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Erythropoietin Production in Cultures of Goat Renal Glomeruli

(renal erythropoietic factor/Fe-uptake assay)

H. BURLINGTON*, EUGENE P. CRONKITE†, URSULA REINCKE†, AND ESMAIL D. ZANJANI*

* Department of Physiology, Mt. Sinai School of Medicine of the City University of New York, New York, N.Y. 10029, and
† Medical Department, Brookhaven National Laboratory, Upton, L.I., New York 11973

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ABSTRACT Cultures initiated with isolated goat-kidney glomeruli produce erythropoietin over periods of 7 months. Production seems dependent upon a minimum interval of about 30 days between changes of medium. Experiments distinguishing between erythropoietin, renal activator, and serum substrate support the conclusion that the factor released to the medium of long-term glomerular cultures is erythropoietin itself. The system offers promising opportunities for studying regulation of erythropoietin production, and possibly could be developed as a source of significant quantities of the hormone.

The kidney is established as a primary source of erythropoietin in numerous species (1–3). Unestablished is the intrarenal site of erythropoietin production. Evidence exists supporting locales that include a diffuse tubular origin (4), proximal tubules (5), juxtaglomerular apparatus (6, 7), and glomerulus (8). These reports, coupled with suggestions that kidney tissue in culture might yield erythropoietic agents (9, 10), prompted us to investigate cultures of isolated renal glomeruli as a source of erythropoietin. We found that cultures of goat glomeruli are capable of producing an erythropoietically active substance in significant quantity for as long as 7 months. The activity is ascribable to erythropoietin, and appears to be unrelated to other factors that may be involved in erythropoiesis.

METHODS

Cultures

A detailed description of procedure and culture characteristics appears elsewhere (11). Briefly, a modification of the method of Greenspon and Krakower (12) is used to isolate renal glomeruli, which are then plated in flasks or dishes in growth medium containing 10–15% serum. ‡ Glomeruli that attach to the flask surface give rise to cellular outgrowth, usually within 3–9 days, generally forming a confluent monolayer by 21–30 days. Fig. 1 shows the appearance of the outgrowth on day 15 in a typical culture of goat glomeruli. Medium, collected at different intervals during culture, was assayed for erythropoietin both *in vivo* and *in vitro*

Erythropoietin assay

In vivo. Ex-hypoxic polycythemic mice (Brookhaven strain) (13) or transfused plethoric mice (Swiss–Webster strain) (14) were used to determine erythropoietic activity. Activity was related to uptake of ^{59}Fe into circulating erythrocytes after an intraperitoneal injection of test substance. In addition to test groups, all assays included saline-injected controls and at least three groups receiving different levels of a standard preparation of erythropoietin. A minimum of two separate assays were done on all media.

In vitro. Short-term cultures of rat or human bone-marrow cells were used; the method of Krantz and coworkers (15) was followed. This method uses incorporation of ^{59}Fe into extractable heme as an index of erythropoietic activity. Assays were done in triplicate.

Pertinent details of procedures related to specific experiments are included where appropriate in legends accompanying tables and figures.

RESULTS

Erythropoietic activity of culture media

The level of erythropoietic activity in goat glomerular culture media collected after a 31-day interval between culture days 156 and 187 is indicated in Fig. 2. Significant activity was associated with media harvested from primary and first-passage cultures (column *1*). Second-passage (column *2*) and pooled first- and second-passage (column *3*) media were without activity in this culture, however, we have seen abundant activity in media of first, second, and third passage subcul-

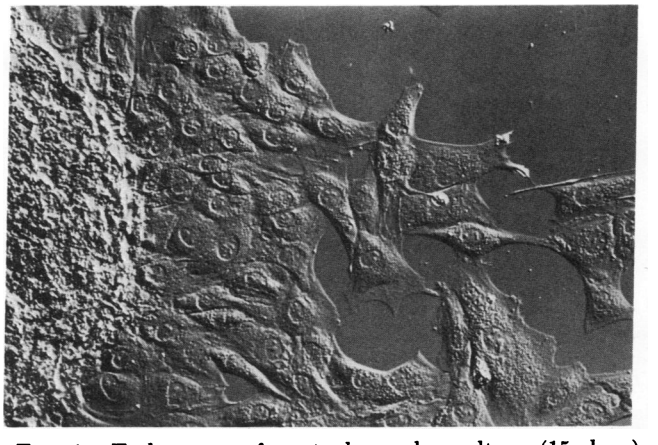

FIG. 1. Early stage of goat glomerular culture (15 days) viewed with Nomarski interference optics. Monolayer is seen developing from intact glomerulus. (×400)

‡ Results reported here were obtained with McCoy's 5A medium (modified) and fetal-calf serum.

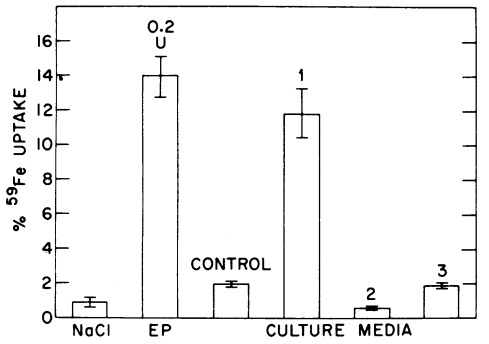

FIG. 2. Activity determined in ex-hypoxic polycythemic mice. All mice received a total of 2 ml of test substance administered in 2 equal doses on days 3 and 4 after hypoxia. ^{59}Fe (0.5 μCi per mouse) was given on day 5 and the % of ^{59}Fe uptake into erythrocytes was determined on day 7. *1*, primary + first passage; *2*, second passage; *3*, first + second passage. Control = McCoy's 5A medium + 15% fetal-calf serum, incubated 31 days at 38°. Responses to 0.05 units and 0.8 units of erythropoietin standard were 5.89 ± 0.76% and 28.13 ± 3.11%, respectively. Primary culture through second passage required 187 days in culture.

tures in other instances. Such variability appears to be related to the age and history of the cultures.

The ability of glomerular culture media, in this instance derived from first- and second-passage subcultures, to stimulate heme synthesis in short-term cultures of rat bone-marrow is evident from results shown in Fig. 3. Attention is directed to the 38-day collection interval involved.

The *in-vitro* response was shown to be dose-related in a separate experiment (Table 1).

Nature of culture factor

Early work led to suggestions that the erythropoietic factor released from kidney is not erythropoietin, but rather an agent that reacts with a substance in blood to produce the hormone (16). Substantial evidence has since been amassed in support of a renal–hepatic axis (17, 18) involving inter-

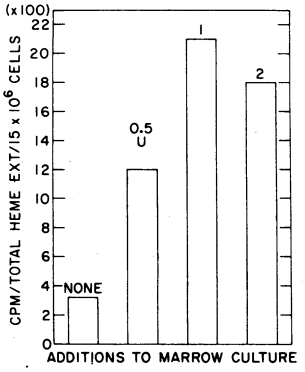

FIG. 3. 1 ml of culture medium lyophilized and redissolved in 0.2 ml of NCTC-109 medium was incubated with rat marrow cells at 37° for 42 hr. ^{59}Fe was then added and incubation was continued an additional 6 hr. *None*, control medium (McCoy's 5A modified + 12% fetal-calf serum, incubated at 38° for 38 days). *Ordinate*, cpm in total heme extracted from 15 × 10^6 cells. *1*, first-passage subculture; *2*, second-passage subculture. 38-day collection; 187 culture days for combined first and second passages.

TABLE 1. *Effect of erythropoietin and various doses of goat glomerular culture medium on heme synthesis by rat and human marrow cells in vitro*

Additions to culture	^{59}Fe Rat marrow (cpm/15 × 10^6 cells)	^{59}Fe Human marrow (cpm/1.5 × 10^6 cells)
None	560 ± 42	148 ± 21
0.01 units of erythropoietin*	590 ± 24	—
0.02	610 ± 21	—
0.04	1214 ± 53	—
0.06	1697 ± 32	—
0.10	3112 ± 64	880 ± 30
0.02 ml of culture medium†	516 ± 49	—
0.04 ml	600 ± 31	—
0.08 ml	1397 ± 63	—
0.10 ml	2091 ± 103	1138 ± 88
0.16 ml	3420 ± 192	976 ± 42

* Step III Sheep-plasma erythropoietin (Connaught Labs., Toronto, Canada).

† Medium collected on culture day 53; 29-day collection interval.

action of renal erythropoietic factor with a liver-generated substrate to yield erythropoietin in peripheral blood.

It was important to determine whether the factor produced in glomerular cultures is erythropoietin *per se*, a precursor substrate, or an activator analogous to renal erythropoietic factor. The following series of experimental results support the conclusion that the culture product is erythropoietin.

(*a*) Fig. 4 summarizes the effect of antibody to erythropoietin (anti-erythropoietin) on the activity of culture media. Activity is clearly abolished by anti-erythropoietin.

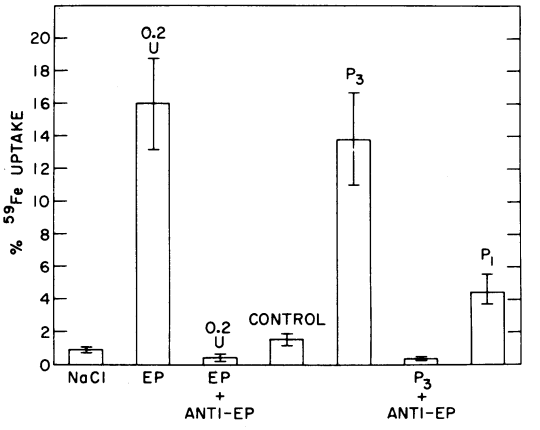

FIG. 4. Activity determined in ex-hypoxic polycythemic mice, as described in Fig. 2. Anti-erythropoietin prepared in rabbits against human urinary erythropoietin, injected with complete Freund's adjuvant into foot pad (3 weeks, 2 injections/week; 30 units/injection). P_1, first-passage subculture; P_3, third-passage subculture; Control = McCoy's 5A modified + 12% fetal-calf serum, incubation for 46 days at 38°. Responses to 0.05 units and 0.8 units of erythropoietin standards were 8.21 ± 0.90% and 31.25 ± 3.92%, respectively. 46-day collection; 234 culture days from P_1–P_3. *Ep*, erythropoietin.

TABLE 2. *The nature of erythropoietic activity in glomerular culture medium*

Material assayed*	% ^{59}Fe incorporation into erythrocytes ±SEM
(*a*) Saline	0.24 ± 0.03
(*b*) 0.2 units of erythropoietin	3.61 ± 0.36
(*c*) 0.2 units of erythropoietin + anti-erythropoietin†	0.19 ± 0.03
(*d*) Renal erythropoietic factor alone	0.48 ± 0.12
(*e*) Goat serum alone	0.30 ± 0.04
(*f*) Goat serum + anti-erythropoietin	0.25 ± 0.05
(*g*) Culture medium alone	2.03 + 0.25
(*h*) Culture medium + anti-erythropoietin	0.26 ± 0.25
(*i*) Renal erythropoietic factor + (*f*) above	3.18 ± 0.58
(*j*) Renal erythropoietic factor + (*h*) above	0.54 ± 0.20
(*k*) (*h*) above + goat serum	0.29 ± 0.20

* Activity assayed in transfused plethoric mice.

† Procedure as follows whenever anti-erythropoietin was used: Test substance and anti-erythropoietin incubated at 37° for 60 min, then at 4° for an additional 6 hr, followed by addition of goat antiserum to rabbit IgG to react with unbound anti-erythropoietin at room temperature for 30 min. The mixture was then centrifuged and the supernatant was collected for assay.

(*b*) The combination of results displayed in Table 2 serve to eliminate renal erythropoietic factor and plasma substrate as agents responsible for erythropoietic activity of culture media, while lending further support for designation of this substance as erythropoietin. Again, culture medium incubated with anti-erythropoietin (group *h*) lost all activity, while at the same time it was possible to show with proven preparations of renal erythropoietic factor that "substrate" is uninvolved (group *i* and *j*), and that renal erythropoietic factor was similarly absent from active culture medium (group *k*).

(*c*) Sialic acid appears to be a needed component of erythropoietin for activity *in vivo* and desialylation by neuraminidase significantly reduces activity in the standard *in vivo* bioassay (19). Table 3 indicates that the same result is true for the activity associated with glomerular culture medium.

TABLE 3. *Effect of neuraminidase on erythropoietic activity of glomerular culture medium*

Material assayed*	% ^{59}Fe incorporation into erythrocytes ± SEM
Saline	0.18 ± 0.0
0.2 units of erythropoietin	3.0 ± 0.41
Medium alone†	1.97 ± 0.21
Medium + neuraminidase‡	0.17 ± 0.06

Sigma Type-VI neuraminidase from *Clostridium perfringens*, stated to be free of protease activity by the supplier, was used.

* Assay in hypertransfused plethoric mice.

† Same medium as in Table 2 (group *g*), incubated 40 min at 37°.

‡ Incubated 40 min at 37°. Neuraminidase removal before assay as described in ref. 19.

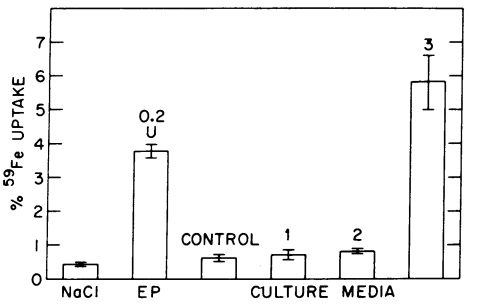

FIG. 5. Assay conditions as described in legend, Fig. 2. Primary culture of goat glomeruli. Media harvested on culture day-29 (*1*), day-35 (*2*), and day-42 (*3*). Responses to 0.05 units and 0.8 units of erythropoietin standards were 1.42 ± 0.031% and 7.85 ± 0.84%, respectively.

Influence of collection interval on Erythropoietin in culture media

Production of detectable erythropoietin in glomerular cultures appears dependent upon an elapsed interval of not less than 29–30 days between medium changes. This result is illustrated in Fig. 5, which shows that only a 32-day collection interval yielded erythropoietin in a culture tested additionally for erythropoietin production at 19- and 25-day intervals. We have consistently seen the need for this minimum period in both newly established monolayer cultures and in older (6 months) cultures, which displayed extensive stacking and overlapping of cells. Regulatory factors involved in erythropoietin production by glomerular cultures may have a threshold level that is only reached after a 30-day period under the culture conditions used.

CONCLUSION

The glomerular culture system appears to have important potentials in several directions. For example, mechanisms governing erythropoietin formation and release are uniquely accessible to experimental manipulation. In addition, if significant quantities of erythropoietin can be routinely produced in such cultures, enhanced opportunities are provided for purification, chemical characterization, and eventual therapeutic application of the hormone.

A preliminary report of this work was presented at the Annual Meeting of the American Society of Hematology (Burlington H., Cronkite, E. P., Reincke, U. & Zanjani, E. D. (1971) *Blood* **38**, 827). Human urinary Ep-A-1-TaLSL was kindly supplied by the NIH Erythropoietin Distribution Committee. This work was supported, in part, by the U.S. Atomic Energy Commission.

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