The Cytological Localization of Erythropoietin in the Human Kidney Using the Fluorescent Antibody Technique[1] (35570)

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It has been well documented that the kidney is the primary site of erythropoietin (ESF) production (1–3). However, the localization of the specific site of ESF production within the kidney has not been elucidated. Using the indirect fluorescent antibody technique, Fisher *et al.* (4) localized ESF in the vascular wall of the glomerular tuft of the anemic sheep kidney. The antigen used in these earlier studies was anemic sheep plasma erythropoietin which was impure, thus, making it difficult to interpret these findings. This work was confirmed by Frenkel *et al.* (5) who found fluorescent staining in the peripheral portion of the glomerular tuft of the anemic sheep kidney.

Due to the controversial nature of these early findings (4,5) concerning ESF localization in the anemic sheep kidney, we have localized ESF in the glomerular tuft of the human kidney using the indirect fluorescent antibody technique and subsequently fluorescent staining was blocked with purified human erythropoietin.

Materials and Methods. Preparation of antiserum. Antierythropoietin serum was prepared by injecting crude human urinary erythropoietin (0.75 units/mg of protein) into a young adult male albino rabbit according to a modification of the method of Kabat and Mayer (6). The immunization schedule was as follows: alum-precipitated erythropoietically active protein from the urine of a patient with a pure red cell aplasia was injected intraperitoneally on alternate days for a period of 5 weeks in doses increasing from 2.1 units of ESF (1.5 mg of protein) to 120.5 units of ESF (127.8 mg of protein). The rabbit was also injected concomitantly im 1 time/week with ESF in complete Freund's adjuvant. One week following the last injection, the rabbit was bled via cardiac puncture and the serum was collected.

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TABLE I. Inhibitory Effect of Erythropoietin Antiserum on Human ESF in Polycythemic Mice.

Treatment	No. of mice	^{59}Fe incorporation (mean %)
Saline	5	0.33 ± 0.079[a]
ESF,[b] 0.2 unit	5	8.11 ± 1.53
+ anti-ESF,[c] 0.1 ml	5	0.29 ± 0.044

[a] ±, standard error of mean.

[b] Human urinary erythropoietin.

[c] Rabbit antiserum to ESF (human) was absorbed with a 1:4 dilution of normal human serum.

Determination of anti-ESF activity. Anti-ESF activity was determined *in vitro* by Ouchterlony gel-diffusion (7) and *in vivo* in the polycythemic mouse assay (8).

Three to four precipitin lines were identified by gel-diffusion when unabsorbed antiserum was reacted with human urinary erythropoietin. However, after absorbing the antiserum with an equal volume of normal human plasma diluted 1:4 in isotonic saline, no precipitin lines were seen. It has been reported that the ESF–antibody complex is nonprecipitating (9). However, using a special Agarose gel preparation Lange and McDonald (10) reported a precipitating antibody to erythropoietin.

Neutralizing activity of the antiserum was tested by measuring percent ^{59}Fe incorporation in red blood cells of exhypoxic polycythemic mice (8). The ESF–antiserum mixture was incubated for 30 min at 37° and overnight at 4° before injection into the assay mice. As shown in Table I, 0.1 ml of the

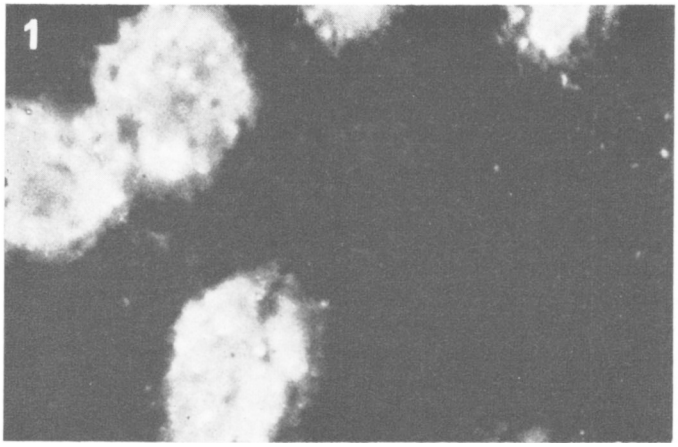

FIG. 1. Low power (100×) fluorescence photomicrograph of an anemic human kidney section treated with antierythropoietin serum.

antiserum blocked completely the effects of 0.2 unit of ESF on ^{59}Fe incorporation in red blood cells of polycythemic mice.

Preparation of kidney sections for fluorescent antibody studies. The indirect fluorescent antibody technique was employed for the localization of ESF. In this procedure the antigen (ESF) was localized by applying the antibody to ESF and a fluorescein-labeled antiglobulin.

A kidney was obtained from a patient at Charity Hospital, New Orleans, Louisiana, who had died from a lower gastrointestinal hemorrhage. At the time of death his venous hematocrit was 15. Small blocks (6–12 × 3 mm) of kidney were frozen rapidly within 30 min after death. Frozen sections were cut at a thickness of 6–8 μ with a cryostat (—20 to —25°), air dried for 15–30 min and fixed in 95% ethanol. The same procedure was also followed in preparing a kidney from a hematologically normal (HCT 48) patient at Mercy Hospital, New Orleans, Louisiana.

Staining of the tissue was carried out on the same day as the sectioning. Frozen sections were treated with 1 drop of antierythropoietin serum and incubated in a humidifying chamber at 37° for 45 min. After rinsing and washing with Difco fluorescent antibody (FA) buffer (pH 7.2) for 5 min, the tissues were stained at room temperature with rhodamine counterstain for 10 min. Following an additional wash with FA buffer, they were treated with fluorescein isothiocyanate-labeled goat antirabbit globulin and incubated at 37° for 45 min. The tissue was then rewashed with buffer, mounted in glycerin, and examined with a fluorescence microscope. Specificity of staining was confirmed by treating the kidney sections with (a) normal rabbit serum, or (b) antierythropoietin serum which was neutralized with a highly purified human urinary erythropoietin.[2] Fluorescent tissue sections were destained and treated with hematoxylin–eosin for histologic examination under light microscopy.

Results. Figure 1 shows a low power (100×) view of the intense fluorescent staining of glomerular tufts in the anemic human kidney treated with absorbed anti-ESF. A high power photomicrograph of a fluorescent glomerulus is shown in Fig. 2A. The same glomerulus after destaining to remove the fluorescent antibody and restained with hematoxylin–eosin is shown in Fig. 2B. In the anemic kidney sections, the specific yellow-green fluorescent staining was only seen in the glomerular tufts. The tubules, collecting ducts, interstitium, juxtaglomerular apparatus, parietal layer of Bowman's capsule, and peritubular capillaries did not show any fluorescent labeling. Anemic kidney sections treated with either normal rabbit serum or with antiserum which had been previously

[2] Purified human ESF (sp act, 8300 units/mg of protein) was supplied by Dr. E. S. Espada, Biochemistry Department, Universidad Nacional del Nordeste, Facultad Medicina, Corrientes, Argentina.

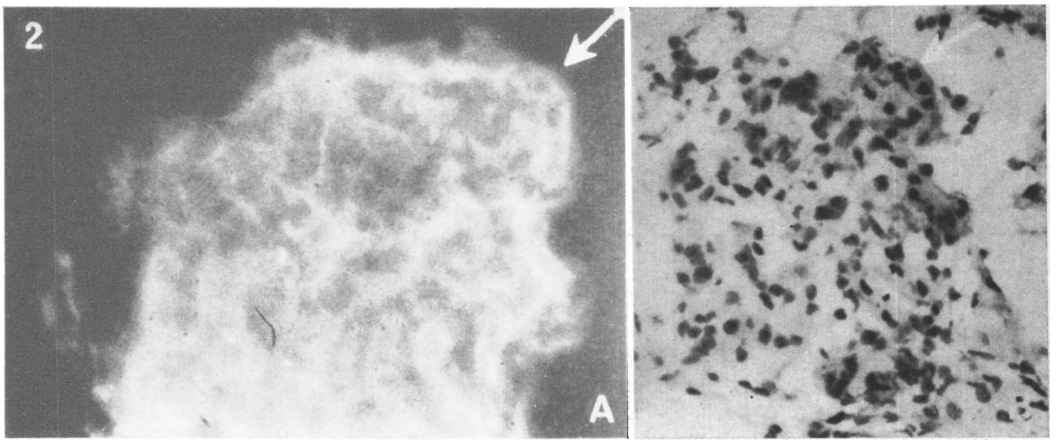

FIG. 2. Fluorescent glomerulus (440×) from an anemic human kidney (A); same glomerulus (360×) stained with hematoxylin–eosin (B); (arrows) identical areas of the same glomerulus.

neutralized with highly purified human urinary erythropoietin did not show any fluorescence. The specific blocking action of purified ESF is further evidence that the cytoplasmic fluorescence seen in the peripheral cells of the glomerular tuft is erythropoietin. A slight amount of fluorescence was also seen in the glomerular tuft cells in a section of kidney removed from a hematologically normal human subject when the ESF antiserum was applied. However, the fluorescence was very faint when compared with that of the anemic kidney section.

Discussion. The specific staining seen in all glomeruli of an anemic human kidney suggests that erythropoietin is either produced or stored in the glomerulus. Intense fluorescence was localized in the cytoplasm of cells found both at the periphery and within the glomerular tuft. Our evidence that the labeling was specific for ESF is based on the absence of fluorescent staining in (a) an anemic human kidney treated with normal rabbit serum, and (b) an anemic human kidney treated with antierythropoietin serum which had previously been neutralized with purified erythropoietin.

Although it is difficult to distinguish epithelial or endothelial cells by light microscopy, the epithelial cell is the most likely site of erythropoietin localization. First, the intense fluorescence which is seen at the outermost periphery and within the glomerular tuft suggests a visceral epithelial cell localization of erythropoietin. Second, areas of the glomerulus which show specific staining correspond to the epithelial cells which were identified after destaining and restaining with hematoxylin–eosin (Fig. 2B). Our studies do not resolve the obvious problem of whether ESF is produced or stored in the visceral epithelial cell of the glomerulus. However, it is not suprising that the epithelial cell of the glomerular tuft with its rich cytoplasm which contains a Golgi apparatus, numerous mitochondria, and an extensive rough surfaced endoplasmic reticulum (11, 12) is capable of producing a substance such as erythropoietin. Further work is necessary to clarify whether the epithelial cells of the glomerular tuft are a site of production or storage of erythropoietin.

A renal erythropoietic factor (erythrogenin) has been found in the nuclear (13) and light mitochondrial (14) fractions of the kidney and is capable of generating erythropoietic activity when reacted with a plasma substrate. Therefore, it is possible that the epithelial cell may be a site in the kidney where erythrogenin acts on a substrate to generate erythropoietin.

Summary. Intense fluorescent staining of cells in the glomerular tuft has been demonstrated in an anemic human kidney following application of an antibody to erythropoietin using the indirect fluorescent antibody technique. The fluorescence was more localized in the cytoplasm of epithelial cells in the

periphery of the glomerular tuft. A slight amount of fluorescent staining in the glomeruli was blocked by a highly purified preparation of human urinary erythropoietin. The present studies suggest that the visceral epithelial cell of the glomerular tuft is the most likely source of erythropoietin.

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