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Insulin-like growth factor I stimulates erythropoiesis in hypophysectomized rats

(body growth/erythrocyte mass/growth hormone)

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ABSTRACT **Stimulation of erythropoiesis during growth is necessary to ensure proportionality between erythrocyte mass and body mass. However, the way by which erythrocyte formation is adapted to body growth is still unknown. Growth arrest in hypophysectomized rats is accompanied by decreased erythropoiesis. We have, therefore, examined whether insulin-like growth factor I (IGF-I), the mediator of growth hormone effects on body growth, is able to restore erythropoiesis in these animals. Subcutaneous infusions of 120 μg of recombinant human IGF-I per day in hypophysectomized rats led to increases in body weight, ^{59}Fe incorporation into erythrocytes, and the number of reticulocytes that were similar to increases caused by infusions of 28 milliunits of human growth hormone per day. Body weight gain and ^{59}Fe incorporation were linearly correlated. Like growth hormone, IGF-I also caused a significant rise in serum erythropoietin concentrations. However, the stimulatory effect on erythropoiesis occurred before serum erythropoietin levels had risen. These results demonstrate that IGF-I mediates the stimulatory effect of growth hormone on erythropoiesis *in vivo* and thus further support the somatomedin concept. They also show that IGF-I can stimulate erythropoiesis in an endocrine manner, and they suggest two possible routes of action: a direct one and an indirect one by means of enhanced erythropoietin production.**

It is well established that erythrocyte (RBC) mass increases in strict proportion to the body mass during the growth period of mammals (1). This proportionality is physiologically important because it ensures that increased oxygen consumption during growth is matched by an increased oxygen transport capacity. However, the way by which this adaptation is brought about is unknown. Since erythropoiesis in the adult is primarily controlled by erythropoietin (Epo) (2), one could assume that Epo is of major importance also within this adaptive process. Epo is mainly produced by the kidneys. Its production rate is thought to be related to the ratio of renal oxygen demand to renal oxygen supply (2). One might therefore speculate that the increase of kidney mass during growth causes an increased renal oxygen consumption and, in consequence, a relative renal deficiency of oxygen. In turn, an enhanced rate of Epo production would lead to stimulation of erythropoiesis and thus adapt RBC mass to body growth. However, the recent observation that massive hypertransfusion of mice and rats during rapid growth does not fully suppress erythropoiesis (3, 4) argues against this concept. Therefore, the question arises whether factors other than Epo may contribute to the stimulation of erythropoiesis during growth. Obvious candidates for such a function are growth hormone (GH) and insulin-like growth factor I (IGF-I).

Growth arrest in hypophysectomized rats is not only accompanied by low IGF-I serum levels (5, 6) but also by reduction of erythropoiesis (7, 8). GH has been reported to stimulate erythropoiesis in hypophysectomized rats *in vivo* (9–11) and *in vitro* (12, 13). IGF-I, a polypeptide hormone of molecular mass 7649 and a structural homologue of insulin (14), might well be a common denominator for the regulation of whole body mass and RBC mass: its synthesis is governed by GH (15), there is unambiguous evidence that it mediates GH effects (6, 16, 17), and it enhances erythropoiesis *in vitro* (18–20). Therefore, we investigated the effect of IGF-I on erythropoiesis *in vivo* and compared it with that of GH. To this end, hypophysectomized rats were infused with human GH or recombinant human IGF-I (rhIGF-I) through subcutaneously implanted miniosmotic pumps, and body weight, ^{59}Fe incorporation into RBCs, the number of reticulocytes, and serum erythropoietin levels were determined.

MATERIALS AND METHODS

Animals. Normal male Tif RAI rats and male Tif RAI rats 6–8 weeks after hypophysectomy were used. The rats were 5 weeks old at the time of hypophysectomy. Hypophysectomy was kindly performed by R. Cortesi (Ciba–Geigy, Basel). The animals had free access to food and water and were matched with respect to body weight in the various experimental groups. Body weight ranged from 120 to 140 g and was recorded daily during the experiments.

Experiments with GH and IGF-I. Hypophysectomized rats were implanted subcutaneously with miniosmotic pumps (Alzet, model 2001) and infused for 6 days with saline (control), human GH [28 milliunits (mu)/day; Nanormon, Nordisk], or rhIGF-I [120 μg/day; gift from W. Rutter (Emeryville, CA) and J. Nüesch (Ciba–Geigy, Basel)]. On the second or fourth day of the infusion five rats per group were injected intraperitoneally with 2 μCi (1 Ci = 37 GBq) of ^{59}Fe per 100 g of body weight. Forty-eight hours after the injection the rats were anesthesized in ether and bled by heart puncture. Blood of each rat was collected in heparin-coated and nonheparinized plastic tubes. The latter was kept on ice for 1 hr and centrifuged for 15 min at 1500 × *g* and 4°C. Serum was stored at −20°C. The fractional ^{59}Fe incorporation into RBCs was determined in heparinized blood and calculated according to the following generally used formula: % ^{59}Fe incorporation = (radioactivity per ml of blood/total radioactivity injected) × blood volume (ml) × 100. Simultaneous determinations of blood volume (^{51}Cr method) and ^{59}Fe

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Abbreviations: IGF-I, insulin-like growth factor I; RBC, erythrocyte; Epo, erythropoietin; GH, growth hormone; rhIGF-I, recombinant human IGF-I: mu, milliunit(s).

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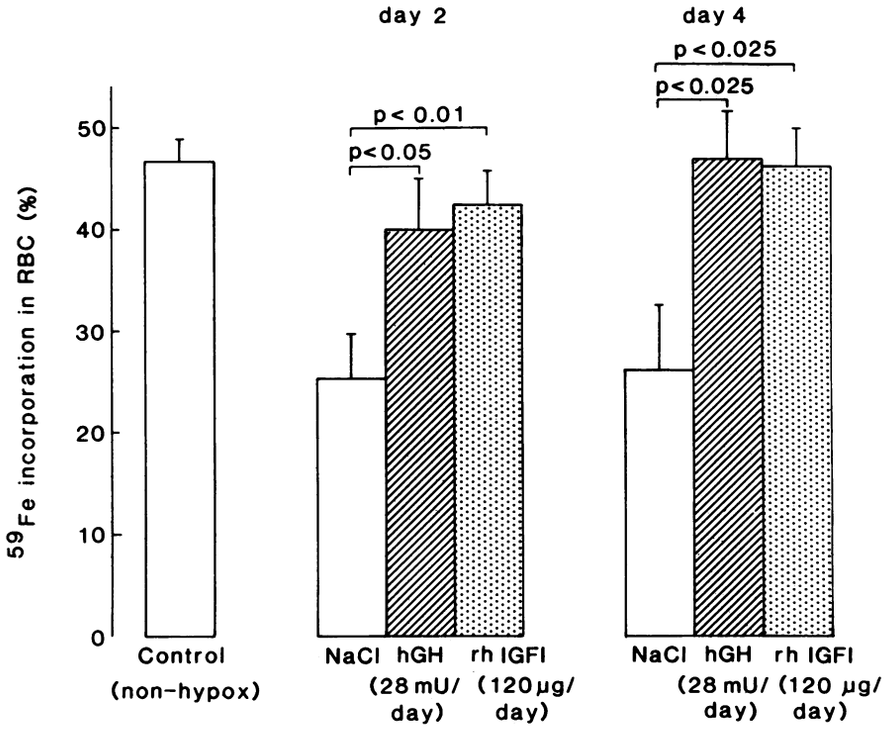

FIG. 1. ^{59}Fe incorporation into RBCs of hypophysectomized rats infused subcutaneously with saline, human GH (28 mu/day), or rhIGF-I (120 µg/day). Control, nonhypophysectomized. Data are means ± SEM of five animals per group. Rats were injected with 2 µCi of ^{59}Fe per 100 g of body weight on days 2 and 4 of the infusion and ^{59}Fe incorporation into RBCs was determined in whole blood 48 hr later.

incorporation in the same animal were not possible because of overlap of the ^{51}Cr and ^{59}Fe spectra. Therefore, blood volume was calculated according to published data (21) as 6.6% and 5.8% of the body weight for normal and hypophysectomized rats, respectively. Aliquots of heparinized blood were also taken for determination of hematocrit, erythrocyte, and reticulocyte counts.

Experiments with Epo. To quantitate the effect of Epo on ^{59}Fe incorporation in hypophysectomized rats, the following experiments were performed. A pool of rat serum containing high levels of Epo was prepared by exposing normal male rats to normobaric hypoxia (8% O_2) for 18 hr. Epo activity of the pool was 1.3 u/ml, as determined with the exhypoxic polycythemic mouse assay for Epo (22). Hypophysectomized rats (five animals per group) were injected subcutaneously every 12 hr for 4 days with 1 ml of different dilutions of the Epo-enriched pool corresponding to 125, 250, 500, and 1000 mu of Epo per injection. Fifty-four hours after the first Epo injection the animals received intraperitoneally 2 µCi of ^{59}Fe per 100 g of body weight. Forty-eight hours later—i.e., 6 hr after the last Epo injection—they were bled by heart puncture. Blood was collected as described above.

Determination of Epo and IGF-I. Serum concentrations of Epo were determined by radioimmunoassay (RIA) as described in detail (23). Epo-enriched rat serum was used as a standard that had been calibrated in the exhypoxic polycythemic mouse assay for Epo (22). Each sample was measured in duplicate in three separate assays.

Serum IGF-I was determined at the end of the infusion experiments by RIA (24) using rabbit antiserum (final dilution, 1:20,000) batch 6656/251074 (gift from the late K. Reber, Hoffmann–La Roche, Basel) against human IGF-I. Pure rat IGF-I was not available during the study to perform standard curves for the determination of endogenous rat IGF-I. The latter was, therefore, expressed in human ng equivalents. Recently, we were able to use pure rat IGF-I (kindly supplied by M. Kobayashi, Fujisawa Pharmaceutical, Osaka, Japan) as a standard and found that endogenous rat IGF-I levels are 4- to 6-fold higher than the corresponding human ng equivalents.

After acidification of the sera, IGF-I was separated from IGF carrier proteins on Sep-Pak C_{18} cartridges (Waters Associates). The extraction protocol supplied with the so-

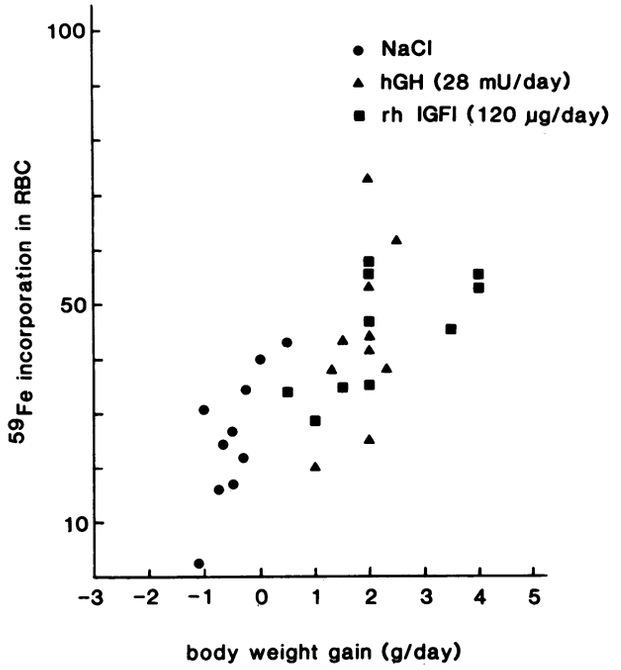

FIG. 2. Correlation between body weight gain and ^{59}Fe incorporation into RBCs of individual hypophysectomized rats infused subcutaneously with saline, human GH, or rhIGF-I. If a linear regression curve is calculated one obtains ^{59}Fe incorporation = 29.5 + 6.7x (body weight gain); r = 0.72.

Table 1. Number and percentage of reticulocytes in the blood of hypophysectomized rats infused with saline, human GH, or rhIGF-I

	Reticulocyte count			
	After 4 days		After 6 days	
Infusion	Cell number $\times 10^{-6}$ per μl of blood	% RBCs	Cell number $\times 10^{-6}$ per μl of blood	% RBCs
Saline	0.22 ± 0.02	4.3 ± 0.3	0.23 ± 0.02	4.5 ± 0.4
Human GH	0.33 ± 0.03*	6.8 ± 0.6*	0.32 ± 0.01*	5.7 ± 0.3*
rhIGF-I	0.30 ± 0.02*	5.6 ± 0.4*	0.39 ± 0.04*	7.7 ± 0.6*

Data are presented as means ± SEM (n = 5). Human GH, 28 mu/day; rhIGF-I, 120 μg/day.
*$P < 0.05$ vs. saline, Student's t test.

matomedin C RIA kit from Immuno Nuclear (Stillwater, MN) was followed. Recovery of added human IGF-I was between 92% and 103%. After reconstitution with 1 ml of phosphate-buffered saline (pH 7.4) containing 0.2% serum albumin, all samples were assayed at three different dilutions.

Statistics. Levels of significance were calculated by using Student's t test.

RESULTS

In saline-infused hypophysectomized rats serum levels of immunoreactive IGF-I were 6 ± 3 human ng equivalents/ml (mean ± SEM; n = 10), as compared to 200–250 human ng equivalents/ml in normal (nonhypophysectomized) rats. Infusion of 28 mu of human GH per day over 6 days in hypophysectomized rats induced an increase of the endogenous IGF-I serum level to 40 ± 8 human ng equivalents/ml (mean ± SEM; n = 6) corresponding to 160–240 ng/ml (see *Materials and Methods*). By the sixth day of the infusion with rhIGF-I, IGF-I serum levels had risen to 208 ± 32 ng/ml (mean ± SEM; n = 10). These increases in serum IGF-I levels were accompanied by a nearly linear increase in body weight between the second and sixth day of infusion: mean body weight gain per day was 2.23 ± 0.17 g for GH and 2.58 ± 0.46 g for rhIGF-I (mean ± SEM; n = 10). Hypophysectomized control animals infused with saline or injected with Epo-enriched rat serum did not gain weight.

The effects of GH and IGF-I on erythropoiesis were assessed by measurement of the hematocrit, the number and percentage of reticulocytes, and ^{59}Fe incorporation into newly formed RBCs on the second, fourth, or sixth day of the infusion as indicated in the corresponding figures and tables. The hematocrit in hypophysectomized control animals was 0.30 ± 0.02 (mean ± SEM; n = 10), as compared to 0.35 ± 0.03 in normal rats (mean ± SEM; n = 15),and did not change during GH or rhIGF-I infusion.

^{59}Fe incorporation into newly formed RBCs under the infusion of GH or IGF-I is shown in Fig. 1. It was significantly decreased (46%) in hypophysectomized rats. Both GH and IGF-I stimulated ^{59}Fe incorporation to near normal already after 2 days of infusion and had restored it completely on day 4. When ^{59}Fe incorporation into RBCs of the individual animals is plotted against body weight gain a positive correlation between the two indices is obtained (Fig. 2).

As shown in Table 1, GH and IGF-I caused a significant increase of the number and percentage of reticulocytes, another index of erythropoiesis, after 4 and 6 days of infusion.

Since Epo is considered as the major determinant of erythropoiesis *in vivo* (2), we tried to find out whether or not the stimulatory effects of GH or rhIGF-I on erythropoiesis are mediated by Epo. Serum Epo levels were, therefore, measured by RIA (23) after different times of infusion of saline, GH or rhIGF-I (Table 2). No change of the serum Epo levels was observed after 2 days of infusion of GH or rhIGF-I. After 4 days, GH and IGF-I had caused a 50% increase of the serum Epo concentration, which rose to >150% over that of saline-treated controls after 6 days of GH or rhIGF-I treatment.

To determine whether the rise of serum Epo was sufficient to account for the stimulation of erythropoiesis during the infusion of GH and IGF-I, we examined the effect of exogenous rat Epo on serum Epo levels and on ^{59}Fe incorporation into RBCs of hypophysectomized rats. Serum Epo concentrations were raised by repeated subcutaneous injections of Epo-enriched rat serum (Fig. 3). This treatment led to a dose-dependent increase of ^{59}Fe incorporation into RBCs (Fig. 3). During Epo as well as during GH or rhIGF-I treatment ^{59}Fe incorporation and serum Epo concentrations were linearly correlated (Fig. 4). However, the correlation coefficients are different for the Epo and the GH or IGF-I treatment. Interestingly, ^{59}Fe incorporation and serum Epo of normal (nonhypophysectomized) rats fit well into the correlation obtained for GH or rhIGF-I-treated hypophysectomized rats.

DISCUSSION

This study was designed to examine whether subcutaneous infusions of GH and rhIGF-I, the mediator of GH effects on body growth, are capable of stimulating decreased erythropoiesis in hypophysectomized rats. We found that resumption of growth by GH and IGF-I treatment was accompanied by a significant enhancement of erythropoiesis as monitored by increased ^{59}Fe incorporation into RBCs and by an increase in the number and percentage of reticulocytes but no change of the hematocrit. It is important to note that body weight gain parallels the increase in tibial epiphyseal width and organ weights during GH and IGF-I treatment of hypophysectomized rats (6). From the above findings one may infer that both hormones increased RBC formation and thus RBC mass to an extent sufficient to refill the expanding intravascular volume during growth. This conclusion is supported by the positive correlation between ^{59}Fe incorporation into RBCs and body weight gain of the individual animals (Fig. 2). Thus, the stimulation of erythropoiesis by GH or IGF-I parallels the increase in body weight.

Our results obtained with GH are consistent with reports in the literature that repeated subcutaneous injections of GH

Table 2. Effects of subcutaneous infusions of saline, human GH, and rhIGF-I on serum concentrations of immunoreactive Epo in hypophysectomized rats

	Serum Epo concentration, mu/ml		
Infusion	2 days	4 days	6 days
Saline	24.1 ± 3.5	22.4 ± 2.4	20.0 ± 2.2
Human GH	28.8 ± 4.1	35.3 ± 10.6	53.5 ± 5.9*
rhIGF-I	25.9 ± 2.0	32.4 ± 4.7†	51.8 ± 7.6*

Data are presented as means ± SEM (n = 5).
*$P < 0.005$ vs. saline.
†$P < 0.025$ vs. saline.

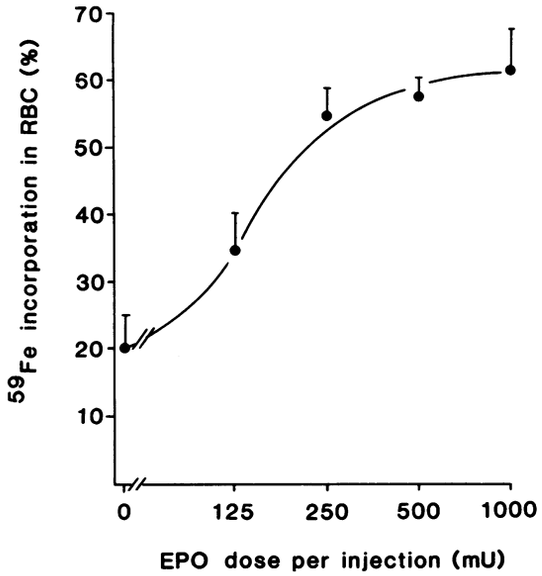

FIG. 3. ^{59}Fe incorporation into RBCs of hypophysectomized rats receiving repeated subcutaneous injections of Epo-enriched rat serum. ^{59}Fe incorporation is plotted as a function of the dose of Epo administered per injection. Data are means ± SEM of five animals per group. Serum Epo levels measured 6 hr after the last injection were 19 ± 11 (no Epo injection) and 23 ± 1.7, 26 ± 2.2, 28 ± 2.8, and 30 ± 3.0 mu/ml (mean ± SEM) for the four administered Epo doses.

in hypophysectomized animals stimulate erythropoiesis in proportion to the increase in body weight and blood volume without affecting the erythrocyte concentration in the blood (10, 11, 21) and lead to a time-dependent increase in serum Epo levels (12). Beyond that, however, this study shows that (*i*) IGF-I itself in the absence of GH stimulates erythropoiesis *in vivo*, (*ii*) IGF-I is able to exert this effect in an endocrine fashion, and (*iii*) IGF-I mediates the effect of GH on erythropoiesis and Epo synthesis, which further supports the somatomedin concept (25). The latter conclusion is based on the following reasoning: infusion of 28 mu of GH per day or 120 μg of rhIGF-I per day raised the plasma levels of endogenous immunoreactive rat IGF-I from 6 ± 3 human ng equivalents/ml (corresponding to 24–36 ng/ml) to 40 ± 8 human ng equivalents/ml (corresponding to 160–240 ng/ml) and that of circulating exogenous human IGF-I to 208 ± 32 ng/ml, respectively. Thus, the endogenous (rat) and exogenous (human) IGF-I levels during GH or rhIGF-I infusion are comparable. They are also comparable with respect to their biological potencies, because partially purified rat IGF-I, standardized in our RIA against human IGF-I, was found to be three to five times more active than human IGF-I on DNA synthesis of rat calvaria cells *in vitro* (26). The similar rise of IGF-I, whether induced endogenously by means of GH administration or effected by infusion of exogenous human IGF-I, is accompanied by increases in ^{59}Fe incorporation, reticulocyte number, and serum Epo levels that are similar for both treatments (Fig. 1, Tables 1 and 2).

A provocative question results from our study: Is the stimulation of erythropoiesis by GH or IGF-I due to a direct interaction of endogenous or exogenous IGF-I with hemopoietic cells or is it due to enhanced Epo production? To clearly distinguish between these two possibilities we would have had to "neutralize" endogenous Epo during GH or rhIGF-I treatment. However, monospecific antibodies against rat Epo are not yet available in sufficient amounts to perform this type of *in vivo* experiment. Therefore, we used another approach—we raised serum Epo levels in hypophysectomized rats not receiving GH or IGF-I by repeated injections of Epo-enriched rat serum and measured ^{59}Fe incorporation into RBCs as a function of the serum Epo levels. It turned out that the correlation between these two indices was strikingly different in the absence from that in the presence of GH or IGF-I (Fig. 4). Epo appeared to be much more effective on ^{59}Fe incorporation in the absence than in the presence of GH or IGF-I, including normal animals, in which GH and IGF-I are physiologically present. Thus, GH or IGF-I decreases the sensitivity of erythropoiesis toward Epo, which may indicate some direct interaction between IGF-I and erythroid precursor cells *in vivo*.

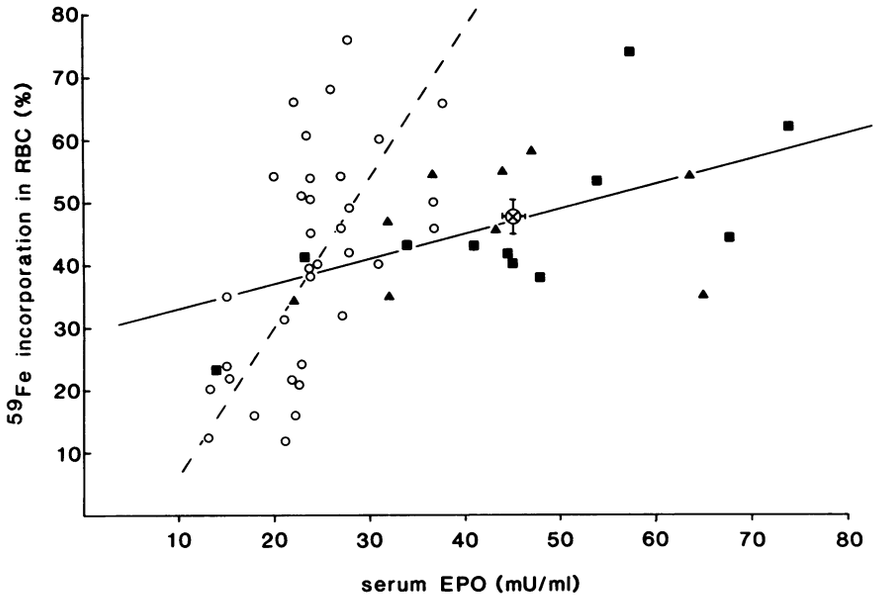

FIG. 4. Correlations between serum Epo concentrations and ^{59}Fe incorporation into RBCs in hypophysectomized rats injected with saline or Epo-enriched rat serum (○), human GH (28 mu/day) (▲), or rhIGF-I (120 μg/day) (■). Open symbols, IGF-I deprived; closed symbols, IGF-I repleted group. The linear regression curves $y = -18.8 + 2.4x$; $r = 0.75$ (dashed line) for Epo- and saline-treated rats and $y = 29.0 + 0.4x$; $r = 0.57$ (solid line) for GH- and IGF-I-treated rats were significantly different ($P < 0.02$). The open circle with a cross represents the means ± SEM of five normal (nonhypophysectomized) male Tif RAI rats.

This latter contention is supported by four further arguments: (*i*) the stimulation of ^{59}Fe incorporation by GH or IGF-I was already increased after 2 days of infusion when serum Epo was still unaltered; (*ii*) IGF-I receptors have been demonstrated on erythroid precursor cells (27); (*iii*) IGF-I has been shown to act as a mitogen on late erythroid precursors *in vitro* (18–20); (*iv*) removal of the kidneys, which are considered as the source of GH-stimulated Epo production (12), decreases but does not prevent the stimulatory effect of GH on erythropoiesis (11).

Taken together, the results obtained in this study suggest that IGF-I may be the factor that governs RBC formation and organ and body growth and would thus account for the adaptation of RBC mass to whole body mass.

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