Inactivation of the Gene for Anticoagulant Protein C Causes Lethal Perinatal Consumptive Coagulopathy in Mice

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Abstract

Matings of mice heterozygous for a protein C (PC) deficient allele, produced by targeted PC gene inactivation, yielded the expected Mendelian distribution of PC genotypes. Pups with a total deficiency of PC ($PC^{-/-}$), obtained at embryonic day (E) 17.5 and at birth, appeared to develop normally macroscopically, but possessed obvious signs of bleeding and thrombosis and did not survive beyond 24 h after delivery. Microscopic examination of tissues and blood vessels of E17.5 $PC^{-/-}$ mice revealed their normal development, but scattered microvascular thrombosis in the brain combined with focal necrosis in the liver was observed. In addition, bleeding was noted in the brain near sites of fibrin deposition. The severity of these pathologies was exaggerated in $PC^{-/-}$ neonates. Plasma clottable fibrinogen was not detectable in coagulation assays in $PC^{-/-}$ neonatal mice, suggestive of fibrinogen depletion and secondary consumptive coagulopathy. Thus, while total PC deficiency did not affect the anatomic development of the embryo, severe perinatal consumptive coagulopathy occurred in the brain and liver of $PC^{-/-}$ mice, suggesting that a total PC deficiency is inconsistent with short-term survival. (*J. Clin. Invest.* 1998. 102:1481–1488.) Key words: protein C deficiency • coagulation • gene deletion • inflammation • embryonic development

Introduction

The blood coagulation system is an intricate cascade of reactions and feedback control mechanisms that ensures a rapid response to vascular injury, yet avoids occlusion of the vasculature. A key component of this system in the regulation of blood clot propagation is the protein C (PC)[1]-dependent anticoagulant pathway. PC circulates in the plasma as a zymogen and exerts its anticoagulant function after activation to its serine protease form, activated PC (aPC).

Human PC is synthesized predominantly in the liver. Recent evidence suggests that human umbilical vein endothelial cells (1) and cells from male reproductive tissues (2) are also sites of synthesis of this protein. In the rat, other minor sites of PC synthesis include kidney, diaphragm, stomach, intestine, uterus, and placenta (3). In human liver, PC is synthesized as a single polypeptide chain, which undergoes a number of intracellular posttranslational modifications, including β-hydroxylation, γ-carboxylation, glycosylation, and endoproteolytic cleavage. The resultant two-chain PC molecule consists of a 251–amino acid residue heavy chain and a 155–amino acid residue light chain, which are linked by a single disulfide bond (4). Mature PC is multimodular and contains structural elements characteristic of other vitamin K–dependent coagulation proteins, such as fVII, fIX, and fX. These distinguishable regions include a Gla-rich motif, two epidermal growth factor–like modules, an activation peptide region, and a serine protease domain (5–8).

Activation of PC to aPC is catalyzed by the thrombin–thrombomodulin (TM) complex (9), or, less efficiently, by thrombin alone (10), and occurs consequent to cleavage of the Arg^{12}-Leu^{13} heavy chain peptide bond, with release of a dodecapeptide from the zymogen (11). In its anticoagulant role, the resulting aPC catalyzes limited proteolytic inactivation of coagulation fV/fVa (10) and fVIII/fVIIIa (12) in the presence of Ca^{2+}, phospholipids, and the cofactor, protein S. aPC also plays an indirect profibrinolytic role by inactivating fVa, thus limiting thrombin formation, and thereby attenuating subsequent thrombin-catalyzed activation of a fibrinolytic inhibitor, TAFI (13). Further, aPC enhances fibrinolysis by direct inactivation of other fibrinolytic inhibitors, such as PAI-1 (14, 15). In addition to these activities, aPC has been implicated in the host–defense reactions that occur during intravascular inflammation (16), possibly by inhibiting production of the tumor necrosis factor, TNF-α, a key mediator of sepsis (17). However, the molecular mechanisms involved in the direct antiinflammatory properties of aPC remain to be elucidated.

Inherited defects in the PC system have been found to be the underlying risk factors in a majority of cases of familial thrombophilia (18). Patients homozygous or heterozygous for PC deficiency are at increased risk for severe thromboembolic

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1. *Abbreviations used in this paper:* aPC, activated protein C; *cda*, cytidine deaminase; DIC, disseminated intravascular coagulation; ES, embryonic stem; fV, fVII, fVIII, fIX, and fX, blood coagulation Factors V, VII, VIII, IX, and X, respectively (a = activated forms of these proteins); H&E, hematoxylin and eosin; HPRT, hypoxanthine-guanine phosphoribosyltransferase; neo^R, neomycin resistance; PC, protein C; TM, thrombomodulin.

disorders, including neonatal *purpura fulminans*, disseminated intravascular coagulation (DIC), and recurrent venous thrombosis (19–23). Acquired PC deficiency can also occur during DIC, liver disease, or surgically induced hypercoagulable states (24–26).

As emphasized by these manifestations of congenital and acquired deficiencies, PC plays a demonstrably important role in anticoagulation and in other physiological and pathological processes. Therefore, it was of interest to develop a biological model of a total PC deficiency state that would facilitate the evaluation of the different roles of PC during and/or after development. This was achieved by the targeted replacement of the murine PC gene via homologous recombination in embryonic stem (ES) cells, which led to the generation of mice totally deficient of PC. The initial characterization of these animals is the subject of this communication.

Methods

Materials

The *PC* gene was cloned from a λFIXII 129 SVJ murine liver library (Stratagene, La Jolla, CA). To assist in the in vivo gene mapping strategies described herein, its entire nucleotide sequence has been determined (27).

The cDNA encoding murine PC, which was used as a probe, was cloned in this laboratory from a λ Zap cDNA library containing liver mRNA from a C57/6XCBA mouse (Stratagene). The basic strategy was to generate a PstI fragment of the cDNA from human PC (28) in order to screen the murine cDNA library. A 1.5-kb mouse *PC* cDNA fragment was thus obtained and used to rescreen the mouse library, from which the entire murine *PC* cDNA was obtained.

The cDNA encoding cytidine deaminase (*cda*) was obtained from Dr. M. Dewerchin (Leuven, Belgium) and has the features previously detailed (29). A cassette, EcoRI-PGKpromoter-cda-PGKpolyA-HindIII, was constructed by the usual techniques, which was then inserted into vectors of choice through suitably placed EcoRI/HindIII cloning sites.

The cDNA encoding murine hypoxanthine-guanine phosphoribosyltransferase (HPRT) was obtained from Dr. M. Dewerchin. The characteristics of this gene have been described (30).

Construction of the targeting vector for homologous recombination in ES cells

To generate mice deficient in PC, a targeting vector was constructed for introduction into R1 ES cells by homologous recombination. The targeting vector contained a 6.5-kb *PC* 5′ flanking region which extended from a 5′ XbaI site to an XhoI site 456 bp upstream of exon 2, and included the first untranslated exon (Fig. 1). The *PC* 3′ flanking region extended from a HindIII site located 390 bp 3′ of the *PC* stop codon to an EcoRI site 2.8 kb downstream of this site (27). A neomycin resistance (neo^R) cassette used for positive selection was cloned between the 5′ and 3′ flanking regions, and replaced the entire PC coding region after homologous recombination into the mouse chromosome. A *cda* cassette cloned upstream of the 5′ *PC* flanking region was used for negative selection against random integrants. The targeting vector was linearized with SacII at the 3′ terminus of the 3′ flank and electroporated into the R1 line (31) of ES cells.

ES cells heterozygous for targeted recombination at the *PC* locus were used for aggregation with Swiss morula-stage embryos to generate chimeric offspring (32). A germline-transmitting male chimeric (> 80%) mouse was test-bred with Swiss mice to generate an F_1 colony of PC heterozygotes ($PC^{+/-}$). Breeding pairs among these mice were mated to produce F_2 progeny for phenotypic analysis. To minimize the generation of congenicity in the mouse lines, breeding partners were regularly intermixed.

Detection of PC-deficient mice

DNA analysis. Genotypic screening of DNA extracted from ES cells and mouse tails was performed by Southern blot hybridization, using a 0.9-kb EcoRI/XbaI 5′ external probe and a 0.5-kb EcoRI/BglII 3′ external probe. EcoRI digests of genomic DNA yielded a differential restriction pattern of 11.5 kb for the wild-type PC gene versus 7.5 kb for recombinants using the 5′ external probe. BamHI digests of genomic DNA yielded a differential restriction pattern of 7.5 and 6.5 kb for wild-type and recombinant alleles, respectively, using the 3′ external probe.

RNA analysis. Total RNA was isolated from E17.5 mouse liver of wild-type, heterozygous, and homozygous animals, using the RNA isolation kit (Stratagene). Approximately 50 μg of RNA was loaded onto the gel. The murine *PC* cDNA or the control murine *HPRT* cDNA were labeled with [α-^{32}P]dCTP and used as the probe for Northern blot analysis.

PC activity in mouse plasma. The amidolytic activity of murine aPC in plasma was assayed using Protac C to activate PC (33), and the chromogenic substrate, S-2366 (Nodia/Chromogenix, Antwerp, Belgium). The *p*-nitroanilide release from the substrate was measured at 405 nm and was found to be proportional to the PC level in the range of 0.1–120% of pooled plasma from normal adult mice. The assay was performed by adding 25 μl of 1:10 (vol/vol) diluted citrated plasma to 25 μl of the activator Protac C (1 U/μl). After a 5-min activation at 37°C, 50 μl of the substrate S-2366 (0.8 μg/μl) was added and the absorbance at 405 nm was monitored over a 2–4 min time interval at 37°C. The amount of aPC present in the plasma samples was determined by comparison of these rates of hydrolysis of the chromogenic substrate to that of a curve generated with use of a PC standard.

Timed matings and embryo harvesting

Timed natural matings of $PC^{+/-}$ heterozygotes were set to generate embryos. Embryos at different stages of development were harvested and dissected free of maternal tissue. DNA extracted from the tails, or from yolk sac, was used to determine genotype of the animals. Whole-body late-stage embryos (≥ E17.5) and neonates were fixed in Bouins fixative (Prosan, Gent, Belgium), whereas earlier stage embryos and dissected organs were fixed in phosphate-buffered 1% paraformaldehyde. The fixed tissues were dehydrated and embedded in paraffin. Routinely, 10-μm sagittal sections were made throughout the body from E17.5 embryos and neonates, while individual organs and earlier stage embryos were sectioned transversally at 7 μm. The E17.5 embryos and neonates were stained with hematoxylin and eosin (H&E) and immunostained with antibodies to fibrinogen.

Histological and immunohistochemical staining

All tissues were stained with H&E. For immunostainings of paraformaldehyde-fixed tissues, epitope retrieval was performed by incubating sections in antigen retrieval buffer (Dako, Copenhagen, Denmark) at 96°C for 90 min.

Fibrin/fibrinogen staining was performed by a three-step procedure using a goat polyclonal antibody raised against mouse fibrinogen (Nordic, Tilburg, The Netherlands; diluted 1:400, vol/vol), followed by rabbit anti–goat IgG (Dako; diluted 1:100, vol/vol), which were preadsorbed with 10% mouse serum at 4°C, overnight, and by a goat anti–rabbit IgG–peroxidase complex (Dako; diluted 1:50, vol/vol). Endothelial cells were stained via a one-step procedure using a peroxidase-labeled polyclonal rabbit antibody raised against human vWf (Dako; diluted 1:50, vol/vol). Peroxidase activity was developed by incubating sections in 0.05 M Tris-HCl/0.06% diaminobenzidine/0.01% H_2O_2, pH 7.0, resulting in a brownish stain of the immunoreactive sites. All sections were briefly counterstained with Harris' hematoxylin.

Fibrin polymerization time clotting assay

A fibrin polymerization time clotting assay was used to assess fibrinogen levels in neonatal plasma samples using an adult wild-type mu-

rine plasma pool as a control. Plasma samples diluted (a 1:5 dilution was used for adult wild-type plasma and a 1:1 dilution was used for neonatal plasma samples) in F.P.T dilution buffer (A. Christiaens, Brussels, Belgium) were added to a glass tube and incubated at 37°C for 30 s. At $t = 0$, an equal volume of bovine thrombin (400 U/ml) was added. The clotting time was determined and converted to the concentration of fibrinogen using a conversion table from F.P.T. Dil. The F.P.T. Dil conversion table ranges from 10 (28 mg%) to 40 s (8.4 mg%). Samples that took > 40 s to clot at the lowest dilution were assumed to contain < 8.4 mg% fibrinogen in plasma.

Results

A targeting vector (Fig. 1 *A*) designed to inactivate the murine *PC* gene by homologous recombination was introduced into R1 ES cells by electroporation. Correctly targeted clones, identified by Southern blot analysis using both 5′ and 3′ external probes (Fig. 1 *B*), were obtained. Aggregation of a recombinant R1 ES cell line with Swiss morula-stage embryos led to the generation of eight chimeric mice. Upon test-breeding with Swiss females, a male chimera (> 80%) proved to be a germline transmittant of the mutated PC allele. Intercross mating of healthy heterozygous ($PC^{+/-}$) F_1 mice produced F_2 littermates for phenotypic analysis (Fig. 1 *B*).

Initial postnatal genotypic analysis at postnatal day 1 showed a significant underrepresentation of $PC^{-/-}$ pups. Of a total of 207 F_2 mice, only 16 (8%) nulls were observed whereas 52 (25%) were expected. The only nulls identified were found dead or partly consumed by the mother on the day of birth. The small number of null neonates prompted an investigation of embryos at gestation day E17.5. This analysis showed the expected Mendelian distribution of genotypes of 84 mice from 12 litters, with 18 (22%) $PC^{+/+}$, 44 (52%) $PC^{+/-}$, and 22 (26%) $PC^{-/-}$ mice observed. Mice at this stage of development were

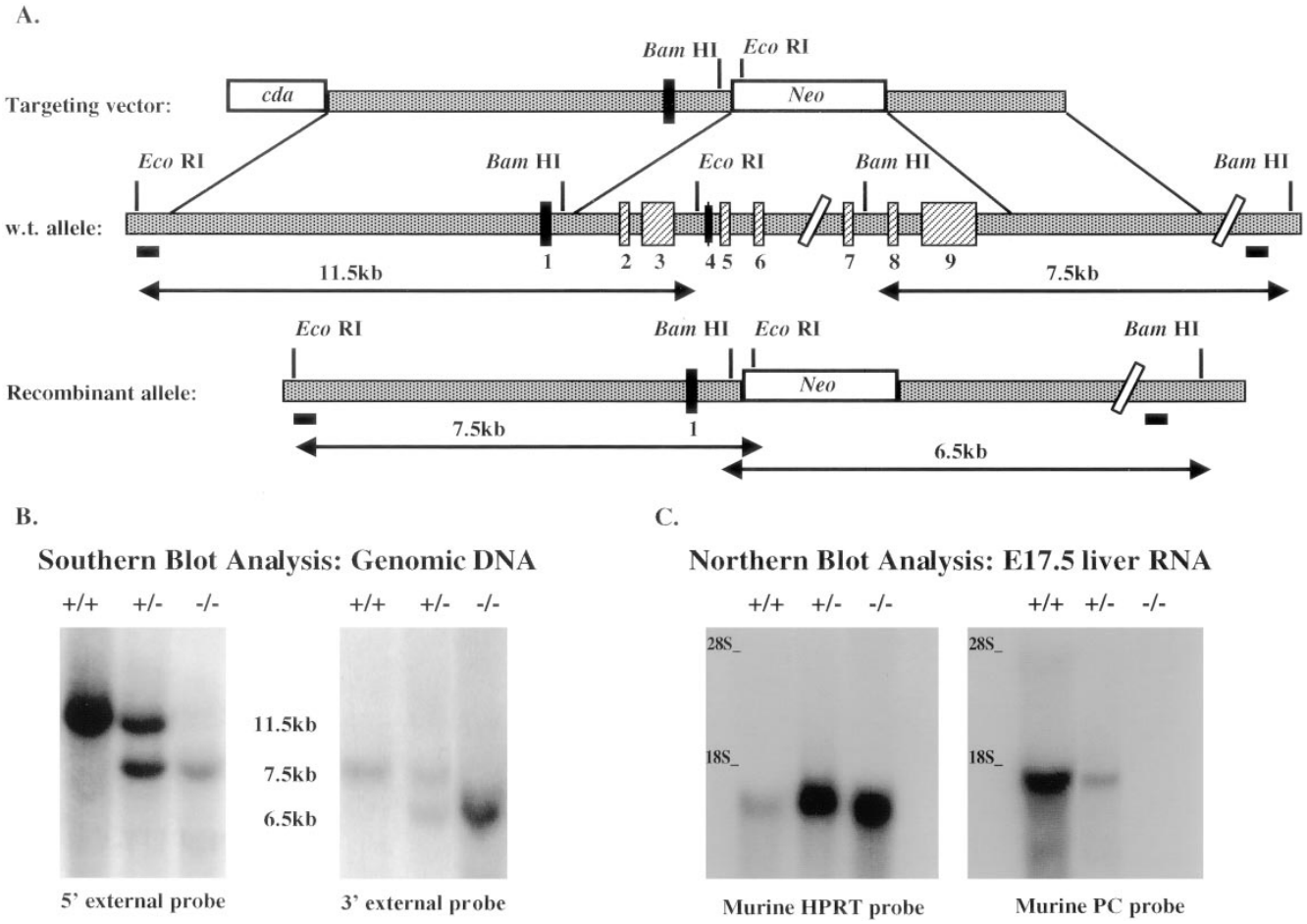

Figure 1. Targeted gene replacement at the murine *PC* locus. (*A*) Targeting vector for *PC* gene disruption. The top line illustrates the targeting vector. Homologous flanking regions are shaded gray. The neo^R selection cassette cloned between the flanking regions was designed to replace the entire coding region of *PC* upon homologous recombination. An EcoRI restriction site was introduced from the polylinker of the neo^R cassette. A *cda*-negative selection cassette cloned upstream of the 5′ flanking region was used to select against random integrants. The second line indicates the wild-type murine *PC* allele. The first untranslated exon is presented as a darkened block while the other exons are presented as lighter-shaded boxes. The 5′ and 3′ extended probes are indicated by the darkened boxes below the gene. The recombinant allele is indicated in the third line. The differential restriction fragments that result from the diagnostic digests are indicated. The slanted bars indicate a location where a break was placed in a large noncoding region. (*B*) Southern blot analysis of DNA extracted from $PC^{+/+}$, $PC^{+/-}$, and $PC^{-/-}$ genomic DNA. EcoRI-digested DNA was used with the 5′ external probe and BamHI-digested DNA was used with the 3′ external probe. (*C*) Northern blot analysis of total RNA extracted from $PC^{+/+}$, $PC^{+/-}$, and $PC^{-/-}$ E17.5 livers. The murine *HPRT* cDNA and murine *PC* cDNA probes were used.

alive and appeared normal macroscopically, suggesting that the fatal challenge to the null embryos must have occurred between E17.5 and birth. The births of a total of 7 litters (44 pups) were continuously monitored to identify whether $PC^{-/-}$ pups survived birthing. A total of 12 $PC^{-/-}$ animals (27%) was obtained. Most null pups (8/12) were born dead whereas the others were severely bruised in the head and expired within a few hours. Since the trauma of birth posed the most severe mechanical stress on null embryos, and perhaps affected survival, E18.5 embryos were delivered by cesarean section and placed with a foster mother. Although the $PC^{-/-}$ pups were macroscopically normal at the time of delivery and were accepted and cared for by the foster mother, they did not nurse. Their survival times were < 24 h, whereas survival times for the $PC^{+/+}$ and $PC^{+/-}$ littermates were normal.

Unlike $PC^{+/+}$ or $PC^{+/-}$ mice, $PC^{-/-}$ mice did not produce detectable PC mRNA in the liver (Fig. 1 *C*) or PC activity in plasma. Using the murine *PC* cDNA as a probe for Northern blot analysis of RNA extracted from E17.5 livers, the absence of a message is evident in $PC^{-/-}$ embryos, whereas PC mRNA is detectable in livers of $PC^{+/+}$ and to a lesser degree in $PC^{+/-}$ embryos (Fig. 1 *C*). *HPRT* RNA was observed in all embryos, indicating that the PC deficiency did not cause generalized defects in RNA synthesis or stability (Fig. 1 *C*). Measurement of PC amidolytic activity in E17.5 embryonic ($n = 3$) and neonatal ($n = 3$) mouse plasma (the latter obtained by intracardial puncture) showed no detectable ($< 0.1\%$) PC activity in $PC^{-/-}$ plasma samples. In contrast, neonatal $PC^{+/+}$ ($n = 3$) and E17.5 $PC^{+/+}$ ($n = 3$) activity levels were present at 34±3 and 42±3%, respectively, of an adult $PC^{+/+}$ plasma pool. A heterozygote ($PC^{+/-}$) adult murine plasma pool contained 63±5% of adult PC, whereas neonatal ($n = 3$) and E17.5 ($n = 3$) plasma PC levels from $PC^{+/-}$ pups were 35±3% and 38±4% of the corresponding $PC^{+/+}$ mice at these developmental stages. The occurrence of full-term $PC^{-/-}$ mice, together with the lack of detectable PC amidolytic activity in $PC^{-/-}$ E17.5 mouse plasma ($n = 3$), suggested that embryonic development was not dependent on fetally synthesized PC, nor on detectable levels of maternally transferred protein.

Macroscopically, $PC^{-/-}$ neonates were clearly bruised predominantly in the head region. Upon removal of the skull, blood was often evident in the subdural space. Signs of thrombosis (bruising) were also observed within the brain. Further, examination of the abdominal cavity showed signs of DIC with depletion of blood from several lobes in the liver.

Blood vessel development in liver, kidney, heart, lung, and brain was normal in embryos of all genotypes as judged by H&E staining (not shown). Organs developed normally during embryogenesis and displayed some thrombotic changes during development. However, the brain (Fig. 2) and liver (Fig. 3) of the $PC^{-/-}$ animals illustrated the most severe progressive thrombotic and necrotic phenotypes as embryonic development proceeded. This may be due to imbalance of TF/TFPI levels in the brain and the high local concentrations of fibrinogen and other clotting factors in the liver. Microscopic analysis was performed on six $PC^{-/-}$ neonates whose deaths occurred shortly after birth. In these animals, widespread coagulopathy was observed manifested by fibrin deposition in the microvasculature of the brain (Fig. 2 *J*). Additionally, severe bleeding in the brain (Fig. 2 *I*), interstitial fibrin deposition in the liver (Fig. 3, *E* and *F*), and necrosis of hepatic tissue (Fig. 3, *E* and *F*) were observed in $PC^{-/-}$ neonates, but not in $PC^{+/+}$ neonates (Fig. 2, *K* and *L*, and Fig. 3, *G* and *H*). Moreover, the dura vessels were normal, but were significantly dilated without signs of bleeding. Some traces of fibrin deposition were evident in the glomeruli and tubuli of the kidneys of the neonates, as well as occasional clotting in the heart and lungs (not shown).

Microscopic analysis at various developmental stages was performed to identify the time point of thrombotic onset in $PC^{-/-}$ embryos. Although extensive thrombosis was not evident until birth, early signs appeared beginning at E12.5 and thereupon progressed. In all of the six embryos examined at these early time points (E12.5 to E14.5), a small degree of fibrin deposition was noted throughout the telencephalic region of the brain in $PC^{-/-}$ embryos (Fig. 2, *A* and *B*), but not in $PC^{+/+}$ embryos (Fig. 2, *C* and *D*). Although bleeding was minimal, it was observed in some cases near the sites of thrombosis. Some interstitial fibrin was observed in the liver of these early stage embryos (not shown), but it was minimal and there were no evident signs of brain or hepatic tissue degradation.

The progression of these symptoms became apparent in E17.5 $PC^{-/-}$ embryos. The hemostatic abnormalities in the brain varied among E17.5 and older embryos, and ranged from only minimal clotting and no bleeding to extensive fibrin deposition throughout the brain, with severe bleedings localized in the forebrain of the $PC^{-/-}$ embryos near the lateral ventricles (Fig. 2, *E* and *F*). No such signs were found in $PC^{+/+}$ embryos (Fig. 2, *G* and *H*), nor in $PC^{+/-}$ embryos.

Beginning signs of tissue necrosis were evident in the liver of nearly all E17.5 embryos analyzed. In most E17.5 $PC^{-/-}$ embryos, interstitial fibrin was present in all hepatic lobes (Fig. 3 *B*), whereas no evidence of fibrin was found in E17.5 $PC^{+/+}$ embryos (Fig. 3 *D*). In some cases, the extent of degradation was exemplified by the loss of hepatocytes and deterioration of the extracellular matrix. Infiltrating leukocytes were also observed in the PC null embryos (Fig. 3 *A*), all alive at the time of harvest, as compared with $PC^{+/+}$ pups, where no bleeding is observed (Fig. 3 *C*). While the observed histology could be due to the effects of a PC deficiency, we cannot discount the possibility that the presence of leukocytes could be due to a response to fibrin deposition and/or bleeding (34).

In cases of severe thrombosis, and without proper inhibition of fibrin generation via control of thrombin levels, coagulation factors may be consumed to the point of depletion. Since PC plays a vital role in anticoagulation by regulating thrombin levels, consumptive coagulopathy could have been expected to occur in $PC^{-/-}$ mice. To establish whether fibrinogen depletion was attained after the trauma of birth, clotting assays to determine relative fibrinogen concentrations were performed on neonatal plasma samples and were compared with those of an adult wild-type murine plasma pool. Neonatal $PC^{+/+}$ mice contained 34±4% of the clottable fibrinogen of adult $PC^{+/+}$ mice. These latter values were essentially the same as those for E17.5 embryos. Plasma samples from $PC^{-/-}$ E17.5 mice and neonatal mice did not clot at all, indicating depletion of fibrinogen due to consumptive coagulopathy, whereas $PC^{+/-}$ mice contained $\sim$ 30–40% of the values found in $PC^{+/+}$ mice at their corresponding stages of development.

Discussion

Targeted gene disruption at the *PC* locus in mice has led to a phenotype similar to the clinical manifestations of human homozygous PC deficiency. Predisposition to neonatal *purpura*

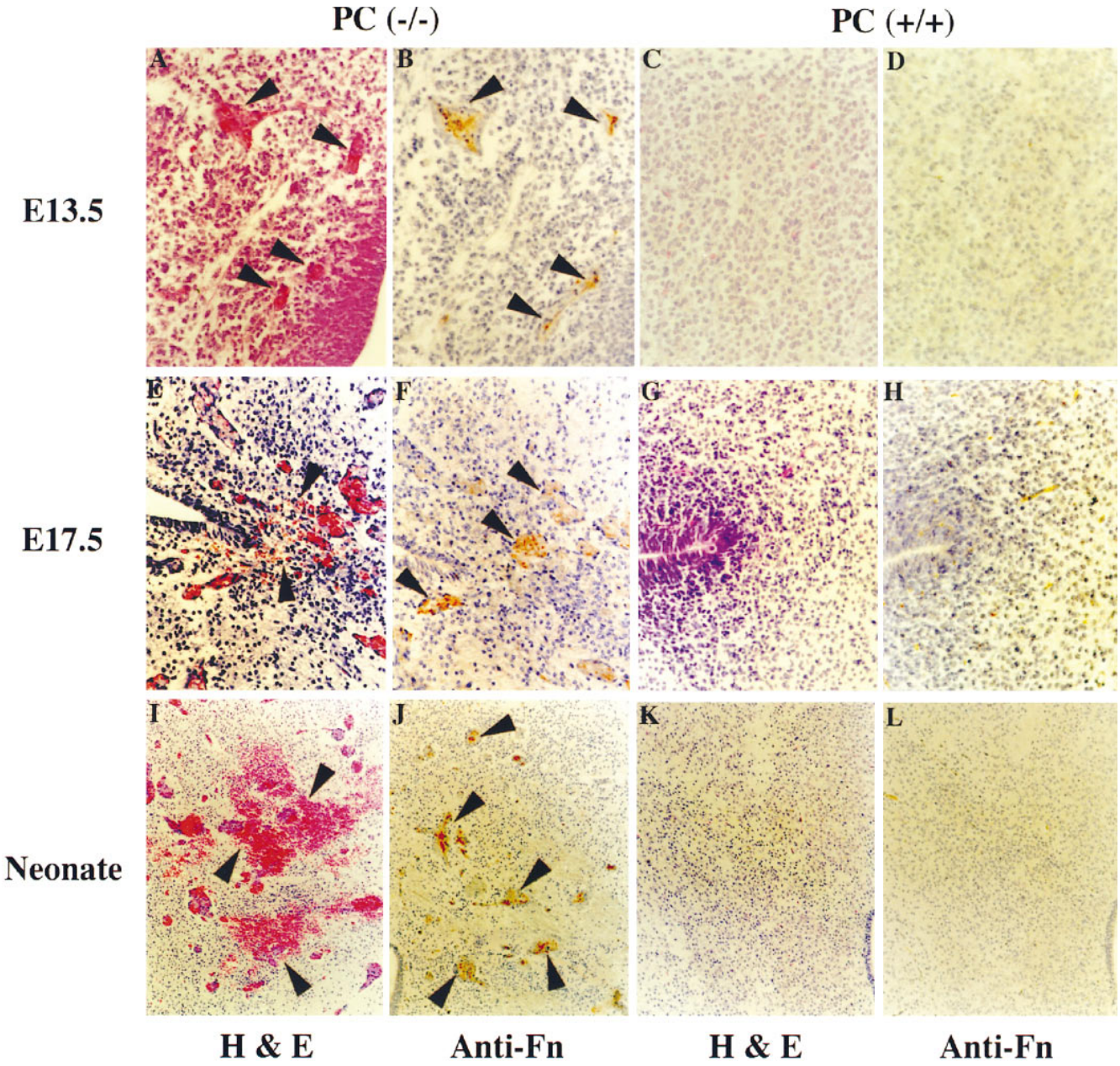

Figure 2. Histological analysis of brain sections of E13.5 (*A–D*), E17.5 (*E–H*), and neonatal (*I–L*) mice. *A*, *E*, and *I* and *C*, *G*, and *K* represent H&E stainings of $PC^{-/-}$ and $PC^{+/+}$ sections, respectively. *B*, *F*, and *J* and *D*, *H*, and *L* represent antifibrin(ogen) stainings of parallel sections to those shown in *A*, *E*, and *I* and *C*, *G*, and *K*, respectively. The arrows in *A* and *B* point to clotted vessels in the telencephalic region. *C* and *D* show no clotting in the corresponding sections of the $PC^{+/+}$ control. The arrows in *E* point to a minor bleeding event near the lateral ventricle. The surrounding clotted vessels are indicated in *F* by the arrows. The $PC^{+/+}$ control stains presented in *G* and *H* show no clotted vessels or bleeding. The arrows in *I* point to areas of extensive bleeding surrounding the clotted vessels indicated by the arrows in *J*. The $PC^{+/+}$ neonatal control sections shown in *K* and *L* demonstrate no bleeding or clotting in the corresponding region of the brain near the lateral ventricle.

fulminans, recurrent venous thrombosis, and DIC is associated with defective PC anticoagulant activity in humans. The severity of the phenotypes in the mouse likely relates to total absence of PC, whereas, even in homozygous PC gene mutations in humans, some residual low level of PC activity is usually observed. The hypercoagulable state of neonatal $PC^{-/-}$ pups emphasizes the vital anticoagulant role of aPC in the maintenance of homeostasis and survival. Progressive hemostatic challenges during embryogenesis crest at the time of birth and thrombotic neonatal death ensues possibly in response to widespread occlusion of the microvasculature in the brain. The combination of thrombosis and bleeding observed in the brain of $PC^{-/-}$ neonates is indicative of a consumptive coagulopathy. With the PC system compromised, control of procoagulant activity appears to be lost and the subsequent depletion of coagulation factors leads to bleeding. Clearly, the PC anticoagulation system provides the balance needed for the regulation of the hemostatic response.

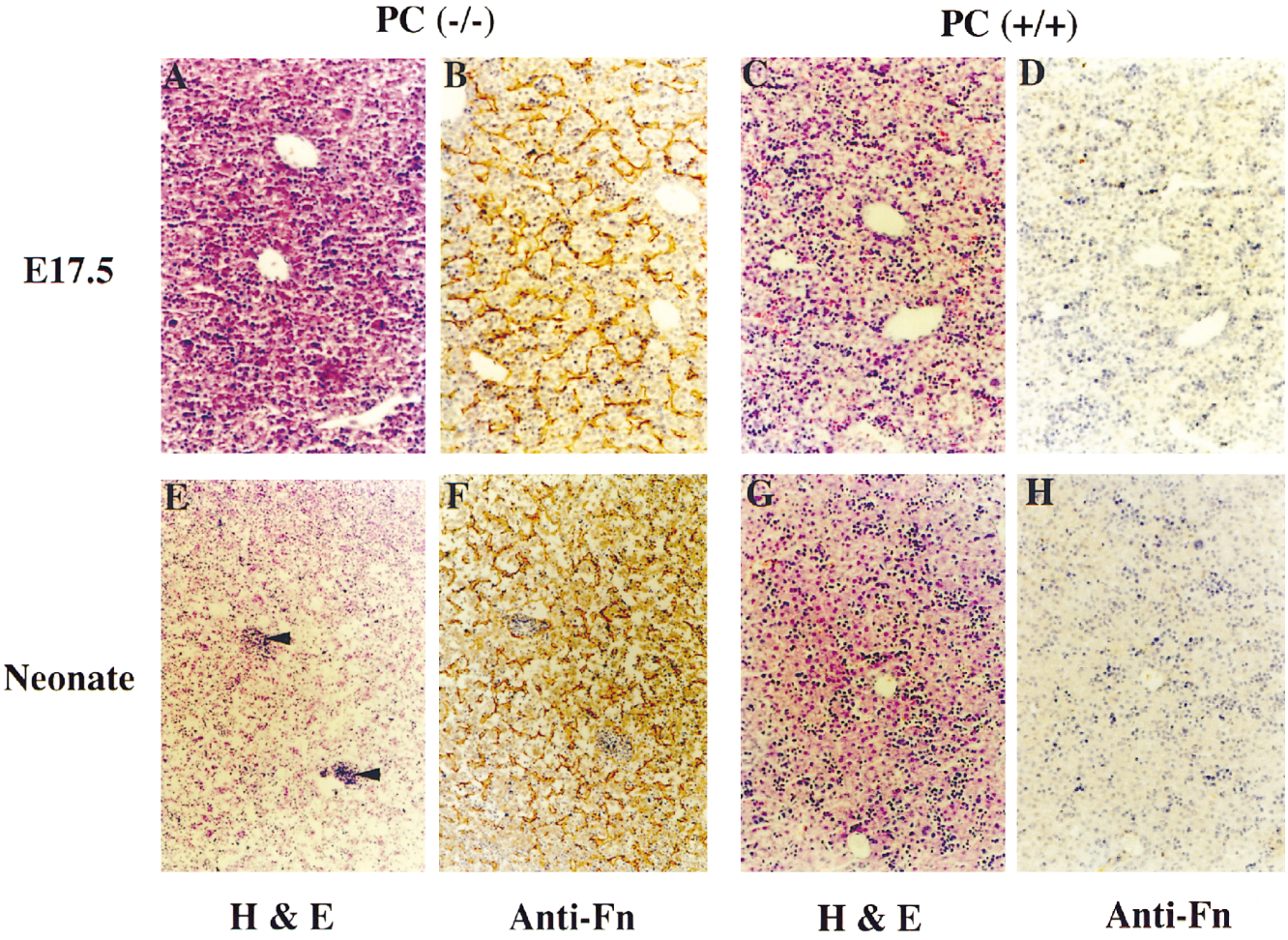

Figure 3. Histological analysis of liver sections of E17.5 (*A–D*) and neonatal (*E–H*) mice. *A* and *E* and *C* and *G* represent H&E stainings of $PC^{-/-}$ and $PC^{+/+}$ sections, respectively. *B* and *F* and *D* and *H* represent antifibrin(ogen) stainings of parallel sections to those shown in *A* and *E* and *C* and *G*, respectively. Interstitial fibrin(ogen) deposition is evident at E17.5 in the $PC^{-/-}$ liver as shown in *B*. The E17.5 $PC^{+/+}$ control section shown in *D* demonstrates no fibrin(ogen) deposition. The $PC^{-/-}$ neonatal liver sections shown in *E* and *F* demonstrate advanced tissue necrosis with the depletion of red blood cells and increased levels of fibrin(ogen) deposition. Tissue necrosis and fibrin(ogen) deposition are not observed in the corresponding neonatal $PC^{+/+}$ stained sections shown in *G* and *H*.

Embryos deficient for TM, another key component of the anticoagulant pathway, are arrested at E8.5 (34), a developmental time at which RT-PCR analyses of normal embryos display signals for the mRNA encoding both PC and prothrombin (Conway, E.M., personal communication). Thus, the embryonic lethality associated with the lack of the *TM* gene, in contrast to the normal embryonic development of mice with a total deficiency of the *PC* gene, implies that the anticoagulant pathway is not required for embryonic development. These observations also suggest that TM may have an essential developmental function not involving its role in the initiation of the PC anticoagulant pathway. However, we cannot completely rule out the possibility that in $PC^{-/-}$ mice TM provides an essential anticoagulant function by interacting with maternally derived PC. This could result from maternal transfer of PC to the embryo, at a level lower than the detectable limit in our assays. Alternatively, since embryonically derived TM at E8.5 is expressed on trophoblasts and parietal endoderm on embryonic membranes (34), it may interact with PC and thrombin in the maternal circulation, thus providing essential anticoagulant functions in the microenvironment surrounding the embryo. Thus, it is possible that the essential developmental role of TM is to inhibit further thrombin generation by interacting with PC derived from maternal sources. On the other hand, it has been demonstrated that mice expressing a mutant form of TM, which fails to effectively activate PC, develop normally (35), although they suffer from a mild thrombotic phenotype. This underscores the possibility that the essential physiologic function of TM in development is other than its role in the initiation of the PC anticoagulant pathway. Further, since the phenotypes of these animals exhibit far less severe symptoms than those of $PC^{-/-}$ mice, where PC activation is not possible, other mechanisms may exist and contribute to activation of PC such that anticoagulant functions needed for survival can be achieved. In this regard, it has been shown recently in vitro that fXa or thrombin can effectively activate PC in the presence of heparin or dextran sulfate in systems lacking TM (36).

Investigations with other targeted gene alterations fortify the concept that hemostasis in E9.5 embryos may not require,

or may only minimally require, fibrin formation. Targeted disruption of the fibrinogen α chain, which prevents normal fibrinogen assembly, does not lead to developmental defects in affected mice (37), nor do complete disruptions of the fVII (38), fVIII (39), and fIX (40) genes, which are also needed to different extents for fibrin formation. Furthermore, in an earlier study, its was shown that fibrin clots do not form in any of the embryonic or vitelline vessels after intracardial injection of thrombin in E9.5 embryos cultured in vitro at levels three orders of magnitude higher than those required to induce lethal thrombosis in adult mice (38). Taken together, these results suggest that fibrin formation is not required for normal development in early embryos. While fetuses completely deficient in fVII, fVIII, fIX, α-fibrinogen, or PC display different degrees of bleeding and tissue necrosis at later times in development, these appear to be indirect effects of the coagulopathy rather than a primary effect of the genetic deficiency on embryogenesis and development. In addition, platelet-mediated hemostasis is probably also not essential at these early developmental times, since transcription factor NF-E2–deficient mice, in which platelet counts are drastically reduced to the point that postnatal animals suffer a severe and fatal bleeding diathesis, develop normally (41).

On the other hand, a total deficiency of the prothrombin (42, 43) and fV (44) genes leads to a substantial percentage of animals exhibiting early embryonic death. These results suggest that thrombin formation may be important to embryogenesis in nonhemostatic roles that do not lead to fibrin or thrombus formation. In this regard, it is pertinent that deficiency of the thrombin receptor, PAR-1, causes partial embryonic lethality (45). It is also relevant that thrombin is required for embryogenesis, whereas genes involved in the known pathways leading to thrombin generation (fVII, fVIII, and fIX) are not. Perhaps the TF/fVIIa and fVIIIa/fIXa pathways provide somewhat redundant mechanisms for the activation of fX leading to thrombin generation. Alternatively, these results suggest that there may be other essential pathways leading to thrombin production during embryogenesis. In this regard, cancer procoagulant, a cysteine protease that is expressed on embryonic membranes and activates fX (46), may provide such a function.

Normal birth rates of $PC^{-/-}$ mice also contrast with the 60% embryonic lethality observed at E9.5–E11.5 in animals containing a targeted mutation of the fVIIa/TF binding region of TFPI (47). This observation is of interest because TFPI also serves an anticoagulant role through its fXa-dependent inhibition of fVIIa/TF. Thus, it is possible that a loss of anticoagulant activity at the level of fVIIa or fXa may be more inconsistent with survival than that produced by a PC deficiency because of possible differences in the relative efficiencies with which the PC deficiency and TFPI mutation interfere with the inhibition of thrombin generation. On the other hand, TFPI may serve other primary functions during development, perhaps through neutralization of other fXa-dependent functions.

In addition to its known role as an anticoagulant, other less well-characterized physiological and pathological processes that involve the PC system might have appeared in the pathology of the $PC^{-/-}$ mouse. On a molecular level, aPC has been implicated in modulating host–defense reactions that occur during inflammation (16). In E18.5 $PC^{-/-}$ mice that are spared the birthing trauma and its associated induction of more severe thrombosis and bleeding states, inflammation, as indicated by the infiltration of leukocytes in the liver interstitia, together with the onset of necrosis, was observed. While the very extensive brain necrosis, with secondary edema and bleeding (resulting in increased intracranial pressures), with associated suppression of vital cardio-respiratory functions, is the most likely cause of death of the $PC^{-/-}$ animals, the liver necrosis and inflammation might have also played direct or indirect roles in the acute lethality.

This study has demonstrated that although PC does not appear to play a significant role in embryogenesis, it does exhibit its requisite activity as an anticoagulant and/or profibrinolytic agent as early as E12.5. Thrombotic onset, evidenced by slight fibrin deposition, is localized primarily in the brain at the early developmental stages, but progresses and is dramatically exaggerated after birth. Later stage embryos (≥ E17.5) and neonates display thrombotic changes in tissues other than the brain, such as liver, kidneys, heart, and lungs, albeit to a much lesser degree. This is suggestive of early signs of DIC. In conclusion, the PC system is a key component in the regulation of hemostasis, and is vitally important for survival. Total PC deficiency presents a severe pathological state and is inconsistent with a favorable long-term outcome.

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