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Epididymis is a novel site of erythropoietin production in mouse reproductive organs

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Abstract

The epididymis consists of the interstitial tissue and the ductus epididymidis, an extremely tortuous duct, in which spermatozoa exported from the testis gain motility and fertilizing capacity. We found that the cultured mouse epididymis produces erythropoietin (Epo). The content of Epo mRNA in the epididymis from the adult mouse (8-week-old) amounts to 40% of that in the kidney. The epididymal Epo mRNA dramatically increased upon growth; its level increased 120-fold from the age of 3 weeks to 7 weeks when they complete sexual maturation, while the increase in the total RNA was 3-fold. Hypoxia induced a 5-fold increase in the epididymal Epo mRNA transiently, which is much lower than the induction in the kidney (28-fold). In situ hybridization technique elucidated that the site of Epo production was located in the interstitial space between ductus epididymidis. The epididymal Epo may have an unidentified function in the male reproductive organ.

Keywords: Erythropoietin; Male reproductive tract; Epididymis; Testis; Hypoxia; In situ hybridization

Erythropoietin (Epo) produced by the fetal liver and adult kidney functions as a primary stimulator of red blood cell formation. Oxygen concentration is the major regulator of Epo production; Epo mRNA expression is induced under hypoxia through accumulation of a transcriptional factor HIF-1 [1–4].

Besides these two sites, the brain produces Epo in a hypoxia-inducible manner [5–8]. Neurons express Epo receptor [9–12] and the brain Epo protects neurons from ischemic death [13,14]. Findings that the subcutaneous administration of Epo [15,16] as well as the intracerebroventricular infusion [13,14,17–19] is neuroprotective in the experimental models of various brain injuries including ischemia have launched a clinical trial with recombinant human Epo [20].

Reproductive organs of female mouse also have been shown to produce Epo [21–23]. The uterine Epo is implicated in the capillary formation required for the uterine endometrial growth that occurs periodically in an estrogen-dependent manner [22]. Interestingly, the hypoxic induction of Epo mRNA in the uterus needs the presence of estrogen [22,24]. A recent paper of humans has indicated that the human uterus expresses Epo mRNA in a menstrual cycle-dependent manner [25], which supports our findings of mice.

These results prompted us to explore Epo production in the male counterparts. Here, we report that the epididymis is a primary site of Epo production in the mouse reproductive organs. Developmental increase of epididymal Epo mRNA and localization of Epo-producing cells are also shown.

Materials and methods

Animals. Animals were maintained and handled in accordance with the guidelines for the care and use of laboratory animals at Kyoto University. Unless otherwise indicated, 8-week-old outbred mice (35–40 g) of the ICR strain (Japan SLC, Shizuoka, Japan) were used.

Culture of the testis and epididymis. The bilateral testes and epididymides from each mouse were extirpated. One testis or epididymis

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of mouse was cultured in a 24-well culture plate in 700 μl medium containing the test substance and the contralateral organ was cultured without the substance as a control. They were incubated in a humid 5% CO_2 atmosphere at 33 °C in DMEM supplemented with 10% FCS. Epo protein in the culture media was measured with an enzyme-linked immunoassay using two monoclonal antibodies that bind Epo at different epitopes [26,27]. This assay can measure Epo as low as 1 pg/ml or 0.1 mU/ml. Recombinant human Epo was used as a standard. Recombinant human Epo was produced and isolated as described previously [26,28].

Hypoxic exposure. For hypoxic stimulation, we used an airtight cabinet in which the premixed gas was introduced. The gas flow rate was adjusted so that 7% O_2 was achieved approximately at 30 min after the animals were placed into the cabinet. The effects of hypoxia on Epo mRNA levels in the kidney, testis, and epididymis were examined with mice exposed to normobaric hypoxia (7% O_2/93% N_2). At various time points after hypoxic exposure, the animals were killed, and then the tissues were quickly removed and frozen in liquid nitrogen until used for RNA extraction.

Cobalt chloride stimulation. Cobaltous ion induces hypoxia-inducible genes. Cobalt(II) chloride hexahydrate was dissolved in saline (4.5 mg/ml). Each mouse was subcutaneously injected 100 μl of the cobalt solution (12 mg/kg body weight). The control group was given 100 μl of saline and left under normoxia. At 4 h after cobalt administration, the kidney and epididymis were removed for measurement of Epo mRNA.

Standard plasmid containing the cDNA fragment of Epo. Sequence coordinates of mouse Epo cDNA are based on the definition of the transcription start site as +1 [29]. A 451 bp fragment encompassing 272–722 of the mouse Epo cDNA was ligated into a vector pCR3.1-Uni using a Eukaryotic TA Cloning Kit (Invitrogen, Carlsbad, CA). The resulting plasmids were used as a standard for PCR of Epo cDNA. These cDNA fragments contained the 112-bp nucleotide sequences, which correspond to the PCR products amplified from the mRNA-derived cDNAs using the primers described below.

RT and real-time PCR. Total RNA was prepared from the frozen tissues according to the protocol of the RNA Isolation System Kit (Promega, Madison, WI). RT was carried out at 45 °C for 60 min in 20 μl RT mixture containing 1 μg total RNA, 200U reverse transcriptase (Invitrogen), 20U RNase inhibitor, 0.5 mM each dNTPs (TaKaRa, Kyoto, Japan), and 2.5 μM random nonamer primer. One μl of the RT mixture was used for real-time PCR.

The PCR product of Epo mRNA-derived cDNA was quantified in real time, using a double dye-labeled fluorogenic oligonucleotide probe and an automated fluorescence-based system for detection of PCR products as described previously [24]. Briefly, the probe was labeled at its 5′ end with a fluorogenic reporter dye, 6-carboxy-fluoresceine (FAM) and at its 3′ end with a quencher dye, 6-carboxy-tetramethylrhodamine (TAMRA). The nucleotide sequence in the probe 5′-(FAM)-TGCAGA AGGTCCCAGACTGAGTGAAAATA-3′-(TAMRA) correspond to 397–425 in mouse Epo cDNA. This double dye-labeled probe was obtained from PE Applied Biosystems (Foster City, CA). The Epo-specific sequences used for PCR were the forward primer 371F, 5′-GAGGCAG AAAATGTCACGATG-3′ and the reverse primer 482R, 5′-CTTCCAC CTCCATTCTTTTCC-3′. The forward and reverse primers correspond to the nucleotides 371–391 and 462–482 in mouse Epo cDNA. Similarly, the other probe and primers set were designed to detect mouse Epo receptor mRNA. The nucleotide sequence in the probe 5′-(FAM)-CG ATATGAAGTGGACGTGTCGGCAGG-3′-(TAMRA) corresponds to 559–584 in the mouse Epo receptor cDNA. The Epo receptor-specific sequences used for PCR were the forward primer 530F, 5′-CTCCTGGA GCACCTATGACCAC-3′ and the reverse primer 614R, 5′-TCCACCC TTTGTGTCCCTCCT-3′. The forward and reverse primers correspond to the sequences 530–551 and 594–614 in Epo receptor cDNA, respectively. For PCR, we used TaqMan Universal PCR Master Mix containing dUTP instead of dTTP (PE Applied Biosystems). PCR consisting of 50 cycles at 95 °C for 15 s and 60 °C for 1 min was performed. All procedures including data analysis were performed on the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) using the software provided with the instrument.

Probes and in situ hybridization. Three non-overlapping antisense oligonucleotides were designed for in situ hybridization to detect mouse Epo mRNA. They were complementary to nucleotide residues 67–111 (AACTCGACTGTCGCAGATGAGGCGTGGGGGAGCA CAGAGGACTGG), 329–373 (TGGCTTTGTCTATATGAAGCTG AAGGGTCTCTGGTGGCTGGGAGG), and 1190–1234 (CACTGT GAGTGTTCGGAGTGGAGCAGGTGGGGTGGTATCTGGAGG) of mouse Epo cDNA.

Eight-week-old male mice were used for in situ hybridization. The animals were killed by cervical dislocation, and the epididymis, testis, and kidney were rapidly removed and frozen in liquid nitrogen. Cryostat sections, about 14 μm in thickness, were prepared and mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Shinetsu Chemical Industry, Tokyo, Japan). The sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 15 min and acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0). The sections were prehybridized for 2 h in a buffer containing 50% formamide, 0.1 M Tris–HCl (pH 8.0), 4× SSC (1× SSC; 150 mM NaCl and 15 mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.6 M NaCl, 0.25% sodium dodecyl sulfate (SDS), 200 μg/ml tRNA, 1 mM EDTA, and 10% dextran sulfate. Hybridization was performed at 42 °C for 10 h in the prehybridization buffer supplemented with 10,000 cpm/μl of ^{35}S-labeled oligonucleotide probes. The slides were washed at room temperature for 30 min in 2× SSC containing 0.1% sarkosyl and twice at 55 °C for 40 min each in 0.1× SSC containing 0.1% sarkosyl. The sections were dipped in Kodak NTB2 nuclear track emulsion (Kodak, Rochester, NY) and exposed for 1 month.

Results

In vitro production of Epo by the male reproductive organs

The testes and epididymides obtained from 8-week-old mice were cultured and at intervals Epo in the culture media was assayed by the enzyme immunoassay method. The assayable Epo in the media increased in a culture time-dependent manner (Fig. 1). Surprisingly, Epo in the culture media of mouse epididymis is 4-fold higher than that of testis. The presence of a protein synthesis inhibitor, cycloheximide, severely inhibits the appearance of Epo in culture media, indicating that Epo in the culture media largely resulted from secretion of the newly synthesized Epo but not from leakage of Epo accumulated in the tissues. Production of Epo and expression of Epo mRNA in the cultured female reproductive organs were induced by estrogen (17β-estradiol) [14]. But the induction was not found when testis and epididymis were cultured in the presence of 1 μM 17β-estradiol and testosterone.

Epo mRNA in the kidney, testis, and epididymis

Epo mRNA in the kidney, testis, and epididymis was measured by the quantitative RT-PCR method. Fig. 2 shows Epo mRNA contents in the reproductive organs relative to the renal content that was defined as 100%.

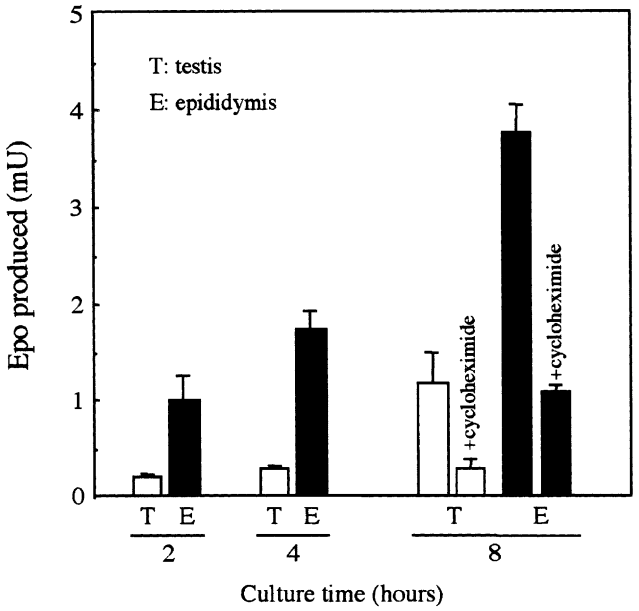

Fig. 1. Epo production by cultured testis and epididymis. Testes (T; open columns) and epididymides (E; filled columns) from 8-week-old mice were cultured for indicated times. Secreted Epo was measured by enzyme immunoassay. Cycloheximide was added at 20 μM just before culture starts and the organs were cultured for 8 h. Bars represent means ± SE ($n = 3$–5).

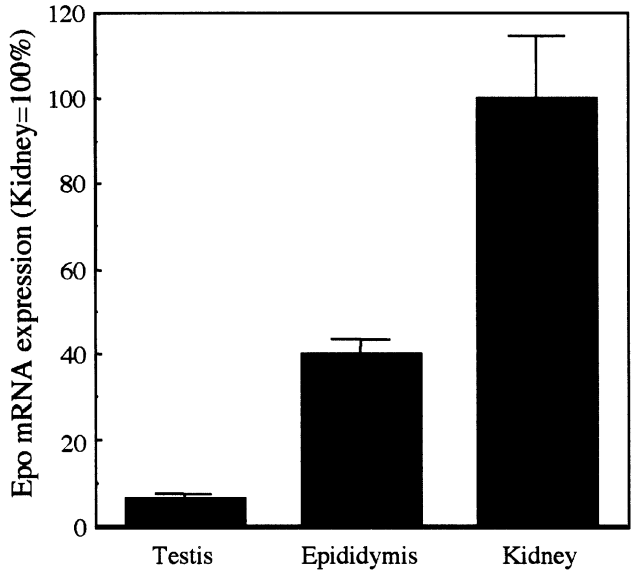

Fig. 2. Content of Epo mRNA in the testis, epididymis, and kidney. The testis, epididymis, and kidney were removed from 8-week-old mice and the total RNA was extracted. Epo mRNA contents were measured by quantitative real-time PCR. The ordinate indicates the copies of testicular and epididymal Epo mRNA relative to that in the kidney, which was 2.7×10^6 copies/kidney. Bars represent means ± SE ($n = 5$–6).

Epo mRNA content in the epididymis and that in the testis was approximately 40 and 6% of that in the kidney, respectively. The epididymis contains 7-fold more Epo mRNA than the testis, which is comparable with the difference in Epo production by the organs cultured in vitro (see Fig. 1).

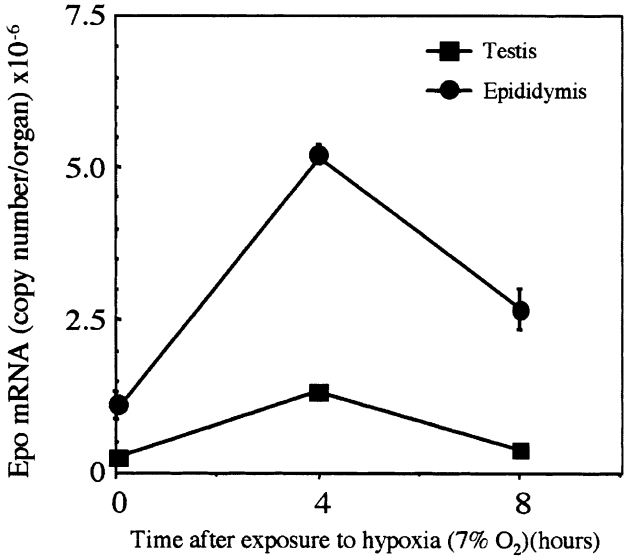

Fig. 3. Temporal patterns of hypoxic induction of Epo mRNA in testis and epididymis. Eight-week-old mice were exposed to hypoxia (7% O_2). The tissues were extirpated at the indicated time points and removed for measurement of Epo mRNA. (■) Testicular Epo mRNA; (●) epididymal Epo mRNA. Bars represent means ± SE ($n = 4$–5).

Temporal pattern of the hypoxic induction of testicular and epididymal Epo mRNA

We have shown that there are two types of temporal patterns in Epo mRNA induction upon exposure to hypoxia [24]. Induction in the kidney and female reproductive organs reached the maximum at 2–4 h after animals were exposed to hypoxia (7% oxygen) and then markedly declined within 8 h despite the continuous hypoxia. By contrast, the elevated level in the brain was sustained as long as hypoxia continued. Although the mechanism remains unknown, this notable difference is conformable with the tissue-specific functions of Epo; Epo supports neuron survival under ischemia, and therefore a high level of Epo expression is required as long as hypoxia continues, whereas the continuous activation of Epo gene expression in the kidney would cause erythrocytosis, and therefore the downregulation must operate even under hypoxia. To examine the temporal pattern of male reproductive organs, we quantified Epo mRNA in the testis and epididymis from mice that were exposed to hypoxia (7% oxygen) for various durations. At 4 h after exposure to hypoxia, Epo mRNA in both organs was induced 5–6-fold but the levels were decreased at 8 h (Fig. 3). Thus the temporal pattern of hypoxic induction in reproductive organs of both sexes is the kidney type. Hereafter, we focused on the mouse epididymis because expression of Epo mRNA in the testis is much lower than that in the epididymis.

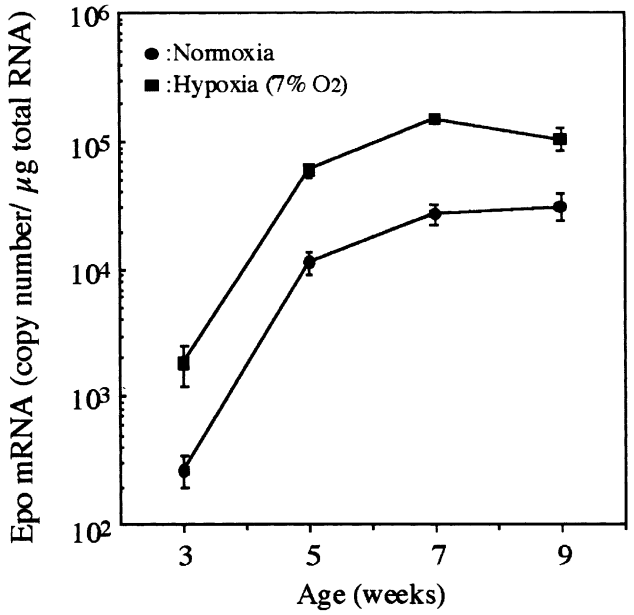

Fig. 4. Developmental change of epididymal Epo mRNA. The epididymis was removed for measurement of Epo mRNA from mice at ages of 3–9 weeks. Hypoxic inducibility of Epo mRNA was examined in mice exposed to hypoxia (7% O_2) for 4 h. (●) Normoxic group; (■) hypoxic group. Bars represent means ± SE ($n = 3$).

Developmental change of mRNAs for Epo and its receptor in the epididymis

Since we found that the Epo mRNA level in the epididymis of young mice (3-week old) was detectable but very low as compared with that in the mature mice (8-week old), we examined the developmental change. Extractable total RNA per one epididymis was 23 μg in 3-week-old mouse and increased with growth to 50, 62, and 68 μg in 5-, 7-, and 9-week-old mouse, respectively ($n = 6$ for all experiments). Epo mRNA content (copy number per μg total RNA) dramatically increased upon growth and reached a plateau at the age of 7 weeks (Fig. 4). The total RNA increased 3-fold from 3 weeks to 7 weeks of age, while an increase of Epo mRNA level was 120-fold. This specific elevation of Epo mRNA is consistent with the sexual maturation of male mice; they are capable of fertilizing at the age of 6 weeks. Hypoxia inducibility of the epididymal Epo mRNA was nearly unchanged during growth (Fig. 4).

The epididymal Epo receptor mRNA was definitely detected with RT-PCR but there was no specific increase during sexual maturation; it increased in proportion to the rise in total RNA (data not shown).

Localization of Epo mRNA in the epididymis by in situ hybridization

Induction of the mouse epididymal Epo mRNA under 7% O_2 is 5-fold, which is lower than that in the kidney (28-fold) (see Table 1). Cobaltous ion mimics the effect of hypoxia on Epo through HIF-1 activation [30]. To elevate Epo mRNA as much as possible for in situ hybridization experiments, additional effect of Co^{2+} injection on hypoxia induction of Epo mRNA was examined. As administration of Co^{2+} elevated Epo mRNA level more profoundly than the hypoxia alone (Table 1), the epididymis and kidney (as a control) obtained from animals that had received both stimuli (hypoxia + Co^{2+} injection) were subjected to the in situ hybridization.

Table 1
Induction of Epo mRNA in the epididymis and kidney by hypoxia and Co^{2+}

Treatment	Fold induction (average of $n = 5$)	
	Epididymis	Kidney
7% O_2	5	28
Co^{2+}	9	14
7% O_2 + Co^{2+}	11	59

The Epo mRNA level in the organs from 8-week-old mice without stimulation was regarded as 1. Cobalt was given just before animals were exposed to hypoxia for 4 h.

Previous in situ hybridization studies of the kidney have shown that peritubular interstitial cells in the cortex or outer medulla are responsible for Epo production [31,32]. Consistent with these results, hypoxia treatment yielded signal-positive cells in the renal cortex (Fig. 5b) and the dual stimulation (hypoxia + Co^{2+}) enhanced not only signal intensity but also increased the number of positive cells (Fig. 5c). By the conditions used here, we were unable to find definite signal in the kidney from mouse that had received no stimuli (Fig. 5a). The epididymis from mouse that received both stimuli (hypoxia + Co^{2+}) yielded clear signals (Fig. 5e), while no significant signals were found from mouse that had received no stimuli (Fig. 5d). A high power view of the epididymis indicates that the Epo-producing cells are located in the interstitial space of ductus epididymidis but not present in the duct epithelium (Fig. 5f). The specificity of in situ hybridization was confirmed by that the hybridization using three non-overlapping anti-sense probes exhibited consistent labelings, and by that the presence of an excess amount of the unlabeled probe in the hybridization reactions abolished positive signals (data not shown). The cells expressing Epo mRNA distributed throughout the epididymis. Consistent with this in situ hybridization results, Epo mRNA was detected in all sections (caput, corpus, and cauda) with RT-PCR technique.

Discussion

In this paper we have demonstrated that the epididymis is a primary site of Epo production in mouse reproductive organs, but a physiological function of the

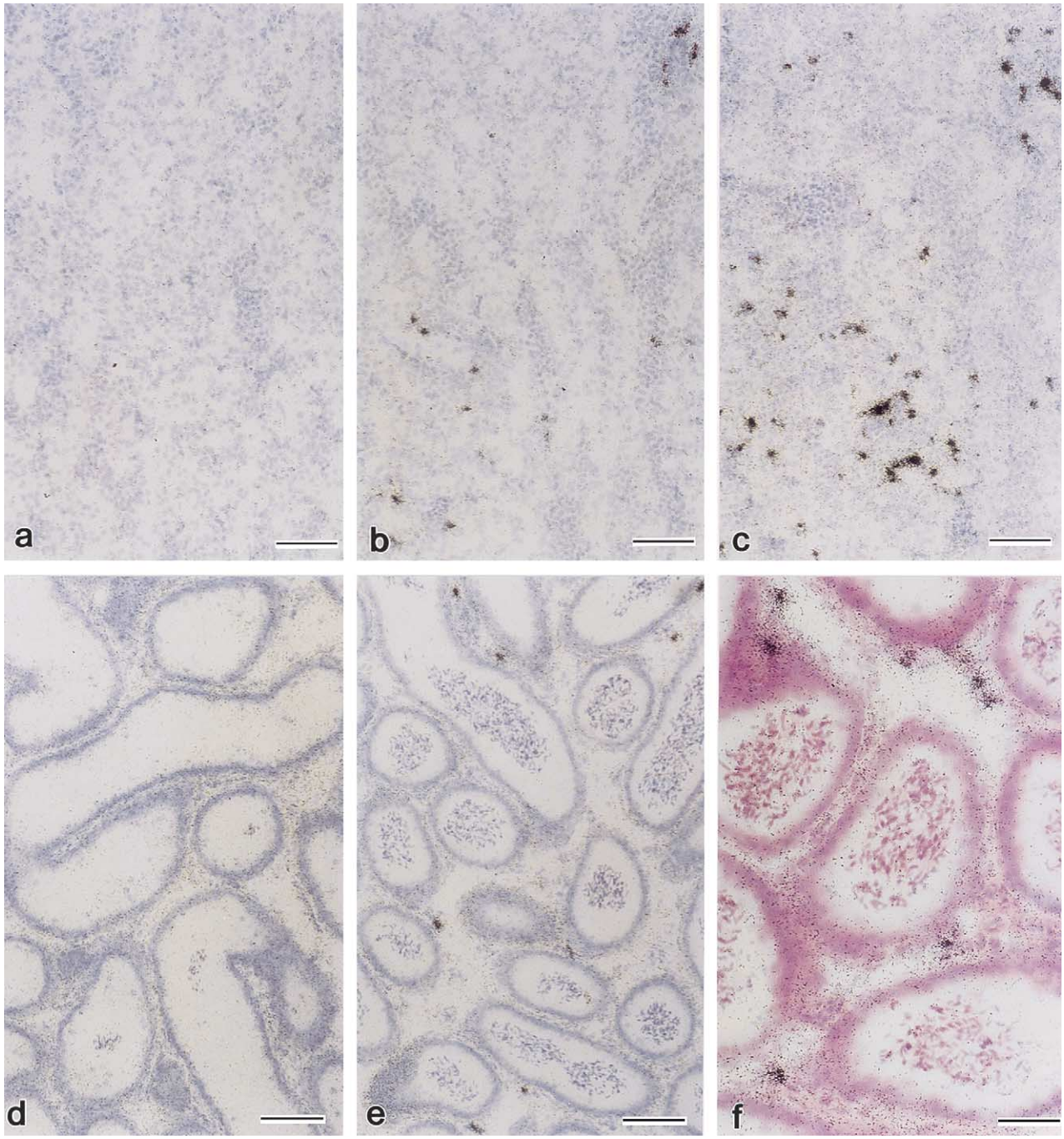

Fig. 5. Detection of Epo-producing site in the kidney and epididymis by in situ hybridization. (a)–(c) Kidney; (d)–(f) epididymis. (a) Untreated; (b) induced by hypoxia for 4 h; (c) induced by hypoxia and Co^{2+} injection for 4 h. (d) Untreated; (e) induced by hypoxia and Co^{2+} injection; (f) a high power view of the epididymis induced upon hypoxia and Co^{2+} injection. Note that signals in the epididymis are seen in the interstitial space of ductus epididymidis. Bars are 100 μm in (a)–(e) and 50 μm in (f). Since in situ hybridization performed by the use of three probes (see Materials and methods) gave similar results, the photographs obtained by the use of the probes 329–373 are presented.

epididymal Epo remains to be studied. The main tissue comprising the epididymis is ductus epididymidis, a long and highly convoluted duct. The caput epididymidis is connected to the rete testis via several ductuli efferentes [33]. The caput and corpus are involved in the maturation process of spermatozoa. Spermatozoa leaving the testis are immotile and infertile, but spermatozoa leaving the epididymis have gained motility and fertility. After spermatozoa in the caput and corpus epididymidis undergo a series of morphologic and functional changes that lead to the acquisition of full fertilizing capacity, they reach the cauda epididymidis, which serves as the main storage place of matured spermatozoa. The epithelium that lines the ductus epididymidis consists of

two types of cells: principal cells and basal cells. The principal cells have been shown to secrete glycoproteins into the lumen, which are believed to be necessary for sperm maturation, while the basal cells may be precursors of principal cells [33]. The presence of Epo production site in the interstitial tissue, however, suggests that Epo is not directly involved in sperm maturation but rather supports the duct functions through paracrine action. Identification of the site where Epo receptor is expressed is crucial for understanding a role of the epididymal Epo. But low expression of Epo receptor made it difficult to find the site and further examination with more sensitive ways is needed.

There are some papers suggesting that Epo action operates in the male reproductive organs. Rat testis has been shown to express Epo mRNA [34] and more recently it has been reported that Sertoli and peritubular myoid cells may be responsible for the testicular expression of Epo mRNA [35]. Rat Leydig cells express Epo receptor and binding of Epo stimulates testosterone production [36]. Infusion of recombinant human Epo in men elevated the spermatic venous level of testosterone [37]. Thus, all findings of Epo in the male reproductive organs have been restricted to the testis. To our knowledge, there are no previous reports showing that the epididymis expresses Epo mRNA and secretes Epo protein. In this context, it is of interest that administration of Epo to rats stimulated sperm maturation and improved fertilizing capacity [38], because the epididymis is the site where spermatozoa mature.

The content of Epo mRNA in the mouse epididymis amounts to 40% of the kidney, suggesting that the male reproductive organ-derived Epo may contribute to the circulating Epo. There is no difference in serum Epo levels between normal men and women even though there is a significant difference in their hemoglobin levels [39,40], but this fact does not exclude the possibility that the male reproductive organs may contribute to total Epo production. Since the epididymis not only expresses Epo mRNA but also secretes Epo protein, this possibility should be examined further. A comparative survey on experimental animals and patients with the renal anemia may provide an important clue; if there is some contribution, the fall of serum Epo level in males would be more refractory to the progression of renal failure than that in females.

Epo is produced by mouse female reproductive organs such as the uterus, ovary, and oviduct [22–24]. Epo production by these organs undergoes unique regulation; it is enhanced by estrogen. An increased serum level of Epo in pregnant women may be derived from Epo produced by these organs because estrogen level is maintained to be high during pregnancy [41]. Many malignant tumors from the ovary and uterus express Epo and its receptor [42]. Inhibition of Epo signaling destroyed ovarian and uterine tumors in grafted nude mice, suggesting that Epo may be involved in the initiation and/or development of these tumors. Epo level is elevated in the peritoneal fluid of women with endometriosis and the elevation is significant in the initial stage of the disease [43]. Taken together, Epo seems to exert so far unrecognized physiological and pathological roles in reproductive organs of both sexes.

Further studies remain to be done to develop techniques by which Epo-producing cells can be isolated from epididymis and propagated in vitro. Males have a pair of epididymis and therefore the unilateral organ from an anemic patient with renal failure can be used for preparation of these cells. These cells are transplanted back to the patient without immunological rejection, which would lead to Epo production under control of physiological regulators.

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