The Kidney Is a Major Site of α_2-Antiplasmin Production

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Abstract

The serpin α_2-antiplasmin (α_2-AP) is the major circulating inhibitor of plasmin; it plays a determining role in the regulation of intravascular fibrinolysis. In addition to blood plasma, plasmin formation occurs in various organs where it is thought to fulfill a spectrum of functions not restricted to clot lysis. α_2-AP is synthesized by hepatocytes, but other possible sites of production have not been investigated. To explore the potential extravascular contribution of α_2-AP in the regulation of proteolysis, we have isolated the murine α_2-AP cDNA and determined its mRNA distribution in adult tissues.

In addition to liver, kidneys are major sites of α_2-AP mRNA accumulation in the mouse. The transcript is present in epithelial cells lining the convoluted portion of proximal tubules, and its accumulation is under androgen control. Human kidneys also contain high levels of α_2-AP mRNA. Moderate amounts of α_2-AP mRNA are detected in other murine tissues such as muscle, intestine, central nervous system, and placenta. Our observations indicate that α_2-AP can be synthesized in a number of tissues, where it could function as a distal regulator of plasmin-mediated extracellular proteolysis. (*J. Clin. Invest.* 1996. 97:2478–2484.)
Key words: fibrinolysis • plasmin • plasminogen • proteolysis • serpins

Introduction

α_2-Antiplasmin (α_2-AP)[1] is a serine-protease inhibitor (serpin) that is regarded as the primary physiological inhibitor of plasmin. Structural and kinetic studies have shown that α_2-AP has three functional sites: a plasminogen/plasmin binding site, a reactive site that binds covalently the catalytic serine residue of plasmin, and a cross-linking site to the fibrin α chain (1–4). α_2-AP is abundant in plasma, where it exerts its antifibrinolytic properties by competing with fibrin for plasminogen binding and through plasmin inhibition. The functional importance of α_2-AP is illustrated by the rare reported cases of congenital α_2-AP deficiency who exhibit severe lifelong hemorrhagic tendency. Hepatocytes are considered to be the site of production of circulating α_2-AP. To our knowledge no other cell type has been demonstrated to synthesize the protein; α_2-AP released by glioblastoma organ cultures (5) and cultured monocytes (6) may originate from the circulating pool of the protein, and α_2-AP in platelet α-granules (7), like other constituents of these organelles, could have been taken up from plasma.

A wide body of evidence suggests that the role of plasmin may not be limited to intravascular fibrinolysis. Plasmin is a serine protease generated by an enzymatic cascade that is controlled by the combined actions of proteases, plasminogen activators (PAs), and antiproteases, plasminogen activator inhibitors (PAIs) (8). In contrast to PAs, plasmin is a proteolytic enzyme of broad specificity that is capable of catalyzing the degradation not only of fibrin but also, directly or indirectly, of most extracellular proteins. Therefore, plasmin is thought to fulfill a large spectrum of biological functions (8). For instance, plasmin-mediated proteolytic activity is postulated to participate in tissue repair processes (9, 10), to help maintain tubular patency in renal tubules (11, 12), and to be involved in extracellular metabolism in the central nervous system (13, 14). Therefore, in addition to preventing excessive intravascular fibrin dissolution, plasmin inhibition could influence biological phenomena in multiple tissues. In this view, a more complete understanding of the sites and circumstances of α_2-AP production in vivo is mandatory to assess the potential role of this antiprotease in modulating physiological and pathological processes associated with expression of the PA/plasmin system.

We report here the cloning of the murine α_2-AP cDNA and show that its RNA transcription is not restricted to hepatocytes. In particular, we identify the kidney as a major source of α_2-AP production and demonstrate that testosterone modulates α_2-AP mRNA accumulation in epithelial cells lining the convoluted segments of proximal tubules. Similarly, we provide evidence that α_2-AP is produced by the human kidney.

Methods

Murine and human tissues. Murine tissues were obtained from NMRI mice killed by cervical dislocation and processed as described elsewhere (12). Normal human kidney samples were obtained from nephrectomy specimens performed for carcinomas and normal liver tissue was obtained from hepatectomies. Macroscopically uninvolved areas were carefully dissected, frozen in liquid nitrogen, and stored at −70°C until analyzed. Tissue integrity was verified by conventional histological evaluation. Serum-free culture supernatant and RNA from human kidney tumor–derived cells, UOK_1 and Ge1, were kindly provided by Dr. P.-Y. Dietrich (Hôpital Universitaire de Genéve, Geneva, Switzerland).

Cloning of α_2-AP cDNA. Oligonucleotides of degenerate sequences (5′-ATGTAYYTNCARAARGGNTT-3′ and 5′-TCRAAYTTRTTNCKCCARAA-3′), based on the human α_2-AP cDNA sequence (amino acids 166–172 and 240–246) (4), were prepared. We used 1 μg of total murine liver RNA to synthesize cDNA with the oligonucleotide spanning the 3′ region as a primer (15). The mouse α_2-AP cDNA was then amplified by PCR, yielding a fragment of 242 bp which was subcloned into pBluescriptKS$^-$ vector (Stratagene, La Jolla, CA) and sequenced. This fragment was labeled by random

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Received for publication 27 December 1994 and accepted in revised form 12 March 1996.

1. *Abbreviations used in this paper:* α_2-AP, α_2-antiplasmin; PA, plasminogen activator; PAI, plasminogen activator inhibitor; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

J. Clin. Invest.

0021-9738/96/06/2478/07 $2.00
Volume 97, Number 11, June 1996, 2478–2484

priming (16) and further used to screen a lambda-ZAP murine liver cDNA library (Stratagene). Inserts from three positive phage clones were excised from the lambda-ZAP vector according to the instruction manual and sequenced on both strands by the chain termination method using successive primers or exonuclease III/mung bean nuclease unidirectional deleted recombinant plasmids (17). The full-length cDNA-containing plasmid was called pmuα_2-AP_fl. Nucleic acid and deduced amino acid sequences were compared with EMBL/GenBank and Swissprot databases using the FASTA program (18). Nucleic acid and deduced amino acid sequences have been deposited in the EMBL data library under accession number Z36774.

Probe preparation and RNA analysis. Complementary and sense RNA probes were synthesized with T3 or T7 RNA polymerases using the HindIII- and BamHI-digested pmuα_2-AP.811 plasmid, respectively. This plasmid is derived from one clone of the lambda-ZAP liver cDNA containing a 704-bp cDNA fragment of the murine α_2-AP corresponding to bases 199–902 of pmuα_2-AP_fl. For in situ hybridization the probes were either labeled with [^{33}P]UTP or [^{3}H]UTP and [^{3}H]CTP.

Plasmids containing murine urokinase-type PA (uPA) (19) or tissue-type PA (tPA) (20) cDNA sequences were linearized and used as templates for in vitro transcription as described (12). The human α_2-AP cDNA was obtained by reverse transcription–PCR of total RNA extracted from normal liver, using the same degenerate oligonucleotides as mentioned above.

Total RNAs were extracted from murine and human tissues according to the guanidium/acid phenol method (21). For Northern blot analyses, 5 μg of total RNA was separated by formaldehyde gel electrophoresis and blotted onto nylon membranes as described (22). Blots were then probed with ^{32}P-labeled cRNA transcribed from pmuα_2AP.811, in 50% formamide, 2.5× Denhardt's solution, 0.05 M Pipes, pH 6.8, 0.8 M NaCl, 2 mM EDTA, 0.1% SDS, and 0.1 mg/ml of salmon sperm DNA, at 65°C for 18 h. Washes were carried out for 3 × 20 min at 70°C in 0.2× SSC, 0.1% SDS.

RNase protection assays were performed following standard methods (17) with slight modifications. Total RNA, 0.5–2 μg, isolated from murine tissues (21), was hybridized at 45°C to 300,000 cpm (10^8 cpm/μg) of a 360-base ^{32}P-labeled murine α_2-AP cRNA probe (XmnI-digested α_2-AP cDNA fragment subcloned in pBluescriptKS$^-$). The hybridization was carried out in 20 μl of buffer containing 80% formamide, 0.4 M NaCl, 40 mM Pipes, pH 6.4, 2 mM EDTA, and yeast tRNA to make up a final RNA concentration of 0.5 μg/μl. After 4 h of hybridization, 200 μl of 0.3 M NaCl, 20 mM Tris, pH 7.4, 4 mM EDTA, 50 ng/μl RNase A were added to the hybridization mixture and the incubation was continued for 30 min at 37°C. Then, protected fragments were precipitated for 30–60 min at −20°C after addition of 50 μl of a denaturing solution (4 M guanidine-thiocyanate, 2% Na-lauroylsarcosinate, 25 mM Na-citrate, pH 7.0, 0.1% β-mercaptoethanol) and 250 μl isopropanol. After centrifugation at 14,000 *g* for 15 min at 4°C, the pellets were resuspended in 8 μl of RNA-loading buffer (80% formamide, 1 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene-cyanol) and heated at 70°C for 2 min. Samples were loaded on a 16 × 20 cm 8 M urea/6% polyacrylamide gel and electrophoresed at 25 V/cm for 120 min in 1× TBE. After electrophoresis, the gel was fixed with acetic acid/ethanol/water (1:2:7), dried, and exposed to autoradiographic film or phospho-imager screen for counting.

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ma2ap     1  MALLRGLLVLSLSCLQGPCFT.FSPVSAVDLPGQQPVSEQAQQKLPLPALFKLDNQDFGD
ha2ap     1  MALLWGLLVLSWSCLQGPCSV.FSPVSAMEPLGWQLTSGPNQEQVSPLTLLKLGNQEPGG
ba2ap     1  MALLWGLLALILSCLSSLCSAQFSPVSTMEPLDLQLMDGQAQQKLPPLSLLKLDNQEPGG

ma2ap    60  HATLKRSPGHCKSVPTAEETRRLAQAMMAFTTDLFSLVAQTSTSSNLVLSPLSVALALSH
ha2ap    60  QTALKSPPGVCSRDPTPEQTHRLARAMMAFTADLFSLVAQTSTCPNLILSPLSVALALSH
ba2ap    61  QIAPKKAPEDCKLSPTPEQTRRLARAMMTFTTDLFSLVAQSSTRPNLILSPLSVALALSH

ma2ap   120  LALGAQNQTLHSLHRVLHMNTGSCLPHLLSHFYQNLGPGTIRLAARIYLQKGFPIKDDFL
ha2ap   120  LALGAQNHTLQRLQQVLHAGSGPCLPHLLSRLCQDLGPGAFRLAARMYLQKGFPIKEDFL
ba2ap   121  LALGAQNQTLQRLKEVLHADSGPCLPHLLSRLCQDLGPGAFRLAARMYLQKGFPIKEDFL

ma2ap   180  EQSERLFGAKPVKLTGKQEEDLANINQWVKEATEGKIEDFLSELPDSTVLLLLNAIHFHG
ha2ap   180  EQSEQLFGAKPVSLTGKQEDDLANINQWVKEATEGKIQEFLSGLPEDTVLLLLNAIHFQG
ba2ap   181  EQSEQLFGAKPMSLTGMKGEDLANINRWVKEATEGKIEDFLSDLPDDTVLLLLNAIHFQG

ma2ap   240  FWRTKFDPSLTQKDFFHLDERFTVSVDMMHAVSYPLRWFLLEQPEIQVAHFPFKNNMSFV
ha2ap   240  FWRNKFDPSLTQRDSFHLDEQFTVPVEMMQARTYPLRWFLLEQPEIQVAHFPFKNNMSFV
ba2ap   241  FWRSKFDPNLTQRGAFHLDEQFTVPVDMMQALTYPLHWFLLEQPEIQVAHFPFKNNMSFV

ma2ap   300  VVMPTYFEWNVSEVLANLTWDTLYHPSLQERPTKVWLPKLHLQQQLDLVATLSQLGLQEL
ha2ap   300  VLVPTHFEWNVSQVLANLSWDTLHPPLVWERPTKVRLPKLYLKHQMDLVATLSQLGLQEL
ba2ap   301  VLMPTRFEWNASQVLANLTWDILHQPSLSERPTKVQLPKLHLKYQLDLVATLSQLGLQEL

ma2ap   360  FQGPDLRGISEQNLVVSSVQHQSTMELSEAGVEAAAATSVAMNRMSLSSFTVNRPFLFFI
ha2ap   360  FQAPDLRGISEQSLVVSGVQHQSTLELSEVGVEAAAATSIAMSRMSLSSFSVNRPFLFFI
ba2ap   361  FQAPDLRGISDERLVVSSVQHQSALELSEAGVQAAAATSTAMSRMSLSSFIVNRPFLFFI

ma2ap   420  MEDTIGVPLFVGSVRNPNPSALPQLQEQRDSPDNRLIGQNDKADFHGGKTFGPDLKLAPR
ha2ap   420  FEDTTGLPLFVGSVRNPNPSAPRELKEQQDSPGNKDFLQSLKGFPRGDKLFGPDLKLVPP
ba2ap   421  LEDSTSLPLFVGSVRNPNPGAQPERKEQQDSPDGKDSFQDHKGLPRGDKPFDPDLKLGPP

ma2ap   480  MEEDYPQFSSPK*
ha2ap   480  MEEDYPQFGSPK*
ba2ap   481  SEEDYAQPSSPK*
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Figure 1. Amino acid sequences of murine, human, and bovine α_2-AP. Amino acid sequences deduced from the cDNA sequences of mouse, human, and bovine were aligned. Shading indicates residues conserved in mouse and human and/or bovine α_2-AP.

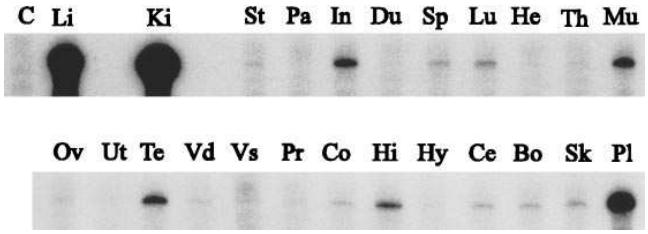

Figure 2. Tissue distribution of murine α_2-AP mRNA. 1 μg (liver and kidney) or 10 μg of total RNA from various murine tissues was analyzed by RNase protection using a murine 360-base α_2-AP ^{32}P-labeled cRNA probe. *C*, yeast RNA; *Li*, liver; *Ki*, kidney; *St*, stomach; *Pa*, pancreas; *In*, intestine; *Du*, duodenum; *Sp*, spleen; *Lu*, lung; *He*, heart; *Th*, thymus; *Mu*, striated muscle; *Ov*, ovary; *Ut*, uterus; *Te*, testis; *Vd*, vas deferens; *Vs*, seminal vesicle; *Pr*, prostate; *Co*, brain cortex; *Hi*, hippocampus; *Hy*, hypothalamus; *Ce*, cerebellum; *Bo*, bone; *Sk*, skin; *Pl*, placenta.

In situ hybridizations. Kidneys were obtained from 3–6-mo-old NMRI male mice, frozen in methyl butane, and kept at −80°C until analyzed. 5-μm cryostat tissue sections were fixed in 1% glutaraldehyde in PBS. The hybridization and posthybridization washes were carried out as described previously (12). Macroscopic mRNA localizations were revealed by film autoradiography (Kodak-SB) whereas microscopic detection was performed by emulsion autoradiography (Kodak-NTB2).

Castration and testosterone injection. Males were anesthetized and castrated as described by Vassalli et al. (23). After 2 wk of recovery, mice were daily injected subcutaneously with 0.5 ml of corn oil alone or containing 100 μg of testosterone-propionate per gram of body weight. After 5 d, the animals were killed and kidneys, liver, and seminal vesicles were dissected and their RNA was extracted as mentioned above.

Protein analysis. Kidneys from wild-type (C57/B6) and uPA-deficient mice (24) were flushed with 15 ml of serum-free defined medium (25, 26). After dissection, kidneys were minced and cultured for 4 h in 2 ml of serum-free defined medium. Protease-inhibitor binding assays were performed with either diluted human or murine plasma or supernatant of primary kidney cultures. Kidney tissue was also homogenized in PBS buffer containing 0.1% Triton X-100, centrifuged at 800 *g* for 5 min, and the supernatant was collected for further processing. Inhibitor-containing fluids were incubated with ^{125}I-labeled purified human plasmin for 10 min at 37°C. Plasmin–inhibitor complexes were immunoprecipitated with either rabbit anti–human α_2-AP or rabbit anti–human plasminogen, according to standard procedures (17). Samples were subjected to SDS-PAGE in 8% polyacrylamide gels and the gels were analyzed by autoradiography.

Results

Isolation and characterization of murine α_2-AP cDNA clones. Degenerate primers were prepared on the basis of the human α_2-AP cDNA sequence, in a region devoid of strong amino acid homology with other serpins; they corresponded to the sequence coding for amino acid residues 166–172 and 240–246 (4, 27). PCR amplification of reverse-transcribed mouse liver RNA yielded the expected 242-bp DNA fragment. This fragment was subcloned into pBluescriptKS$^-$ plasmid and both strands were sequenced. The nucleic acid sequence showed 80% identity with its human counterpart. The product of in vitro transcription of this 242-bp cDNA was used to probe Northern blots of RNAs from murine tissues and recognized single-size transcripts (data not shown). This 242-bp fragment was labeled with [^{32}P]dCTP and used to screen 0.5×10^6 pfu of

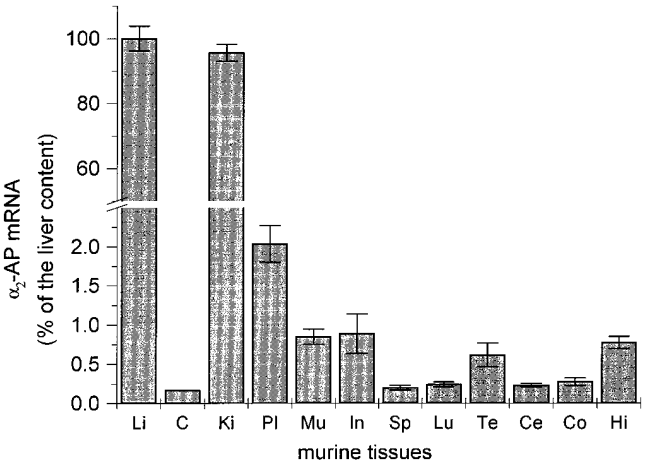

Figure 3. Quantification of α_2-AP mRNA in adult murine tissues. Results were obtained by phospho-imager quantification of RNase protection assays performed as in Fig. 2. Bars represent the mean value±SD, expressed as percentage of the liver signal, from at least three independent experiments.

lambda-ZAP mouse liver cDNA library. Among five positive clones, three were excised from the phage vectors, converted to plasmid vectors, and sequenced. Two contained the full-length cDNA (2,202 bp) and one contained a fragment of 704 bp spanning the region corresponding to the base pairs 198–902. Step deletions of ~ 200 bp each were obtained by the ExoIII/mung bean nuclease digestion method on the plasmid pmuα_2-AP_fl linearized with XhoI and KpnI. The EcoRI insert was subcloned in the reverse orientation and the same deletion procedure was repeated to obtain overlapping sequences of both strands. The longest open reading frame starts at position 37 and extends to a stop codon at position 1510. A canonical polyadenylation signal (AATAAA) (28) is found at position 2162. The 3′ untranslated region of the murine α_2-AP cDNA sequence has 69% identity with the corresponding human cDNA sequence and contains 18 gaps.

The amino acid sequence deduced from the murine α_2-AP cDNA displays an overall identity of 74 and 73% with its human and bovine counterparts, respectively (Fig. 1). A predicted signal-sequence cleavage site, which fulfills the criteria required by the (−3,−1) rule (29), is present at amino acid residue 27; thus the mature murine inhibitor would consist of a 464–amino acid protein and would start with a valine (Fig. 1, residue 28), unlike the human or bovine proteins which start with a methionine or phenylalanine, respectively (30) (Fig. 1, human residue 28 and bovine residue 23). In the amino-terminal region, the glutamine residue 41 (Fig. 1) of the fibrin α chain–binding site is conserved (31); similarly, in the carboxy-terminal region, the lysines (residues 475 and 491) described as important plasmin-interacting sites are conserved in the three proteins (32). The region around the reactive site (Arg^{402}-Met) is particularly well conserved among the three proteins, notably the P_{14}-P_{10} residues which are believed to be important for the inhibitory activity of serpins (33).

In addition, five potential N-glycosylation sites are present in the murine protein (Asn^{125}, Asn^{295}, Asn^{309}, Asn^{316}, and Asn^{437}); these sites have been demonstrated to be glycosylated in human and bovine proteins, suggesting that this is also the case in murine α_2-AP.

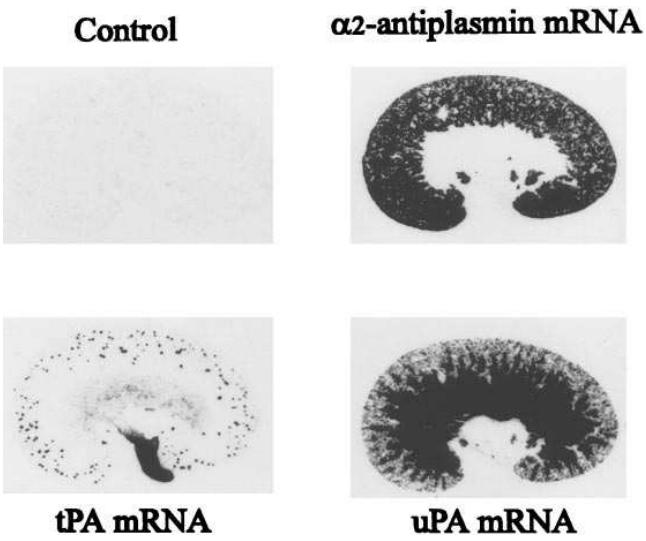

Figure 4. Regional localization of α_2-AP mRNA in the murine kidney. [^{33}P]UTP-labeled α_2-AP mRNA (*top left*), α_2-AP cRNA (*top right*), uPA cRNA (*bottom left*), and tPA cRNA probes (*bottom right*) were hybridized to consecutive cryostat kidney tissue sections. α_2-AP accumulation is limited to circumscribed zones in the cortex. Autoradiograms were exposed for 4 d at room temperature. ×2.

Tissular and cellular distribution of murine α_2-AP mRNA. The presence of α_2-AP mRNA in adult murine tissues was assessed by RNase protection: α_2-AP mRNA was detected in liver, kidney, intestine, spleen, lung, muscle, ovary, testis, cerebral cortex, hippocampus, cerebellum, bone, skin, and placenta (Fig. 2). The highest levels of α_2-AP mRNA were observed in liver and kidney, while the abundance of α_2-AP mRNA in the other tissues ranged between 0.3 and 2% of that in the liver (Fig. 3).

The renal sites of α_2-AP mRNA accumulation were identified by hybridization of cRNA probes to kidney cryostat tissue sections. Macroscopic localizations performed with a ^{33}P-labeled probe revealed circumscribed zones of α_2-AP mRNA accumulation in the cortex (Fig. 4). The specificity of in situ hybridization was confirmed by the lack of signal when hybridizing consecutive sections to an α_2-AP sense probe and by the nonoverlapping distributions of uPA and tPA mRNAs (Fig. 4) (12). Microscopic localization studies performed with a ^{3}H-labeled probe showed that hepatocytes are responsible for α_2-AP synthesis in the liver (not shown). In the kidney, they confirmed the restricted distribution observed macroscopically and ascribed α_2-AP mRNA accumulation to epithelial cells present in the cortex. The labeled epithelial cells displayed a brush border and were contained within a single type of tubule that, on transverse sections, had a large diameter without a visible lumen and that predominated around glomeruli. Therefore, α_2-AP mRNA-containing cells were identified as epithelial cells lining the convoluted portion of proximal tubules (Fig. 5).

Incubation of supernatants from murine renal tissue short-term cultures with ^{125}I-labeled plasmin and analysis of the samples by SDS-PAGE after immunoprecipitation with human antiplasminogen revealed the presence of a 140-kD radiolabeled plasmin–inhibitor complex (Fig. 6, *lane 3*); this complex comigrated with the α_2-AP–plasmin complex formed in murine plasma (Fig. 6, *lane 2*). When renal tissue was obtained from uPA-deficient mice (24), the amount of 140-kD α_2-AP–plasmin complex was higher (Fig. 6, *lane 4*); uPA may interact with α_2-AP (34, 35), and in samples from uPA-deficient mice the amount of free α_2-AP available for reaction with ^{125}I-plasmin may be higher. These results suggest that α_2-AP is pro-

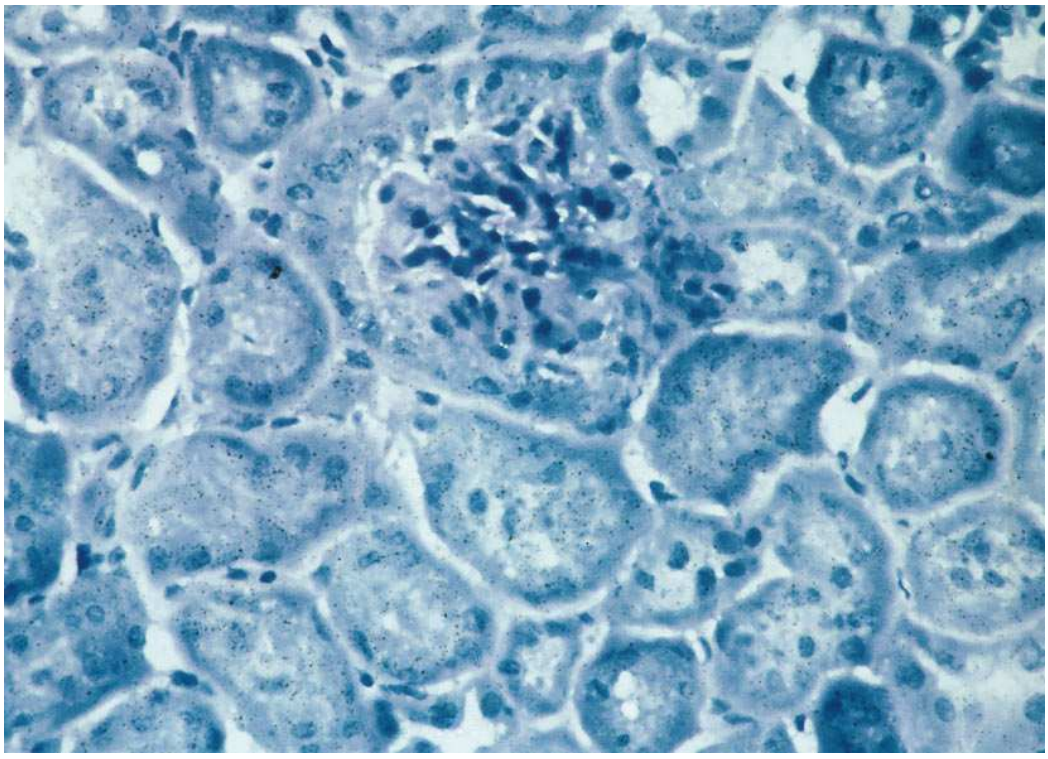

Figure 5. Cellular localization of α_2-AP mRNA in the murine kidney. 5-μm cryostat sections of a male murine kidney were hybridized to a ^{3}H-labeled murine α_2-AP cRNA probe. α_2-AP mRNA is detected in epithelial cells lining the convoluted portion of proximal tubules. Slides were exposed for 6 mo at 4°C. ×600.

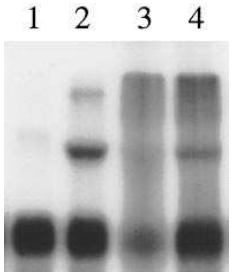

Figure 6. α_2-AP in murine kidney. 1 ml of serum-free supernatant of murine kidney short-term cultures was incubated with ^{125}I-labeled plasmin and the α_2-AP/plasmin complex was immunoprecipitated using rabbit anti–human plasminogen polyclonal antibodies. Lane *1* shows immunoprecipitate of ^{125}I-labeled plasmin. Lane *2* shows immunoprecipitate of 1 μl of murine plasma (dilution 1:50). Lane *3* shows immunoprecipitate of wild-type kidney culture supernatant. Lane *4* shows immunoprecipitate of uPA-deficient murine kidney culture supernatant.

duced in cultured renal tissue, and therefore that kidney α_2-AP mRNA can be translated into an active inhibitor.

α_2-AP in human kidney. Total RNA from human kidney tumors and normal cortex was analyzed by Northern blot using a human α_2-AP cDNA-derived probe (Fig. 7 *a*). A single band of ~ 2,200 bases, i.e., of a size comparable with that of α_2-AP mRNA in human liver tissue (Fig. 7 *a*, *lane 10*), was detected in all samples (Fig. 7 *a*, *lanes 1–9*); the abundance of α_2-AP mRNA varied between samples. α_2-AP mRNA was also detected in two different established human cell lines, UOK_1 and Ge1, prepared from kidney tumors (Fig. 7 *a*, *lanes 11 and 12*). To determine whether these tumor-derived cell lines produced α_2-AP, the cells were cultured for 12 h in serum-free medium. Culture supernatants were incubated with ^{125}I-labeled plasmin, immunoprecipitated with rabbit anti–human plasminogen antibodies, and analyzed by SDS-PAGE (Fig. 7 *b*). The presence of a 140-kD radiolabeled plasmin–inhibitor complex (Fig. 7 *b*, *lane 4*), comigrating with the α_2-AP–plasmin complex formed in human plasma (Fig. 7 *b*, *lane 2*), suggests that Ge1 cells synthesize α_2-AP; UOK_1 cells produced less inhibitor (Fig. 7 *b*, *lane 3*) than Ge1 cells, in accord with their lower content in α_2-AP mRNA (Fig. 7 *a*, *lanes 11* and *12*). Taken together these results demonstrate that, just as murine kidneys, human kidneys contain high levels of α_2-AP mRNA, and that human kidney cell lines can produce active α_2-AP.

Modulation of α_2-AP mRNA accumulation in the murine kidney. The abundance of kidney α_2-AP mRNA was fivefold higher in male mice than in females (Fig. 8, *top*), whereas no difference in α_2-AP mRNA levels was observed between male and female livers (Fig. 8, *bottom*). Furthermore, the abundance of α_2-AP mRNA in the kidneys of neonates was much lower than in adults. To explore a possible hormonal modulation of α_2-AP expression, we assessed the effects of castration and of testosterone injection. Kidneys of castrated males contained α_2-AP mRNA levels similar to those of females, and testosterone substitution restored the renal α_2-AP mRNA levels of castrated males to those of control males. In addition, testosterone administration to females induced a three- to fourfold increase in renal α_2-AP mRNA content when compared with that of control females (Fig. 8, *top*). By contrast, neither castration nor injections of testosterone affected the accumulation of α_2-AP mRNA in liver (Fig. 8, *bottom*). Moreover, transcription rate of α_2-AP mRNA was assessed by run-on transcription assay. The results (not shown) suggest that testosterone regulates α_2-AP gene expression at a posttranscriptional level.

Northern blot analysis of liver and kidney mRNAs from tissues taken every 6 h over a 24-h period did not reveal nyctohemeral variation in the abundance of α_2-AP mRNA (not shown).

Discussion

Most studies on the cellular origin and regulation of expression of the components of the PA/plasmin system have been focused on PAs and PAIs. Much less is known regarding plasminogen and α_2-AP, which are produced by hepatocytes and circulate at relatively high concentrations in blood plasma and other extracellular fluids. In particular, possible extrahepatic sources of these distal components of a proteolytic system implicated in a large number of physiological and pathological circumstances have not been systematically explored. To address this issue, we have cloned and sequenced murine α_2-AP cDNA. The deduced amino acid sequence of murine α_2-AP shows a high degree of identity with its human and bovine counterparts. The putative signal peptide cleavage site appears to be similar between the murine and human and bovine proteins, although the mature forms of the inhibitor would have a different amino-terminal residue. The three functional sites of the inhibitor (i.e., the fibrin-binding site, the reactive site, and the plasminogen-binding site) are also very well conserved.

We have found that the α_2-AP gene is expressed in several adult murine tissues in addition to the liver. The kidney is a major site of α_2-AP mRNA accumulation, and in situ hybridization localizes the transcript exclusively in epithelial cells lining the convoluted segments of proximal tubules. Since the contribution of these cells to total kidney RNA is certainly much less than that of hepatocytes to total liver RNA, the relative abundance of α_2-AP mRNA is most likely higher in these tubular cells than in hepatocytes.

The human kidney, in particular its cortical region, also contains high levels of α_2-AP mRNA. The variable amounts of α_2-AP mRNA detected in tumors and normal tissues analyzed

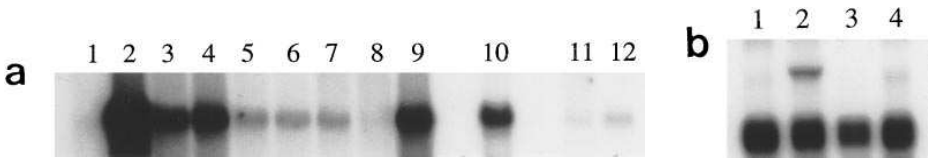

Figure 7. (*a*) α_2-AP mRNA in human kidney. 5 μg of total RNA prepared from human liver and from five different human kidneys were analyzed by Northern blot using a human α_2-AP ^{32}P-labeled cRNA probe. Lanes *1*, *3*, *5*, and *7* show RNA from dissected normal cortex. Lanes *2*, *4*, *6*, *8*, and *9* show RNA from dissected renal tumors. Lane *10* shows RNA from human liver. Lanes *11* and *12* show 5 μg of total RNA from UOK_1 and Ge1 kidney tumor cell lines. (*b*) α_2-AP production in human kidney cell lines. 1 ml of serum-free supernatant of UOK_1 (lane *3*) and Ge1 (lane *4*) cell lines was incubated with ^{125}I-labeled plasmin and immunoprecipitated using rabbit anti–human plasminogen polyclonal antibodies. Lane *1* shows immunoprecipitated ^{125}I-labeled plasmin. Lane *2* shows immunoprecipitate of 1 μl of murine plasma (dilution 1:50). Lane *3* shows immunoprecipitate of UOK_1 cell culture supernatant. Lane *4* shows immunoprecipitate of Ge1 cell culture supernatant.

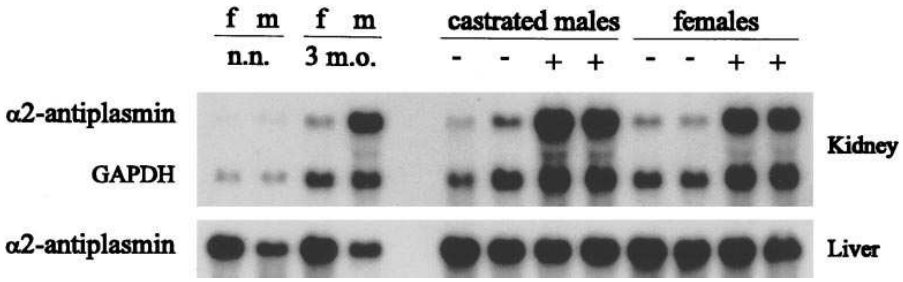

Figure 8. Testosterone-elicited modulation of kidney α_2-AP mRNA. 5 μg of total kidney (*top*) and liver (*bottom*) RNAs was analyzed by Northern blot hybridization using murine α_2-AP and GAPDH ^{32}P-labeled cRNA probes. Total RNA was prepared from male (*m*), and female (*f*) kidneys. Animals were either untreated or castrated and injected with corn oil alone (−) or 100 μg/gram of testosterone-propionate (+). Females were injected the same way.

suggest that α_2-AP expression may be modulated depending on physiological or pathological parameters. Although direct evidence for the translation of kidney α_2-AP mRNA is lacking, the detection of free α_2-AP (as revealed by the formation of ^{125}I-plasmin–α_2-AP complexes) in supernatants of short-term mouse kidney cultures and of Ge1 cells (derived from human kidney tumor) strongly suggests that α_2-AP can be synthesized by renal tissues. Interestingly, this observation corroborates the postulated histogenesis of renal cancers which are thought to arise from proximal tubules (36).

Whether kidney-derived α_2-AP may also contribute to the circulating pool of the protein cannot be decided at this time. The identification of the kidney as a site of high level α_2-AP production should be considered in the context of our present understanding of the PA/plasmin system's involvement in renal biology. Kidneys have long been recognized as a major source of PA production; more recently, uPA and tPA have been shown to be produced by epithelial cells lining distinct portions of renal tubules and to be secreted in urine (12). The present study documents the accumulation of α_2-AP mRNA in cells lining portions of tubules located upstream of the segments that produce uPA. Though the functional relevance of plasmin formation in the tubular compartment remains to be elucidated, these findings suggest that α_2-AP could exert a control over plasmin-mediated proteolysis in renal tubules. As illustrated by experiments performed with tissues of uPA-deficient mice, α_2-AP may interact not only with plasmin but also uPA, thereby providing powerful means to regulate plasmin formation and plasmin activity within tubules. Exploration of experimental pathologies indicates that plasmin-catalyzed proteolysis may prevent intratubular protein deposition and thereby may help maintain tubular patency (11). It will be of interest to investigate a possible implication of α_2-AP in tubular dysfunctions.

Male mouse kidneys were found to contain larger amounts of α_2-AP mRNA than female kidneys, and this sexual dimorphism was ascribed to the effect of testosterone. Other androgen-responsive genes are known to be expressed by the same epithelial cells within proximal tubules, such as the kidney androgen-regulated protein (37), β-glucuronidase, ornithine-decarboxylase (38, 39), and P450 cytochromes (40, 41). Testosterone is considered to control the expression of the β-glucuronidase and ornithine-decarboxylase genes at a post-transcriptional level, whereas it increases the transcription rates of the kidney androgen-regulated protein and P450 cytochrome genes. Since the rates of α_2-AP gene transcription estimated by nuclear run-on experiments are equivalent in both sexes, testosterone appears to regulate α_2-AP gene expression at the level of transcript processing and/or stability. Of interest, mouse liver α_2-AP mRNA accumulation was not influenced by androgens, demonstrating a tissue-specific endocrine regulation of α_2-AP production. Another serpin, protease-nexin I (PN-I), is also under androgen control in a subset of the tissues in which it is expressed (23). Further studies are required to determine if the accumulation of α_2-AP mRNA in the human kidney is also sexually dimorphic. In any event, our results indicate that the production of α_2-AP, like that of many other serpins, can be modulated as a function of the hormonal status of the animal.

In addition to liver and kidney, a number of other murine tissues also contain α_2-AP mRNA, albeit at much lower levels; however, without knowledge of the cells that contain the transcript in these other tissues, it is not possible to determine whether the α_2-AP gene is expressed at low levels in many cells or at high levels in a fraction of the cells. It is noteworthy that these other tissues, for instance the placenta and the central nervous system, are recognized sites of PA synthesis. The presence of α_2-AP mRNA in the central nervous system is of particular interest since, while most neurons produce tPA, plasmin-mediated proteolysis is limited to defined cerebral areas (13, 14). This apparent discrepancy is not attributable to the three known PAIs, raising the possibility that other inhibitors, including α_2-AP, are involved in the local control of plasmin activity. In conclusion, our observations provide novel evidence to suggest that a distal regulation of the activity of the PA/plasmin system may operate to prevent excessive proteolysis in discrete extracellular compartments, through the local production of α_2-AP.

Acknowledgments

We are grateful to Dr. J. Huarte for his help and advice in animal handling and to Dr. P. Carmeliet for providing uPA-KO animals.

This work was supported by grants from the Swiss Fonds National de la Recherche Scientifique.

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