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Downregulation of Urokinase-Type and Tissue-Type Plasminogen Activators in a Rabbit Model of Renal Ischemia/Reperfusion[1]

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Urokinase-type and tissue-type plasminogen activators (uPA, tPA) are key enzymes for starting the plasminogen system, which plays important roles in various physiological and pathological conditions. In order to examine the gene regulation in rabbit pathophysiological models we attempted to clone full-length cDNAs encoding uPA and tPA from kidney extracts of rabbit (*Oryctolagus cuniculus*) by reverse transcription-polymerase chain reaction and rapid amplification of cDNA ends. The cloned rabbit uPA and tPA cDNAs were 2,350 and 2,561 bp in length, respectively, and the basic molecular structures predicted from the cDNAs were well-conserved compared with human uPA and tPA. In a rabbit model of renal ischemia/reperfusion (I/R), the expression of uPA and tPA mRNAs was down-regulated and that of their physiological inhibitor, type 1 plasminogen activator inhibitor, mRNA was up-regulated in ischemic kidney compared to non-ischemic kidney. In addition, fibrinolytic activity in ischemic kidney was lower than that in non-ischemic kidney. It is suggested that repression of fibrinolysis in the kidneys in rabbit I/R may contribute to the progression of renal damage in the model.

Key words: cDNA, rabbit, renal ischemia/reperfusion, tissue-type plasminogen activator, urokinase-type plasminogen activator.

Plasminogen activator (PA) plays a prominent role in the regulation of intra- and extra-vascular fibrinolysis through the activation of the inactive proenzyme, plasminogen, to the active enzyme, plasmin, which is implicated in the degradation of the extracellular matrix as well as fibrin. Two physiological PAs have been identified: tissue-type (tPA) and urokinase-type (uPA). The tPA-mediated pathway is primarily involved in the resolution of blood clots, and the uPA-mediated pathway in a variety of other biological processes, including cell migration, tissue remodeling and fibrosis formation (*1*). Their physiological inhibitor, type 1 plasminogen activator inhibitor (PAI-1), is also implicated in such processes through the inhibition of plasmin formation.

Renal ischemia/reperfusion (I/R) injury is often encountered in surgery, especially in vascular procedures and transplantation. Free radicals, leukocytes, cytokines, and proteinases are implicated in the onset mechanism of the injury (*2*). However, the implication of the plasminogen system in renal I/R injury has not been reported. It was suggested that the functional plasma levels of activators and inhibitors of the plasminogen system in rabbit were similar to those in man (*3*). Moreover, rabbit has been widely used as an experimental animal for studying the plasminogen system *in vitro* (*4, 5*) and *in vivo* (*6, 7*). In particular, rabbits seem to be useful as a model for surgery-associated I/R injury studies because of their suitable size (*8*). We previously reported that the plasminogen system was involved in the onset of I/R injury through induced endothelial cell damage and increased vascular permeability in a rabbit lung I/R injury model (*9*). To clarify the role of the plasminogen system in rabbit pathological experimental models in more detail, it is necessary to determine the characteristics and fluctuation of the components of the rabbit plasminogen system, such as PAs and PAI-1.

In the present paper, we report the sequences of rabbit cDNAs encoding uPA and tPA, and the expression of the mRNAs of uPA, tPA, and PAI-1 in a rabbit renal I/R model.

MATERIALS AND METHODS

Reverse Transcription (RT)–PCR—Total cellular RNAs were isolated from the kidneys of normal Japanese white rabbits after treatment with a guanidinium isothiocyanate-sarcosyl solution followed by phenol extraction and ethanol precipitation (*10*). In order to obtain gene-specific primers for tPA, RT-PCR was performed with degenerate primers [sense, 5′-GACTGGAC(A/G/C/T)GAGTG(C/T)GAGCT-3′; antisense, 5′-AGGTA(A/G)TT(A/G/C/T)GT(A/G/C/T)ACCT-T(A/G/C/T)GTGTA-3′], which were designed based on the well-conserved regions of the known cDNAs of other mammals (*11–13*). One microgram of normal rabbit kidney total

[1] The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers AY029517 (uPA) and AY029518 (tPA).

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Abbreviations: EGF, epidermal growth factor–like domain; FN1, fibronectin type 1 domain; I/R, ischemia/reperfusion; K, kringle domain; ORF, open reading frame; PA, plasminogen activator; PAI-1, type-1 plasminogen activator inhibitor; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription–PCR; SPC, serine protease catalytic domain; tPA, tissue-type PA; uPA, urokinase-type PA; UTR, untranslated region.

RNA was reverse-transcribed with a random hexamer, an oligo (dT) 15 primer and Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD). After denaturation at 94°C for 2 min, PCR was performed with Taq DNA polymerase (Fermentas, Hanover, MD), *i.e.* 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 40 s, followed by final extension at 72°C for 2 min. The 341-bp PCR product was purified with Microcon PCR (Millipore, Bedford, MA), subcloned into pBluescript II (Toyobo, Osaka) constructed for TA-cloning (*14*), and sequenced using M13 forward and reverse primers. Subsequently, gene-specific primers for tPA (sense, 5′-GAGGCTCACGTCCGGCTGTACCCCTCCA-3′, antisense, 5′-TCCTTCTGCCCACAGCCCAGCCCCCA-GC-3′) and nested primers (sense, 5′-GCACGCCCCAGC-AGCTGAAGAACCGAACCG-3′; antisense, 5′-GGGTCCTC-CTGAGTCACCCTGGCAGGCGT-3′) were designed. Gene-specific primers for uPA (sense, 5′-GGCGGCAGCCTCAT-CAGTCCTTGCTGGGT-3′; antisense, 5′-GTATGGACCGG-GATGGCTGAGCACATTGGC-3′) were designed based on partial cDNA sequences of rabbit uPA (AF 069711 and AF 097647). The nucleotide sequences determined with an ABI PRISM 310 Genetic Analyzer using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA). All sequencing were performed once or more in both directions to obtain maximum accuracy for the sequence in question.

3′ and 5′ Rapid Amplification of cDNA Ends (RACE) for Rabbit uPA and tPA—3′ and 5′ RACE was performed using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). In brief, 1 μg of total RNA extracted from normal rabbit kidneys was reverse-transcribed with the oligo(dT)-anchor primer, and with the oligo(dT) primer and 5′-anchor oligonucleotide in the kit for 3′ and 5′ RACE, respectively. After denaturation of the 3′ or 5′ first strand cDNA at 98°C for 2 min, PCR was performed with the uPA or tPA gene–

A

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   1                                                                  CACAAGGCGGAATCTGGAGCGCCGGATCT
  30  GGGCTGAGCGTCCACCTACACCCGCAGCGCAGGTCCGCATCCTCCTGCCGCAGCCACTGAGCCGCTGTCTAGGGCCCCAGCCTCGCCACC

 120  ATGAGAGTCCTCCTGGTATGCCTGCTTCTCTGCGCCCTGGTCGTGAGCGACTCCGAAGGAAGCCATGAACTTCACGGAGTGTCTGATGCT
(-20)  M  R  V  L  L  V  C  L  L  L  C  A  L  V  V  S  D  S  E  G  S  H  E  L  H  G  V  S  D  A

 210  TCAAACTGCGGCTGTCTGAATGGAGGAACATGTGTGACCTACAAGTACTTCTCCAACATTTGGCGATGTAACTGCCCAAAGAAATTCCAA
( 11)  S  N  C  G  C  L  N  G  G  T  C  V  T  Y  K  Y  F  S  N  I  W  R  C  N  C  P  K  K  F  Q

 300:  GGAGAGCACTGTGAGATAGATACATTGAAAACCTGCTATCATGGGGATGGTCACTCTTATCGTGGAAAGGCCAACACTGACATCATGGAT
( 41):  G  E  H  C  E  I  D  T  L  K  T  C  Y  H  G  D  G  H  S  Y  R  G  K  A  N  T  D  I  M  D

 390  CGACCCTGCCTTGCCTGGAACTCTGCCAATGTTCTTACGAAAACATACCATGCCCACAGACCTGATGCCCTTCAGCTGGGCTTGGGGAAA
( 71)  R  P  C  L  A  W  N  S  A  N  V  L  T  K  T  Y  H  A  H  R  P  D  A  L  Q  L  G  L  G  K

 480:  CACAATTACTGCAGGAACCCAGACCACCAGAGAAGACCCTGGTGCTATGTGCAGGTTGGCCTAAAGCAGCTCATCCAAGAGTGCAAGGTG
(101)  H  N  Y  C  R  N  P  D  H  Q  R  R  P  W  C  Y  V  Q  V  G  L  K  Q  L  I  Q  E  C  K  V

 570  CATGACTGCTCTTCAGGAAAAAAACCTGCCCTTCCTCCAGGAAAATTGGAGTTTCAGTGTGGCCAGAAGGCTCTGAGGCCCCGTTTTAAG
(131)  H  D  C  S  S  G  K  K  P  A  L  P  P  Q  K  L  E  F  Q  C  G  Q  K  A  L  R  P  R  F  K

 660  ATTATTGGGGGAGAATTCACCATCATTGAGAACCAGCCCTGGTTTGCAGCCATCTACAGGAGGCACCGGGGAGGCTCTGTCACTTATGTG
(161):  I  I  G  G  E  F  T  I  I  E  N  Q  P  W  F  A  A  I  Y  R  R  H  R  G  G  S  V  T  Y  V

 750·  TGTGGCGGCAGCCTCATCAGTCCTTGCTGGGTGGTCAGCGCCACACATTGCTTCATTAATCACCAAAAGAAAGAGGACTACATTGTCTAC
(191)  C  G  G  S  L  I  S  P  C  W  V  V  S  A  T  H  C  F  I  N  H  Q  K  K  E  D  Y  I  V  Y

 840.  CTGGGTCGGTCAAGGCTTAACTCCATGACACCTGGGGAGATGAAGTTTGAAGTGGAGCAACTCATCTTGCATGAGGGCTATAGAGCTGAC
(221)  L  G  R  S  R  L  N  S  M  T  P  G  E  M  K  F  E  V  E  Q  L  I  L  H  E  G  Y  R  A  D

 930:  ACACTGGCTCATCACAACGATATTGCCTTGCTGAAGATACTTTCCAACAATGGCCAATGTGCTCAGCCATCCCGGTCCATACAGACTATT
(251).  T  L  A  H  H  N  D  I  A  L  L  K  I  L  S  N  N  G  Q  C  A  Q  P  S  R  S  I  Q  T  I

1020.  TGCCTGCCCCCATGGAATGCTGATCCCAATTTTGGCACAAGCTGTGAGATCACCGGCTTTGGAAAAGAGAATTCCACCGACTACTTGTAT
(281):  C  L  P  P  W  N  A  D  P  N  F  G  T  S  C  E  I  T  G  F  G  K  E  N  S  T  D  Y  L  Y

1110·  CCAGAGCAGCTGAAAATGACTGTGGTGAAGCTGGTTTCCTACCAGGAGTGTCAGCAACCCCATTACTATGGCTCTGAAGTCACCACCAAA
(311)  P  E  Q  L  K  M  T  V  V  K  L  V  S  Y  Q  E  C  Q  Q  P  H  Y  Y  G  S  E  V  T  T  K

1200  ATGCTTTGTGCCGCTGACCCACAGTGGGAAACAGATTCCTGCCAGGGAGACTCAGGAGGGCCCCTAGTCTGCTCCGTTCAAGCCGTATG
(341):  M  L  C  A  A  D  P  Q  W  E  T  D  S  C  Q  G  D  S  G  G  P  L  V  C  S  V  Q  G  R  M

1290·  ACTCTGACTGGGATTGTGAGCTGGGGCCGTGGATGTGCCTTGAAGAATAAGCCAGGTGTCTATACAAGAGTCTCACGCTTCCTGCCCTGG
(371)  T  L  T  G  I  V  S  W  G  R  G  C  A  L  K  N  K  P  G  V  Y  T  R  V  S  R  F  L  P  W

1380  ATCCGTAGTCACATTGGGGAAGAGAATGGCCTGGCCCTCTGAAAACTTCCAGGGAGCCAAGGAGGAAATGGGCACCACCCACTTCCATAC
(401)·  I  R  S  H  I  G  E  E  N  G  L  A  L  *

1470  TGACCGTCCTTTTTGCAGTAGGATCATCTCCATCAGCCACAAGGAAGAGAATACGGGGATAGGCTCTGCAAAGATGGTTTTGCTTGTGCT
1560  GCCCAGCAGGGCAAGTGACAATAGCTTCAACCTCAGACATGTGTGCCTGGGTGCTGGCCACCCAGACCCCCCTGGCCAGTTGGAAGGGT
1650:  GGTCCTGATTCAGGATGGTAGTTTGTCTTTTTCTGGACTAAAGCTTCCTGGAGTTGAACAATGGCTTCTCCTGTGCATGGGTGCGATTGC
1740  TAGCTCCCTCAACAATGGTCATTCATGAGGCCCACTCTTGGGAAATGAGTAATTTCCCACTTAGGAAGTGTAACCGTTGAAGTCTCTTGA
1830:  AGGAACTTGGCTGTTGGGGGAATAGCAGTCTGGGAAGCAGTGACACTAATAACTTAAGGGAAGGGCTCTGATACTCCATGAATGTATCTA
1920.  CAAATACTATGTATGTGTATATATTTGTGCCCTGATGCTTGGGCTATGAGTGTTAAGTGTGAGCAAGAGCTGGTGTGTTCCTGTGACAAA
2010  AAGTCTAGATATTTCCCTAAATTTTGTGGACTGTGACACCACAAAGAGGGTCTGTCTAGAGAGGCTGTGGGTCAACTCAGGTCTCTTGGG
2100.  TCTCCCACATGATGCCTGTGAATGTATCATTCTGAGGCACAACCTGTGACCAGCACTAAATGTCATGATTTCATTTCACATAGTTTCTCT
2190  TTCTTGGCCAGTTACCCTCAAACCTAGTTCATCCAATCCTCACTGGGTGGGGTGAGGACCACTCCTGTACACTAAATATTTAATAATTAT
2280:  ATTCTACTATTTTAATTTATATCTATTTTTGTAATTCTGAACAAAAACAATCAATAAAATCTGATTTTTTG
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Fig. 1. **The nucleotide and deduced amino acid sequences of rabbit uPA (A) and tPA (B).** The predicted signal peptide (A), or signal and pro-peptides (B) are underlined and indicated by negative numbering. The stop codon is indicated by an asterisk. The polyadenylation signal is double-underlined.

specific primer and two universal primers recognizing both the 3′ and 5′-anchor regions, using TaKaRa LA Taq polymerase (Takara, Otsu), with 35 cycles of denaturation at 98°C for 20 s and annealing/extension at 65°C for 3 min, followed by final extension at 72°C for 7 min. To obtain the 3′ and 5′ RACE products for tPA, nested PCR with the gene-specific nested primer and nested universal primer provided in the kit was performed under the same conditions. The amplified products were gel-purified with a Prep-A-Gene DNA Purification System (Bio-Rad, Hercules, CA) and sequenced as described above.

Renal I/R Model—We followed the Guide for Animal Experimentation issued by Miyazaki Medical College, which is based on the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication no. 86-23, 1985). Male Japanese white rabbits, weighing 2.0–2.5 kg, were anesthetized with intravenous sodium thiopental (30 mg/kg). During the experiment, a warming blanket was used to maintain the rec-

Fig. 1B

B

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   1                                                                    AGACTGGCCCGGGGCAG
  18  GAGAGAAAGCCACACCAAGCAGCCGAGGAGCCGGCGCACCGGCAGCAGCCAGAGGCACGAACTCCAGCAGCTGTCTGCAGAGACGCGGAG

 108  ATGTGGAACCCAGGGGTGAGAGAGCTCTTGTGTGTGTTGTTGCTGTGTGGAGCAGCCTGCGCTCGGCCCAGCCAGGAAGCCCCAGCCCGA
(-33)  M  W  N  P  Q  V  R  E  L  L  C  V  L  L  L  C  Q  A  A  C  A  R  P  S  Q  E  A  P  A  R

 198  GTCAGAAGAGGAGCCAGATCCTTCAGAGTGACCTGTGTGGACCAGCAGACGCAGCTGACGTACCAGCGCCGGGAGTCGTGGCTCCGCCCG
( -3)  V  R  R  Q  A  R  S  F  R  V  T  C  V  D  Q  Q  T  Q  L  T  Y  Q  R  R  E  S  W  L  R  P

 288  GGGCTCCGAGGCAACCGTGTGGAATACTGCAGGTGCAGCAGCGGCGGGCCGCGGTGCCACTCGGTGCCCGTCCAAAGCTGCAGCGAGCCC
( 28)  G  L  R  G  N  R  V  E  Y  C  R  C  S  S  G  G  P  R  C  H  S  V  P  V  Q  S  C  S  E  P

 378  AGGTGCCTCAACGGTGGCACGTGTTCGCAGGCTCTGTATTTCTCGGATTTTGTCTGCCAGTGCCCCGAAGGATTTGTCGGGAAACGCTGC
( 58)  R  C  L  N  G  G  T  C  S  Q  A  L  Y  F  S  D  F  V  C  Q  C  P  E  G  F  V  G  K  R  C

 468  GAAGTCGATACCCGTGCTAGGTGCTACGAGGACAGGGGCATCGGCTACAGAGGCACGTGGAGCACAACAGAGAGTGGGGCACAGTGTGTC
( 88)  E  V  D  T  R  A  R  C  Y  E  D  R  G  I  G  Y  R  G  T  W  S  T  T  E  S  G  A  Q  C  V

 558  AACTGGAACAGCAGCTGGCTGGCCCTGAAGCCCTACAGCGGGCGGAAGCCGAACGCCCTCCGGCTGGGCCTGGGCAACCACAACTACTGC
(118)  N  W  N  S  S  W  L  A  L  K  P  Y  S  G  R  K  P  N  A  L  R  L  G  L  G  N  H  N  Y  C

 648  AGAAACCCAGACCGAGACACAAAGCCCTGGTGCTACGTCTTTAGGGCAGGGACGTACAGCCCAGAGTTCTGCAGCACGCCCGCCTGCTCC
(148)  R  N  P  D  R  D  T  K  P  W  C  Y  V  F  R  A  G  T  Y  S  P  E  F  C  S  T  P  A  C  S

 738  AAGGAAAAAAATGGGAACTGCTACTTGGGAAAGGGGCAAGCGTACCGCGGCACCCACAGCCTCACCACGTCGGGTGCCTCATGCCTCCCG
(178)  K  E  K  N  G  N  C  Y  L  G  K  G  Q  A  Y  R  G  T  H  S  L  T  T  S  G  A  S  C  L  P

 828  TGGAATTCCATGCTCCTGGTGGGCGAGAAGTACACAGCGCGGCAGAGCAACGCGGAGGCCCTGGGCCTGGGCAAGCATAATTACTGCAGG
(208)  W  N  S  M  L  L  V  G  E  K  Y  T  A  R  Q  S  N  A  E  A  L  G  L  G  K  H  N  Y  C  R

 918  AACCCAGACGGAGACTCCAAGCCCTGGTGCCACGTGTTGAAGAACCGCAAGTTGGTGATAGAGTACTGCGACGTGCCCCAGTGCGCCACC
(238)  N  P  D  G  D  S  K  P  W  C  H  V  L  K  N  R  K  L  V  I  E  Y  C  D  V  P  Q  C  A  T

1008  TGTGGCCTGCGACAGGACAAGCAGCCCCAGTTCCGCATCAAAGGGGGGCTGTTCACCGACATCACCGCTCACCCCTGGCAGGCGGCCATC
(268)  C  G  L  R  Q  D  K  Q  P  Q  F  R  I  K  G  G  L  F  T  D  I  T  A  H  P  W  Q  A  A  I

1098  TTCACCAACAACAGGAGGTCGCCCGGGGAGCGGTTTCTGTGCGGGGGTATCCTGATCAACTCCTGCTGGGTTCTGTCTGCTGCCCACTGC
(298)  F  T  N  N  R  R  S  P  G  E  R  F  L  C  G  G  I  L  I  N  S  C  W  V  L  S  A  A  H  C

1188  TTCCTGGAGAGGTTCCCACAGCAGAAACTTAGGGTGATCCTGGGCAGAACGTACCCCCTGGTGTCGGCAGAGGAGGAGCAGATATTCGAA
(328)  F  L  E  R  F  P  Q  Q  K  L  R  V  I  L  G  R  T  Y  P  L  V  S  A  E  E  E  Q  I  F  E

1278  GTGGAACAGCCCATCCTGCATGAGAGATTTGATGAAGGCACCTACGACAACGACATCGCGCTGCTAAAGCTGAAATCCACTTCAGGATCC
(358)  V  E  Q  P  I  L  H  E  R  F  D  E  G  T  Y  D  N  D  I  A  L  L  K  L  K  S  T  S  G  S

1368  TGTGCCCAGGAGAGCCAGGCCGTGCGCCTCGTCTGCCTGCCCGACGCCAGCCTGCAGCTGCCCGACTGGACAGAGTGCGAGCTGTCCGGC
(388)  C  A  Q  E  S  Q  A  V  R  L  V  C  L  P  D  A  S  L  Q  L  P  D  W  T  E  C  E  L  S  G

1458  TATGGCAAACACGAGGAGTTTTCGCCTGTCTTCTCCGAGCAGCTGAAGGAGGCTCACGTCCGGCTGTACCCCTCCAGCCGCTGCACGCCC
(418)  Y  G  K  H  E  E  F  S  P  V  F  S  E  Q  L  K  E  A  H  V  R  L  Y  P  S  S  R  C  T  P

1548  CAGCAGCTGAAGAACCGAACCGTCACGGGCAACATGCTGTGTGCTGGAGACACGCGGAGTGGGGGGGCCCAGGTGAACCTGCATGACGCC
(448)  Q  Q  L  K  N  R  T  V  T  G  N  M  L  C  A  G  D  T  R  S  G  G  A  Q  V  N  L  H  D  A

1638  TGCCAGGGTGACTCAGGAGGACCCCTGGTGTGCATGACAGACGGCCACATGACCTTGATCGGCATCATCAGCTGGGGGCTGGGCTGTGGG
(478)  C  Q  G  D  S  G  G  P  L  V  C  M  T  D  G  H  M  T  L  I  G  I  I  S  W  G  L  G  C  G

1728  CAGAAGGACGTGCCGGGCGTGTACACCAAGGTGGTTAATTACCTCGGCTGGATCCAGCAACACGTGGGACCGTGACCAGGAGACACAGTG
(508)  Q  K  D  V  P  G  V  Y  T  K  V  V  N  Y  L  G  W  I  Q  Q  H  V  G  P  *

1818  CCCCGAGGGTGCGTGAGGGGCCGCCGAGACTCCCACAGCAGCTGTGGGGCAGAGAGCCCCTCCCCAGGAGAGGGACGGGTGCACATGCCA
1908  CAGGGGGACTTTCCAGGCTGACGTCACAGGGTGTCAGTCAGGTGGGAGACGGATGGACCAGGGCCGGCCCCTCGGGAACACCGTCCGGTT
1998  TCAAGAGTGGGAGCCCCACCTCCCAGTGTGTCCCAGGCCGGCACAAGAACTGGATCGCTGCTGCAGGGGTATCGCAACAGCCCGAGGAAT
2088  AGCAAGCGCGGCGGCAGTCGCAGGAGGCCTCCCCCGAAGCAGGCCTGCCTCCCCCTGGGCAGTCGCAGGAGGCCTCCCCCGGACGCAGGC
2178  CTGCCTCCCCCTGGGCAGTCGCAGGAGGCCTCCCCTGGACGCAGGCCTGCCTCCCCCTGGGCAGTCGCAGGAGGCCTCCCCTGGGCGCAG
2268  GCCTGCCTCCCCTGGGCAGTCGCAGGAGGCCTCCCCCGGACGCAGGCTTTCCTCCCCCTGGGCAGTCGCAGGAGGCCTCCCCTGGATGCA
2358  GGCCCGCCTCCCCTGGGCGCTCCGTAGCTCCTGGAAGGCTCCTTTCCTTTCGTGTACAGTGTAAATCTTCAGGAGAATGTATCATTTGGA
2448  TCGCTGACAAAAGCAGAGCTGTAGCATATTTTTATGGATCCATGCTAGTGTGTCCTGTTGCCACACTGTGTATTTATACTGTACTTAATA
2538  AATGTAGATGTATTTTTCACATTT
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tal temperature at 38–40°C. The left renal artery was exposed and cleared through a median incision in the abdomen. Five minutes after the administration of heparin (700 U/kg) into an ear vein, the left renal artery was clamped for 60 min. In successful experiments, the kidney turned dark within 1–2 min, and the normal color returned within 2–3 min after removal of the clamp, indicating reperfusion. After 120 min reperfusion, rabbits were sacrificed, and their left and right kidneys were removed for Northern-blot and fibrin-zymography analysis. The clamped left kidney was taken as "ischemic," and the nonclamped right one as "non-ischemic."

Northern-Blot Analysis—Aliquots of total RNA, each 15 μg, were subjected to electrophoresis, transferred to a Hybond N nylon membrane (Amersham, Buckinghamshire, UK), and fixed to the membrane by exposure to UV. Hybridization was performed with a Gene Images Random Prime Labelling and Detection System (Amersham) as described previously (*15*). The fluorescein-labeled cDNA probes used corresponded to the following nucleotide positions: 447–1291 of uPA (Fig. 1A), 387–1271 of tPA (Fig. 1B), 585–1295 of PAI-1 (*16*), and 124–835 of 18S rRNA (*17*). The levels of mRNA were determined with a Luminescent Image Analyzer LAS-1000 plus (Fuji, Tokyo).

Fibrin-Zymography Analysis—Plasminogen activators were extracted from the kidneys with 5 volumes of 75 mM potassium acetate buffer (pH 4.2) containing 0.3 M NaCl, 0.1 M L-arginine, 10 mM EDTA, and 0.25% Triton X-100 (*18*). After centrifugation of the homogenates for 15 min at 15,000 ×*g*, fibrinolytic activity in the supernatants was assayed by fibrin-zymography as described previously (*9*).

RESULTS

Approximately 1.6 and 1.0 kbp products were obtained on 3′ and 5′ RACE for uPA, and 1.1 and 1.6 kbp products for tPA, respectively. By sequencing these products directly, the full-length sequences of both PAs were clarified. In addition to direct sequence analysis, we also cloned these products into pBluescript II constructed for TA-cloning (*14*), and sequenced at least 4 individual clones using M13 forward and reverse primers to determine the 3′ and 5′-end sequences in both directions.

The 2,350- and 2,561-bp final nucleotide sequences encoding rabbit uPA and tPA, and their deduced amino acid sequences are presented in Fig. 1. The nucleotides consist of a 119-bp 5′-untranslated region (UTR), a 1,299-bp open reading frame (ORF), and a 932-bp 3′-UTR, followed by a

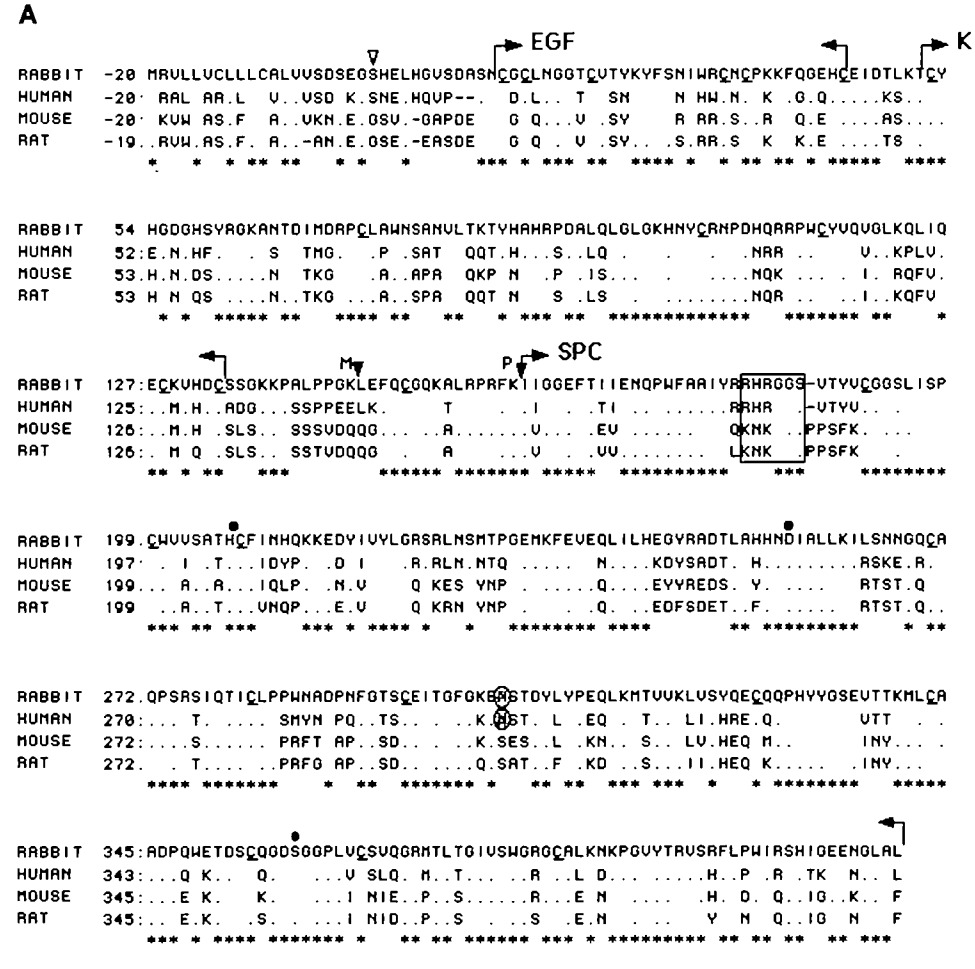

Fig. 2. **Comparison of deduced amino acid sequences of rabbit uPA (A) and tPA (B) with those of man, mouse, and rat.** The human, mouse and rat uPA sequences are taken from Refs. *42*, *43*, and *44*, and those of tPA from Refs. *11*, *12* and *13*, respectively. Identical amino acid residues among the four species are indicated by asterisks. The open triangle indicates the predicted N-terminus of rabbit PAs. Cysteine residues in the sequence after removal of the putative signal peptide (A), or signal and pro-peptides (B) are underlined. The closed triangle with "M" or "P" indicates the site of cleavage by a matrix metalloproteinase or plasmin in human PAs, respectively. The open box indicates the important residues for PAI-1 binding in human PAs and the counterparts in other species. The shadowed boxes indicate the important residues for interaction with ω-amino acid ligands in human tPA and the counterparts in other species. The shadowed circles indicate a potential *N*-glycosylation site. The closed circle indicates the triad amino acid residue that forms the catalytic site of serine protease. Domain positions corresponding to those in the human sequence are shown between two arrows. EGF (residues 11–42) and K (residues 50–131) in human uPA (*20*); FN1 (residues 9–46), EGF (residues 54–87), K1 (residues 95–176), and K2 (residues 183–264) in human tPA (*45*); SPCs (residues 159–411 in human uPA and residues 279–530 in human tPA) (*46*).

27-bp poly A tail in uPA, and a 107-bp 5′-UTR, a 1692-bp ORF and a 762-bp 3′-UTR, followed by a 21-bp poly A tail in tPA. Thus, peptides of 433 and 564 amino acids are encoded by the single ORFs in uPA and tPA, respectively. Based on the statistical distribution of residues around the cleavage site for signal peptidase (*19*) and the known N-terminus of human uPA (*20*), the first 20 amino acids in the uPA sequence probably represent the signal peptide. In the tPA, the first 33 amino acids probably represent the signal and pro-peptides, in analogy with purified human tPA (*21*). Analysis of the deduced amino acid sequences using the SMART program (*22*) revealed that rabbit uPA and tPA contained distinct domains, such as an epidermal growth factor-like domain (EGF), a kringle domain (K), and a serine protease catalytic domain (SPC) in uPA, and a fibronectin type 1 domain (FN1), a EGF, two kringle domains (K1 and K2), and a SPC in tPA, from the NH_2 terminus. The amino acid homologies relative to the human counterparts are EGF, 78%; K, 80%; and SPC, 88% in uPA, and FN1, 61%; EGF, 88%; K1, 83%; K2, 78%; and SPC, 78% in tPA.

Alignment of the rabbit uPA and tPA amino acid sequences with their human, mouse and rat counterparts is shown in Fig. 2. The sequence of rabbit uPA or tPA after removal of the putative signal peptide or signal and pro-peptides contains 24 or 34 cysteine residues at the same positions as in other molecules, respectively. The sequences also contain triad amino acid residues that form the catalytic site of serine protease (His^{206}, Asp^{257} and Ser^{358} in uPA, and His^{326}, Asp^{375} and Ser^{482} in tPA), potential *N*-glycosylation sites (Asn^{304} in uPA, and Asn^{120} and Asn^{452} in tPA) (*23*), and the cleavage site for plasmin (Lys^{160}-Ile^{161} in uPA, and Arg^{279}-Ile^{280} in tPA) (*20, 21*) at conserved positions.

Figure 3 shows the expression of uPA, tPA, and PAI-1

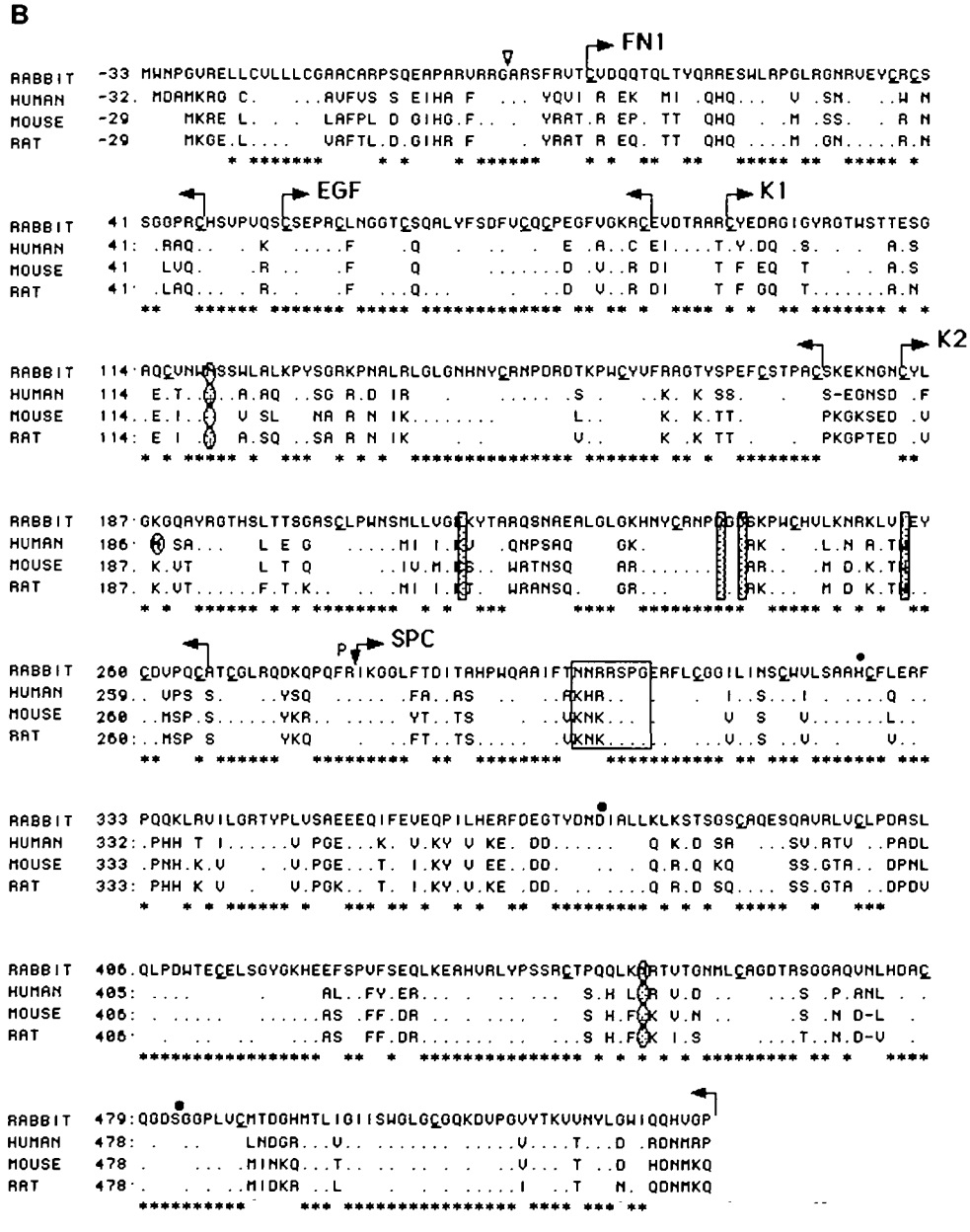

Fig. 2B

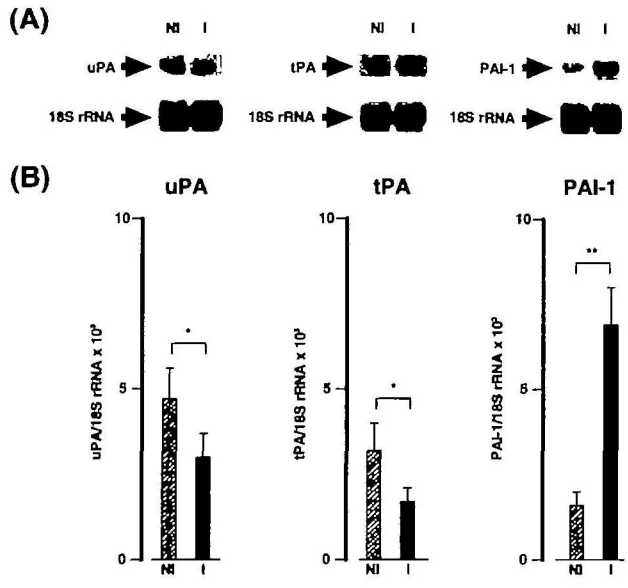

Fig. 3. **Quantitative analysis of the expression of uPA, tPA, and PAI-1 mRNAs in renal I/R.** Total RNA isolated from non-ischemic kidney (NI) or ischemic kidney (I) was subjected to Northern-blot analysis. (A) A Northern blot of uPA, tPA, PAI-1, and 18S rRNA is shown. The exposure time was 20min for uPA, tPA, and PAI-1, and 30 s for 18S rRNA. (B) Statistical analysis was performed with the paired Student's *t*-test (*$p < 0.05$; **$p < 0.01$). Absorption values, obtained by densitometric scanning of blots, were normalized as to the 18S rRNA expression. A value of $p < 0.05$ was considered statistically significant. Data are shown as means ± SE for four rabbits.

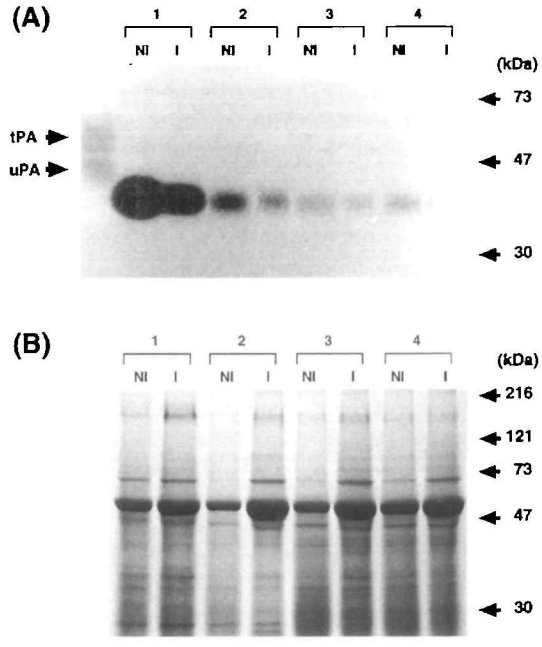

Fig. 4. **Fibrin-zymography (A) and protein staining pattern (B) of kidney extracts from renal I/R.** (A) Extracts of non-ischemic kidney (NI) or ischemic kidney (I) obtained from four renal I/R rabbits were analyzed for fibrinolytic activity. Recombinant human tPA (Toyobo, Osaka) and purified human uPA (Green Cross, Osaka) were used as standards. The molecular masses of the bands were estimated by comparison with prestained protein molecular mass standards (Gibco BRL, Gaithersburg, MD). (B) Samples for fibrin-zymography from the rabbits were subjected to SDS/PAGE and stained with Coomassie Brilliant Blue R-250.

mRNAs in renal I/R. The uPA expression is dominant in non-ischemic kidney, while that of PAI-1 is dominant in ischemic kidney. The levels of uPA and tPA in ischemic kidney were significantly decreased, the ratios being 0.6 and 0.5, respectively, in comparison to non-ischemic kidney. In contrast, the PAI-1 level showed an increase of 4.3 times. We also measured the fibrinolytic activity in a kidney extract obtained from 4 renal I/R rabbits. As shown in Fig. 4A, one principal lytic band corresponding to an estimated molecular mass of 37 kDa was observed. Although the level of fibrinolytic activity greatly varied between individuals, the activity in ischemic kidney was lower than that in non-ischemic kidney in all cases. The similarity in the protein staining patterns on SDS/PAGE of the fibrin-zymography samples among the four rabbits (Fig. 4B) suggests that the individual differences in the fibrinolytic activity were not caused by different sample amounts used for the zymography study. As fibrin-zymography reflects the amount of fibrinolytic enzymes in samples, the variation in fibrinolytic activity may be due to differences in the basic contents of fibrinolytic enzymes in the kidneys among individuals. No lytic band was detected with plasminogen-poor fibrin-agar plates, indicating that these lytic bands were due to PA(s). Moreover, the lytic bands disappeared on zymography with plasminogen-rich fibrin-agar plates containing 1 mM amiloride, a potent inhibitor of uPA (*24*), indicating that the lytic bands were due to uPA (data not shown).

DISCUSSION

We demonstrated here the repression of fibrinolysis in ischemic kidney compared to in non-ischemic kidney using a rabbit renal I/R model. As shown in Fig. 3, the mRNA expression of uPA and tPA is down-regulated, and that of PAI-1 is up-regulated in ischemic kidney. Recently, similar results, *i.e.* an increase in the expression of PAI-1 mRNA, were obtained for human liver I/R (*25*) and a murine lung I/R injury model (*26*). However, the expression of tPA mRNA was up-regulated in the murine model. Although the differences in species, organs and I/R time schedule might be considered to explain this discrepancy, the most possible reason is the effect of clot formation in the organs during vascular clamping. It is well known that thrombin up-regulates gene expression of tPA, uPA, and PAI-1 (*27, 28*). In the present study, rabbits were injected with heparin before clamping to avoid thrombin formation in the ischemic kidney, while the mice were not. It has been reported that tPA mRNA decreased in human endothelial cells during reoxygenation following anoxia (*29*), and tPA and uPA mRNAs also decreased in murine lungs under hypoxic conditions (*30*), supporting our present data.

The extensive fibrin deposition, and subsequent obstruction of vessels and tubules may reflect an imbalance in renal fibrinolysis in several kidney diseases, such as glomerulonephritis (*31*), thrombotic microangiopathy (*32*), disseminated coagulopathy (*33*), and transplant rejection (*34*). Especially gene expression of uPA, not tPA, is suggested to play a critical role in local fibrin deposition/dissolution in a murine model of sepsis (*33*). In the present study, downregulation of uPA was observed in the ischemic kidney at the

protein level as well as the mRNA level (Figs. 3 and 4), suggesting that repression of the fibrinolysis could possibly promote the formation of renal microthrombi, and thus contribute to the progression of renal damage in renal I/R injury.

We also analysed the primary structures of rabbit PAs. As expected for a serine protease, the characteristic active-site triad residues are present in the rabbit PAs. In addition, the basic domains and cystein residues are also perfectly conserved, suggesting that the rabbit PAs possibly possess a tertiary conformation similar to those of man and other animals. Although the basic molecular structures of rabbit uPA and tPA are well conserved, there are several similarities and dissimilarities in the sequences compared with those of man, mouse and rat (Fig. 2). As shown in Fig. 4, a low molecular mass form of uPA was observed in the rabbit kidney extracts. Glu^{143}-Leu^{144} is cleaved by a matrix metalloproteinase to yield a low molecular mass human uPA (*35*). The site in rabbit uPA relative to the human sequence is Lys-Leu. It was suggested that proline at the –3 position of the cleavage site was essential for collagenase cleavage (*36*). Since the rabbit uPA sequence contains a proline residue at the same position as in the human counterpart, rabbit uPA seems to be cleaved by the metalloproteinase. Possible uPA– and tPA–PAI-1 complexes with molecular masses of 100 and 110 kDa, respectively, were observed in rabbit plasma (*3*). It was reported that positively charged residues (Arg^{179}-Ser^{184} in human uPA and Lys^{299}-Gly^{305} in human tPA) played an important role in the binding of PAI-1 (*37, 38*). The six amino acid residues are perfectly conserved in the rabbit uPA sequence. In the tPA sequence, two arginine residues corresponding to Arg^{301} and Arg^{302} in human tPA, which play a prominent part in the interaction, are conserved (*38*). The most striking dissimilarity of the rabbit tPA sequence from others is in a part of a lysine binding site in the K2 domain. It is known that tPA efficiently converts plasminogen to plasmin in the presence of fibrin. The K2 domain has a lysine binding site that participates in binding fibrin (*39*). Residues K^{215}, D^{239}, D^{241}, and W^{256} in the human K2 domain were demonstrated to be of great importance as to its ability to interact with ω-amino acid ligands (*40, 41*). These amino acid residues are perfectly conserved in the human, mouse and rat sequences. However, the amino acids corresponding to K^{215} and W^{256} are glutamate and isoleucine, respectively, in the rabbit sequence, although the two aspartate residues at positions 239 and 241 (as numbered in man) are conserved. Since nonaromatic mutations at W^{256} of the isolated K2 domain abolished its ability to interact with ω-amino acids (*40*), it is likely that rabbit tPA may exhibit less lysine and fibrin binding capacity than human, mouse and rat tPAs.

In conclusion, we isolated cDNAs encoding rabbit uPA and tPA, and demonstrated that fibrinolysis was repressed in the ischemic kidney of a renal I/R model. The present results will be advantageous for investigating the roles of the plasminogen system, not only in I/R injury but also in various pathophysiological conditions with rabbit as the experimental animal.

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