# *In Vivo* and *In Vitro* Erythropoietin Activities in Cultures of a Hepatocellular Carcinoma Cell Line (43577)

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*Abstract.* **The present studies were undertaken to characterize erythropoietin (Ep) production in an Ep-producing hepatocellular carcinoma (Hep3B) cell line. Hep3B cells which had been maintained in culture were transplanted under the renal capsule and subcutaneously in nude mice. The Hep3B xenograft doubling time is approximately 7 days. The mean hematocrit value of the Hep3B tumor-bearing nude mice was 33.2 ± 1.1% ($n$ = 8), which was significantly lower than that of control nongrafted nude mice (40.8 ± 1.7%, $n$ = 5). The Hep3B tumor-bearing nude mice showed significantly higher Ep levels in the sera (37.5 ± 5.5 munits/ml, $n$ = 8) than control nude mice (13.5 ± 2.7 munits/ml, $n$ = 5). Ep levels in the sera were correlated ($R$ = 0.714) with the total Ep in the tumor extracts, whereas no Ep was detectable in any of the kidney extracts. On the other hand, an inverse linear relationship ($R$ = −0.811) between the hematocrit values and Ep levels in the sera was demonstrated in the Hep3B tumor-bearing nude mice. The Hep3B cells recultured after growing in the nude mice were capable of enhancing Ep production in response to hypoxia, very similar to the original Hep3B cells which had been maintained in culture during the same time period. In addition, 15-methylprostaglandin $E_1$ at a concentration range of 4–400 ng/ml produced significant increases in Ep secretion and cAMP accumulation in Hep3B cultures under hypoxic conditions (1% $O_2$). The Ep produced by Hep3B cells expressed 3.7 times higher *in vitro* bioactivity than immunoactivity. The bioactivity of Hep3B Ep was completely neutralized by an antibody to highly purified human recombinant Ep. In contrast, the *in vivo* bioactivity of the Hep3B Ep was less than one tenth of its immunoactivity. These results indicate that the Hep3B tumor-bearing nude mice and the *in vitro* Hep3B culture system may provide a reproducible model system which should be useful for studies of the mechanism of Ep production.** [P.S.E.B.M. 1993, Vol 203]

Erythropoietin (Ep) is a glycoprotein hormone that is the primary regulator of erythropoiesis, causing an increase in the proliferation and differentiation of marrow erythroid progenitor cells. The kidney is well known to be the primary site of Ep production in the adult (1, 2). The liver is the main source of extrarenal Ep in the fetus (3) and anephric adult mammal (4). Ep production has also been demonstrated in cultures of human renal carcinoma cells (5, 6), human testicular germ tumor cells (7), mouse macrophages (8), and human hepatic carcinoma cells (9). Kurtz *et al.* (10) have demonstrated Ep production in cultured rat kidney mesangial cells, and in fetal mouse liver cells (11). However, the difficulties in establishing continuously growing normal kidney or liver cell lines from animals and the cloning of Ep-producing tumor cell lines emphasize the need for a reproducible system as a model to study the mechanism of Ep production. A cloned hepatocellular carcinoma cell line (Hep3B) has been demonstrated to secrete a number of plasma proteins (12), including Ep (13). The present studies were undertaken to evaluate the *in vivo* and *in vitro* erythropoietic responses of a Hep3B cell line to hypoxia and some autacoids.

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## Materials and Methods

**Transplantation of a Human Hepatocellular Carcinoma Cell Line (Hep3B) into Nude Mice.** A Hep3B cell line (12, 14) was carried in a monolayer cell culture

*in vitro*. Inbred female BALB/c athymic nude mice, 6–8 weeks old (Charles River Co., Wilmington, MA), were used for successive passaging of those Hep3B tumors. Nude mice were housed in a laminar air flow hood unit. Hep3B tumors were transplanted under the kidney capsules of nude mice. The kidney was approached through a posterior lumbar incision under sterile conditions using ether anesthesia. A single-cell suspension of Hep3B cells (approximately $1 \times 10^7$ cells) was inoculated into the subrenal capsular space using a 25-gauge needle. Approximately 8 weeks later, the Hep3B tumors were removed aseptically, placed on ice, minced into 2-$mm^3$ fragments, and then implanted subcutaneously on the back of the nude mice using a No12 trocar needle. When new tumor growths of more than 1 cm in diameter were developed, they were removed and transplanted aseptically to other nude mice subcutaneously on the back. Tumors were measured at the width ($a$) and length ($b$) using micrometer calipers and volume of the tumors ($V$) was estimated using micrometer calipers according to the formula $V = (a^2 \times b)/2$ (15) and expressed in $mm^3$.

**Preparation of Samples from Hep3B Tumor-Bearing Nude Mice.** Blood samples for hematocrit and serum erythropoietin (Ep) determinations were drawn via cardiac puncture at the time of sacrifice. Serum samples were obtained after centrifugation and were frozen at −80°C until tested in the Ep assay. The tumors from nude mice were weighed, minced, and placed in cold phosphate-buffered saline immediately after extirpation. These preparations were then washed with phosphate-buffered saline and homogenized for 90 sec at 4°C with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 0.02 $M$ cold phosphate-buffered saline at a ratio of 5 ml buffered to 1.0 g of tissue. This mixture was centrifuged for 30 min at 12,000 $g$ at 4°C and the supernatant was collected and frozen until tested in the Ep assay. The kidney extracts were also prepared using the same procedure.

**Cell Culture Technique.** The Hep3B tumors were recultured *in vitro* as reported previously (5). Briefly, the tumors were removed aseptically from the nude mice, minced, and pressed through a No. 100 stainless steel sieve with a spatula and continuously rinsed with cold Eagle's minimal essential medium. The resulting cell suspension was passed through a 25-gauge needle to dissociate the cell aggregates. The nude mouse Hep3B cells recultured from Hep3B tumor-bearing nude mice were maintained in 75-$cm^2$ Corning culture flasks containing Eagle's minimal essential medium supplemented with 10% fetal bovine serum (FBS), 0.1 m$M$ nonessential amino acids, 1 m$M$ sodium pyruvate, 100 units/ml of penicillin G, and 200 $\mu$g/ml of streptomycin in a humidified atmosphere of 5% $CO_2$ and 95% air at 37°C, as original Hep3B cells had been maintained *in vitro*. The culture medium was renewed every 2 days. The effects of hypoxia on Ep production in the nude mouse Hep3B cell cultures were compared with those in the original Hep3B cells. Aliquots of $3 \times 10^5$ viable cells were dispensed into 4.5-$cm^2$ multiwell plates. After a 24-hr preincubation period, the cells were exposed to hypoxia (1% $O_2$, 5% $CO_2$, and 94% $N_2$) for 20 hr in a humidified atmosphere at 37°C, as reported previously (16). In addition, the effects of 15-methyl-prostaglandin $E_1$ (15-methyl-$PGE_1$) (Fisher) on Ep production in a Hep3B cell culture under hypoxic conditions were assessed. At the end of the incubation period, the supernatants were harvested and frozen at −80°C prior to the Ep assay.

**Radioimmunoassay for Erythropoietin.** The levels of Ep in the mouse serum, kidney extracts, Hep3B tumor extract, and culture medium were determined by using a radioimmunoassay (RIA) for human Ep. The details of the RIA used in the present studies have been published previously (17, 18). The Ep standard curve was prepared using highly purified recombinant human Ep (18).

**Cyclic AMP Determinations.** Effects of 15-methyl-$PGE_1$ on cAMP production in a Hep3B cell culture exposed to hypoxia were measured according to a modification of the procedure described by Ueno *et al.* (16). Briefly, Hep3B cells were incubated with 15-methyl-$PGE_1$ for 1 hr in a hypoxic atmosphere and cAMP was extracted as reported previously (16). cAMP was measured by RIA using a cAMP assay kit (Du Pont-NEN, Boston, MA).

**Fetal Mouse Liver Erythroid Colony-Forming Technique.** *In vitro* bioactivity of Hep3B Ep was determined using the fetal mouse liver erythroid colony-forming unit assay according to the method of Iscove and Sieber (19). Fetal mouse (CD-1) liver cells from 12- to 13-day-old fetuses, which are known to be predominantly erythroid progenitors, were disaggregated by passing the liver through hypodermic needles and suspended at a final concentration of $10^5$ cells/ml in $\alpha$-medium containing 0.8% methylcellulose, 20% FBS, $10^{-4}$ $M$ mercaptoethanol, 100 units/ml of penicillin, 10 $\mu$g/ml of streptomycin, and Hep3B culture medium. One milliliter of the cell suspension was plated in 35-mm petri dishes and incubated in a humidified atmosphere of 5% $CO_2$ and 95% air at 37°C for 48 hr. Highly purified human recombinant Ep served as the Ep standard. The cultures were stained with 3′,3′-diaminobenzidine, and colonies of eight or more benzidine-positive cells were counted as erythroid colony-forming unit type colony with an Olympus inverted microscope.

**Exhypoxic Polycythemic Mouse Assay.** Exhypoxic polycythemic mice were employed to determine the *in vivo* bioactivity of Hep3B Ep, as previously reported (20, 21). CD-1 strain mice (Charles River Breeding Laboratories) were placed in a hypobaric chamber at 0.42 atm for 22 hr/day for 2 weeks. On the

sixth and seventh days after removal from the hypoxia chamber, the mice were injected subcutaneously with two equally divided doses of Hep3B Ep samples and 0.5 $\mu$Ci of $^{59}$Fe ferrous citrate was injected intravenously 24 hr later. The mice were exanguinated after 48 hr and radioiron incorporation into circulating red blood cells was determined. The log dose response curve was linear between 100 and 800 munits Ep/ml.

## Results

Hepatocellular carcinoma cells (Hep3B) at approximately $10^7$ cells inoculated under the renal capsule of nude mice produced tumors within 8 weeks and an approximately 90% success rate was obtained. The Hep3B xenograft doubling time is approximately 7 days (Fig. 1). The Hep3B tumor-bearing nude mice were sacrificed 3 weeks after recognition of increases in tumor volumes. The mean hematocrit value of nude mice carrying Hep3B tumors was 33.2 ± 1.1%, which was significantly lower than that of control nongrafted nude mice (40.8 ± 1.7%). On the other hand, serum Ep levels in the Hep3B tumor-bearing nude mice showed significantly ($P < 0.05$) higher values (37.5 ± 5.5 munits/ml) when compared with nongrafted control nude mice (13.5 ± 2.7 munits/ml). No significant difference was seen in the body weights between the two groups of nude mice. Figure 2 illustrates the relation between total tumor Ep levels and serum Ep levels in the Hep3B tumor-bearing nude mice. An almost linear correlation ($R = 0.714$) was determined between the two parameters, whereas no Ep was detectable in any kidney extract, suggesting that the increase in serum Ep levels may have been attributed to Ep production by the Hep3B tumors. As shown in Figure 3, an inverse linear relation ($R = -0.811$) between hematocrit values and serum Ep levels was noted in the Hep3B tumor-bearing nude mice. It seems likely that the anemia caused by factors produced in the Hep3B tumor-bearing mice can trigger these tumor cells to enhance Ep production. Therefore, *in vitro* experiments were conducted to confirm the response of Hep3B cells to hypoxia (1% $O_2$). Hep3B tumors carried in nude mice were able to grow again in a monolayer culture. As shown in Figure 4, these nude mouse Hep3B cells in culture retained their capability of enhancing Ep production in response to

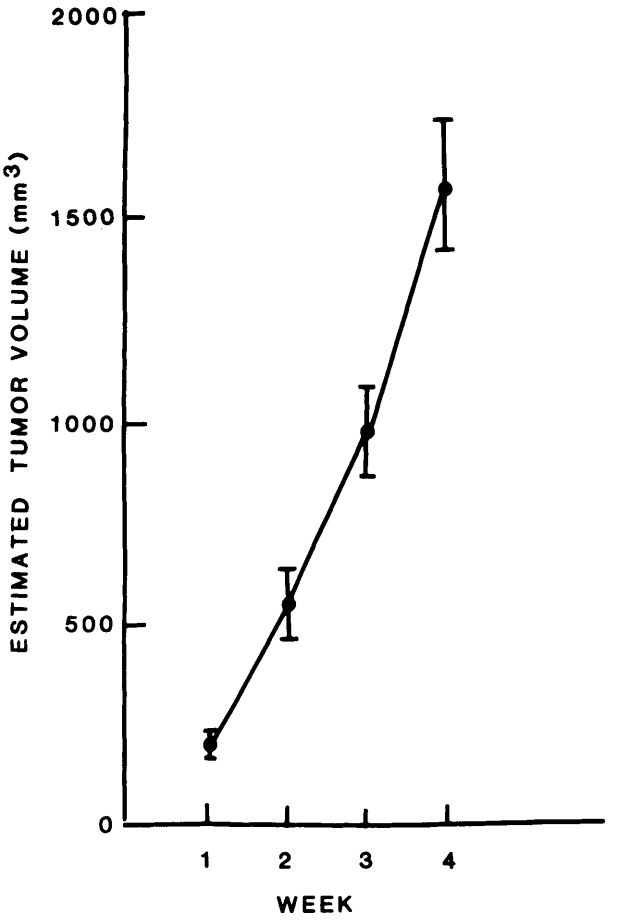


**Figure 1.** Growth curve of Hep3B tumors in nude mice. Hep3B tumors were transplanted subcutaneously on the back of nude mice. The tumor volume was determined with calipers (15). Each value represents the mean ± SE from eight nude mice.

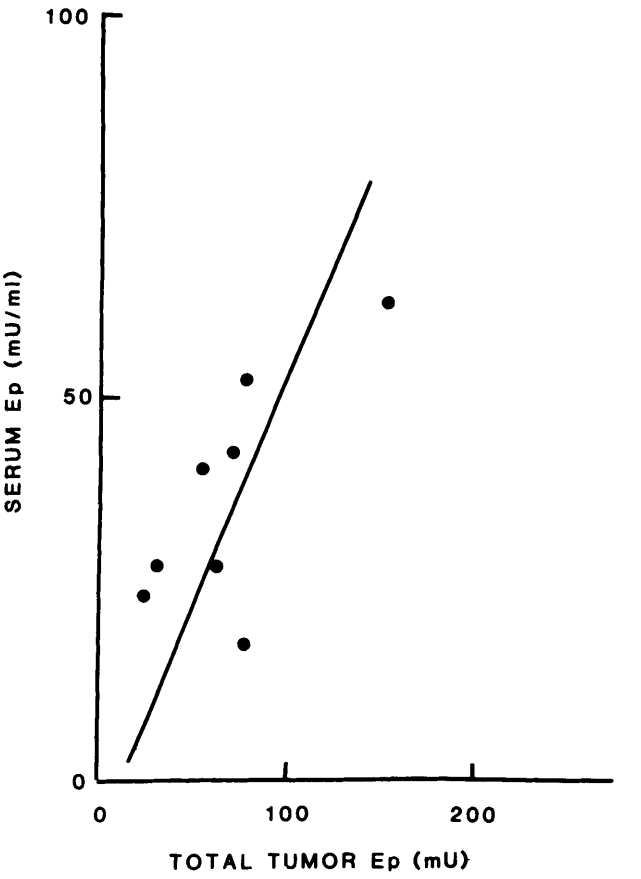


**Figure 2.** Total tumor Ep and serum Ep levels in Hep3B tumor-bearing nude mice. The total tumor Ep levels were measured as described in Materials and Methods. The linear regression coefficient was 0.714.

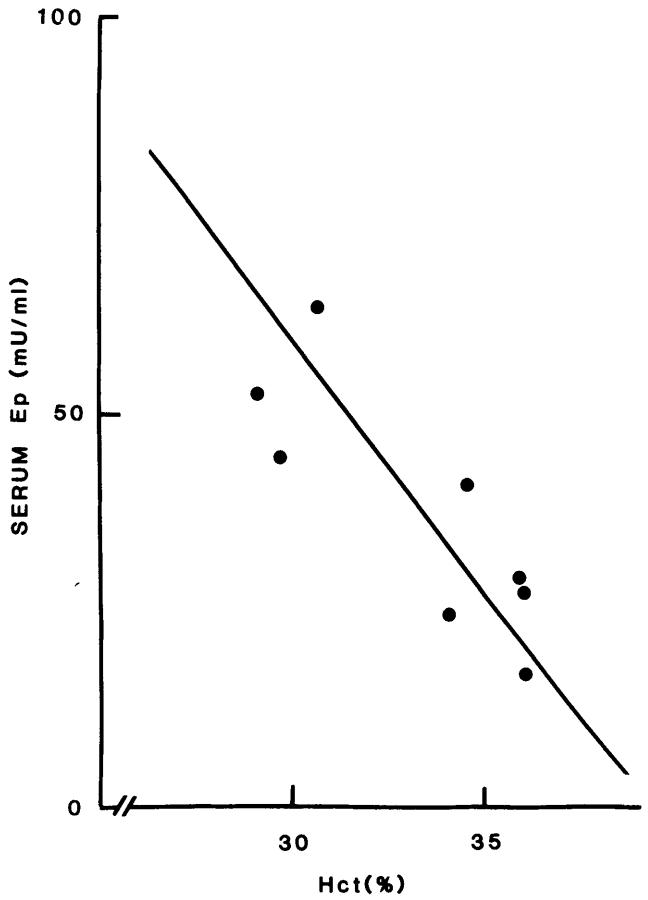


**Figure 3.** Hematocrit versus serum Ep level in Hep3B tumor-bearing nude mice. The linear regression coefficient was −0.811.

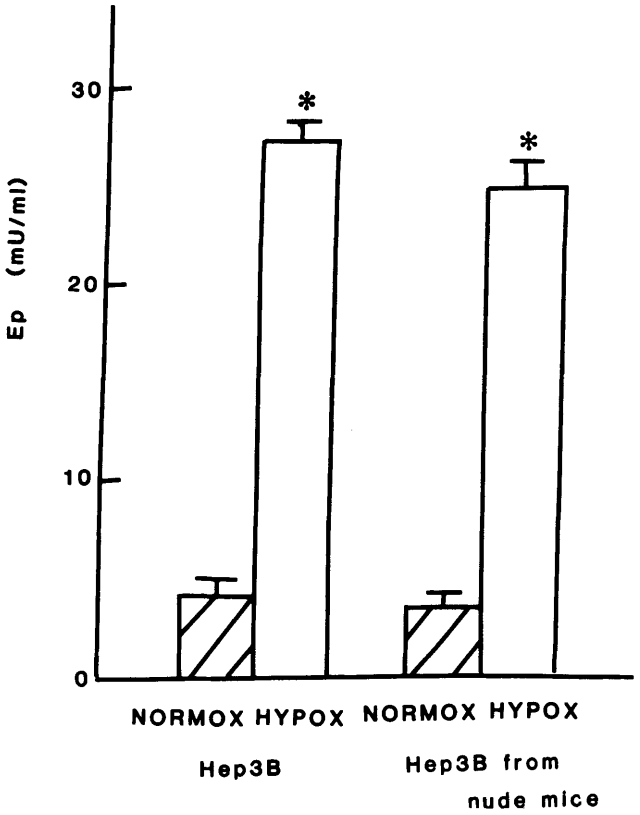


**Figure 4.** *In vitro* effects of hypoxia on Ep production by Hep3B cells maintained *in vitro* and Hep3B cells recultured from Hep3B tumors in nude mice. The original Hep3B cells were maintained *in vitro* and the nude mouse Hep3B cells which were recultured from Hep3B tumor-bearing nude mice were incubated at low density for 20 hr in a hypoxic atmosphere (1% $O_2$, 5% $CO_2$, and 94% $N_2$). Each value represents the mean ± SE of eight different samples and the asterisk indicates the value is significantly different from respective normoxia control ($P < 0.05$).

hypoxia (medium Ep, normoxia: 3.6 ± 0.4 munits/ml; hypoxia: 26.9 ± 1.1 munits/ml) as well as the original Hep3B cells which had been maintained in culture during the corresponding period (normoxia: 4.2 ± 0.8 munits/ml; hypoxia: 27.4 ± 0.8 munits/ml).

In addition, 15-methyl-$PGE_1$ at a concentration range of 4–400 ng/ml significantly ($P < 0.05$) enhanced Ep production in Hep3B cells under hypoxic conditions (Fig. 5). In parallel experiments, 15-methyl-$PGE_1$ in the same concentrations produced significant dose-dependent increases in cAMP accumulation in Hep3B cell cultures in a hypoxic atmosphere (Fig. 6).

The *in vitro* and *in vivo* bioactivity of Hep3B Ep was estimated using the fetal mouse liver erythroid colony-forming technique (FMLC) and exhypoxic polycythemic mice assays and compared with their immunoreactivity. The bioactivity of the spent culture media of Hep3B cells on erythroid colony growth in FMLC was completely neutralized by an antibody to highly purified human recombinant Ep (Fig. 7). The dose-response regression line for the Hep3B Ep bioactivity in FMLC was parallel to that seen in the RIA and a linear relationship ($R = 0.98$) was also found between the immunoreactivity and *in vitro* bioactivity (Fig. 8). However, the Hep3B Ep expressed higher *in vitro* bioactivity (3.7 ± 0.5-fold, $n = 4$) in comparison with the immunoreactivity. In contrast, the *in vivo* bioactivity of the Hep3B Ep in exhypoxic polycythemic mouse assay (EHPCMA) was less than one tenth of its immunoactivity (Table I). It is possible that the anemia in the Hep3B tumor-bearing mice is due to a lack of *in vivo* bioactivity of Hep3B Ep.

## Discussion

The Hep3B cell line was originally isolated from a liver biopsy of a patient with hepatocellular carcinoma (12, 14). This cell line has been found to secrete a number of plasma proteins (12) including Ep (13), serving as a valuable tool for erythropoietin research. In the present studies Hep3B cells formed tumors in nude mice when injected under the renal capsule, where there is a rich vascular bed, ensuring adequate nutrients for tumor growth (22). These solid tumors were successfully transplanted to other nude mice subcutaneously. The Hep3B xenograft doubling time is approxi-

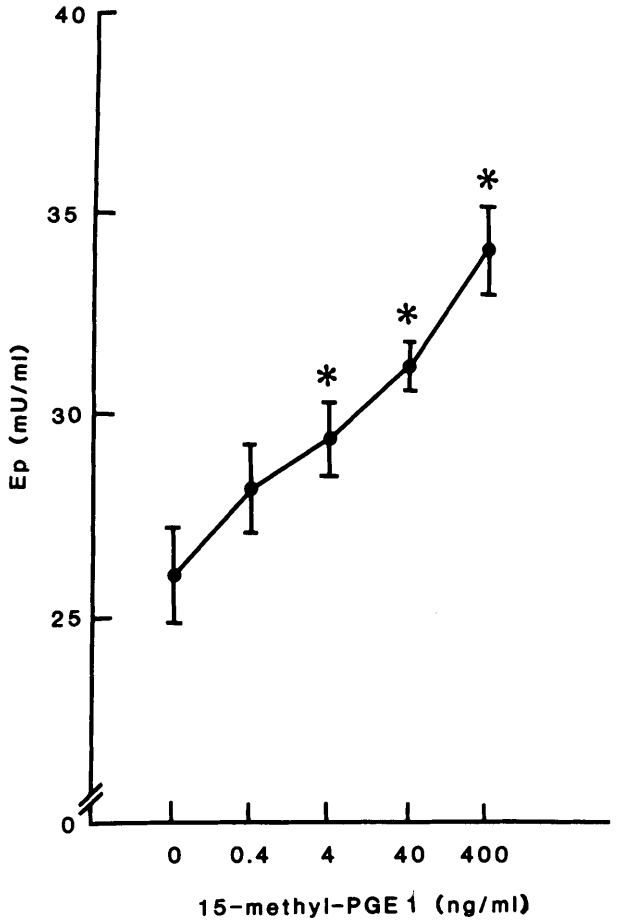


**Figure 5.** Effects of 15-methyl-$PGE_1$ on Ep secretion in Hep3B cells exposed to hypoxia. Hep3B cells were incubated with 15-methyl-$PGE_1$ in a concentration range of 0.4–400 ng/ml for 20 hr under hypoxic (1% $O_2$) conditions. Each value represents the mean ± SE of 12 samples and the asterisk indicates that the value is significantly different from hypoxia alone ($P < 0.05$).

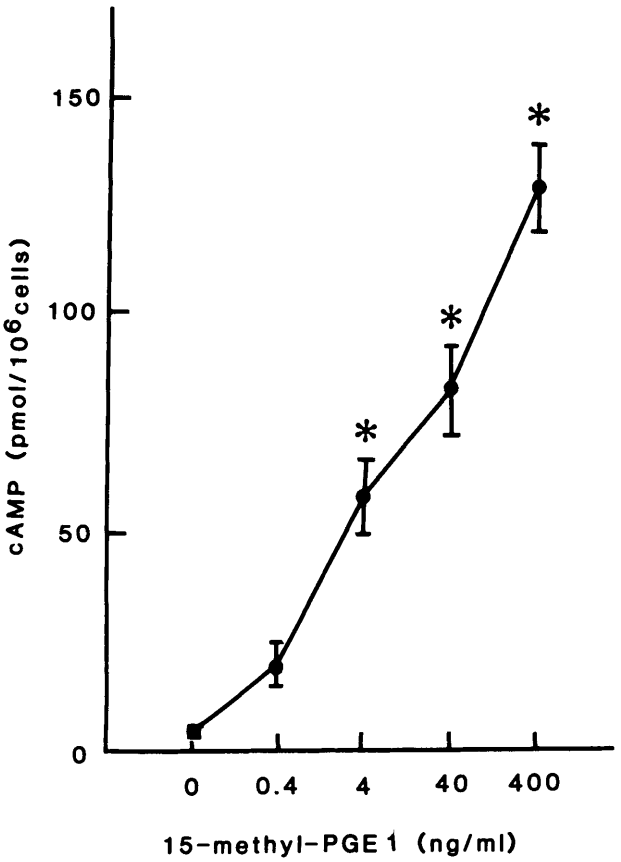


**Figure 6.** Effects of 15-methyl-$PGE_1$ on cAMP accumulation in Hep3B cells exposed to hypoxia. Hep3B cells were incubated with 15-methyl-$PGE_1$ at a concentration range of 0.4–400 ng/ml for 60 min under hypoxic (1% $O_2$) conditions. Each value represents the mean ± SE of six to nine samples and the asterisk indicates that the value is significantly different from hypoxia controls ($P < 0.05$).

mately 7 days, which is quite similar to the doubling time reported in sublethally irradiated nude mice (23).

The hematocrit values of nude mice transplanted with Ep-producing Hep3B tumors were significantly lower than those of control nongrafted nude mice. It is not surprising that these Hep3B tumor-bearing nude mice did not show an increase in their hematocrit values, because unlike previous reports (5, 6) in which Ep-producing cell lines were derived from renal carcinomas of patients with erythrocytosis, the patient from which the Hep3B cell line was established was not reported to have erythrocytosis.

Anemia is a common symptom observed in patients with malignant neoplasms. There are many possible causes of this anemia in patients with cancer, including hemorrhage, abnormalities in iron metabolism, bone marrow failure, hemolysis, cachexia, malnutrition, and concurrent infections (24). It is also possible that erythropoietin levels are inappropriately low in the anemia associated with cancer and that erythropoietin deficiency may contribute to the development of this form of anemia (25). On the other hand, tumor necrosis factor (TNF) is suggested to be involved in the cause of anemia in patients with malignancies. Aderka *et al.* (26) reported that continuous production of TNF by peripheral blood mononuclear cells may occur in cancer patients. It has also been reported that serum levels of TNF are elevated in patients with cancer (27). Blick *et al.* (28) reported that repeated injections of TNF produced anemia in humans. Recently, the effects of TNF on erythropoiesis have been evaluated and TNF is reported not only to suppress erythroid progenitors (29), but also to inhibit Ep production *in vitro* (30). Therefore, it is conceivable that cytokines such as TNF may be involved in the cause of the anemia in patients with malignancies.

In the present studies, we found a linear relationship between total Ep levels of Hep3B tumor extracts and serum Ep levels in the Hep3B tumor-bearing nude mice, whereas Ep was undetectable in the kidney extracts. Furthermore, an inverse linear relation between hematocrit values and serum levels of Ep was demonstrated. In contrast, Miller *et al.* (25) have recently reported that there was not an inverse linear relation

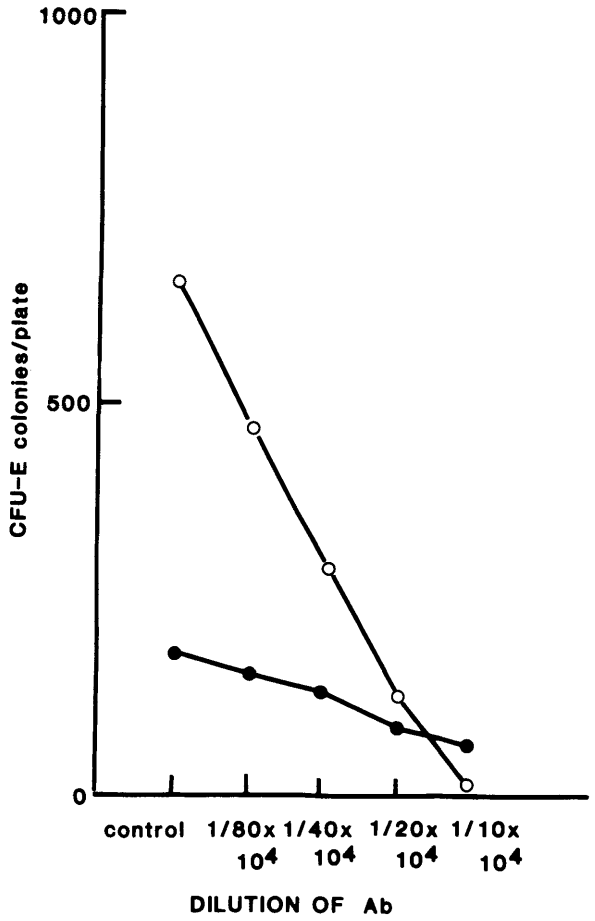


**Figure 7.** Neutralization of the bioactivity of Hep3B Ep in FMLC by a specific antibody to Ep. Mouse fetal liver cells were incubated with serially diluted specific antiserum to purified human Ep in the presence (○) or absence (●) of Hep3B cell culture medium (final Ep activity by FMLC, 13.9 munits/ml) for erythroid colony-forming unit studies.

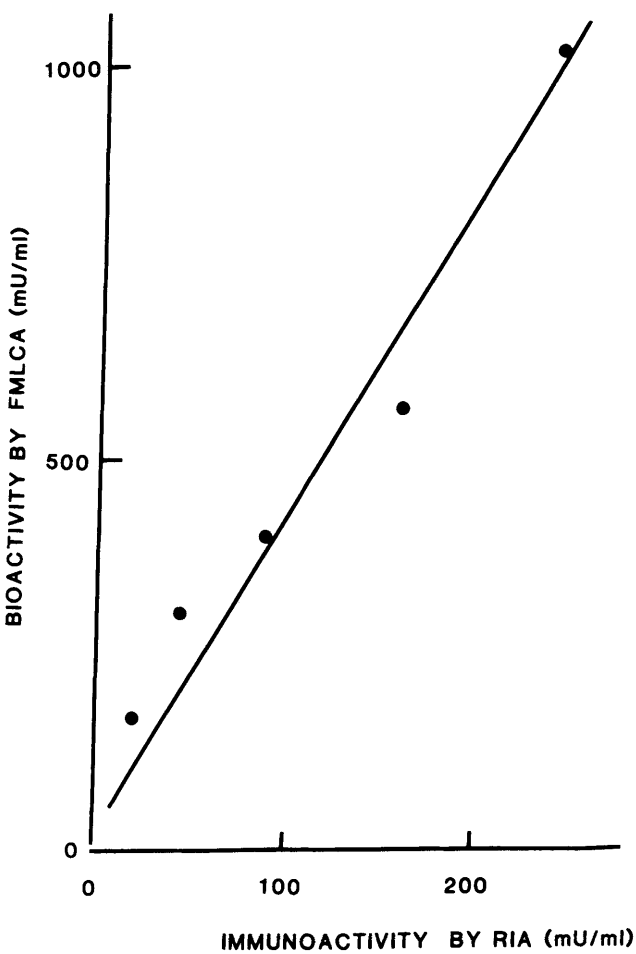


**Figure 8.** Correlation between immunoactivity and *in vitro* bioactivity of Hep3B Ep. The immunoactivity was determined in the Ep RIA and the *in vitro* bioactivity was estimated using FMLC. The linear regression coefficient was 0.977.

between serum levels of Ep and hemoglobin in their investigation of patients with cancer. Therefore, it seems most likely that the increase in serum levels of Ep in the Hep3B tumor-bearing nude mice was attributed to Ep production by Hep3B tumors. It is also conceivable that a feedback mechanism that stimulates Ep production in response to anemia is present in the Hep3B tumors.

*In vitro* cultures of Hep3B cells from nude mice were carried out successfully in order to assess *in vitro* Ep production by Hep3B cells in response to hypoxia. The Hep3B cells recultured from nude mice were capable of enhanced Ep production in response to hypoxia just as well as the original Hep3B cells. Nevertheless, the Hep3B tumor-bearing nude mice remained anemic. The biologic activity of the Ep produced by Hep3B cells was measured *in vitro* using FMLC and *in vivo* using EHPCMA. The medium of Hep3B cell cultures clearly supported erythroid colony growth in FMLC, which was completely inhibited by an antibody to purified human recombinant Ep. Hence, the erythropoietic activity in the Hep3B cell culture medium

**Table I.** Comparison between Immunologic and *In Vivo* Biologic Activities of Ep in Hep3B Cell Culture Medium[a]

| Experiment | RIA (mU/ml) | EHPCMA[b] (mU/ml) |
|---|---|---|
| 1 | 2591.1 | 171.4 ± 5.0 ($n$ = 4) |
| 2 | 2896.1 | 196.7 ± 11.8 ($n$ = 4) |

[a] Hep3B cell culture media were concentrated 10 times by Centriprep-10 (Amicon, Lexington, MA). Recovery of Ep after concentration was approximately 90%. A known amount of purified recombinant Ep was added to the culture medium and concentrated using Centriprep-10 (<10% was lost by the concentration procedure when measured via RIA before and after concentration).
[b] Data are expressed as mean ± SE.

was demonstrated to be Ep dependent. On the other hand, the control culture medium without Hep3B cells showed very low activity in the erythroid colony growth assay, which was also inhibited by the antibody to Ep. Since it has been reported that fetal mouse liver cells

are capable of producing and releasing an erythropoietically active substance into the culture medium (11, 31), the bioactivity of our control medium may be due to a low basal level of erythropoietic activity produced by fetal mouse liver cells. It is also conceivable that growth factors present in FBS containing medium such as testosterone (32) may have a synergistic action with the erythropoietic substances on erythroid colony growth, as reported previously (33). A linear relationship was found between the immunoactivity and the *in vitro* bioactivity of Hep3B Ep, and the *in vitro* bioactivity was demonstrated to be more than 3-fold higher than the immunoactivity. The reason for this difference may be due to a synergistic action of Hep3B Ep with some of the factors, such as albumin and transferrin, which are produced by Hep3B cells (12) and have been reported to support erythroid colony growth (34, 35). In contrast to the *in vitro* bioactivity, the *in vivo* bioactivity of Hep3B cell culture medium assayed in EHPCMA was less than one tenth of its immunoactivity. Barone-Verelas *et al.* (36) have reported an inhibitor of erythropoiesis in concentrated human serum. In these studies, concentrated normal human serum inhibited *in vivo* bioactivity of erythropoietin. In the present studies, it seems unlikely that the concentration of medium containing 10% FBS inhibited *in vivo* bioactivity of Epo because the concentration of FBS was probably not high enough to inhibit erythropoiesis in that it did not inhibit *in vitro* bioactivity. There was also a significant correlation found between *in vitro* bioactivity and immunoactivity of erythropoietin in Hep3B culture media. Therefore, it is not likely that Hep3B culture medium contains a significant amount of an inhibitor of Epo bioactivity. Sherwood and Goldwasser (37) have postulated in earlier studies using renal carcinoma cells that the discrepancy between *in vitro* and *in vivo* bioactivities of their renal carcinoma cell Ep may be because renal carcinoma cell Ep may be a mixture of native and asialo-erythropoietin. It is well known that erythropoietin-lacking terminal sialic acid residues can be detected by the *in vitro* assay, but asialoerythropoietin is inactive *in vivo*. This has been supported by the recent report that the bulk of the desialylated recombinant Ep was cleared from the plasma immediately after it was injected into animals and allowed to accumulate in the liver, where it was rapidly metabolized (38).

It has also been reported that glycosylation of the Ep molecule may play an important role in its metabolism as well as its production and biologic activity (39). Thus, it is possible that the Hep3B is metabolized or cleared from plasma more rapidly than native Ep. Further physicochemical characterization studies are needed to elucidate the structural difference between native Ep and Hep3B Ep.

Prostaglandins have been reported to stimulate radioiron incorporation into newly formed red cells of plethoric mice (40–42). Accordingly, $PGE_1$ has been shown to stimulate Ep production in isolated perfused dog kidneys (41). Hagiwara *et al.* (43) have also reported that $PGE_2$ plays an important role in stimulating Ep production in cultured renal carcinoma cells. On the other hand, it has been reported that incubation of Hep3B cells with $PGE_2$ for 24 hr under normoxic conditions did not result in an increase in erythropoietin secretion (13). In the present studies, *in vitro* cultures of 24 hr under normoxic conditions did not result in an increase in erythropoietin secretion (13). In the present studies, *in vitro* cultures of Hep3B cells with 15-methyl-$PGE_1$ in a hypoxic atmosphere successfully produced significant increases in Ep secretion and cAMP production. A concomitant rise in cAMP levels in an isolated dog kidney perfused with $PGE_1$ has also been reported in parallel with the increase of Ep levels (41). It is most likely that the stimulatory effect of $PGE_1$ on Ep secretion is mediated through the activation of adenylyl cyclase.

The present experiments indicate that Hep3B cells can secrete immunoreactive Ep, which is similar to native Ep, although it is not active *in vivo*. The Hep3B cell line provides a reproducible model system that is useful for studies of the mechanism of ectopic erythropoietin production.


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