Role of the renin-angiotensin system in primitive erythropoiesis in the chick embryo

Katia Savary, Annie Michaud, Judith Favier, Etienne Larger, Pierre Corvol, and Jean-Marie Gasc

Inactivation of the gene encoding mouse angiotensin I-converting enzyme (ACE), which converts angiotensin I into angiotensin II, results in anemia in adult animals. This anemia is corrected by angiotensin II, demonstrating the involvement of angiotensin II in adult (definitive) erythropoiesis. We investigated the possible role of the renin-angiotensin system (RAS) in primitive erythropoiesis in the yolk sac of the chicken embryo. Enzymatically active ACE was detected in the yolk sac endoderm, concomitantly with the differentiation of blood islands in the adjacent yolk sac mesoderm. The simultaneous presence of all the other components of the RAS (renin, angiotensinogen, angiotensin II receptor) in the vicinity of the blood islands suggests that this system is involved in erythropoiesis. This role was confirmed by in vivo blockade of the RAS with fosinoprilate, a specific inhibitor of chicken ACE, which decreased hematocrit by 15%. A similar decrease in hematocrit was observed following treatment with the angiotensin II receptor antagonist Sar1-Ile8-Angiotensin II, suggesting that this effect was mediated by angiotensin II. Both treatments affected hematocrit by decreasing erythroblast proliferation. Thus, the RAS, and its effector peptide angiotensin II in particular, modulates primitive erythropoiesis. [Blood. 2005;105:103-110]

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Introduction

Angiotensin I-converting enzyme (ACE) is a key element of the renin-angiotensin system (RAS), an integrated system of peptides, enzymes, and membrane receptors, the principal role of which is the regulation of blood pressure and hydromineral homeostasis. This function is accomplished by generation of the octapeptide angiotensin II (Ang II) from the inactive decapeptide angiotensin I (Ang I).1 Ang II exerts a strong vasopressor effect, but evidence is accumulating that this molecule has several other important functions: it has been found to be proinflammatory,2 to facilitate the generation of reactive oxygen species (ROS),3 and to possess hypertrophic and hyperplastic properties in various cell types.4-7

The RAS also appears to regulate hematopoiesis, in both experimental models and humans. Indeed, ACE knock-out mice present isolated normochromic anemia,8 whereas in humans, anemia and leukopenia have been reported following the initiation of treatment with high doses of ACE inhibitors.9,10 RAS blockade with Ang II receptor antagonists or ACE inhibitors has even been used to treat patients with posttransplantation erythrocytosis or polycythemia vera.11,12 Finally, further indirect evidence for involvement of the RAS in hematopoiesis is provided by the marked increase in plasma renin concentration reported in patients with chronic obstructive pulmonary disease accompanied by secondary erythrocytosis,13 suggesting a common causal relationship.

This demonstration of a role for ACE in the regulation of adult hematopoiesis led us to investigate the possible role of this enzyme in another important hematopoietic event: primitive hematopoiesis. We used a simple and comprehensive model: the chicken embryo yolk sac. Primitive hematopoiesis, which is restricted to the erythroid and macrophage lineages, is gradually completely replaced by definitive hematopoiesis, originating from the embryo itself. However, gene inactivation studies in mice have shown that some transcription factors, such as SCL/tal-1,14,15 GATA-1,16,17 and GATA-2,16,18 are involved in both primitive and definitive hematopoiesis, whereas others, such as AML-1,19 and the erythropoietin receptor,20 are specifically involved in definitive hematopoiesis.

Primitive hematopoiesis occurs in the yolk sac, from structures called blood islands. At stage HH8 (stage 8, Hamburger and Hamilton table of embryonic development), the first blood islands differentiate within the mesoderm, in the extraembryonic area. Two cell lineages arise from these blood islands: endothelial cells and hematopoietic cells. The very first cells containing hemoglobin are detected at this stage, in the caudal part of the extraembryonic area. Very few of these cells are present at first, and they begin to increase considerably in number only at stage HH9 to HH10. At stage HH12 to HH13, circulation between the embryo and the yolk sac is established, and at stage HH15 to HH16, complete maturation of the blood islands is accompanied by vascular remodeling.21 Until day 5 or 6 of development, primitive erythroblasts are released from the yolk sac blood islands into the bloodstream. These precursor cells divide and mature in the bloodstream.22

We thought that the RAS might be involved in erythropoiesis and therefore investigated the ontogeny and functionality of this system, and particularly of a key enzyme of this system, ACE, in the chicken yolk sac model. We carried out this study between the laying of the egg and the fourth day of development, a period encompassing most of the morphogenetic events and tissue interactions required for embryonic organ and tissue patterning. The basic processes
occurring during this period of development are readily accessible and have been extensively studied in the chick embryo, whereas they are difficult to access and more complex in the mammalian embryo.

We began by studying the expression of ACE and the other components of the RAS in the chicken embryo, in the first few days of development. We detected the expression of ACE, renin, angiotensinogen (AGT), and the chicken angiotensin II receptor (cAT) as early as stage HH8. At the same time, blood islands were differentiating in the extraembryonic area adjacent to the sites of RAS component production. We then investigated the effects on primitive erythropoiesis of blocking the RAS at 2 levels, using an ACE inhibitor and a cAT receptor antagonist. We found that ACE had a positive effect on primitive erythropoiesis and that this effect required the generation of angiotensin II.

### Materials and methods

#### Embryonic material: chicken embryos

Fertilized eggs (from White Leghorn chickens) were provided by a commercial breeder (Haas, Kaltenhouse, France). They were incubated at 38°C in a humid atmosphere (70% to 80% relative humidity), as previously described.23

The developmental stages of chicken embryos were determined according to the Hamburger and Hamilton (HH) table of development24 or were defined according to the duration of incubation in hours or days.

#### Reverse transcription and polymerization chain reaction

Total RNA was prepared from the whole blastodisc or from the extraembryonic area, using the Nucleospin RNA L Kit (Macherey-Nagel, Horrjet, France). The last stage of the RNA purification procedure consisted of treatment with DNase to eliminate potential genomic DNA contamination. First-strand cDNAs were then synthesized, using 1 μg total RNA, and 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) was performed with 1/20 of the reverse transcription reaction mixture. Control experiments without reverse transcriptase were performed to ensure that no genomic DNA was present. The primers used for PCR are shown in Table 1. Reverse transcriptase (RT)-PCR was made semiquantitative by including cGAPDH primers in the PCR mixture, to make it possible to normalize expression of the gene of interest. The relative amounts of amplified products were analyzed with a Bio-Rad Phosphorimager and Quantify One software (Bio-Rad, Hercules, CA).

#### Riboprobes for in situ hybridization (ISH)

We used the following cDNAs to generate labeled riboprobes for in situ hybridization: the somatic chicken ACE23 cDNA was inserted into the Bluescript KS vector (Stratagene, Amsterdam, The Netherlands). The cAT receptor cDNA was inserted into pCDNA3 (Stratagene). The chicken vascular endothelial growth factor (VEGF) cDNA was inserted into pcRII (Stratagene). The qual receptor VEGF receptor cDNA (VEGFR2) was inserted into pCDNAI/Amp (Stratagene). The chicken AGT and the chicken renin cDNAs were cloned by RT-PCR from total RNA from embryonic day 15 (E15) kidneys. Sequences homologous to the mammalian AGT cDNA sequence were identified in the chick expressed sequence tag (EST) database (Delaware Biotechnology Institute [http://www.chickost.udel.edu/]), and primers were designed to clone the cAGT cDNA. The chicken renin cDNA was cloned using primers designed from sequences known to be conserved between species. PCR products were cloned with the TA cloning kit (Invitrogen), such that the cDNA was inserted into pcRII and sequenced. Radioactive riboprobes were generated with RNA polymerase (Roche, Mannheim, Germany) from linearized plasmids.

The ACE riboprobe for whole-mount in situ hybridization was generated with the DIG-RNA Labeling Kit (Roche).

#### In situ hybridization

The ISH technique used has been described in detail elsewhere.26 Whole-mount in situ hybridization was performed as described by Henrique et al.27

#### Antibodies

Anti–chicken ACE antibodies were produced by immunizing a rabbit against a mixture of 2 peptides corresponding to 2 different selected sequences in the ACE N-terminal extracellular domain, peptide 1: RSWYDSTTFEDDDLE (amino acid [AA] 126 to 139), peptide 2: VRRKLYDRYGPKYIC (AA 156 to 169) according to the chicken ACE sequence numbering used by Esther et al.25 Antibodies were obtained after 4 injections of the mixture over a 10-week period. Only serum obtained from the last bleeding was used. Preimmune serum was used as a control, and the specificity of antibodies was assessed by prior incubation with the corresponding peptides (3 μg/mL, approximately 50-fold molar excess).

#### Immunohistochemistry

ACE protein was detected on paraffin sections by incubation with the anti–ACE serum generated in rabbit (dilution, 1/200), with routine signal amplification by ABC Elite (Vector, Burlingame, CA), and diaminobenzidine (DAB) was used as the chromogen for detection.

#### Apoptosis

Apoptotic cells were stained using the transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) technique, as previously described for paraffin section.28

#### Preparation of membrane proteins and determination of ACE enzymatic activity

Whole embryos, corresponding to about 200 mg tissue, were dissected and homogenized in 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; Sigma, St Louis MO), pH 7.2, 8.8 mM 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS; Sigma) and centrifuged for 30 minutes at 15 000 g and 4°C. The supernatant was used for the determination of total protein concentration (Lowry colorimetric assay) and ACE enzymatic activity. ACE activity was measured on 10 to 25 μL supernatant containing the solubilized enzyme from total embryo, as described by Wei et al.29 ACE activity was determined using the synthetic substrate hippuryl-histidine-leucine (HHL). The production of hippuric acid from HHL by hydrolysis was detected after incubation at 37°C for 4 hours. The product was resolved and quantified by reverse-phase high-performance liquid chromatography (HPLC). All enzymatic activities were tested in the presence or absence of 10−5 and 10−3 M ACE inhibitor lisinopril, which had an IC50 (inhibitory concentration of 50%) for native chicken ACE estimated at approximately 4.2 nM in preliminary experiments. For inhibition experiments, enzyme and inhibitor were incubated

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**Table 1. Sequence of the primers for PCR amplification of the chicken ACE, AT receptor, renin, AGT, and GAPDH cDNAs**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sense and antisense primer sequences</th>
<th>PCR product, length</th>
</tr>
</thead>
<tbody>
<tr>
<td>cACE</td>
<td>5′ GCC TGT GCC ACT CCT GGA GTA TT 3′ 5′ GGA CCA TGA CTD GGC CCA CAT 3′</td>
<td>630 bp</td>
</tr>
<tr>
<td>cAT</td>
<td>5′ CTGTTTGCAGGAAGCCGACATTAC 3′ 5′ TGCTATACCAAAACCCACGCAC 3′</td>
<td>519 bp</td>
</tr>
<tr>
<td>cRenin</td>
<td>5′ CCGGTGACGACGGACGAGGG 3′ 5′ GACGTTCGCTGACGGCAGG 3′</td>
<td>495 bp</td>
</tr>
<tr>
<td>cAGT</td>
<td>5′ CAGACACACCTGCGACGACC 3′ 5′ CCCTGATACGAGCACAGGAG 3′</td>
<td>546 bp</td>
</tr>
<tr>
<td>cGAPDH</td>
<td>5′ TGT TAT CCA TGT TCC CCA CAT CACTAT 3′ 5′ GCC CTA TCA TGC GCT TTG 3′</td>
<td>822 bp</td>
</tr>
</tbody>
</table>

GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; bp, base pair.
together for 1 hour at 37°C before adding the substrate to allow steady-state equilibrium between the enzyme and its inhibitor to be reached.

ACE inhibition activity

We assessed the ability of 3 inhibitors of mammalian ACE (captopril, lisinopril, and fosinoprilate) to inhibit recombinant chicken ACE obtained from stably transfected Chinese hamster ovary (CHO) cells, as described by Wei et al. 30

In vivo treatment and blood sample collection

Embryos were incubated for 42 or 50 hours. They were then cracked open, and the embryo was deposited in a plastic Petri dish. Embryos were then treated with 20 μL of either the ACE inhibitor fosinoprilate or the cAT receptor antagonist Sar1-Ile8-Angiotensin II (Sar1-Ile8-Ang II) at various concentrations (0.1 to 1 mg/mL in mineral oil). They were incubated for a further 48 hours, and blood was then obtained by section of the vitelline vein, using a calibrated glass micropipette, with 2.5 to 5 μL blood taken from each embryo. Blood samples were used for hematocrit or mitotic index measurements.

Hematocrit and mitotic index

Hematocrit was determined after brief centrifugation (5 minutes, 800g) of the blood samples in a calibrated micropipette (inner diameter, 0.58 mm). Mitotic index was determined under a microscope, for blood smears briefly fixed in methanol and then stained with May-Grünwald Giemsa.

Capture and manipulation of the images

Slides used for in situ hybridization, immunohistochemistry, and TUNEL techniques were counterstained with toluidin blue and mounted in Eukitt. Images were analyzed under an Ortholux II microscope (Leica, Rueil-Malmaison, France) using IPLab software (Roper Scientific, Evry, France) and captured with a Polaroid DMC camera (Leica) using Adobe Photoshop software (Adobe, San Jose, CA). Images of whole mount in situ hybridization were analyzed under MZLF III stereomicroscope and captured with the CoolSNAP camera (Roper Scientific, Evry, France) using IPLab software. Images were improved (luminosity and contrast) using the Photoshop software without altering the scientific information contained in the original picture.

Statistical analysis

The results are expressed as means ± SEM. Differences between experimental groups were analyzed with the nonparametric Mann-Whitney test. Values of P less than .05 were considered statistically significant.

Results

Early expression of ACE and the other components of the RAS in the chicken embryo yolk sac

We used semiquantitative RT-PCR to determine the stage at which ACE mRNA first became detectable during chicken embryogenesis. A faint band (630 bp) corresponding to ACE mRNA was already detectable in the unincubated egg (in birds, the laid egg already contains more than 10^8 cells). Semiquantitative RT-PCR showed that the amount of ACE mRNA present increased by a factor of more than 20 during the first 56 hours of development (Figure 1A).

At 24 hours of development (stage HH6), RT-PCR on mRNA extracted from the yolk sac after removal of the embryo itself showed that mRNAs encoding ACE, AGT, renin, and cAT receptor were present in the yolk sac (Figure 1B).

Figure 1. ACE expression and enzymatic activity during the first few days of chicken embryo development. (A) Expression and semi-quantification of ACE mRNA by RT-PCR in whole embryo including the yolk sac. The amount of ACE mRNA from 0 to 56 hours of development (HH0 to HH16) was normalized according to cGAPDH mRNA levels. (B) Levels of ACE, AGT, renin, and cAT mRNA, as determined by RT-PCR (35 cycles) in the yolk sac at 24 hours of development (stage HH6). (C) ACE activity in embryos with their yolk sacs from 18 to 56 hours of development (HH4 to HH16), evaluated by hydrolysis of the highly specific synthetic substrate HHL (see "Material and methods"). Each point represents ACE activity in a pool of n = 30 (stage HH4) to n = 3 (stage HH16) embryos (duplicate values).

Ontogeny of ACE: enzymatic activity, spatiotemporal expression pattern, and distribution in the various cell types

During the first 56 hours of development, we measured ACE activity in extracts of whole blastodisc (embryo plus yolk sac). ACE activity was detectable from 18 hours of development onward and increased by a factor of 30 between 18 hours (stage HH4) and 56 hours (stage HH16) of development (Figure 1C). This activity was inhibited by lisinopril (75% inhibition at 10^-6 M and full inhibition at 10^-3 M) and can therefore be attributed to ACE itself.

Whole-mount in situ hybridization with a non–radioactive probe was used to determine the detailed spatio-temporal pattern of ACE synthesis at stages HH4, HH6, HH9, and HH18. In situ hybridization and immunohistochemistry on sections were used to identify the cell types producing ACE.

During the first 2 days of development, ACE was expressed in a precisely controlled pattern. At stage HH4, ACE was detected exclusively in the anterior part of the extraembryonic area (Figure 2A). ACE synthesis then rapidly extended laterally and caudally in the extraembryonic area, on both sides of the embryo (stage HH6), forming a horseshoe arch (Figure 2B). It finally covered the entire extraembryonic area at stage HH9 (Figure 2C), when the heart was the only site of intraembryonic ACE production. On day 3 of embryonic development, ACE was expressed throughout the vascular area (Figure 2D).

Thus, ACE was expressed and enzymatically active in the extraembryonic area, from stage HH4 onward.

ACE expression eventually reached the caudal part of the yolk sac endoderm at 24 hours of development (stage HH6), before the start of blood island differentiation in the adjacent extraembryonic mesoderm, as shown on a caudal section of the embryo after in situ
hybridization with the ACE riboprobe (Figure 3A-B). The ACE protein was also detected at the same location as its mRNA, as shown by in situ hybridization (Figure 3C) and immunohistochemistry (Figure 3D).

The blood islands emerge from the splanchnopleural mesoderm of the extraembryonic area by condensation of stem cells called hemangioblasts.30-33 These cells are bipotent: cells situated at the periphery of the aggregates differentiate into endothelial cells, whereas those within the core of the blood islands are committed to the hematopoietic lineage. VEGF and its receptor VEGFR2 are key factors in this differentiation. We therefore compared the spatio-temporal patterns of VEGF and VEGFR2 expression with that of ACE, by in situ hybridization. At stage HH6, ACE was located in the endodermal layer of the extraembryonic area (Figure 3E-F), whereas VEGF (Figure 3G) and VEGFR2 (Figure 3H) were expressed in the embryonic and extraembryonic mesoderm.

Localization of all the components of the RAS during blood island differentiation

At stage HH8, in situ hybridization (Figure 4A-C), and immunohistochemistry (data not shown) demonstrated a high level of ACE production in the yolk sac endoderm, which is in contact with the aggregates of hemangioblasts in the mesoderm. The spatio-temporal pattern of ACE expression thus suggests that this enzyme may be involved in regulating the process of blood island differentiation.

The distributions of AGT, renin, and cAT were determined by in situ hybridization. AGT was expressed in small amounts in both the mesoderm and, more markedly, in the extraembryonic endoderm (Figure 4D-E). Renin (Figure 4F-G) and cAT receptor (Figure 4H-I) mRNAs were detected in both the embryonic and extraembryonic mesoderm.

Effect of ACE inhibition

The pattern of ACE expression in the endodermal layer at early stages of embryonic development suggests that this enzyme is involved in the differentiation of blood islands during the first wave of hematopoiesis. We evaluated the putative functional role of ACE during these early differentiation events, using a potent, specific inhibitor to block its enzymatic activity.

We tested several mammalian ACE inhibitors, lisinopril, captopril, and fosinoprilate, for ability to inhibit chicken ACE in vitro. The IC50 values for these 3 inhibitors were similar (7.94 ± 1.36 × 10^–9 M, 2.1 ± 0.62 × 10^–9 M and 1.04 ± 0.5 × 10^–9 M, respectively) (Figure 5). These results were consistent with those obtained in preliminary experiments with native chicken ACE (data not shown), fosinoprilate being the most efficient in any case.

We therefore used this inhibitor to abolish chicken ACE activity in vivo. It was administered on day 2 of development (stage HH11 or HH13), and its effect on circulating red blood cells was evaluated 48 hours later by measuring hematocrit. Hematocrit was found to be 15% lower (24.67% ± 0.59% versus 28.97% ± 0.59%; P < .0001) in fosinoprilate-treated embryos than in control vehicle-treated embryos if the inhibitor was administered at stage HH11 (Figure 6A). The period of sensitivity to ACE inhibitor was limited, and hematocrit was unaffected (26.07% ± 1.05% versus 27.05% ± 0.84%) if the embryos were treated later in development, at stage HH13 (P = .53).

Role of the RAS in primitive erythropoiesis

The decrease in hematocrit following ACE inhibition may result from the lack of Ang II or from the activation or degradation of another peptide processed by the same enzyme. If the hematocrit-lowering effect of ACE inhibition was actually due to the abolition of Ang II production, blocking the RAS at any other level should produce the same effect. We therefore carried out the same experiment with the specific chicken Ang II receptor antagonist,
Sar1-Ile8-Ang II. The application of Sar1-Ile8-Ang II to stage HH11 embryos (Figure 6A) resulted in an 18% decrease in hematocrit 48 hours later (23.23% ± 0.63% versus 28.48% ± 0.8%; \( P = .002 \)), that is, a decrease similar to that observed in fosinoprilate-treated embryos. As observed with ACE inhibitor treatment, embryos treated at stage HH13 were not sensitive to the Ang II receptor antagonist (27.2% ± 1.02% versus 28.48% ± 0.77%; \( P = .34 \)), confirming the existence of a short-time window in which erythropoiesis was dependent on RAS and Ang II.

In another series of experiments, to show that the effect of the treatment depends not only on the stage of the application but also on the dose, we applied 3 concentrations of Ang II receptor antagonist: 0.1, 0.3 and 1 mg/mL. As shown in Figure 6B, at the lowest dose of Ang II receptor antagonist, the effect was minimal and not significant (\(-6.09% \pm 4.05%; \ P = 1\)), and, at 0.3 mg/mL, the effect reached 13.98% ± 3.12% (\( P < .001 \)). At the highest dose tried (1 mg/mL), we observed a toxic effect leading to 40% mortality, thus precluding a valid conclusion, since one can assume embryos affected by the treatment are more fragile and more prone to die than those not affected.

Then, we investigated the mechanisms by which these changes in hematocrit occurred by determining the proliferation index of erythroid cells in the bloodstream, in embryos treated with fosinoprilate or Sar1-Ile8-Ang II (Figure 7). The percentage of erythroid cells dividing in the bloodstream was significantly and similarly lower in embryos treated with either the ACE inhibitor (2.16% ± 0.05% versus 3.18% ± 0.18%; \( P < .002 \)) or the cAT receptor antagonist (1.67% ± 0.096% versus 3.18% ± 0.18%; \( P < .002 \)) than in the control embryos.

We also assessed the possible effects of RAS blockade on the apoptosis of endothelial cells and circulating blood cells. Very few

![Figure 4. Expression of ACE, AGT, renin, and cAT mRNAs during blood island differentiation (stage HH8). Transverse sections in the posterior half of the blastodisc at 29 hours of development (stage HH8) for in situ hybridization. (A-B) ACE expression in the extraembryonic endoderm. (C) Blood islands differentiating in the mesoderm (me), between the ectoderm (ec), and ACE-producing cells in the endoderm (en). Asterisks indicate blood islands (higher magnification of the view from panel A). For comparison, panels D and E show the expression of AGT, panels F and G, the expression of rennin; and panels H and I, the expression of cAT. (A-C,D,F,H) Bright-field illumination. (B,E,G,I) Dark-field illumination. (A-B) Original magnification, \( \times 9 \); type of the objective lenses, EF 4; numerical aperture 0.12. (C) Original magnification, \( \times 60 \); type of the objective lenses, PL APO \( \times 25 \); numerical aperture 0.70. (D-I) Original magnification, \( \times 22 \); type of the objective lenses, EF \( \times 10 \); numerical aperture 0.26. Scale bar: 500 \( \mu m \) (A-B), 100 \( \mu m \) (C), 200 \( \mu m \) (D-I).

![Figure 5. Potency of various specific ACE inhibitors. Determination of the IC50 of various ACE inhibitors: captopril (○), lisinopril (■) and fosinoprilate (▲). Chicken ACE activity was measured in the presence of various concentrations of inhibitor. Each point is the mean of 2 values.

![Figure 6. Effect of RAS blockers on hematocrit. (A) Percentage of hematocrit change in fosinoprilate- and Sar1-Ile8-Ang II–treated embryos, relative to control embryos. Embryos treated with fosinoprilate (Fos, III) at stage HH11 (n = 19) or at stage HH13 (n = 8), and embryos treated with Sar1-Ile8-Ang II (Sar, ▲) at stage HH11 (n = 13) or at stage HH13 (n = 7), in comparison to control embryos treated with vehicle only at stage HH11 (n = 17) or at stage HH13 (n = 8). (B) Dose-dependent effect of Sar1-Ile8-Ang II on hematocrit. Results are expressed as a percentage of control hematocrit values (c, n = 32, ■, in embryos treated with 0.1 mg/mL (n = 7) or 0.3 mg/mL (n = 16) Sar1-Ile8-Ang II (▲). Each bar is a mean \( \pm \) SEM. * \( P < .006 \), ** \( P < .002 \), and *** \( P < .001 \).]
apoptotic cells (< 1%) were detected in the yolk sac on day 3 of development, 24 hours after treatment with vehicle only, the ACE inhibitor or the cAT receptor antagonist.

Discussion

The results reported here show that ACE mRNA was expressed very early in the development of the chicken embryo. ACE mRNA was detected by RT-PCR in unincubated eggs and rapidly increased in abundance during the first hours of development, in line with ACE enzymatic activity. ACE was present within the endoderm, initially in the anterior half of the extraembryonic area at stage HH4, and then, at stage HH9, throughout the extraembryonic area. ACE was found in close contact with the blood islands, which first differentiate in the posterior part of the extraembryonic mesoderm (stage HH8). The precocity and pattern of ACE synthesis is not specific to the chicken embryo. In the mouse embryo on day 7 of development, before the appearance of blood islands in the yolk sac, ACE is also present in the extraembryonic endoderm, as shown by immunohistochemistry (K.S., K.B., P.C., J.-M.G., unpublished observations, 2004).

Blood island differentiation is regulated by factors located in the vicinity of the blood islands. These factors include VEGF and its tyrosine kinase receptor VEGFR2, which play a critical role in endothelial commitment. Inactivation of the VEGFR213,35 or VEGF36,37 gene affects both primitive hematopoiesis and vasculo-genesis, as these 2 processes are closely linked. VEGFR2 is also commonly used as an early marker of differentiation of the endothelial lineage because the gene encoding this factor is among the first to be expressed during the formation of the vascular system.38 Comparison of the distribution of ACE with that of VEGF and its receptor VEGFR2 revealed 2 complementary expression patterns overlapping caudally from stage HH6 onward. The expression of ACE in the anterior part of the extraembryonic area at stage HH4 is consistent with a possible role of ACE in the differentiation of blood islands. Indeed, the endoderm of the anterior extraembryonic area has been shown to be an effective stimulus for blood island formation in the posterior mesoderm.39 In addition, GATA-2, which has been reported to be involved in blood cell proliferation, displays a similar rostrocaudal pattern of synthesis at this early stage.40

On the basis of observation of the spatio-temporal pattern of ACE synthesis and the distribution of ACE, in the endoderm in close contact with the blood islands, we thought that ACE might affect hematopoiesis and/or vasculogenesis, depending on the substrates present in the environment of the enzyme. We therefore investigated the effect of a potent and specific ACE inhibitor on primitive erythropoiesis. No major effect of ACE inhibition on the vascular network was observed, but a single application of the specific ACE inhibitor significantly decreased the hematocrit, suggesting that ACE has a positive effect on the first generation of erythrocytes during embryogenesis. The apparent 15% decrease in hematocrit may actually be an underestimate of much stronger inhibition of erythropoiesis. Indeed, a much larger decrease in erythrocyte production would be required to account for a 15% decrease in the preexisting erythroblast population.

These data raised the question of the substrate involved in the anemia induced by ACE inhibition, as several factors could account for this effect. AcSDKP (N-acetyl-seryl-aspartyl-lysyl-proline), a negative regulator of hematopoietic stem cell differentiation,41-43 is continually degraded by ACE.44,45 It could therefore be involved in the effect of ACE on primitive erythropoiesis. Ang I, the major natural ACE substrate, is also a candidate because Ang II has been shown to exert a proerythropoietic effect in mouse8 and in humans.12 However, this would require the early and simultaneous expression of all the elements of the RAS. RT-PCR and in situ hybridization showed that AGT, renin, ACE, and cAT receptor are expressed early in development in the chicken embryo yolk sac, consistent with the RAS playing a functional role in this tissue during blood island differentiation.

We evaluated the possible role of Ang II in primitive erythropoiesis in chicken, using a specific peptide antagonist of the cAT receptor, Sar1-Ile8-Ang II, as the cAT receptor is not blocked by mammalian nonpeptide Ang II receptor blockers.46

In vivo treatment with Sar1-Ile8-Ang II decreased hematocrit levels to the same extent as fosinoprilate, during the same time window, strongly suggesting that the proerythropoietic effect of ACE specifically involved Ang II.

The low hematocrit in ACE inhibitor-treated or Sar1-Ile8-Ang II–treated embryos may be due to changes in the proliferation, maturation, or survival of erythroid progenitors. Indeed, the mitotic index of erythroid cells indicated that the decrease in hematocrit levels resulted from a marked decrease in erythroid cell proliferation, in the presence of ACE inhibitor (−32%), or Ang II receptor blocker (−47%). This effect on erythroblast proliferation probably results from earlier inhibition, at tissue level, before blood cells are released from the yolk sac into the bloodstream. Indeed, all the components of the RAS are expressed close to the blood islands in the yolk sac. Moreover, the detection of cAT by in situ hybridization in the whole extraembryonic mesoderm suggests a possible autocrine and/or paracrine action of Ang II on blood island differentiation. In adult bone marrow, the Ang II type 1 receptor is present on CD34+CD38− cells, CD34+CD38+ cells, lymphocytes, and stromal cells.47 Ang II may have a positive effect on hematopoiesis in adults by directly activating hematopoietic stem cell proliferation, or indirectly activating the synthesis of growth factors by stromal cells.

In other models, Ang II has been shown to promote the growth of early erythroid progenitors,48 via the Ang II type 1 (AT1) receptor, which is known to activate several intracellular pathways possibly involved in adult hematopoiesis. One of these pathways, the Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway, is activated by several cytokines and growth factors such as interleukin-12 and insulinlike growth factor-1.49,50 This pathway may also be involved in the effect of Ang II on
erythropoietin synthesis in the kidney. 51 The second pathway activated by Ang II, the nuclear factor kappaB (NFκB) pathway, controls the expression of genes involved in hematopoiesis. 52 Finally, Ang II activates the mitogen-activated protein kinase (MAPK) pathway, which has a mitogenic effect. 53 However, primitive hematopoiesis is regulated at least by some factors differing from those involved in adult hematopoiesis. Therefore, the pathway actually involved in the effect of Ang II on primitive erythropoiesis is the Jak/STAT pathway. Indeed, Jak2+/− mice are anemic, due to changes in both primitive and definitive erythropoiesis. Moreover, studies in zebrafish have shown that the gene homologous to Jak2 (jak2a) is transiently expressed during primitive erythropoiesis. 40 Its expression is thus consistent with a potential action of Jak2a on progenitors or immature erythroblasts but not on erythroid cells at later stages of maturation. In our model, this could account for primitive erythropoiesis being sensitive to RAS blockade at stage HH11, but not at stage HH13, when blood island maturation is almost complete.

In conclusion, our results show that ACE is produced and functional during early embryogenesis, at a stage when blood circulation is not yet established and systemic regulation of blood pressure by the RAS is therefore not yet required. The location of ACE in the extraembryonic endoderm, in contact with the first blood islands differentiating in the posterior extraembryonic mesoderm, puts ACE in a strategic position for the modulation of blood island differentiation. At this time, the other elements of the RAS are also present in the yolk sac. It therefore seems likely that the RAS is functional locally. In vivo inhibition of the enzyme by a specific ACE inhibitor or application of a specific antagonist of the chicken Ang II receptor resulted in a significant decrease in both the hematocrit and the mitotic index of erythroid cells, establishing a positive effect of RAS on the first wave of erythropoiesis via the production of Ang II. Thus, our results provide the first demonstration of the existence of a locally active RAS in the yolk sac and probable RAS-dependent involvement of ACE in the modulation of early yolk sac erythropoiesis.

References

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Role of the renin-angiotensin system in primitive erythropoiesis in the chick embryo

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