Production of Erythrogenin by Organ Cultures of Rat Kidney

By J. B. Sherwood, S. H. Robinson, L. R. Bassan, S. Rosen, and A. S. Gordon

Organ cultures of rat kidney produce a factor capable of stimulating erythropoiesis in exhypoxic polycythemic mice. This material appears to be renal erythropoietic factor or erythrogenin, since biologic activity was observed only after incubation of the culture media with rat serum. Production of this material was similar in cultures prepared from four different anatomic regions of the kidney, suggesting that erythrogenin may be produced diffusely throughout this organ. Production of erythrogenin was usually insignificant during the first 3/2 days in culture but became significant in all four anatomic regions during the second 3/2 days. Histologic evaluation of the explants revealed primarily autolysis in the early period followed by the development of epithelial elements, many of which comprised renal tubular structures, during the later culture interval. The newly developed cells had a markedly hypertrophic Golgi apparatus, extensive rough-surfaced endoplasmic reticulum, and numerous polyribosomes, findings consistent with active protein synthesis. This organ culture technique provides direct evidence that the kidney produces an erythropoietic factor and represents a promising model for future studies of this problem.

Recent observations indicate that the kidney elaborates a factor—renal erythropoietic factor (REF) or erythrogenin—that interacts with a serum protein to produce biologically active erythropoietin. Isolated kidneys perfused with hypoxic blood release significantly increased titers of erythropoietin, suggesting that this organ is capable of autonomous response to a hypoxic stimulus and that it contains the biosynthetic pathways for erythrogenin production. However, the anatomic structures and the precise mechanisms involved in erythrogenin production have not yet been elucidated.

A promising approach to these problems appears to lie in studies of erythrogenin production by renal tissue in vitro. McDonald et al. have reported that monolayer cultures of bovine kidney are able to release an erythropoietically active factor. We have recently examined elaboration of an erythropoietic...
factor by organ cultures of rat kidney, with particular attention to the activity of various anatomic regions of the kidney and to the histologic concomitants of production of this factor.

MATERIALS AND METHODS

Culture Methods

Kidneys were obtained from male Sprague-Dawley rats (CD rats, Charles River Breeding Labs., Wilmington, Mass.), average weight 250 g. Kidneys from three donor animals were used for each experiment. The animals were exposed to 0.4 atmospheres of air for 20 hr and were sacrificed immediately after removal from the hypobaric chamber. The kidneys were excised, immediately placed in ice-cold physiologic saline, and divided into four anatomic regions: outer and inner cortex, and outer and inner medulla. Each of these segments was then cut into pieces approximately 1 cu mm in volume, and seven to ten such pieces were placed in a polystyrene, organ culture dish, 60 mm in diameter (Falcon Plastics, Oxnard, Calif.). In each experiment, 7-15 such culture dishes were prepared from each of the four kidney segments. Except as indicated, the explants were incubated in Trowell’s T8 medium supplemented with 10% fetal calf serum, and 50 U of penicillin and 50 μg streptomycin/ml in a humidified atmosphere of 95% air-5% CO2 at 37°C. One milliliter of medium was used in each culture dish. Medium was changed after 3½ days of culture and was harvested again when the cultures were terminated at 7 days. The media from all cultures of each anatomic region were pooled and stored at -20°C until the time of bioassay. Thus, there were eight pools of medium for each experiment, representing the first and second 3½ days in culture for each of the four anatomic regions studied.

Erythrogenin Assay

The pools of media were dialyzed against doubly distilled water, lyophilized, and resuspended in the original volume of distilled water. Fresh medium, treated in the same manner, served as the control preparation. To generate erythropoietin, 1 volume of this fluid was then incubated with 1 volume of normal rat serum (obtained from normal male Sprague-Dawley rats and then dialyzed against 0.005 M EDTA, followed by dialysis against doubly distilled water). To determine whether the cultures were producing erythrogenin or erythropoietin, in one experiment pooled media from cultures of whole kidney were collected over the usual two 3½-day periods, but each pool was then divided into two aliquots. One aliquot from each pool was incubated with rat serum prior to bioassay, while the other aliquot was not.

Erythropoietin activity in each incubation mixture was assayed in four to six exhypoxic polycythemic mice (CF-1 virgin female mice, Carworth Farms, New City, N.Y.). Each mouse received a single, intraperitoneal injection of 2 ml of the incubation mixture on day 3 posthypoxia. This is equivalent to 1 ml of the original culture medium, the amount used in each culture dish. On day 5, each mouse received 0.5 μCi 59FeCl3 intravenously. Per cent 59Fe incorporation into red cells was determined from cardiac blood collected on day 7,12 and this was converted to erythropoietin units by reference to a standard curve prepared from the International Reference Preparation. Hematocrits were determined, and 59Fe incorporation was considered valid in mice with hematocrits of 53 or greater.

Morphologic Evaluation

Tissue was examined after 1, 3, and 7 days in culture. For electron microscopy, explants were fixed for 12 hr in buffered 3% glutaraldehyde and postfixed in osmium tetroxide. Tissues were then dehydrated in a graded series of alcohols and embedded in Epon. Thick (0.5 μ) sections were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Phillips
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RESULTS

Erythropoietin Production

In three experiments, explants of all four anatomic segments of kidney released an erythropoietic factor into the medium. Erythropoietic activity was low during the first 3½ days of culture, and except for inner cortex in experiment 1, $^{59}$Fe incorporation did not significantly exceed the values for control preparations derived from fresh culture medium ($p > 0.2$). With this same exception, activity in supernatant fluids collected during the next 3½ days was higher than that from the first period, and $^{59}$Fe incorporation was significantly greater than control values (Table 1). $^{59}$Fe incorporation with these control preparations did not differ significantly from that observed with injections of saline ($p > 0.2$) (Table 1). Values for the later period in culture corresponded to 0.025–0.09 ESF U/ml of original culture medium, i.e., per individual culture which is equivalent of 5.8–33.7 U/g of tissue. There were no significant differences among the four regions studied. It is not clear why the inner cortex in experiment 1 showed a reverse pattern, with higher activity during the early period in culture (Table 1).

In the experiment in which the effect of incubation with rat serum was

Table 1. Erythropoietic Activity in Culture Medium from Organ Cultures of Rat Kidney

<table>
<thead>
<tr>
<th>Region</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
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<tbody>
<tr>
<td></td>
<td>$^{59}$Fe incorporation (per ml medium)</td>
<td>$^{59}$Fe incorporation (per ml medium)</td>
<td>$^{59}$Fe incorporation (per ml medium)</td>
</tr>
<tr>
<td>Outer cortex</td>
<td>1.4±0.5</td>
<td>1.3±1.0</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td></td>
<td>3.9±0.2</td>
<td>3.8±0.5</td>
<td>5.1±1.0</td>
</tr>
<tr>
<td>Inner cortex</td>
<td>4.7±0.3</td>
<td>2.0±1.6</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td></td>
<td>1.8±0.6</td>
<td>5.1±0.7</td>
<td>4.9±0.6</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>0.9±0.04</td>
<td>2.8±1.2</td>
<td>1.8±0.5</td>
</tr>
<tr>
<td></td>
<td>3.0±0.8</td>
<td>5.3±2.4</td>
<td>4.2±1.2</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>1.7±0.3</td>
<td>2.8±1.2</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Control medium</td>
<td>1.2±0.2</td>
<td>1.6±0.5</td>
<td>1.6±0.5</td>
</tr>
<tr>
<td>Saline</td>
<td>1.0±0.1</td>
<td>0.9±0.2</td>
<td>0.9±0.2</td>
</tr>
</tbody>
</table>

Values shown are means ± SE. Media were examined in two assays: experiment 1 in one and experiments 2 and 3 in the other. Each value refers to a total of 7–15 cultures, medium from which was pooled, incubated with rat serum, and assayed in four to six assay mice. Each mouse received equivalent of 1 ml of original medium, the amount used in each culture. Erythropoietin standards gave values of 3.61±0.18% for 0.05 U and 7.24±0.27% for 0.2 U and were similar in both assays.

* Culture period 1 represents first 3½ days in culture; period 2 the second 3½ days.
† Significantly different from control medium ($p < 0.05$). ESF units are not shown if $p > 0.2$.
§ $p < 0.1$
‡ $p < 0.2$
tested, culture supernatants incubated with serum induced $^{59}$Fe incorporation of 4.7 ± 1.4%, whereas the identical medium not incubated with serum had no detectable activity (1.1 ± 0.1%, as compared to 0.9 ± 0.2% for the saline control). These values refer to the second 3 1/2 days of culture; erythropoietic activity was not detectable in medium from the first 3 1/2 days whether or not it had been incubated with rat serum.

In an additional experiment, explants where maintained in Neuman and Tytell's serum-free medium, while other explants from the same pool of donor kidneys were cultured as usual in the presence of serum. Erythropoietic activity was detectable in medium from the routine cultures after incubation with rat serum: $^{59}$Fe incorporation was 2.1 ± 1.7% for control medium, 4.2 ± 0.8% for medium obtained from the first 3 1/2 days in culture, and 5.7 ± 1.3% for medium from the second 3 1/2 days in culture. By contrast, no erythropoietic activity was found with medium from the serum-free cultures, whether or not it had been incubated with rat serum: $^{59}$Fe incorporation was 1.7 ± 0.1% for control medium and 1.2 ± 0.4% and 1.5 ± 0.3%, respectively, for the early and late periods of culture (values are for medium incubated with serum). Histologically, the explants in the serum-free medium showed complete necrosis at the end of the experiment, while those in the serum-supplemented medium showed viable cells as described below. This extensive autolysis precluded use of these serum-free cultures to differentiate between erythrogenin and erythropoietin production. However, the findings have implications concerning the role of autolysis in the experiments in which erythrogenin activity was found. Although it is possible that erythrogenin is produced and then deteriorates in the absence of serum, the results more likely indicate that autolysis is not a significant source of erythrogenin in this culture system.

**Histologic Studies**

By light microscopy all four renal segments showed extensive autolysis after 1 day in culture. The few tubular elements that appeared still intact were most prominent in the inner medulla and were situated at the periphery of the cultured tissue. After 3 days in culture the cortical segments still showed almost complete autolysis, but the medullary segments now contained considerable numbers of viable cells within recognizable tubular structures. At 7 days, all segments contained large numbers of cells that appeared viable by morphologic criteria. However, a prominent core of central necrosis remained. This represents a substantial increase in cell number as compared to cultures examined at 1 and 3 days. The new cells were found in two locations (Figs. 1 and 2): within the substance of the explant, comprising renal tubular structures, and lining the periphery of the cultured tissue.

Electron microscopic evaluation of 1- and 3-day cultures disclosed only a few viable tubular cells and capillaries. Most of the tubular structures found at 7 days (Figs. 2 and 3) contained central necrotic debris and were lined by flattened cells, which occasionally had short irregular microvilli. The nuclei of these cells often had prominent nucleoli. Mitochondria were often numerous and contained small granules. Most of the cells contained several
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Fig. 1. Explant of rat kidney maintained in organ culture for 7 days. Viable cellular elements are noted in tubules and also lining periphery of tissue. More central regions (lower) are totally necrotic. Outer medulla. Hematoxylin and eosin. X 320.

zones of Golgi complex, appearing as a tubular, vesicular, and saccular structure in the juxtanuclear area. Endoplasmic reticulum was present in three forms: slightly dilated, elongated cisternae with numerous ribosomes (most common); more vesicular profiles containing finely granular, relatively electron-lucent material; and markedly distended cisternae with invaginations forming small intracisternal vesicles with few ribosomes (least common). Free ribosomes and polyribosomal configurations were conspicuous in most of these tubular cells. Many of the cells contained numerous lipid droplets.

The peripheral cells were similar to those comprising the tubules, except for a few details. They were often much more flattened, with smaller and more rounded nuclei. The endoplasmic reticulum was more often of the type with marked dilatation of extensively branched cisternae, the form least commonly observed in the tubular cells. Finally, there were fewer fat droplets, and intercellular junctions were less conspicuous than in the tubular elements.

DISCUSSION

In these experiments, organ cultures of rat kidney consistently released into the medium a factor capable of stimulating erythropoiesis in exhypoxic polycythemic mice. That this factor is erythrogenin (REP) rather than erythropoietin is suggested by the presence of significant erythropoietic activity in the culture supernatant fluids only after incubation with serum. However, the findings do not exclude the possibility\textsuperscript{17} that erythropoietin itself is elaborated by the kidney in an inactive form and is then activated by a serum factor.

It is possible that the low levels of erythrogenin activity observed during
Fig. 2. (A) Two types of cell seen in 7-day cultures: comprising tubular structures (T) and lining periphery of explant (P). Inner medulla. Electron micrograph. X 4900. (B) Portion of typical tubule from 7-day culture showing cells with well-maintained nuclei and multiple lipid droplets. Inner cortex. Electron micrograph. X 4900.

the early period in culture may have been due to release of material that had been synthesized in vivo. However, several lines of evidence indicate that there was active synthesis of erythrogenin in vitro: (1) Increased erythrogenin activity in the medium during the second 3\( \frac{1}{2} \) days of culture suggests de
Fig. 3. Higher-power electron micrographs from 7-day culture showing endoplasmic reticulum (A–D), hypertrophic Golgi apparatus (E), and numerous ribosomal elements (A–E). Endoplasmic reticulum was characterized by elongated profiles (A,B), vesicular forms (C), and distended cisternae forming small intracisternal invaginations (arrow). Electron micrographs. (A) inner cortex, $\times$ 31,280; (B) outer cortex, $\times$ 31,280; (C) inner cortex, $\times$ 21,960; (D) outer medulla, $\times$ 25,800; (E) outer cortex, $\times$ 25,800.

novo synthesis. If the erythrogenin activity had been due to release of preformed material from autolyzed tissue, activity should have been highest during the first few days in culture when there was extensive tissue breakdown. Moreover, no erythrogenin was detectable with cultures maintained in serum-free medium in which there was complete necrosis. (2) The quantity of erythrogenin recovered from the medium during the second interval of culture exceeds the quantity that can be extracted directly from equivalent amounts of noncultured renal tissue, i.e., 5.8–33.7 U/g of tissue (Table 1) as compared to 0.3 U/g,$^{30}$ respectively. However, such comparisons may be misleading,
since erythrogenin is probably degraded or inactivated both during incubation of the cultures and during the process of extraction from whole kidney. (3) Recovery of erythrogenin from cultured tissue coincides with histologic changes suggesting active protein synthesis, i.e., markedly hypertrophic Golgi apparatus, numerous polyribosomes, and extensive rough-surfaced endoplasmic reticulum (Fig. 3). The last finding is suggestive of synthesis of protein bound for export from the cell.2' (4) Finally, preliminary experiments indicate that these explants incorporate $^{14}$C-leucine into a labeled fraction, isolated by column chromatography, with erythropoiesis-stimulating activity after incubation with serum.

It has yet to be ascertained whether normal regulatory factors mediate the production of erythrogenin by these cultures. Experiments are currently under way to determine whether the hypoxic preparation of the donor animals is a necessary stimulus for this process and to evaluate the direct effect of variations in oxygen tension in vitro. It has been suggested that organ cultures maintained in 95% air–5% CO$_2$, as in these experiments, are invariably somewhat hypoxic.$^{10,18,19}$

The similar findings with the four anatomic segments of kidney (Table 1) are consistent with the observation$^{20}$ that erythrogenin is present diffusely throughout the kidney and suggest that this factor may be synthesized by a cell type or types common to all segments studied. However, it is possible that the culture conditions provide a selective advantage to certain cells capable of producing erythrogenin and that the organ cultures may no longer be representative of the anatomic regions from which they were derived. The morphologic findings suggest that renal tubular cells are capable of synthesizing erythrogenin (Figs. 1–3). (Cultures of renal glomeruli from goats have also recently been found to elaborate erythropoietically active material.$^{26}$)

It seems unlikely that the peripheral lining cells were exclusively responsible for this process. However, the relationship of the cells observed in vitro to those responsible for erythrogenin production in vivo still requires clarification. The abundance and type of endoplasmic reticulum, the extensive Golgi zones, and the numerous ribosomes found in the epithelial cells developing during the later period of culture differentiate these cells from normal renal elements. These ultrastructural characteristics are reminiscent of the regenerating epithelial cells found in experimental tubular necrosis.$^{22,23}$ The origin of these cells is also unclear. It is possible that they were derived from surviving cells within the confines of the old tubular basement membranes, structures particularly resistant to autolysis.$^{24}$

A number of problems concerning the production of erythropoietin remain unresolved. For example, evidence differs regarding the importance of the kidney itself as compared to extrarenal sites (e.g., the hypothalamus, the carotid body, or the liver) in the regulation of this function.$^{4,17,55}$ The present experiments provide firm evidence that the kidney itself elaborates an erythropoietic factor, and investigations are now in progress using this organ culture system to evaluate the mechanisms involved in this process.
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REFERENCES

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