject, but has been poorly elucidated thus far [4]. For the first time, using this 16-week-long model, we have further explored the protein expression of possible inflammatory mediators IL-1β, caspase-1, and caspase-3, and investigated the long-term effects of I/R and immunosuppressants on the development of inflammation and the associated changes in renal function and morphologic structure.

Table 1. Correlation matrix showing the r value derived from a linear correlation analysis

r value	Inflammation (ED-1 staining)	24 kD Caspase-3	Caspase-3 activity
32 kD Caspase-3	0.457[b]	0.194	0.438[b]
24 kD Caspase-3	0.814[b]		0.532[b]
17 kD Caspase-3	0.314[a]	0.672[b]	0.275
Caspase-3 activity	0.484[b]	0.532[b]	
45 kD Caspase-1	0.405[a]	0.470[b]	0.400[a]
20 kD caspase-1	0.285	0.349[a]	0.184
35 kD Interleukin-1β	0.406[a]	0.386[a]	0.313
17 kD Interleukin-1β	0.223	0.135	0.236
Serum creatinine	0.463[b]	0.537[b]	0.539[b]
Urinary protein	0.537[b]	0.529[b]	0.517[b]
Tubulointerstitial damage	0.794[b]	0.618[b]	0.712[b]
Fibrosis (Sirius Red staining)	0.651[b]	0.567[b]	0.469[b]

ED-1 is endothelin-1. Correlation matrix showing the *r* value derived from a linear correlation analysis.
[a] $P < 0.05$; [b] $P < 0.01$.

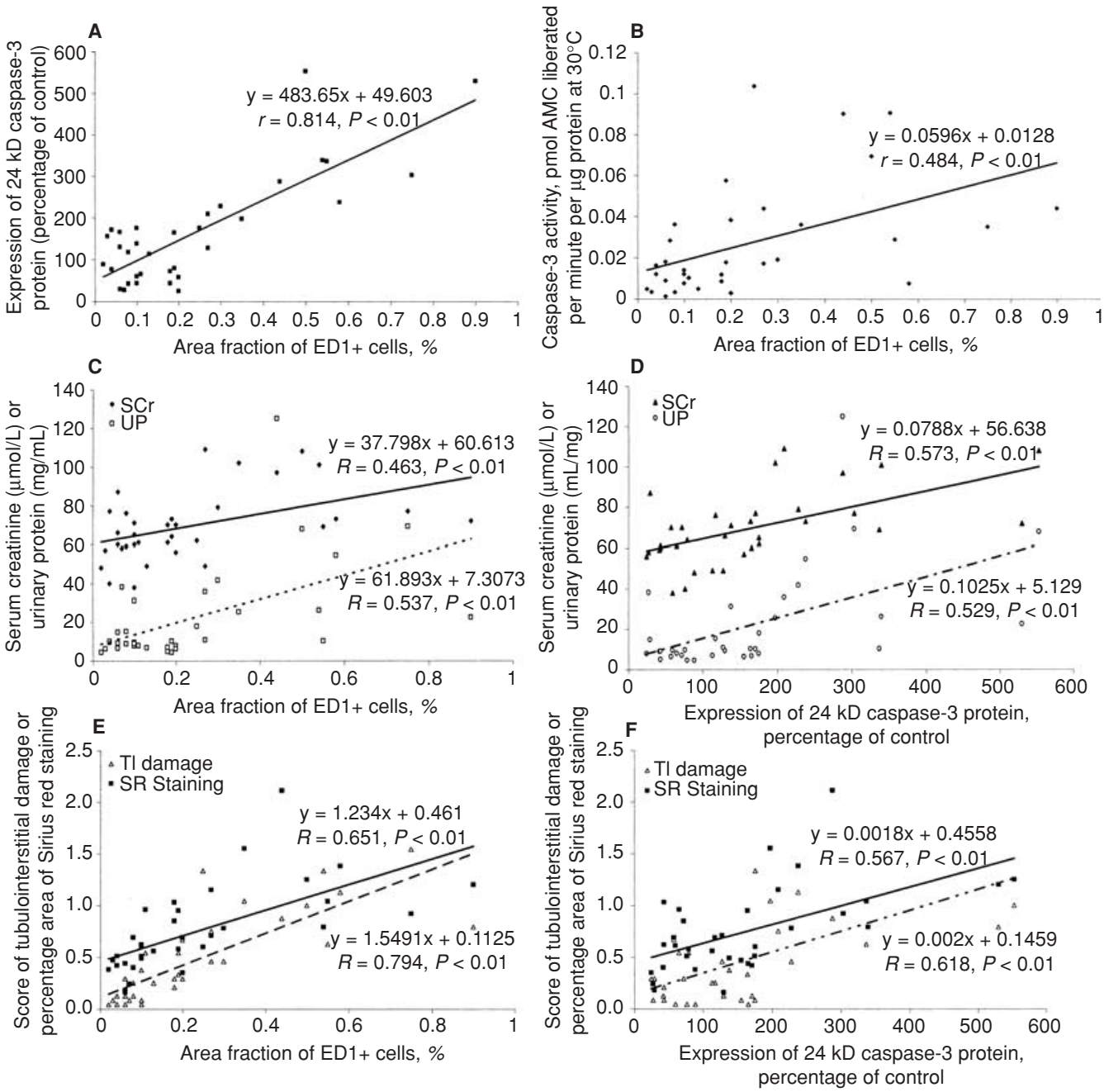


Fig. 10. Correlation between endothelin-1 (ED1+) cells, 24 kD caspase-3 protein or activity and other parameters. (*A*) ED1+ cells vs. the protein expression of 24 kD caspase-3. (*B*) ED1+ cells vs. caspase-3 activity. (*C*) ED1+ cells vs. serum creatinine and urinary protein. (*D*) 24 kD caspase-3 protein vs. serum creatinine and urinary portiein. (*E*) ED1+ cells vs. tubulointerstitial damage and Sirus Red staining. (*F*) 24 kD caspase-3 protein vs. tubulointerstitial damage and Sirus Red staining in the kidneys of various groups.

We have found that I/R induced a significant increase of ED-1+ cells that localized mainly in the tubulointerstitium and periglomerular areas. Different immunosuppressants had opposite impacts on I/R associated inflammation. CsA further increased the infiltration of ED-1+ cells, but tacrolimus, rapamycin, and MMF decreased the number of ED-1+ cells. The inflammatory molecule IL-1β provides a possible mediator of the change in ED-1+ cells induced by I/R injury or immunosuppressants. This would be supported by the finding that the 35 kD IL-1β was numerically increased by I/R and significantly decreased by rapamycin treatment. It may be that there was simultaneous activation and consumption of IL-1β during the time course of I/R injury. This could

lead to continuous cleavage of IL-1β precursor and so maintain the overall level of 17 kD active IL-1β in different groups.

We also showed a significant increase in 45 kD caspase-1 precursor in the I/R group and 20 kD active caspase-1 in the CsA group, both of which were significantly decreased by rapamycin. Caspase-1 is a member of caspase family of cysteine proteases and plays an important role in the maturation of proinflammatory cytokines [46, 47]. For instance, caspase-1 [IL-1β–converting enzyme (ICE)] is involved in the processing of pro-IL-1β and IL-18 [14–18] and all of these molecules have been implicated in the pathogenesis of renal I/R injury [16, 18]. Caspase-1 knockout mice treated with lipopolysaccharide have a blunted IL-1β response [48] and reduced ischemic brain injury [49]. In this model, there may be a simultaneous activation and consumption of caspase-1 in the I/R or immunosuppressant treated groups. Once the consumption of active caspase-1 was inhibited by rapamycin, the increased caspase-1 precursor also reduced. This continuous consumption of active caspase-1 may have been involved in the processing of IL-1β or other biologic function such as its engagement in apoptosis. It has been suggested that changes in IL-1β protein can occur through the activation of caspase-1 [14–16] and active IL-1β could in turn contribute to renal inflammation, although we cannot categorically confirm or rule out a direct link between the activation of caspase-1 and IL-1β in this study. The caspase-1 activity assay, however, did not show significant differences between any groups, which might indicate at least that caspase-1 was not a major player in this model.

On the other hand, 20 kD active caspase-1 or 17 kD active IL-1β was not significantly increased in the I/R group compared to the controls and neither of them was directly correlated with the interstitial inflammation though their precursors were. This may indicate that activated caspase-1 and its inflammatory product are involved in, but not key factors for, the induction of inflammation in this long-term renal I/R injury. This is consistent with data from the acute renal I/R study (45 minutes renal ischaemia followed by 24 hours reperfusion) carried out by Daemen et al [50] using caspase-1−/− and caspase-1+/+ mice. Therefore, other activated caspases and inflammatory mediators, other than caspase-1 and IL-1β, are likely to be more prominently involved in this model.

The activation of caspase-3 was evident in this model. As another important member of the caspase family, it occupies a critical position in the signal transduction cascade associated with immune responses, apoptosis, and inflammation. Active caspase-3 is also responsible for the maturation of some proinflammatory mediators, such as IL-1β, IL-18, and MIP-2 [19–21]. The 32 kD caspase-3 precursors require two proteolytic cleavage events to become functionally active enzymes. The removal of NH_2 terminal prodomain generates a 29 kD processing intermediate that is subsequently cleaved into 17 and 12 kD subfragments, which then heterodimerize to form activated protease [51–54]. However, other active fragments, such as 18 to 20 kD, have been reported as well [55–59]. The efficacy of full-length caspase-3 antibody has also been approved in our previous studies. This antibody was able to bind 17 and 12 kD recombinant human caspase-3 proteins, 20 kD band in human renal tissues, and 32, 29, 24, and 17 kD bands in rat renal tissues and cells [41, 43, 44, 60]. In this study we have shown for the first time that the 24 kD caspase-3, an active subunit of caspase-3 that is specific for the species of rat [41, 43], was significantly increased in the I/R and CsA groups but deceased by tacrolimus, rapamycin, or MMF at the end point of 16 weeks. This result was consistent with the change trend in caspase-3 activity. Analysis of the electroeluates not only confirmed that the 24 kD protein was an active subunit of caspase-3, but also demonstrated that the 24 kD caspase-3, rather than the 17 kD protein, predominantly contributed to the activity of caspase-3. The 24 kD caspase-3 was the molecule that most closely associated with the changes of caspase-3 activity in comparison with the 32 kD and 17 kD caspase-3. Similarly, the 24 kD caspase-3 correlated with episodes of inflammation when compared to 32 kD or 17 kD caspase-3, 45 kD or 20 kD caspase-1, and 35 kD or 17 kD IL-1β. This may indicate that 24 kD caspase-3, rather than the other isoforms of caspase-3 or caspase-1, was predominantly involved in the progression of inflammation, as well as being involved in the apoptosis per se, such as processing proinflammatory mediators [19–21] or cleavage of calpastatin to activate calpain [61, 62]. The inflammation driven by the increase of 24 kD caspase-3 may be a crucial mediator directly linked with the changes in renal function and structure or the nephrotoxic effect of CsA that was characterised by interstitial inflammation and fibrosis, which was well documented [61–63].

The immunostaining of active caspase-3 was scattered in kidneys and was mainly localized in tubular and interstitial areas. This was consistent with the distribution of ED-1+ cells. Although the identity of the cells staining positive with active caspase-3 antibody cannot be defined, they could be, according to their location, both tubular and inflammatory cells. The co existence of active caspase-3–positive cells and ED-1+ cells in the interstitial areas of the kidney appears to imply the involvement of active caspase-3 in inflammation. Some active caspase-3–stained cells exhibit the morphologic changes of apoptosis especially in tubular areas indicating their involvement in apoptosis. In addition, the staining pattern of active caspase-3 was different to that of full-length caspase-3 in our previous study, which had wider staining

areas including normal tubular and medullary areas and rare apoptotic morphologic features [43]. This revealed, therefore, that the localization of caspase-3 active form is more actively to be linked with its biologic functions such as in inflammation and apoptosis.

Furthermore, the effect of caspase-3 on inflammation may link with calpain, another cysteine protease, which plays a pivotal role in ischemic injury to liver, heart, brain, and kidney [66, 65]. Calpain inhibitor-1 reduces renal dysfunction and injury associated with I/R [66]. Calpain activation has been associated with the down-regulation and cleavage of calpastatin, a calpain inhibitor, by caspase-3 and caspase inhibitor Z-D-DCB normalized these changes [64]. The relationship between caspase-3 and calpain, and the effect of immunosuppressant on calpain in the long-term renal I/R injury model warrants further investigation.

Different immunosuppressants had varying degrees of effect on inflammation in this long-term rat I/R injury model. CsA, tacrolimus, and MMF dosages were similar to those used in the clinical setting, whereas the rapamycin dosage was approximately tenfold higher. Rather than reducing the inflammatory effects of I/R, CsA caused further inflammation, in contrast with the other agents. A comparison of CsA with tacrolimus, rapamycin, or MMF also demonstrated other differences with regard to the expression of 24 kD caspase-3, renal function, tubulointerstitial damage, and renal fibrosis in long-term renal I/R injury. However, in spite of these different effects of CsA and other immunosuppressants, changes in inflammation, renal function, tubulointerstitial damage, and renal fibrosis were closely correlated with the expression of 24 kD caspase-3 in all groups. Rapamycin proved to be the drug with the greatest effect in these areas though this may be due to its high dosage. Tacrolimus and MMF were lesser than rapamycin, but both of them are equivalent effectiveness, although they belong to different categories. We cannot exclusively conclude that rapamycin is the optimal immunosuppressant for long-term renal I/R injury because of the high dosage used. The evidence here does at least suggest that tacrolimus and MMF might prove to be better immunosuppressive drugs than CsA.

From a clinical perspective, chronic immunosuppressive treatment needs to be a combination of drugs with different mechanisms of action to enhance efficacy, reduce dosage, and minimize side effects. We have reported that a pan caspase inhibitor, B-D-FMK, inhibited caspase activity, decreased apoptosis and inflammation, and subsequently ameliorated renal injury and fibrosis in an immune-medicated chronic glomerulonephritis model [44]. It has also been reported that the caspase inhibitor ZVAD-FMK reduced caspase-3 activity and prevented the onset of not only apoptosis, but also inflammation and tissue injury in a renal I/R mouse model, though that analysis was based on the I/R kidneys of 24 hours [29]. The evidence from these studies suggests that caspase-3 inhibition may be a useful therapeutic intervention to ameliorate the long-term effects of I/R injury and CsA-induced nephropathy. Caspase-3 inhibition may exert additive or synergistic effects with various immunosuppressants especially CsA and minimize their side effects.

CONCLUSION

The mechanism of long-term I/R injury and various immunosuppressant treatment may include inflammation, which is closely correlated with the expression of 24 kD caspase-3. Active 24 kD caspase-3 may be a major factor that alters both renal function and morphologic structure of I/R rat kidneys treated with various immunosuppressants.

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