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

Short title: ACE and primitive erythropoiesis

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ABSTRACT

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.
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). 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, to facilitate the generation of reactive oxygen species (ROS), and to possess hypertrophic and hyperplasic properties in various cell types.

The RAS also appears to regulate hematopoiesis, in both experimental models and humans. Indeed, ACE knockout mice present isolated normochromic anemia, whereas in humans, anemia and leukopenia have been reported following the initiation of treatment with high doses of ACE inhibitors. RAS blockade with Ang II receptor antagonists or ACE inhibitors has even been used to treat patients with post-transplantation erythrocytosis or polycytemia vera. Finally, further indirect evidence for involvement of the RAS in hematopoiesis is provided by the marked increase in plasma renin concentration reported in a patient with chronic obstructive pulmonary disease accompanied by secondary erythrocytosis, 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, GATA-1 and GATA-2, are involved in both primitive and definitive hematopoiesis whereas others, such as AML-1 and the erythropoietin receptor, 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 extra-embryonic 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 extra-embryonic area. Very few of these cells are present at first and they begin to increase considerably in number only at stage HH9-10. At stage HH12-13, circulation between the embryo and the yolk sac is established, and at stage HH15-16, complete maturation of the blood islands is accompanied by vascular remodeling. 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.

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 much 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 extra-embryonic area adjacent to the sites of RAS component production. We then investigated the effects on primitive erythropoiesis of blocking the RAS at two 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.
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 described23.

The developmental stages of chicken embryos were determined according to the Hamburger & 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 extra-embryonic area, using the NucleoSpin RNA L Kit (Macherey-Nagel, Hoerdt, 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 of total RNA, and 200U of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). 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. RT-PCR was made semi-quantitative 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 Biorad Phosphorimeter and Quantify One software (Biorad, Hercules, CA).

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’ CGC TGG GGC ACT CCT GGA GTA TT 3’ &lt;br&gt; 5’ GGA CCA TGA CTG GGC CCA CAT 3</td>
<td>630 bp</td>
</tr>
<tr>
<td>cAT</td>
<td>5’ CTGTTCAGGAAAGGCACAGTTAC 3’ &lt;br&gt; 5’ TGTCATATCGAAAGCCACAGAC 3</td>
<td>519 bp</td>
</tr>
<tr>
<td>cRenin</td>
<td>5’ CCCTGATACGACAGGGAG 3’ &lt;br&gt; 5’ GCACCTTGCTTGACCTACGCC 3</td>
<td>495 bp</td>
</tr>
<tr>
<td>cAGT</td>
<td>5’ CATGACTGACACCAGTGAGACC 3’ &lt;br&gt; 5’ GGTGATGGGAAATGTGCATCC 3</td>
<td>546 bp</td>
</tr>
<tr>
<td>cGAPDH</td>
<td>5’ TGT GAT CAA TGG GCA CGC CAT CACTAT 3’ &lt;br&gt; 5’ GCT GTG TGC TTG GCT CAC TCC TTG G 3</td>
<td>822 bp</td>
</tr>
</tbody>
</table>
Riboprobes for *in situ* hybridization (ISH)

We used the following cDNAs to generate labeled riboprobes for *in situ* hybridization: the somatic chicken ACE cDNA was inserted into the Bluescript KS vector (Stratagene). The cAT receptor cDNA was inserted into pCDNA3 (Stratagene). The chicken vascular endothelial growth factor (VEGF) cDNA was inserted into pCRII (Stratagene). The quail receptor VEGFR2 was inserted into pcDNAI/Amp (Stratagene). The chicken AGT and the chicken renin cDNA were cloned by RT-PCR from total RNA from E15 kidneys. Sequences homologous to the mammalian AGT cDNA sequence were identified in the chick EST database (Delaware Biotechnology Institute (http://www.chickest.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, California, US), 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. Whole-mount *in situ* hybridization was performed as described by Domingos Henrique *et al.*

Antibodies

Anti-chicken ACE antibodies were produced by immunizing a rabbit against a mixture of two peptides corresponding to two different selected sequences in the ACE N-terminal extracellular domain, peptide 1: RSWYDSTTFEDDLE (AA 126 to 139), peptide 2: VRRKLYDRYGPKYIC (AA 156 to 169) according to the chicken ACE sequence numbering used by Esther *et al.* Antibodies were obtained after four 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-cACE serum generated in rabbit, (dilution: 1/200), with routine signal amplification (ABC Elite, Vector) and DAB 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\textsuperscript{28}.

Preparation of membrane proteins and determination of ACE enzymatic activity
Whole embryos, corresponding to about 200 mg of tissue, were dissected and homogenized in 50 mM Hapes (Sigma, Saint Louis MO), pH 7.2, 8.8 mM CHAPS (Sigma) and centrifuged for 30 min at 15,000g 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 of the supernatant containing the solubilized enzyme from total embryo, as described by Wei \textit{et al.}\textsuperscript{29}. ACE activity was determined using the synthetic substrate hippuryl-histine-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 HPLC. All enzymatic activities were tested in the presence or absence of 10^{-6} and 10^{-3} M of the ACE inhibitor lisinopril, which had an IC\textsubscript{50} for native chicken ACE estimated at approximately 4.2 nM in preliminary experiments. For inhibition experiments, enzyme and inhibitor were incubated 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 three inhibitors of mammalian ACE (captopril, lisinopril and fosinoprilate) to inhibit recombinant chicken ACE obtained from stably transfected CHO cells, as described by Wei \textit{et al.}\textsuperscript{29}.

\textit{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 Sar\textsuperscript{1}-Ile\textsuperscript{8}-Angiotensin II (Sar\textsuperscript{1}-Ile\textsuperscript{8}-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 of 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 min., 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 a microscope Ortholux II (Leica) and captured with a Polaroid DMC camera (Leica) using Photophop sofware. Images of whole mount *in situ* hybridization were analyzed under MZLF III stereomicroscope and captured with the CoolSNAP camera (Princeton Instrument) using the IPLab software. Images were improved (luminosity and contrast) using the Photophop 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 non parametric Mann-Whitney test. Values of p<0.05 were considered statistically significant.
RESULTS

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

We used semi-quantitative 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 $10^4$ cells). Semi-quantitative 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).

Figure 1:
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).

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

**Figure 2:**

Thus, ACE was expressed and enzymatically active in the extra-embryonic area, from stage HH4 onwards.

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 extra-embryonic 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).

**Figure 3:**

The blood islands emerge from the splanchnopleural mesoderm of the extra-embryonic area by condensation of stem cells called hemangioblasts. 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 extra-embryonic area (Figure 3E, F),
whereas VEGF (Figure 3G) and VEGFR2 (Figure 3H) were expressed in the embryonic and extra-embryonic mesoderm.

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

At stage HH8, *in situ* hybridization (Figure 4A, B, 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.
Figure 4:
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 extra-embryonic endoderm (Figure 4D, E). Renin (Figure 4F, G) and cAT receptor (Figure 4H, I) mRNAs were detected in both the embryonic and extra-embryonic 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 three inhibitors were similar (7.94 +/- 1.36 x 10⁻⁹M, 2.1 +/- 0.62 x 10⁻⁹ M and 1.04 +/- 0.5 x 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.

**Figure 5:**

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 +/- 0.56 % vs. 28.97 +/- 0.59 %) (p<0.0001) 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 (26.62 +/-
1.05 % vs. 27.05+/-.84 %) if the embryos were treated later in development, at stage HH13 (p=0.53).

**Figure 6:**

![Graph A](image)

![Graph B](image)

**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+/-.63 % vs. 28.48+/-.8 %)(p<0.002), i.e. 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 % vs. 28.48 +/- 0.77 %)(p=0.34), 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 +/- 4.05% (p=0.1) and at 0.3 mg/ml, the effect reached 13.98 +/- 3.12% (p<0.001). At the highest dose tried (1mg/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 % vs. 3.18 +/- 0.18 %)(p<0.002) or the cAT receptor antagonist (1.67 +/- 0.096 % vs. 3.18 +/- 0.18 %)(p<0.002) than in the control embryos.

Figure 7:

We also assessed the possible effects of RAS blockade on the apoptosis of endothelial cells and circulating blood cells. Very few apoptotic cells (less than 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 extra-embryonic area at stage HH4, and then, at stage HH9, throughout the extra-embryonic area. ACE was found in close contact with the blood islands, which first differentiate in the posterior part of the extra-embryonic 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 extra-embryonic endoderm, as shown by immunohistochemistry (unpublished observations of the authors).

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 VEGFR2\textsuperscript{34,35} or VEGF\textsuperscript{36,37} gene affects both primitive hematopoiesis and vasculogenesis, as these two 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\textsuperscript{38}. Comparison of the distribution of ACE with that of VEGF and its receptor VEGFR2 revealed two complementary expression patterns overlapping caudally from stage HH6 onwards. The expression of ACE in the anterior part of the extra-embryonic area at stage HH4 is consistent with a possible role of ACE in the differentiation of blood islands. Indeed, the endoderm of the anterior extra-embryonic area has been shown to be an effective stimulus for blood island formation in the posterior mesoderm\textsuperscript{39}. In addition, GATA-2, which has been reported to be involved in blood cell proliferation, displays a similar rostro-caudal pattern of synthesis at this early stage\textsuperscript{40}.

Based on 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, a negative regulator of hematopoietic stem cell differentiation\textsuperscript{41-43}, is continually degraded by ACE\textsuperscript{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 pro-erythropoietic effect in mouse\textsuperscript{8} and in human\textsuperscript{12}. However, this would require the early and simultaneous expression of all the elements of the RAS. RT-PCR and \textit{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, Sar\textsuperscript{1}-Ile\textsuperscript{8}-Ang II, as the cAT receptor is not blocked by mammalian non peptide Ang II receptor blockers\textsuperscript{46}.

\textit{In vivo} treatment with Sar\textsuperscript{1}-Ile\textsuperscript{8}-Ang II decreased hematocrit levels to the same extent as fosinoprilate, during the same time window, strongly suggesting that the pro-erythropoietic effect of ACE specifically involved Ang II.

The low hematocrit in ACE inhibitor-treated or Sar\textsuperscript{1}-Ile\textsuperscript{8}-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 \textit{in situ} hybridization in the whole extra-embryonic 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\textsuperscript{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, via the AT1 receptor, which is known to activate several intracellular pathways possibly involved in adult hematopoiesis. One of these pathways, the Jak/STAT pathway, is activated by several cytokines and growth factors such as interleukin-12 and insulin-like growth factor-1. This pathway may also be involved in the effect of Ang II on erythropoietin synthesis in the kidney. The second pathway activated by Ang II, the nuclear factor NFκB pathway, controls the expression of genes involved in hematopoiesis. Finally, Ang II activates the MAPK pathway, which has a mitogenic effect. 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 in chicken remains to be identified. One of the pathways that might account for 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, recent studies in zebrafish have shown that the gene homologous to Jak2 (Jak2a) is transiently expressed during primitive erythropoiesis. 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 extra-embryonic endoderm, in contact with the first blood islands differentiating in the posterior extra-embryonic 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.
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Table

Sequence of the primers for PCR amplification of the chicken ACE, AT receptor, Renin, AGT, and GAPDH cDNAs.
Figure Legends

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 sac 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).

Figure 2: Progression of ACE synthesis from stage HH4 to HH18
Whole-mount in situ hybridization with ACE Dig-labeled riboprobe (dorsal view) at stage HH4 (A), HH6 (B), HH9 (C) and HH18 (D). A: ACE expression is initially limited to a horseshoe-shaped pattern in the anterior extra-embryonic area. B: The expression spreads laterally and caudally on both sides of the embryo. C: Finally, ACE covers the extra-embryonic area. D: Ventral view of the embryo on day 3 showing ACE labeling in the entire area vasculosa.

Figure 3: VEGF and VEGFR2 expression at stage HH6: comparison with ACE
Transverse sections, in the posterior half of a blastodisc, at 24 hours of development (stage HH6) were used both for in situ hybridization with ACE, VEGF or VEGFR2 35S riboprobes (A, B, C, E, F, G, H) and for immunohistochemistry with antibodies against ACE (D). A, B: ACE mRNA levels in the extra-embryonic area (e.a.). C: ACE expression is restricted to the endoderm (en). Ectoderm (ec) and mesoderm (mes) lateral to the primitive streak (p.s) are not labeled. D: Immunohistochemistry with antibodies against ACE, on the adjacent section, as shown in (C). E and F: pattern of ACE expression in the yolk sac. For comparison: G and H show the expression of VEGF and its receptor VEGFR2, respectively. Unlike ACE mRNA, the VEGF and VEGFR2 mRNAs are restricted to the mesoderm both in the extra-embryonic and embryonic area.
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 extra-embryonic endoderm. C: Blood islands differentiating in the mesoderm (mes), between the ectoderm (ec) and ACE-producing cells in the endoderm (en). Asterisks indicate blood islands (higher magnification of the view in panel (A)). For comparison: D and E show the expression of AGT, F and G the expression of renin, H and I the expression of cAT.

(A, B, original magnification, x9; type of the objective lenses, EF x4; numerical aperture, 0.12); (C, D, original magnification, x60; type of the objective lenses, PL APO x25; numerical aperture, 0.70); (E, original magnification, x 22; type of the objective lenses, EF x10; numerical aperture 0.26); (F, G, H, original magnification, x 22; type of the objective lenses, EF x10; numerical aperture 0.26).
Scale bar: 500 µm. (A, B), 100 µm (G, H), 200 µm (C, D, E, F).

Figure 5: Potency of various specific ACE inhibitors

Determination of the IC₅₀ of various ACE inhibitors: captopril (circles), lisinopril (squares) and fosinoprilate (triangles). Chicken ACE activity was measured in the presence of various concentrations of inhibitor. Each point is the mean of two values.

Figure 6: Effect of RAS blockers on hematocrit

A: Percentage of hematocrit change in fosinoprilate- and Sar¹-Ile⁸-Ang II-treated embryos, with respect to control embryos. Embryos treated with fosinoprilate (Fos., grey bars) at stage HH11 (n= 19) or at stage HH13 (n=8) in comparison to control embryos treated with vehicle only at stage HH11 (n=17) or at stage HH13 (n=8), and embryos treated with Sar¹-Ile⁸-Ang II (Sar., white bars) 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-Ile\textsuperscript{8}-AngII on hematocrit. Results are expressed as a percentage of control hematocrit values (c, n=32, black bar), in embryos treated with 0.1 mg/ml (n=7) or 0.3 mg/ml (n=16) Sar-Ile\textsuperscript{8}-AngII (white bars). Each bar is a mean +/- SEM. Asterisks (*), (**), and (***) indicate a significant difference (p=0.006), (p<0.002) and (p<0.001), respectively.

**Figure 7: Effect of RAS blockers on erythroid cell proliferation**

Percentage of mitotic erythroid cells in embryos treated with vehicle only (c, n=8, black bar), fosinoprilate (Fos., n=6, grey bar), or Sar\textsuperscript{1}-Ile\textsuperscript{8}-Ang II (Sar., n=7, white bar). Each bar is a mean +/- SEM. Asterisks (**) indicate a significant difference (p<0.002).
Role of the renin-angiotensin system in primitive erythropoiesis in the chick embryo

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