

## Cathelicidin Gene Expression in Porcine Tissues: Roles in Ontogeny and Tissue Specificity†

HUA WU, GUOLONG ZHANG, CHRISTOPHER R. ROSS, AND FRANK BLECHA\*

*Department of Anatomy and Physiology, Kansas State University, Manhattan, Kansas 66506-5602*

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**Cathelicidins constitute a family of mammalian antimicrobial peptides that are synthesized in the bone marrow as propeptides, stored in neutrophil granules as propeptides, and released as active, mature peptides upon neutrophil degranulation. We investigated the developmental expression of two porcine cathelicidins, PR-39 and protegrin. Both cathelicidins were expressed constitutively in the bone marrow of all pigs at all of the ages tested. Peripheral blood neutrophils from young pigs expressed PR-39 and protegrin mRNA, which were not detectable at 42 days of age. At earlier ages, expression of PR-39 mRNA was detected in the kidney and liver and several lymphoid organs, including the thymus, spleen, and mesenteric lymph nodes, but disappeared at 4 weeks of age. These data provide the first evidence of cathelicidin gene expression in peripheral leukocytes and may indicate a role for these antimicrobial peptides in the development of host defense mechanisms.**

Biological organisms have evolved many strategies to defend against pathogens, but clearly one of the oldest and most conserved mechanism of host defense is the elaboration of antimicrobial peptides (4, 9). These natural antibiotics are produced by a diverse array of cells and species in both the plant and animal kingdoms (5, 6, 15, 18, 19, 24, 33). Molecular cloning of mammalian bone marrow cDNA has revealed a family of antimicrobial peptides that share a highly conserved preprosequence followed by structurally variable mature peptide sequences (36). Because the prosequence is very similar to that of cathelin, a 96-amino-acid peptide originally isolated from porcine neutrophils, these antimicrobial peptides are called cathelicidins (36). Members of this family of antimicrobial peptides have been found in several mammalian species, including humans, mice, pigs, cattle, sheep, and rabbits (1, 2, 7, 11–14, 17, 20, 23, 26, 29, 30, 38, 39).

In general, cathelicidins are synthesized by bone marrow progenitor cells (36), stored as proforms in neutrophil granules (34), and processed to mature peptides by enzyme cleavage (25, 28, 35). Although cathelicidin gene expression or protein synthesis by circulating neutrophils has not been reported, human FALL-39/LL-37 mRNA was detected in the testis (2); LL-37 mRNA was found in human keratinocytes (8); immunoreactivity for the proline-arginine-rich 39-amino-acid peptide PR-39 was reported in human colon adenocarcinoma cells (3); and expression of PR-39 mRNA was observed in rat macrophages (21). Here we report the developmental gene expression of two porcine cathelicidins, PR-39 and protegrin, in peripheral blood neutrophils and detection of PR-39 protein in secondary lymphoid organs.

To investigate the developmental gene expression of porcine cathelicidins, total RNA was obtained from bone marrow progenitor cells and peripheral blood neutrophils from littermate pigs at 2, 7, 14, 28, 35, and 42 days of age. Neutrophils were obtained from peripheral blood by venipuncture of the ante-

rior vena cava by using heparinized blood collection tubes, followed by density gradient centrifugation and hypotonic lysis as previously described (31). Pigs then were euthanized by electrocution, femurs were dissected, and bone marrow cells were aspirated and separated into low-density mononuclear cells and granulocytic-lineage cells by density gradient centrifugation as previously described (31). Total RNA was extracted from bone marrow cells and peripheral blood neutrophils by using TRIzol (GIBCO-BRL, Gaithersburg, Md.). First-strand cDNA synthesis was performed by using 1.0 µg of each RNA sample primed with antisense primers (described below) in a 20-µl reaction volume containing 1 mM deoxynucleoside triphosphates, 10 U of RNasin (Perkin Elmer, Foster City, Calif.), and 25 U of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer). All PCRs were performed with half of each resulting cDNA in a 50-µl reaction volume containing 0.2 mM deoxynucleoside triphosphates, 2 mM Mg<sup>2+</sup>, 0.5 µM each sense and antisense primer, and 1.25 U of Jump-Start *Taq* DNA polymerase (Sigma). The PCR profile included 94°C denaturation for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Primer sequences for PR-39, protegrin, and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene were designed by using the Prime program of the Wisconsin Sequence Analysis Package (Genetics Computer Group, Inc., Madison, Wis.) based on known sequences deposited in GenBank. Cathelicidin primers were designed to specifically amplify the mature peptides, rather than the conserved preprosequence. The primers used and the resultant cDNA sizes were as follows: PR-39 sense, 5'-ACC CAT CCA TTC ACT CAC-3'; PR-39 antisense, 5'-AGC CAC AAC AAT AAG ATC C-3' (262 bp); protegrin sense, 5'-TGG ATC AGA TCA AGG ACC-3'; protegrin antisense, 5'-ACA CAG ACG CAG AAC CTA C-3' (100 bp); GAPDH sense, 5'-ACC ACA GTC CAT GCC ATC AC-3'; GAPDH antisense, 5'-TCC ACC ACC CTG TTG CTG TA-3' (452 bp). Specific PCR products for PR-39, protegrin, and GAPDH were obtained by using these primers and confirmed by direct sequencing.

Bone marrow granulocytic cells showed abundant expression of PR-39 and protegrin in pigs at all of the ages that were studied (Fig. 1). Similarly, during the first month of age, ex-

\* Corresponding author. Mailing address: Department of Anatomy and Physiology, VMS 228, 1600 Denison Ave., Kansas State University, Manhattan, KS 66506-5602. Phone: (785) 532-4537. Fax: (785) 532-4557. E-mail: blecha@vet.ksu.edu.

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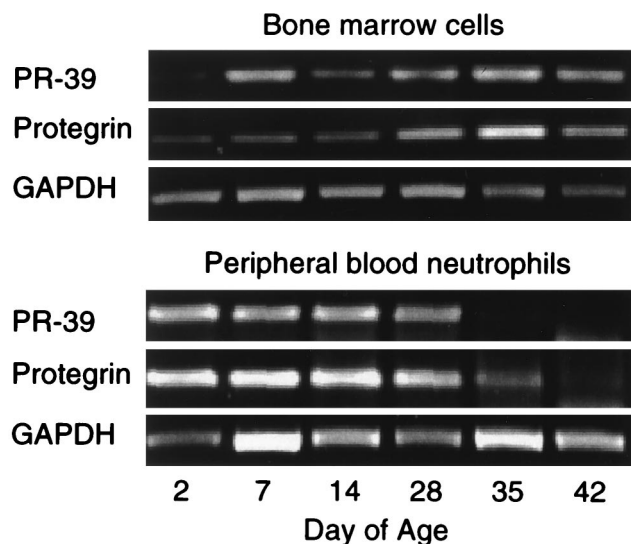


FIG. 1. Developmental gene expression of PR-39 and protegrin in peripheral blood neutrophils. RT-PCR, using primers for PR-39, protegrin, and the GAPDH housekeeping gene, was conducted on bone marrow cells and peripheral blood neutrophils obtained from littermate pigs on 2, 7, 14, 28, 35, and 42 days of age.

pression of mRNA for both cathelicidins in peripheral blood neutrophils was unequivocal (Fig. 1). To our knowledge, this is the first evidence of cathelicidin gene expression in circulating leukocytes and, specifically, peripheral blood neutrophils. However, expression of PR-39 and protegrin mRNAs disappeared at 35 and 42 days of age, respectively (Fig. 1). This expression pattern may reflect the maturation process of granulocytic cells in the young pig. Furthermore, these findings suggest the existence of regulatory elements controlling cathelicidin expression in newborns and raise the possibility of altering these control elements to modulate cathelicidin expression.

Because of the novel finding of cathelicidin gene expression in peripheral blood neutrophils, we wondered if various organs also expressed mRNA for PR-39 and protegrin. Northern analysis, reverse transcription (RT)-PCR, and Southern analysis of RT-PCR products were conducted on bone marrow cells, peripheral blood neutrophils, and tissue samples from the thymus, mesenteric lymph nodes, spleen, liver, kidney, jejunum, and colon. For Southern analysis, PCR products from various porcine tissues were subjected to electrophoresis on 1.5% agarose gels and then capillary transferred overnight onto nitrocellulose membranes (Micro Separations, Westboro, Mass.) with  $20\times$  standard saline citrate (SSC;  $1\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate). For Northern analysis, total RNA (15  $\mu$ g) was denatured and separated on 1.2% agarose-formaldehyde gels and then blotted onto nitrocellulose membranes (Micro Separations). Southern and Northern blots were prehybridized for 2 h in  $5\times$  SSC–50% formamide–0.1% sodium dodecyl sulfate (Ambion, Austin, Tex.) at 42°C and hybridized overnight under the same conditions with a  $^{32}$ P-labeled cDNA probe for PR-39. Posthybridization washes were  $2\times 10$  min at 42°C with  $2\times$  SSC, followed by  $2\times 20$  min at 50°C with  $0.1\times$  SSC–0.5% sodium dodecyl sulfate. Blots were exposed to Kodak X-Omat films (Eastman Kodak, Rochester, N.Y.) with intensifying screens at  $-70^\circ\text{C}$ . The PR-39 cDNA probe was generated by cloning the 262-bp cDNA into pGEM-T vectors (Promega, Madison, Wis.). Positive clones

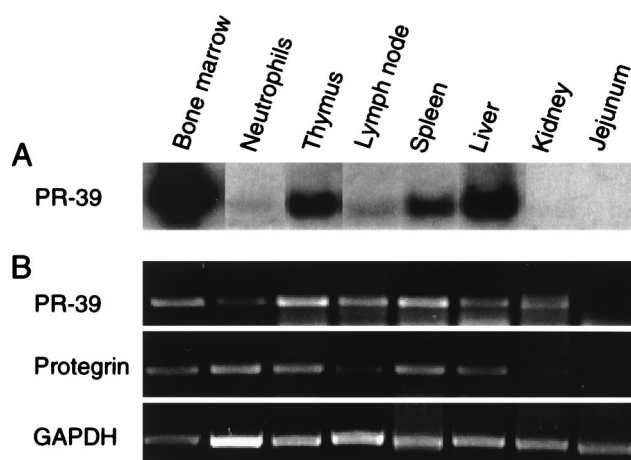


FIG. 2. Tissue distribution of PR-39 and protegrin in 7-day-old pigs. (A) Northern analysis of PR-39 mRNA expression in porcine cells and tissues. Total RNA (15  $\mu$ g per sample) was separated on a 1.2% agarose-formamide gel, blotted onto nitrocellulose membranes, and hybridized to a  $^{32}$ P-labeled PR-39 cDNA probe. (B) RT-PCR, using primers for PR-39, protegrin, and GAPDH, was conducted on the same samples that were used for Northern analysis.

were selected and sequenced. The correct cDNA fragment was cut, randomly labeled with  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  using Ready-To-Go DNA Labeling Beads (Pharmacia Biotech, Piscataway, N.J.), purified of unincorporated nucleotide using ProbeQuant G-50 Micro Columns (Pharmacia Biotech), and used as probes.

As expected, the PR-39 cDNA probe hybridized strongly with bone marrow mRNA from 7-day-old pigs (Fig. 2A). However, thymus, spleen, and liver samples from 7-day-old pigs also showed intense Northern blot signals. Fainter but positive bands also were observed in the Northern analysis of mesenteric lymph node and neutrophil samples. Expression of PR-39 mRNA was not found in the jejunum and kidney in 7-day-old pigs. RT-PCR of the same RNA samples confirmed our Northern analysis findings of PR-39 gene expression in bone marrow, neutrophils, thymus, mesenteric lymph nodes, and liver and also indicated its expression in the kidneys of 7-day-old pigs (Fig. 2B). Moreover, gene expression for a different cathelicidin, protegrin, also was observed in all of the same tissues, except kidney tissue (Fig. 2B).

We then investigated whether these antimicrobial peptides in other tissues also were expressed developmentally in young pigs as was observed in peripheral blood neutrophils. Indeed, Southern analysis of RT-PCR products showed that this was the case for PR-39. As previously shown, expression of PR-39 mRNA was always observed in bone marrow cells and also peripheral blood neutrophils until 35 days of age (Fig. 3). However, the liver and kidney and other lymphoid organs, including the thymus, mesenteric lymph nodes, and spleen, showed clear gene expression of PR-39 at 2, 7, and 14 days of age but not at 28 days of age or older (Fig. 3). PR-39 gene expression was not detected in the gastrointestinal tract (jejunum and colon; Fig. 3), the tissue from which this cathelicidin was isolated originally (1).

We were intrigued by the finding of PR-39 mRNA expression in primary and secondary lymphoid tissues and wondered if these tissues might have been the source of PR-39 described in the original report (1). To address this question, we probed immunoblots of mesenteric lymph node, neutrophil, and spleen proteins and synthetic PR-39 with a monoclonal antibody to PR-39 and a polyclonal antibody to the cathelin domain of PR-39 as previously described (37). Western blot anal-

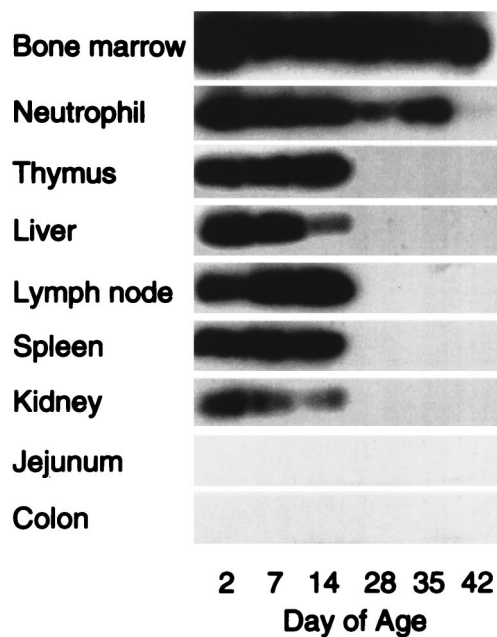


FIG. 3. Developmental gene expression of PR-39 in bone marrow cells, peripheral blood neutrophils, and various other lymphoid tissues. For Southern analysis, the RT-PCR-amplified cDNA fragments were blotted onto nitrocellulose membranes, hybridized with the specific PR-39 cDNA probe, and exposed to autoradiography.

ysis showed that PR-39 was indeed present in mesenteric lymph nodes (Fig. 4). It was present in both the propeptide and mature forms in lymph nodes; the neutrophil preparation used in this blot contained only the propeptide (PMN lane). As reported previously, the polyclonal antibody to cathelin recognized only the propeptide forms of the neutrophil cathelicidins and did not recognize synthetic PR-39 (37). We have consid-

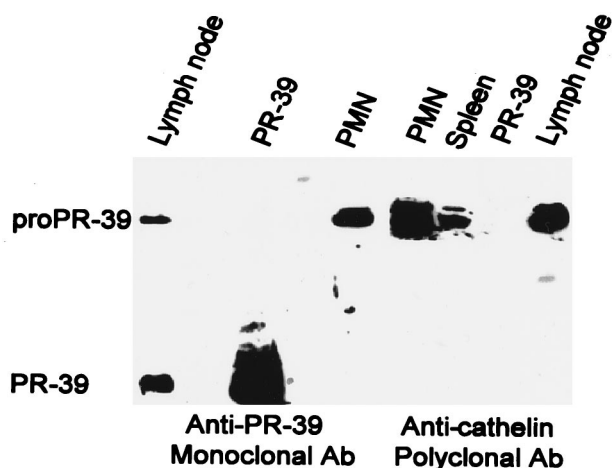


FIG. 4. Western blot analysis of synthetic and native PR-39. Mesenteric lymph node (1-mg tissue equivalent/lane), synthetic PR-39 (1  $\mu$ g/lane), polymorphonuclear neutrophil (PMN) granule extracts ( $0.5 \times 10^6$  cell equivalents/lane), and spleen (1-mg tissue equivalent/lane) samples were subjected to acid-urea polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The immunoblot was developed with a mouse anti-PR-39 monoclonal antibody (Ab) (1  $\mu$ g/ml) or rabbit anti-cathelin serum (1:5,000). A chemiluminescent peroxidase substrate (SuperSignal; Pierce) was used to visualize antibody-specific binding.

ered the possibility that the source of PR-39 in mesenteric lymph nodes may be resident tissue neutrophils or peripheral blood neutrophils. However, we have not observed this signal in other highly vascularized tissues (e.g., kidney and gastrointestinal tract tissues), and therefore, the PR-39 signal in mesenteric lymph nodes probably was not caused by neutrophils.

To our knowledge, these data provide the first evidence of cathelicidin gene expression in peripheral blood neutrophils. Others have reported the presence of PR-39 mRNA in rat macrophages and infarcted rat hearts (21) and PR-39 immunoreactivity in fully differentiated human colon cells (3). However, we have not been able to repeat those findings (data not shown) and have found PR-39 mRNA and protein only in porcine tissues. The repression of gene expression of these antimicrobial peptides as the animal ages may be a reflection of increased numbers of immature neutrophils in the neonatal pig. However, the presence of PR-39 in primary and secondary lymphoid tissues in the neonate also suggests other functions for this peptide. Studies have shown that PR-39 is a multifunctional peptide, with activities that include involvement in wound healing through the induction of syndecan expression (10), anti-inflammatory properties through the specific inhibition of NADPH oxidase production of superoxide anion (32), and chemoattractant activity for neutrophils (16). It is unclear how cathelicidins might function in the developmental maturation of the porcine immune system. However, considering the critical early events involved in neonatal tolerance or immunization (27) and the finding that other antimicrobial peptides have been shown to influence T-cell immunity (22), it is possible that cathelicidins could participate in this critical stage of immune development. We are continuing to explore this hypothesis.

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