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Endotoxin Stimulates Expression of the Murine Urokinase Receptor Gene *in Vivo*

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The regulation of urokinase receptor (u-PAR) gene expression during endotoxemia was studied* in vivo *with a murine model system. Northern blot analysis demonstrated relatively high levels of u-PAR mRNA in mouse placenta, with intermediate levels in lung and spleen and very low levels in heart and kidney. No u-PAR mRNA could be detected in liver, gut, thymus, brain, or skeletal muscle. Intraperitoneal injection of endotoxin (lipopolysaccharide) increased the steady-state levels of u-PAR mRNA in most tissues examined. The greatest induction (sevenfold) was observed in the lung at 1 hour after injection. The cellular localization of u-PAR mRNA was assessed by* in situ *hybridization. In control mice, u-PAR mRNA was detected primarily in alveolar macrophages of the lung and lymphocytes of the spleen and thymus, although a specific signal was also present in other cell types. In general, endothelial cells lacked detectable u-PAR mRNA. The induction of u-PAR mRNA by lipopolysaccharide was apparent within 30 minutes and was localized to tissue macrophages, lymphocytes, and endothelial cells lining arteries and veins. At later times (1 to 3 hours), specialized epithelial cells present in gastrointestinal tract, bile ducts, and uterus were also positive for u-PAR mRNA. Induction of u-PAR* in vivo *by lipopolysaccharide may facilitate the extravasation and migration of leukocytes during inflammation. (Am J Pathol 1995, 147:688–698)

Plasminogen activators (PAs) are serine proteases that catalyze the conversion of plasminogen into plasmin. Plasmin plays a central role in thrombolysis and in a variety of other processes.[1] Two PAs have been identified, including the urokinase type (u-PA) and tissue type (t-PA), which are produced by distinct genes. Urokinase accumulates on the surface on many cells bound to the u-PA receptor (u-PAR).[2] Plasminogen also binds to cell surfaces via a receptor,[3] and its interaction with receptor-bound u-PA results in the formation of plasmin. This surface-associated plasmin is able to degrade proteins of the extracellular matrix and basement membranes. These interactions localize extracellular proteolytic activity during tumor cell invasion, tissue remodeling, and cell migration.[4,5] Recently, u-PAR was implicated as a critical component of the metastatic process.[6] The human,[7,8] murine,[9] and bovine[10] u-PAR cDNAs have been isolated and completely sequenced. The human u-PAR protein has been purified[11] and appears to be the single chain glycoprotein of M_r 50,000 to 60,000. The deduced amino acid sequence of human u-PAR predicts a polypeptide of 313 amino acids composed of three 90-amino-acid, cysteine-rich repeats. The first repeat contains the u-PA binding region.[12] The attachment site for the glycosyl-phosphatidylinositol (GPI) anchor is located at the end of the third repeat and is sensitive to a phosphatidylinositol-specific phopholipase C. u-PAR is deficient on peripheral blood monocytes and granulocytes from patients with paroxysmal nocturnal hemoglobinuria, an acquired disorder characterized by a defect in the membrane attachment of proteins normally anchored by GPI.[13] This deficiency in u-PAR may be related to the high incidence of venous thrombosis in these patients. The unique domain structure of u-PAR, and the fact that it is a GPI-anchored protein, suggests that it is a member of the T cell activating proteins or Ly-6 superfamily.[14]

Murine u-PAR resembles human u-PAR with respect to ligand-binding characteristics, molecular mass, glycosylation pattern, and GPI anchorage.[9]

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The amino acid sequence deduced from the murine cDNA is 62% identical with the human u-PAR sequence, and this homology increases to 70% homology when conservative amino acid substitutions are taken into account. Interestingly, a second form of murine u-PAR was detected by cDNA cloning[9] and seems to arise from alternative mRNA splicing. This form, designated mu-PAR-2, codes for a form of the receptor that lacks the sequence for GPI addition and thus is thought to be a soluble form that is secreted from the cells. The first 133 residues of the mu-PAR-2 protein are homologous to human u-PAR, but the next 66 residues appear to be unique. Recently, a variant form of human u-PAR mRNA (u-PAR-2) was identified[15] and is likely to represent a functional analogue of mouse u-PAR-2.

In cells in culture, u-PAR synthesis is regulated by a diverse group of agents, including inflammatory cytokines, growth factors, hormones, tumor promoters, thrombin, and secondary mediators such as protein kinase C and cAMP. For example, interferon, tumor necrosis factor, and u-PA itself have been reported to stimulate the expression of u-PAR on human monocytes,[16] whereas interleukin (IL)-2 and phorbol myristate acetate increased u-PAR expression in T lymphocytes.[17,18] Basic fibroblast growth factor was shown to increase u-PAR expression by human vascular cells.[19] Despite these results, little is known about the tissue distribution and regulation of the u-PAR gene *in vivo*. In the present work, we have employed a murine model system to identify the tissues and cells that constitutively synthesize u-PAR mRNA and to analyze changes in its expression during endotoxemia. Our results demonstrate that LPS induces u-PAR expression in a variety of tissues and cells *in vivo*. The induction of u-PAR may facilitate extravasation and migration of leukocytes during inflammation and contribute to the pathogenesis of gram-negative sepsis.

Materials and Methods

Cloning of Murine u-PAR cDNA with the Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

The human u-PAR cDNA, kindly provided by Dr. E. K. O. Kruithof (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland), was used as a probe to identify murine cells producing u-PAR. In these experiments, RNA samples isolated either from different murine cell lines (eg, Balb-3T3 fibroblasts, hepatoma 1–6 cells, and RAW 264.7 cells) or from various murine tissues were initially analyzed by Northern blotting. RNA extracted from human U937 cells (human hystiocytic lymphoma) was used as a positive control. A murine monocyte-macrophage line transformed by Abelson leukemia virus (RAW 264.7, ATCC TIB 71, American Tissue Culture Collection, Rockville, MD) and known to constitutively express u-PA activity[20] exhibited the strongest hybridization signal for u-PAR mRNA. It was therefore selected as the source of murine RNA for PCR. Two PCR primers were synthesized based on the published sequence of murine u-PAR[9] and designed for directional cloning with different protuding termini as follows: 5′ primer (5′-GGGAGCTCCTGCAGTGCATGCAGTGTGAGA-3′) and 3′ primer (5′-CCTCTAGAAGTCAGGTCCAGAGGAGGACGC-3′). RT-PCR was performed with the Gene Amp RNA kit (Perkin-Elmer Cetus, Norwalk, CT) according to the manufacturer's instructions for 35 cycles. The amplified fragment was resolved by agarose gel electrophoresis and had the predicted size (1.0 kb). The PCR product was purified, digested with *Sac*I and *Xba*I and subcloned into an ampicillin-resistant plasmid vector (pGEM-3Z; Promega, Madison, WI). Restriction analysis of the resulting plasmid DNA indicated an insert of the predicted size, and sequence analysis demonstrated identity with murine u-PAR 1.[9] The cDNA includes bp 80 to bp 997 from the published sequence.

Animal Protocols and Tissue Preparation

Adult male or female CB6.F1 mice (BALB/c/Byj × C57B16/j; Jackson Laboratories, Bar Harbor, ME, or Scripps Clinic Rodent Breeding Colony), aged 6 to 8 weeks, were used for all of the experiments. Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4, (Sigma Chemical Co., St. Louis, MO) was resuspended in sterile saline (Baxter, Deerfield, IL), and 50 μg (approximately 2 mg/kg) was injected intraperitoneally into mice anesthetized by inhalation of metofane (methoxyflurane, Pitman-Moore, Mundelein, IL). Control mice were anesthetized and injected with saline alone. At different time intervals after injection, mice were anesthetized by metofane inhalation and killed by cervical dislocation. Tissues were rapidly removed by standard dissection techniques. The placenta employed for these experiments was purchased (Harlam Bioproducts for Science, Indianapolis, IN). For Northern blot analysis, tissues were minced and stored in liquid nitrogen until RNA extraction. For *in situ* hybridization, the tissues were immersed in cold 4% (w/v) paraformaldehyde in Dulbecco's phosphate-buffered saline

(Whittaker Bioproducts, Walkersville, MD) and fixed at 4°C overnight. The fixed tissues were then embedded in paraffin blocks and sectioned at 2 to 5 μm thickness with a microtome. The sections were mounted onto polylysine slides and stored at room temperature until *in situ* hybridization analysis.

Preliminary dose-response experiments were performed to determine the optimal amount of LPS. Mice were injected with increasing amounts of LPS (from 0.6 to 100 μg), and 1 hour later, RNA was extracted from lung and spleen and resolved by Northern blot analysis. The maximal response for induction of u-PAR mRNA was achieved with 50 μg, and this amount was thus employed for most of the experiments.

Northern Blot Analysis

For each Northern blot experiment, tissues were obtained from three different animals and analyzed independently. Total RNA was extracted from the frozen tissues by the acid guanidinium-thiocyanate-phenol-chloroform method[21] and the concentration of RNA was determined by sample absorbance at 260 nm. Total RNA was analyzed for u-PAR mRNA by Northern blotting essentially as described.[22] Briefly, the RNA (20 μg) was fractionated by electrophoresis under denaturing conditions on a 1.5% agarose gel, stained with ethidium bromide, and transferred overnight onto nylon membranes (Biotrans ICN, Irvine, CA). The blots were prehybridized for 30 minutes in 50 mmol/L Pipes buffer, pH 6.8, containing 100 mmol/L NaCl, 20 mmol/L Na_2HPO_4, 30 mmol/L NaH_2PO_4, 1 mmol/L EDTA, and 5% sodium dodecyl sulfate, and then were hybridized in the same solution for 16 hours at 65°C, with 10^6 cpm/ml murine u-PAR cDNA probe. The probe was radiolabeled by employing a random primer labeling kit (Boehringer-Mannheim Biochemical, Indianapolis, IN) in the presence of α-[^{32}P]dGTP (3000 Ci/mmol, Amersham, Arlington Heights, IL). After hybridization, the blots were washed four times for 15 minutes each with prewarmed (65°C) 0.67X standard saline citrate (SSC) containing 5% sodium dodecyl sulfate and then subjected to autoradiographic analysis with Kodak XAR-5 film and intensifying screens at −70°C for varying times. The resulting autoradiograms were quantified by densitometric scanning by using an LKB Ultrascan XL laser densitometer (LKB, Bromma, Sweden). To verify that densitometric measurements were within the linear range, total RNA from the lung and spleen of LPS-treated mice was prepared, serially diluted, and analyzed by Northern blotting. The densitometric values obtained from this calibration curve demonstrated linearity. To assess variability in sample loading, Northern blots were rehybridized with a ^{32}P-labeled 18S rat cDNA probe.[23] Despite some variability in sample loading, similar results were obtained for all tissues in three independent experiments. Northern blot analysis of total RNA from mouse tissues with the murine u-PAR cDNA probe revealed the presence of a single transcript of approximately 1.4 kb as estimated with a 0.16 to 1.77 kb RNA ladder from GIBCO BRL (Gaithersburg, MD).

Riboprobe Preparation and in Situ *Hybridization*

The pGEM-3Z vector carrying the murine u-PAR cDNA insert was linearized with the restriction endonucleases *Eco*RI or *Hind*III and employed as a template for the synthesis of antisense or sense riboprobes, respectively. *In vitro* transcription reactions were performed with SP6 or T7 RNA polymerases (Promega) in the presence of [^{35}S]UTP (Amersham, 1000 Ci/mmol). The DNA templates were removed by digestion with RNAse-free DNAse I (RQ1 DNAse, Promega) for 15 minutes at 37°C and the riboprobes purified by phenol extraction and ethanol precipitation.

To study the cellular localization of u-PAR mRNA, *in situ* hybridization was performed essentially as described.[24] Paraffin-embedded tissue sections were treated sequentially with xylene (three times for 5 minutes each), 2X SSC (1X SSC is 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0) containing 10 mmol/L 2-mercaptoethanol and 1 mmol/L EDTA (10 minutes), paraformaldehyde (10 minutes at 4°C), and proteinase K (1 μg/ml in 300 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0). All washes and incubations were performed at room temperature unless otherwise specified. The slides were then prehybridized for 2 hours in 100 μl of prehybridization buffer (50% w/v) formamide, 0.3 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 10% (w/v) dextran sulfate, and 10 mmol/L dithiothreitol) at 42°C. Prehybridization buffer (20 μl) containing 2.5 mg/ml yeast tRNA and 600,000 cpm ^{35}S-labeled riboprobe was added, and the slides were hybridized at 55°C overnight. After hybridization, the slides were treated with 2X SSC containing 1 mmol/L EDTA (twice for 10 minutes each), RNAse A (20 μg/ml in 500 mmol/L NaCl and 10 mmol/L Tris-HCl; 30 minutes), 2X SSC containing 10 mmol/L

2-mercaptoethanol and 1 mmol/L EDTA (twice for 10 minutes each), 0.1X SSC containing 10 mmol/L 2-mercaptoethanol and 1 mmol/L EDTA (2 hours at 60°C), and 0.5X SSC (twice for 10 minutes each). Finally, the slides were dehydrated by immersion in a graded alcohol series containing 0.3 mmol/L CH_3COONH_4, dried, coated with NTB2 emulsion (Kodak, Rochester, NY) diluted 1:2 in distilled water, and exposed in the dark at 4°C for 2, 6, and 12 weeks. Slides were then developed for 2 minutes in D19 developer (Kodak), fixed, washed in water (three times for 5 minutes each), and counterstained with hematoxylin and eosin by standard procedures. Positive hybridization signals appeared under microscopic observation as green grains (epiluminescence) or as dark grains (bright field). No specific signal could be detected in parallel sections hybridized with a sense riboprobe as a control for nonspecific hybridization. Because *in situ* hybridization is not a very quantitative technique, care was taken to minimize variations caused by the handling of the tissues or tissue sections. For example, in all instances in which tissues from control and LPS-treated animals were compared, the tissues were isolated, processed, and hybridized in parallel with the same buffers, probe mixtures, emulsions, and where possible, exposure times. In general, quantitative conclusions were based on results obtained by Northern blot analysis.

Results

Tissue Distribution of Murine u-PAR mRNA

To examine constitutive expression of the murine u-PAR gene *in vivo*, total RNA was extracted from tissues of CB6 mice and analyzed by Northern blotting with a murine u-PAR cDNA probe. Results from a representative animal are shown in Figure 1A. Placenta showed by far the highest concentration of u-PAR mRNA, followed by spleen, lung, heart, and kidney. No u-PAR mRNA could be detected in liver, gut, thymus, brain, or skeletal muscle.

To compare the distribution of u-PAR with its ligand u-PA, the blot was rehybridized with a murine u-PA cDNA probe (Figure 1B). The kidney, which had relatively low levels of u-PAR mRNA, showed the highest concentration of u-PA mRNA, in agreement with previous observations.[25,26] The placenta, which had high levels of u-PAR mRNA and exhibits strong immunohistochemical staining for u-PA protein,[27] also had relatively high levels of u-PA mRNA. Interestingly, significant levels of u-PA mRNA were detected in the thymus, and this tissue lacked detectable u-PAR mRNA. Comparison of Figure 1, A and B, reveals that expression of the receptor and its ligand is not necessarily coincident in the tissues studied.

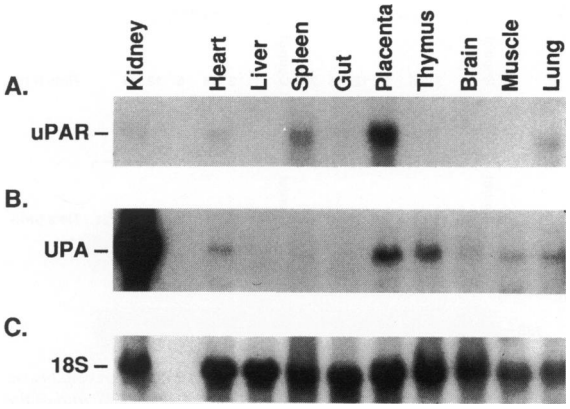

Figure 1. *Relative level of expression of u-PAR and u-PA mRNAs in murine tissues. Total RNA was prepared from the indicated tissues as described in Materials and Methods, and 20 μg were analyzed by Northern blotting with ^{32}P-labeled murine cDNA probes to u-PAR* (**A**) *or to u-PA* (**B**). *The two autoradiograms were exposed for 4 days. To assess variability in sample loading, the blot shown in* (**A**) *was rehybridized with a ^{32}P-labeled rat 18S cDNA probe* (**C**).

Regulation of Murine u-PAR Gene Expression by LPS

Experiments were performed to determine whether u-PAR gene expression was altered *in vivo* in response to endotoxin. Mice were injected intraperitoneally either with saline (controls) or with saline containing 50 μg of LPS (2.0 mg/kg). At various times after injection, selected tissues were removed and analyzed for u-PAR mRNA by Northern blotting. Results from a representative experiment are shown in Figure 2. LPS treatment increased the steady-state levels of u-PAR mRNA in most tissues, with maximal induction observed at 1 to 3 hours. The autoradiographic signals for u-PAR and 18S rRNA were quantitated by scanning densitometry (see Materials and Methods) and the values obtained for u-PAR mRNA normalized to those of 18S rRNA. Maximal inductions of 7-, 5-, 3-, and 2.5-fold were observed in the lung, kidney, spleen, and heart, respectively. The concentration of u-PAR mRNA began to decrease after 1 to 3 hours, reaching baseline levels by 24 hours in most instances. Similar results were obtained for all four organs in three independent experiments. In separate experiments (not shown), the tissues were harvested 15 and 30 minutes after LPS injection and analyzed as above by Northern blotting. Although increased expression of u-PAR mRNA was detected at 30 minutes in the lung, no increase was evident at

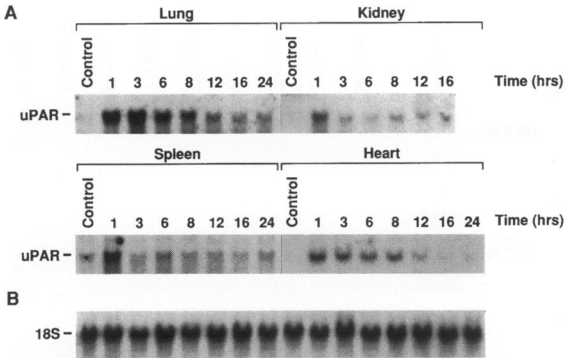

Figure 2. *Changes in the concentration of u-PAR mRNA in response to LPS. Mice were injected intraperitoneally with 50 μg of LPS, and at the indicated times, tissues were removed and total RNA (20 μg) was analyzed by Northern blotting.* A: *Changes in the concentration of u-PAR mRNA in lung, kidney, spleen, and heart at various times.* B: *Variability in sample loading as detected by rehybridizing the spleen and heart blots in* A *with a ^{32}P-labeled 18S cDNA probe. The autoradiograms were exposed for 4 to 6 days.*

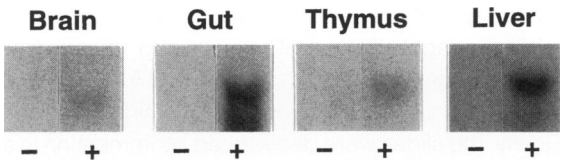

Figure 3. *Induction of u-PAR mRNA in the brain, gut, thymus, and liver by LPS. Mice were injected with 50 μg of LPS and 3 hours later, tissues were removed and total RNA (20 μg) was analyzed by Northern blotting. −, control; +, LPS. The autoradiograms for liver and gut were exposed for 9 days whereas those for brain and thymus were for exposed for 12 days.*

this early time in kidney, spleen, and heart. Upregulation of u-PAR mRNA also was observed in liver and gut, and possibly in the thymus and brain as well (Figure 3). In these instances, the level of induction was not possible to quantitate by densitometric scanning as the control values were very low or not detected (Figures 1 and 3). However, a positive signal was detected in the gut 1 hour after LPS treatment (not shown). Comparison of the 1- and 3-hour time points revealed that LPS induced a 3-fold increase in PAI-1 mRNA during this interval. Thus, the difference between the u-PAR mRNA levels in the gut of untreated controls (not detected by densitometric analysis) and the 3-hour sample must be greater than 3-fold.

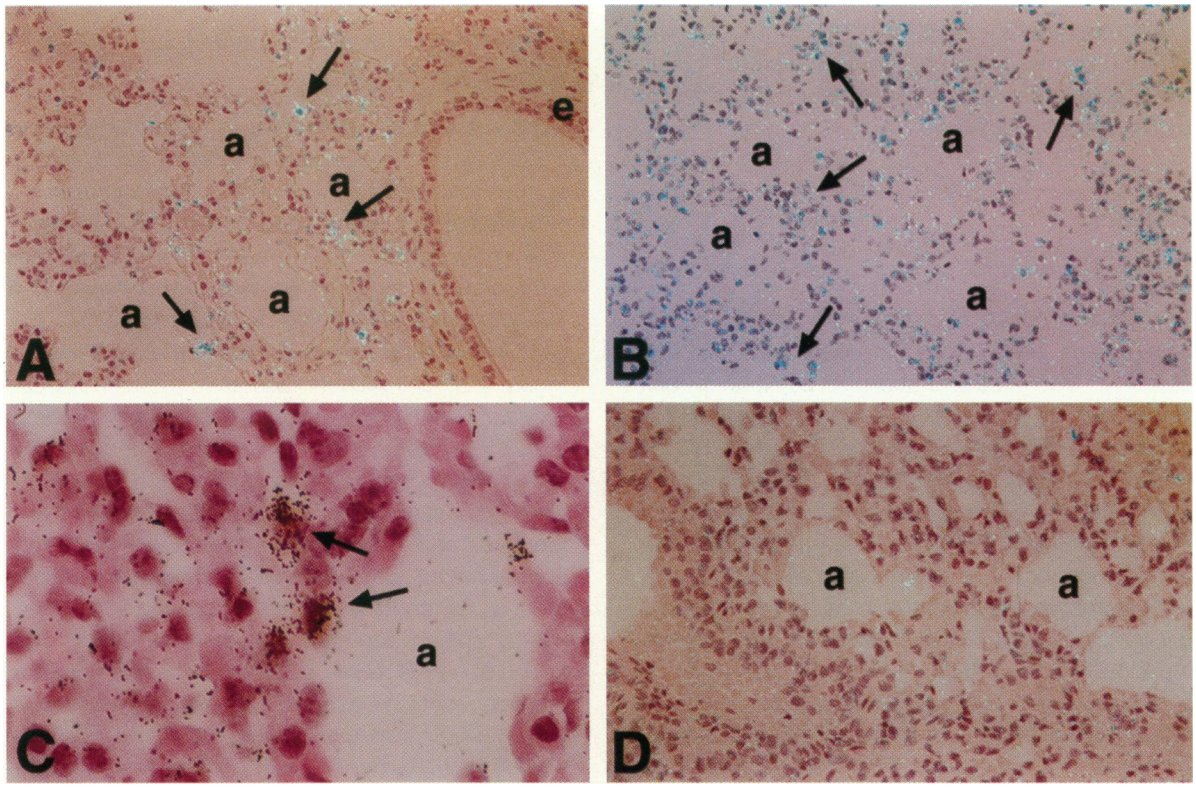

Figure 4. *Localization of u-PAR mRNA in cells of the lung.* A: *Section from a normal lung hybridized with the u-PAR antisense riboprobe. Examples of cells specifically expressing u-PAR mRNA are indicated by the arrows. In general, u-PAR mRNA was detected on cells bordering the alveolar (a) space, whereas bronchial epithelium (e) was negative. Magnification, ×250.* B: *Section of lung from an animal treated with endotoxin for 1 hour showing that the same cells express mRNA. ×250.* C: *A high power, bright-field view of lung from the LPS-treated mouse shown in* B. *×1000. The arrows point to cells at the edge of the alveoli. In* A *to* C, *the slides were exposed for 6 weeks.* D: *Section from the lung of the endotoxin-treated mouse, hybridized with a u-PAR sense riboprobe and exposed for 12 weeks. ×250.*

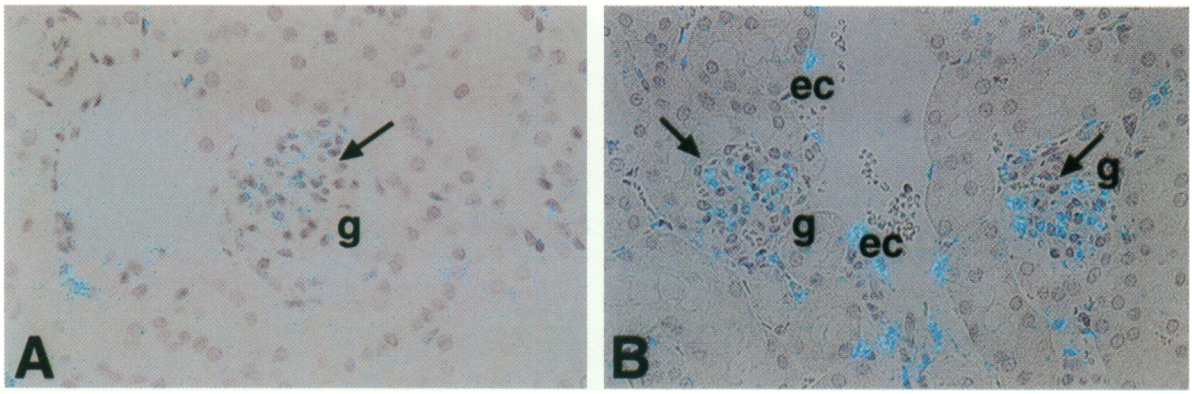

Figure 5. *Localization of u-PAR mRNA in cells of the kidney.* A: *The arrow points to cells within the renal glomeruli that are positive for PAI-1 mRNA.* B: *Section of kidney from an endotoxin-treated animal (1 hour) showing u-PAR mRNA both in the glomeruli (g) and in endothelial cells (ec). Both slides were exposed for 6 weeks and processed in parallel. Magnification, ×400.*

Cellular Localization of Murine u-PAR mRNA

In situ hybridization experiments were carried out to determine the cellular sites of synthesis of u-PAR mRNA in tissues from control and LPS-treated animals. In each instance, tissues from three to four animals in each group were examined in parallel with similar results. Representative sections were selected for each photomicrograph. In these experiments, the specificity of the ^{35}S-labeled u-PAR antisense riboprobe was monitored by hybridizing adjacent tissue sections with a sense riboprobe.

Lung

In the normal lung (Figure 4A), u-PAR mRNA was localized to cells at the edge of the alveoli, whereas the bronchial epithelium was negative. Within 1 hour after LPS administration (Figure 4B), there appeared to be a generalized increase in the density of silver grains in cells bordering the alveolar space. This increase was apparent in all tissue sections examined, consistent with the increase observed by Northern blotting (Figure 2). The bronchial epithelium remained negative after LPS. Analysis of these sections under bright field conditions at higher power shows the specificity of the grains (Figure 4C). The localization of these cells in the alveolar space and their morphology suggests that they are alveolar macrophages.[28] No specific signal was detected when sections were hybridized with the sense riboprobe (Figure 4D).

Kidney

In the kidney of control adult animals, u-PAR mRNA was sometimes detected in cells in the renal glomeruli (Figure 5A), but most glomeruli were negative (not shown). Northern blot analysis reveals that increases in u-PAR mRNA can be detected within 15 to 30 minutes after LPS injection, with the maximal increase at 1 hour (Figure 2B). At this time, most glomeruli and some endothelial cells become positive for u-PAR mRNA (Figure 5B). Additional studies with electron microscopy will facilitate identification of the positive cells in the glomeruli from control and LPS-treated animals. The hybridization signal at 8 hours (not shown) was still higher compared with that of the control, consistent with the data shown in Figure 2A.

Lymphatic Organs

In situ hybridization analysis of the spleen (Figure 6) and other lymphatic organs (eg, thymus and lymph nodes; not shown) suggested increased u-PAR gene expression after endotoxin treatment, consistent with the results shown in Figures 2 and 3. For example, in the spleen, a relatively weak signal was detected in the lymphatic nodules of control animals (Figure 6A), which appeared to be significantly increased in all tissue sections examined by 1 hour after LPS exposure (Figure 6B). In addition, some positive cells were detected in the red pulp. In the thymus of control and LPS-treated mice (not shown), an identical pattern of u-PAR mRNA expression was observed surrounding the germinal center (ie, relatively very weak signal in the medulla of control thymus where it localized primarily over small thymic lymphocytic cells; consistently increased signal in the medulla at approximately 1 to 3 hours after LPS treatment. u-PAR mRNA in control thymus was below

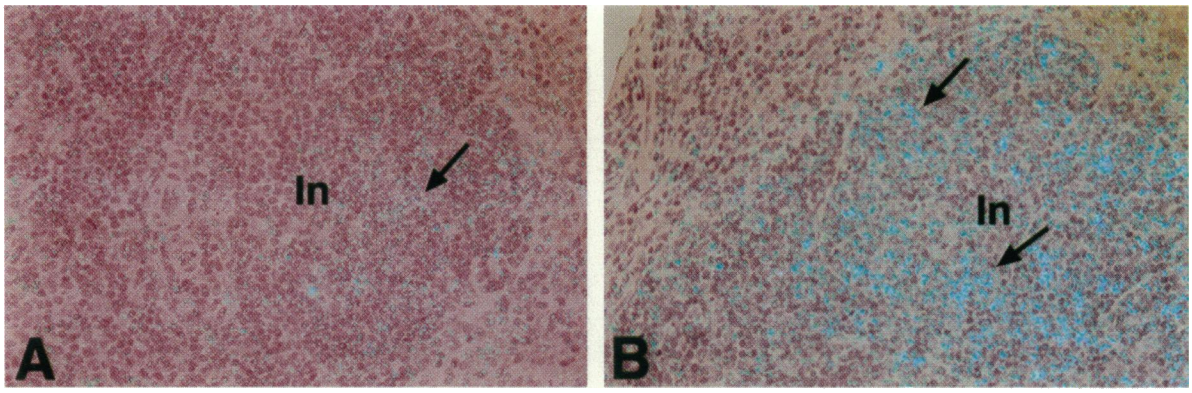

Figure 6. *Distribution of u-PAR mRNA in the spleen.* A: *Section of control mouse spleen showing positive cells in the lymphatic nodules (ln). Magnification, ×250.* B: *Representative section demonstrating a more intense hybridization signal in this area 1 hour after LPS. ×250. Both slides were processed in parallel and exposed for 6 weeks. Arrows indicate examples of positive cells.*

the limits of detection by Northern blot analysis (Figures 1A and 3).

The observations that the lymphoid areas of spleen, the medulla of the thymus, and lymph nodes (not shown) throughout the body were positive for u-PAR raises the possibility that the common cell type responsive to LPS in these tissues is the lymphocyte. Additional immunohistochemical studies are needed to verify this hypothesis.

Heart and Blood Vessels

In the control myocardium, no specific u-PAR mRNA signal was detected in myocytes or veins (Figure 7A) or in endothelium of blood vessels including aorta (Figure 7B). In contrast, after LPS treatment, some of the cardiac myocytes (Figure 7C) and endothelium of the heart vasculature (Figure 7, D and E) became positive. The endothelium of large vessels (eg, aorta; not shown), as well as endothelium lining arteries, veins, and capillaries of a number of organs including the kidney (Figure 5B), brain, and liver (not shown) were also positive after LPS treatment. Smooth muscle cells do not appear to express detectable amounts of u-PAR mRNA.

Gut

Northern blot analysis revealed that endotoxin also induced u-PAR mRNA in the gut of mice (Figure 3), and the cells producing it in the absence and presence of endotoxin appear to be largely the same (Figure 8). For example, in control gut (Figure 8A), u-PAR was observed in cells lining the outer epithelial luminal surface as well as in cells located toward the core of the villus. The basal epithelial cells had very low levels of u-PAR mRNA (not shown). After LPS treatment, u-PAR mRNA was detected primarily over nuclei of these same cells (Figure 8, B and C) and in the basal cells of the crypt (not shown).

Liver

Although the liver of control animals did not contain detectable u-PAR mRNA (Figure 1), it was detected in livers from LPS-treated animals (Figure 3). Three hours after LPS treatment, u-PAR mRNA was detected primarily in the biliary duct epithelium (Figure 9A) and in scattered cells localized in the sinusoidal spaces. Additional immunohistochemical staining is required to identify these latter cells.

Epithelium

u-PAR mRNA was detected in epithelium of a number of organs from untreated control mice, including those in the urinary bladder, liver gallbladder, and gut (not shown). However, u-PAR mRNA was not detected in epithelial cells of the lung, uterus, biliary ducts, and Bowman's capsule of the kidney. The epithelium appears to demonstrate tissue-specific sensitivity to LPS as the bile ducts of the liver (Figure 9A) and uterus epithelium (Figure 9B) were induced by LPS treatment, whereas epithelial cells from the lung and kidney were not (not shown).

Brain

In the brain (not shown), the hypothalamus and cerebellum exhibited a weak hybridization signal for u-PAR mRNA, which did not increase after LPS exposure, consistent with the Northern blots (Figure 3). The meningeal layer also appeared to express u-PAR mRNA and u-PAR mRNA was detected in cap-

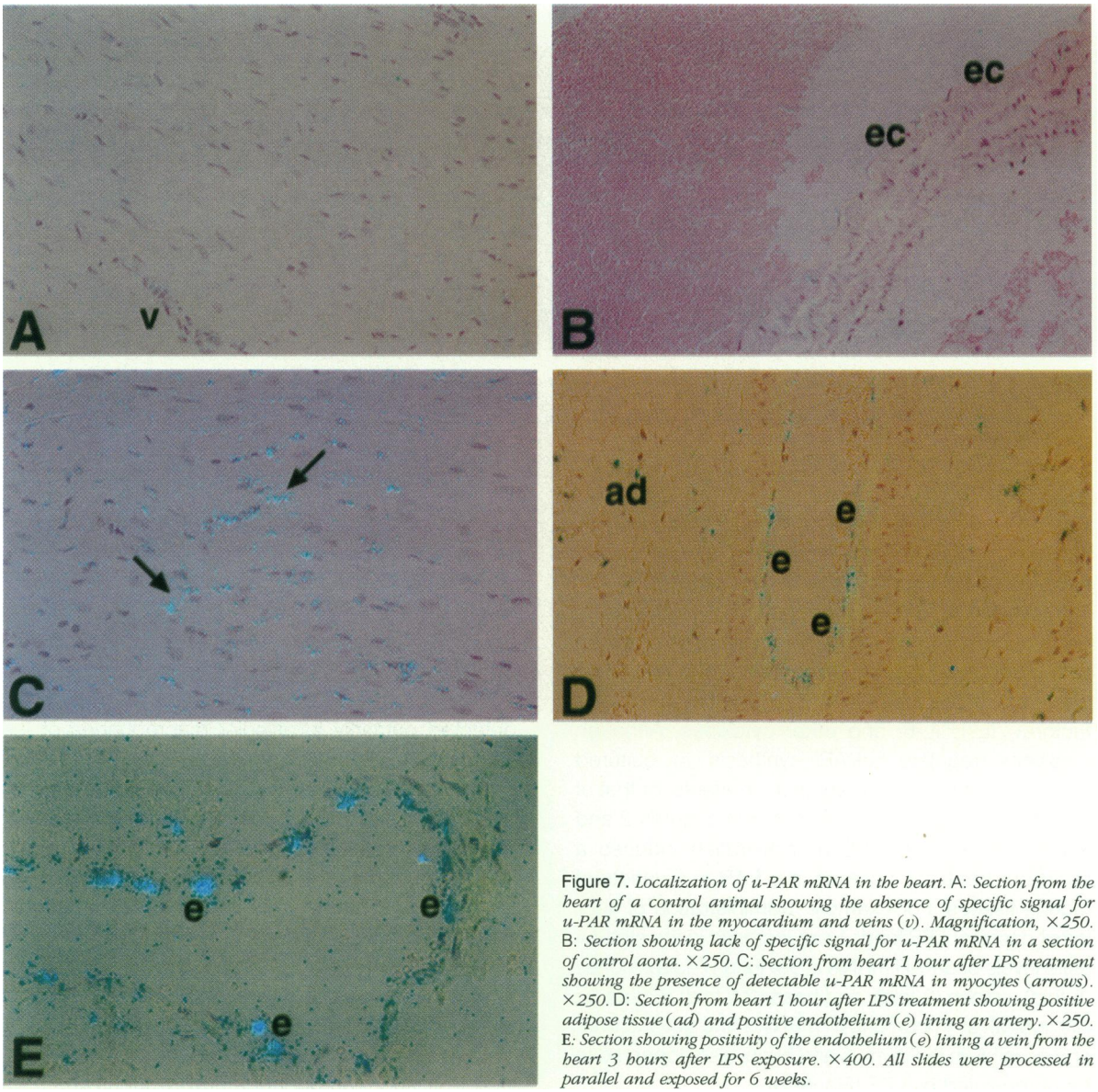

Figure 7. *Localization of u-PAR mRNA in the heart.* A: *Section from the heart of a control animal showing the absence of specific signal for u-PAR mRNA in the myocardium and veins (v). Magnification, ×250.* B: *Section showing lack of specific signal for u-PAR mRNA in a section of control aorta. ×250.* C: *Section from heart 1 hour after LPS treatment showing the presence of detectable u-PAR mRNA in myocytes (arrows). ×250.* D: *Section from heart 1 hour after LPS treatment showing positive adipose tissue (ad) and positive endothelium (e) lining an artery. ×250.* E: *Section showing positivity of the endothelium (e) lining a vein from the heart 3 hours after LPS exposure. ×400. All slides were processed in parallel and exposed for 6 weeks.*

illaries, probably originating from endothelial cells and oligodendroglial cells (macrophage origin).

Adipose Tissue, Skin, and Skeletal Muscle

Very few u-PAR mRNA-positive cells were detected in adipose tissue from untreated mice. However, the amount of u-PAR mRNA-positive cells appeared to increase 1 to 3 hours after LPS treatment (Figure 7D). Establishing the identity of the positive cells in the adipose tissue of LPS-treated mice will require additional studies. No u-PAR mRNA was detected in skin and skeletal muscle from control and LPS-treated animals (not shown).

Discussion

In this report, Northern blot and *in situ* hybridization experiments were performed to investigate the normal tissue distribution and regulation of u-PAR gene expression *in vivo*. The cDNA probes employed for these studies should detect both u-PAR-1 and u-PAR-2 as the first 480 bp of these cDNAs are identical. Northern blot analysis demonstrated relatively high levels of u-PAR mRNA in mouse placenta, with intermediate levels in the lung and spleen and very low levels in the heart and kidney (Figure 1A). No u-PAR mRNA could be detected in the liver, gut, thymus, brain, or skeletal muscle. This distribution

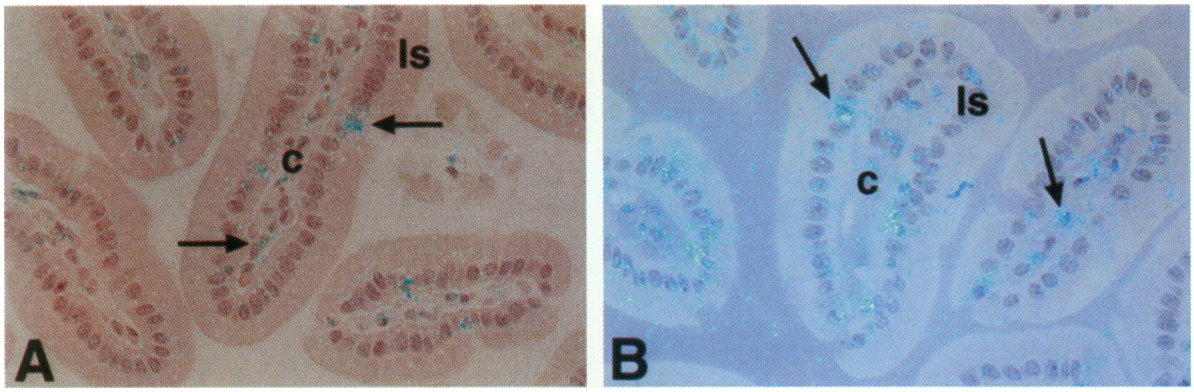

Figure 8. *Distribution of u-PAR mRNA in the gut.* A: *Section from control gut. A positive signal can be detected over scattered cells lining the outer epithelial luminal surface (ls) as well as on cells at the core (c) of the villus.* B: *Section 3 hours after LPS treatment showing PAI-1 mRNA positive cells in the core of the villus and in scattered epithelial cells. Arrows indicate examples of positive cells. Slides were exposed for 6 weeks and processed in parallel.* ×400.

was distinctly different from that of u-PA (Figure 1B). Thus, expression of the receptor and its ligand is not necessarily coincident in the tissues studied.

Endotoxin is a component of the cell walls of gram-negative bacteria that activates many of the cellular phases of acute inflammation.[29] The effects of endotoxin are mediated in part by tumor necrosis factor-α, IL-1, IL-6, and other cytokines. Although cytokines regulate u-PAR synthesis in cultured cells,[16–18] little is known about their effects, or that of LPS itself, on u-PAR synthesis *in vivo*. Figures 2 and 3 demonstrate that LPS administration induces a rapid but transient increase in u-PAR in a variety of murine tissues. For example, administration of sublethal doses of LPS leads to an increase in the steady-state levels of u-PAR mRNA in the lung (7-fold), kidney (5-fold), spleen (3-fold), and heart (2.5-fold) (Figure 2), with a peak at 1 hour. u-PAR mRNA in the gut and liver, and possibly in the thymus and brain, also appeared to be upregulated by LPS (Figure 3).

The widespread induction of u-PAR mRNA by LPS suggested that common vascular cells might be involved. To investigate this possibility, the cellular localization of u-PAR mRNA in tissues from control and LPS-treated mice was assessed by *in situ* hybridization with ^{35}S-labeled sense and antisense u-PAR riboprobes. In control mice, u-PAR mRNA was detected primarily in alveolar macrophages of the lung (Figure 4A), in macrophages and lymphocytes of the spleen (Figure 6A), and in lymphocytes of the thymus (not shown). In addition, a relatively weak but consistently detected signal was observed in some glomeruli of the kidney of control animals (Figure 5A) and in a variety of epithelia (not shown). In general, u-PAR mRNA was not detected in endothelial cells of control tissue (Figure 7B) and no specific signal was apparent in hybridizations with the ^{35}S-labeled sense probe (Figure 4D).

The primary cellular response to LPS was localized to tissue macrophages and lymphocytes (Fig-

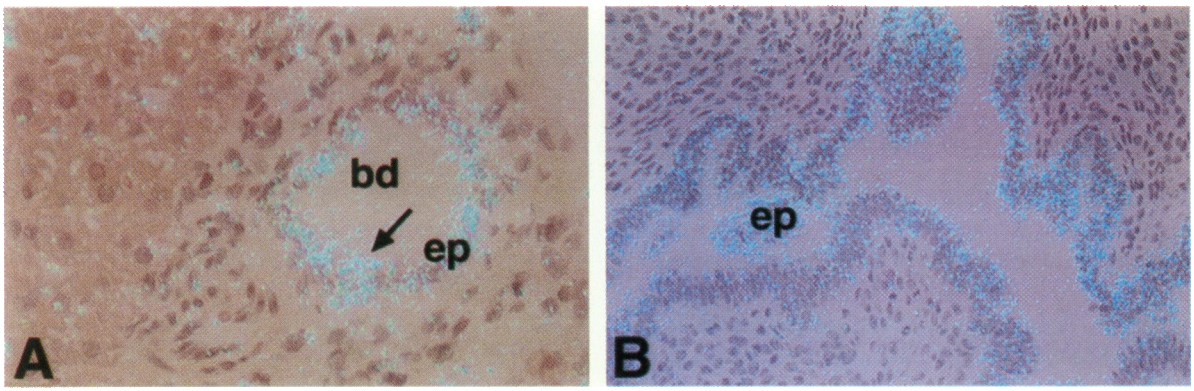

Figure 9. *u-PAR gene expression in the epithelium after LPS treatment.* A: *u-PAR mRNA localization in the epithelium (ep) of the biliary duct (bd) of the liver after 1 hour after LPS treatment.* B: *u-PAR mRNA localization in the uterus epithelium (ep) 1 hour after LPS treatment. Both slides were exposed for 6 weeks.* ×400.

ures 4B and 6B). Only rare neutrophils showed positivity in control or LPS-exposed animals, and the signal was relatively weak. Interestingly, u-PAR mRNA was also markedly enhanced by LPS in the endothelial cells of arteries, veins, and capillaries of a number of organs, including brain and liver (not shown) and blood vessels from the heart (Figure 7, D and E) and kidney (Figure 5B). Some myocytes in the heart expressed u-PAR mRNA in response to LPS (Figure 7C) and cells within most glomeruli of the kidneys showed a marked induction of u-PAR mRNA (Figure 4B). Additional studies are required to identify the specific cell type(s) in the kidney and spleen that express u-PAR mRNA in response to LPS. LPS also markedly induced u-PAR gene expression in a variety of specialized epithelial cells including those present in bile ducts (Figure 9A) and uterus (Figure 8B). Finally, comparison of the results shown in Figures 4A, 5A, and 6A with those in Figures 4B, 5B, and 6B reveal that LPS frequently induced u-PAR mRNA in the same cells that were expressing it in the untreated controls.

The complex effect of endotoxin on mammalian systems includes the activation of the immune system. The ability of macrophages and T lymphocytes to extravasate and reach inflammatory sites is a basic function of cellular immunity and may require the pronounced and rapid induction of the urokinase receptor. For example, antisense nucleotides to u-PAR completely inhibited chemotaxis of human monocytes *in vitro*.[30] Moreover, it was recently shown that u-PAR is present in large human granular lymphocytes and in a small subset of T cells ($CD3^+$). Treatment of T cells with IL-2 (a T-cell-derived lymphokine) caused a large increase in urokinase binding.[18] Thus, induction of u-PAR by LPS may promote local proteolysis and facilitate the extravasation of lymphocytes and monocytes to sites of injury.

Induction of u-PAR may also contribute to the imbalance in the coagulation and fibrinolytic systems frequently observed during gram-negative sepsis. For example, endotoxin appears to activate the coagulation system *in vivo* through the induction of tissue factor and downregulation of thrombomodulin.[31] At the same time, endotoxin suppresses the fibrinolytic system through the induction of type 1 plasminogen activator inhibitor (PAI-1) and the inhibition of t-PA gene expression.[32,33] Collectively, these changes would be expected to create a potent procoagulant state *in vivo*, and disseminated intravascular coagulation is one of the primary life-threatening consequences of endotoxemia. Although it is not yet clear whether induction of u-PAR would also influence these events, it seems likely that the increase in the receptor and its ligand may promote fibrinolysis and the inflammatory state in general. u-PAR appears to participate in the retraction of endothelium *in vitro*.[34] This change may influence endothelial permeability, an important process in the passage of circulating cells through the vessel wall.

In summary, this study provides the first demonstration that u-PAR mRNA is induced during endotoxemia *in vivo*. LPS caused rapid induction of u-PAR in vascular cells including endothelial cells, in cells involved in the inflammatory/immune response, and in epithelial cells. These changes in u-PAR may promote and contribute to the general imbalance in vascular homeostasis associated with gram-negative sepsis.

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