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Transforming Growth Factor-α (TGF-α) Improves Hepatic DNA Synthesis after Hepatectomy in Cirrhotic Rats[1,2]

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Impaired liver regeneration in cirrhosis complicates the surgical treatment of liver tumors which arise in this setting. We developed a rat model to investigate the regenerative response of cirrhotic liver after hepatectomy and studied the effect of exogenous transforming growth factor-α (TGF-α), a potent liver mitogen. Micronodular cirrhosis was established by the simultaneous administration of CCl_4 and phenobarbital. Hepatic DNA synthesis ([^{3}H]thymidine incorporation into DNA) 24 hr after partial hepatectomy in cirrhotic rats was 15.6 ± 3.4 cpm/μg DNA (means ± SEM), which was significantly lower than in normal rats (37.3 ± 3.4 cpm/μg DNA, $P < 0.05$). Exogenous TGF-α (30 nmole/kg, sc every 12 hr) significantly improved [^{3}H]thymidine incorporation (35.6 ± 8.2 cpm/μg DNA, $P < 0.05$). An autoradiographic nuclear labeling index also confirmed increased DNA synthesis (6.7% vs 13.4%). TGF-α had no effect on normal regenerating liver (42.5 ± 8.8 cpm/μg DNA, NS). Although the significance of TGF-α-enhanced liver regeneration in cirrhosis has yet to be assessed, this model may be useful for the study of mechanisms which control hepatic proliferation.

INTRODUCTION

Normal liver has a remarkable capacity to regenerate and allows safe performance of partial hepatectomy in patients with liver tumors [1, 2]. Morphology and function of the liver return to normal once the regenerative process is completed. In liver cirrhosis, liver regeneration is impaired. Cirrhotic patients are at an increased risk of postoperative liver failure and related morbidity and mortality. This problem is not uncommon as 70 to 80% of hepatocellular carcinomas arise in cirrhotic liver [3]. Although there have been a number of efforts to reduce the volume of cirrhotic liver to be resected without sacrificing curability [4], the dilemma of curability versus safety has yet to be overcome.

The impaired regenerative capacity of cirrhotic liver has been investigated since the introduction of a cirrhotic rat model, but the degree of impairment of the regenerative response has not been well quantitated [5]. Little is known about the mechanism of liver growth or how to enhance liver regeneration in cirrhosis [6].

A 50-amino acid peptide, transforming growth factor-α (TGF-α), originally detected in culture fluids of retrovirally transformed cell lines, is known to stimulate DNA synthesis of cultured rat hepatocytes [7, 8]. TGF-α has a 33–44% homology with epidermal growth factor (EGF) and binds to the same cell-surface receptor as EGF [9, 10]. TGF-α is postulated to be more important as a physiological regulator of liver regeneration by means of an autocrine mechanism [11]. Comparative studies indicate that TGF-α is a more effective hepatocyte growth promoter than EGF [12].

In this study, we hypothesized that exogenous TGF-α may improve liver regeneration in cirrhotic rats after hepatectomy and have investigated the effect of TGF-α on DNA synthesis using a carbon tetrachloride-induced model of liver cirrhosis [13].

MATERIALS AND METHODS

Materials

TGF-α (34–43, rat) was purchased from Peninsula laboratories, Inc. (Belmont, CA). Methyl[^{3}H]thymidine, 45 mCi/μmole) and L-U[^{14}C]leucine ([^{14}C]leucine, 310 μCi/μmole) were from Amersham (Arlington Heights, IL).

Establishment of the Cirrhotic Rat Model with Chronic Carbon Tetrachloride Gavage

Micronodular cirrhosis was induced according to Proctor and Chatamra [13]. Briefly, male Wistar rats with a starting weight of 150–175 g were given phenobarbital sodium (35 mg/dl) in their drinking water. After 10–14 days on phenobarbital sodium, when the rats were about 250 g, the first dose of CCl_4 was begun. CCl_4 was administered once a week at midday, using a curved ani-

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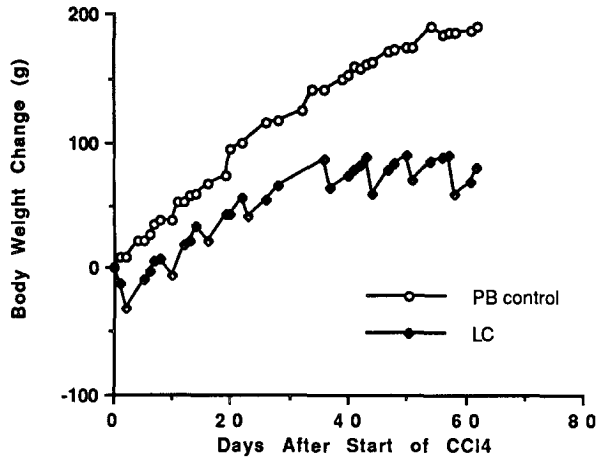

FIG. 1. Body weight change of experimental animals. All animals were weighed every day after starting carbon tetrachloride. Body weight change compared to the starting body weight are expressed on the ordinate. Points are means of LC rats (n = 18) and PB-control rats (n = 9).

mal-feeding needle with a ball-end (16G) under light isoflurane/O_2 anesthesia [14]. The initial dose of CCl_4 was 0.04 ml, and successive doses were thereafter varied individually in order to maintain a critical level of damage, as reflected by the daily body weight fluctuation of each rat (Fig. 1). The administration of CCl_4 lasted for 10 weeks and the dose was increased up to 0.2 ml. The phenobarbital-treated control group (PB-control group) received only anesthesia with isoflurane/O_2 once a week. A minimal delay of 14 days after the last dose of CCl_4 was allowed before operation was undertaken.

At the time of sacrifice, liver remnant and spleen were excised and weighed. Blood samples from the inferior vena cava were collected and serum albumin and serum total bilirubin were measured using a commercial kit (Sigma, St Louis, MO).

As a preliminary study, the left lateral lobe and median lobe of the liver were weighed and compared to the total liver weight after removal from sham-operated rats. The resected lobes (i.e., left lateral lobe plus median lobe) of the liver cirrhosis (LC), phenobarbital (PB)-control, and normal (saline) groups comprised 61.6, 65.8, and 67.2% of the total liver, respectively. The estimated total liver weight (L_{est}) was calculated as follows:

$$L_{est}\ (g) = \text{resected lobes}\ (g)/R$$

(R = 0.616, 0.658, and 0.672 for LC, PB-control, and normal groups, respectively).

DNA Synthesis in the Liver after Partial Hepatectomy in Cirrhotic Rats and in Noncirrhotic Rats

Cirrhotic rats (LC group, n = 18) and normal rats (normal group, n = 18) underwent 70% partial hepatectomy removing the left lateral and median lobes under isoflurane/O_2 anesthesia between 9 and 11 AM [1, 14]. The sham-operated group underwent a similar laparotomy procedure; the liver was exteriorized for a similar amount of time, replaced in the peritoneal cavity, and the incision was closed. Animals were killed 24 hr after the operation. [^{3}H]Thymidine (20 μCi/100 g body wt.) and [^{14}C]leucine (0.5 μCi/100 g body wt.) were injected via the femoral vein 1 hr before sacrifice.

DNA synthesis was estimated by [^{3}H]thymidine incorporation into DNA over the 1-hr period before sacrifice and was expressed as counts per minute incorporated per microgram of DNA. A 0.5-ml aliquot of the liver homogenate was precipitated with 10% trichloroacetic acid and 70% ethanol, and the ^{3}H radioactivity in the precipitate was measured. Another 0.1-ml aliquot was analyzed to measure DNA content using a modified diphenylamine reaction for desoxypentose [15].

To confirm the specificity of measuring [^{3}H]thymidine incorporation on DNA synthesis in hepatocytes, autoradiography was done on one rat from each experimental group. The samples were fixed in 10% buffered formalin and embedded in paraffin blocks. Sections were cut, dipped in Kodak NTB-3 emulsion, and exposed for 4 weeks. Specimens were developed and a labeling index was obtained. To calculate the nuclear labeling index, eight microscopic fields from each specimen were analyzed for both total and [^{3}H]thymidine-labeled nuclei. Approximately 1000 nuclei were analyzed per rat. The data were expressed as the percentage of labeled nuclei.

Protein synthesis was estimated by [^{14}C]leucine incorporation into 10% trichloroacetic acid-precipitable protein over the 1-hr period before sacrifice and expressed as counts per minute incorporated per milligram protein [16]. Protein was measured after the method of Bradford, with a Bio-Rad protein assay kit.

Statistical Analysis

All values shown represent the mean ± SEM. Student's t test was used for comparing group means. The level of significance was established at $P < 0.05$.

RESULTS

CCl_4-treated rats gained significantly less weight than PB-control rats; their weights at the time of operation averaging 420 ± 11 (n = 18, means ± SEM) and 472 ± 26 g (n = 9), respectively. Mortality during the first 3 weeks of treatment was 10.3% (3/29) and overall mortality throughout CCl_4 treatment was 24.1% (7/29). All CCl_4-treated animals had cirrhosis by gross inspection at the time of operation and this was verified histologically (Fig. 2). Ascites was recognized by a transient spike rise of body weight and by gross inspection of the abdomen. Eight out of 29 (27.6%) cirrhotic rats developed appar-

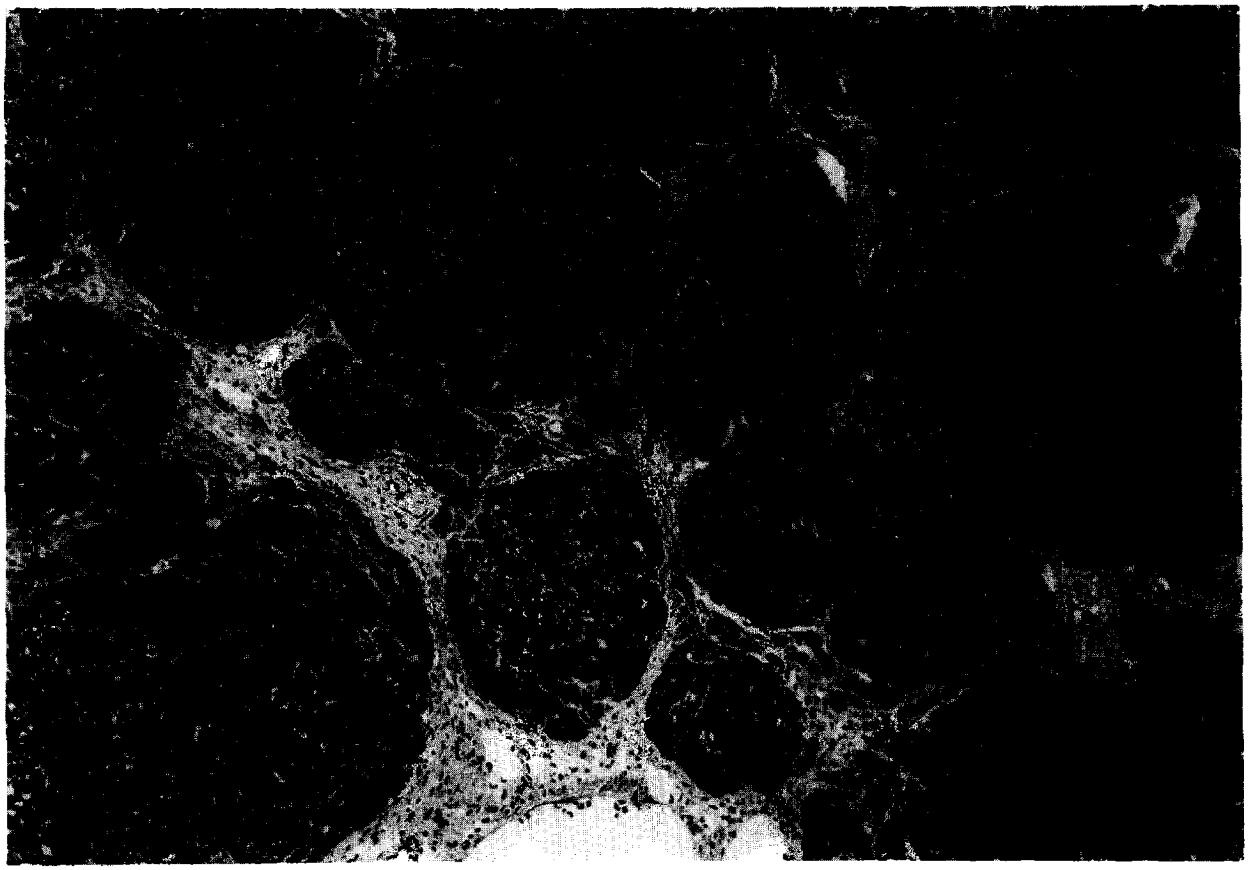

FIG. 2. Histology of CCl_4-induced cirrhotic rat liver. This section demonstrates established micronodules of hepatocytes. Macroscopically, the liver appeared slightly enlarged and finely nodular. Trichrome stain. (×100)

ent ascites during the course of CCl_4 treatment and it resolved by reducing or stopping CCl_4 except in three rats, which were excluded from this study. Another 9 rats developed slight ascites, which resolved without reducing CCl_4. Overall, this cirrhotic rat model was very effective in producing micronodular cirrhosis with a yield of 75.9% (22 out of 29 rats). The data are summarized in Table 1.

TABLE 1

Biological and Biochemical Parameters of Experimental Animals

	Liver cirrhosis (n = 18)	PB-control (n = 9)	Normal (n = 18)
Body wt (g)	420 ± 11*	472 ± 26	318 ± 16
Albumin (g/dl)	2.87 ± 0.08*,**	3.91 ± 0.23	3.70 ± 0.15
T. Bil (mg/dl)	0.40 ± 0.06*,**	0.17 ± 0.05	0.25 ± 0.03
L_{est}[a] (g/100 g BW)	4.29 ± 0.19**	3.71 ± 0.20	3.40 ± 0.11
Splenic wt (mg/100 g BW)	328 ± 28*,**	177 ± 7	197 ± 7

[a] Estimated total liver weight (see Materials and Methods).
* $P < 0.05$ vs PB-control, ** $P < 0.05$ vs normal.

In hepatectomized LC rats, [^{3}H]thymidine incorporation into the liver was significantly increased when compared to sham LC rats. This rate of incorporation 24 hr after hepatectomy was significantly lower than in normal rats, indicating impaired liver regeneration in liver cirrhosis (Table 2). Hepatic [^{14}C]leucine incorporation in sham-operated normal rats was significantly increased 24 hr after hepatectomy. A similar tendency was observed in LC-rats, 24 hr after hepatectomy (Table 2).

TABLE 2

[^{3}H]Thymidine Incorporation ([^{3}H]Thy) and [^{14}C]Leucine Incorporation ([^{14}C]Leu) 24 Hr after Partial Hepatectomy (HTX)

		[^{3}H]Thy (cpm/μgDNA)	[^{14}C]Leu (cpm/mg prot.)
Normal	Sham	5.6 ± 0.4	76.1 ± 10.9
	HTX	37.3 ± 3.4*	122.4 ± 10.5*
LC	Sham	3.9 ± 0.6	65.9 ± 7.2
	HTX	15.6 ± 3.4*¶	103.5 ± 12.0*

Note. n = 5.
* $P < 0.05$ vs sham-operated rats for each group, ** $P < 0.05$ vs Normal HTX.

TABLE 3

Data Summary of Cirrhotic Rats

	Sham (n = 5)	HTX (n = 5)	HTX + TGFα (n = 5)
Body wt (g)	415 ± 44	414 ± 16	442 ± 5
Albumin (g/dl)	2.80 ± 0.17	2.99 ± 0.09	2.87 ± 0.18
T. Bil (mg/dl)	0.23 ± 0.14	0.30 ± 0.08	0.26 ± 0.03
L_{est}[a] (g)	16.4 ± 2.8	17.9 ± 2.0	16.9 ± 0.7
Splenic wt (g)	1.41 ± 0.18	1.37 ± 0.10	1.24 ± 0.11

[a] Estimated total liver weight (see Materials and Methods).

Five rats were randomly chosen from the cirrhotic rat group and given TGF-α (30 nmole/kg, sc) at 0 and 12 hr after hepatectomy. A dose of 30 nmole/kg of TGF-α was chosen based on previous *in vivo* studies with EGF [17]. The parameters presented in Table 1 were similar between these rats and hepatectomized cirrhotic rats which were not given TGF-α (Table 3). Exogenous TGF-α significantly improved [^{3}H]thymidine incorporation in cirrhotic liver 24 hr after hepatectomy (Fig. 3a). TGF-α was also administered to normal rats which underwent partial hepatectomy. TGF-α had no effect on normal liver after hepatectomy vs hepatectomized normal rats without TGF-α. The administration of TGF-α did not significantly change hepatic [^{14}C]leucine incorporation in either cirrhotic or normal rats (Fig. 3b).

To confirm the [^{3}H]thymidine incorporation data seen in Fig. 3a, autoradiography was performed and a nuclear labeling index from normal and cirrhotic rat livers was calculated. Over 1000 nuclei were analyzed in tissue sections from normal rats subjected to sham (0.7%) or 70% (22.3%) hepatectomy. Labeling index analysis was also performed in sham (0.2%) or 70% (6.7%) hepatectomy in rats with cirrhotic liver. Further marked increases in 70% hepatectomized cirrhotic rat liver treated with TGF-α (13.4%) were also noted. Qualitatively, there were essentially no labeled nuclei in sham-hepatectomized cirrhotic rats (Fig. 4a). Increases in labeled nuclei were seen in the 70% hepatectomized cirrhotic rat livers (Figs. 4b and 4c). The autoradiographic nuclear labeling index data support the [^{3}H]thymidine incorporation data.

DISCUSSION

The ultimate goal of these experiments is to determine whether TGF-α (or any other hepatotrophic factor) is capable of enhancing the regenerative response of cirrhotic liver after partial hepatectomy. The rationale for short-term study of peptide stimulation of liver is the finding that [^{3}H]thymidine incorporation into DNA measured *in vivo* has been shown to be a simple and quantitative method for evaluating liver regeneration [18]. We chose to study thymidine incorporation 24 hr after hepatectomy based on previously published reports in both normal and cirrhotic liver [19, 20]. Further studies to define the time course of liver regeneration may be complicated to analyze, due to confounding factors such as nutritional support, but will help define the role of hepatotrophic factors in promoting hepatic growth. Short-term experiments similar to this one have already provided important information about the process of regeneration in normal liver [21–24].

Little quantitative data are available in animal models of regeneration in cirrhotic liver, although several studies have been reported [5, 25–27]. One of the reasons for

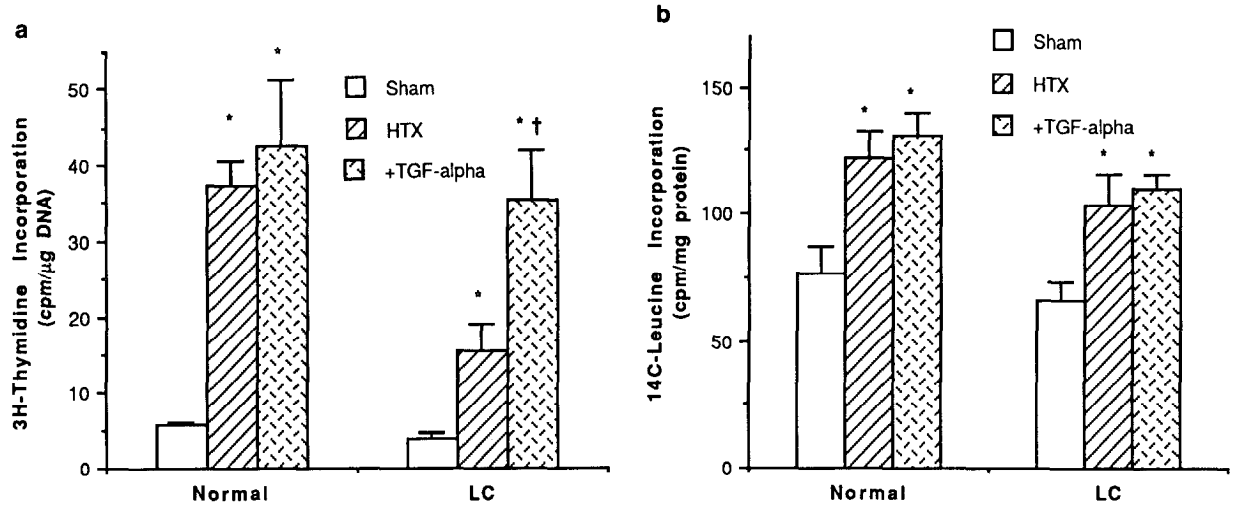

FIG. 3. (a) [^{3}H]Thymidine incorporation into the liver 24 hr after partial hepatectomy. [^{3}H]Thymidine incorporation into the liver was measured 24 hr after partial hepatectomy as described under Materials and Methods. HTX, partially hepatectomized rats; +TGF-α, hepatectomized rats given TGF-α (30 nmole/kg). n = 5–6; error bars = SEM. *$P < 0.05$ vs sham-operated rats for each group. †$P < 0.05$ vs LC-HTX (no TGF-α). (b) [^{14}C]Leucine incorporation into the liver 24 hr after partial hepatectomy. [^{14}C]Leucine incorporation into the liver was measured 24 hr after partial hepatectomy as described under Materials and Methods. HTX, partially hepatectomized rats; +TGF-α, hepatectomized rats given TGF-α (30 nmole/kg). n = 5–6 rats; error bars = SEM. *$P < 0.05$ vs sham-operated rats for each group.

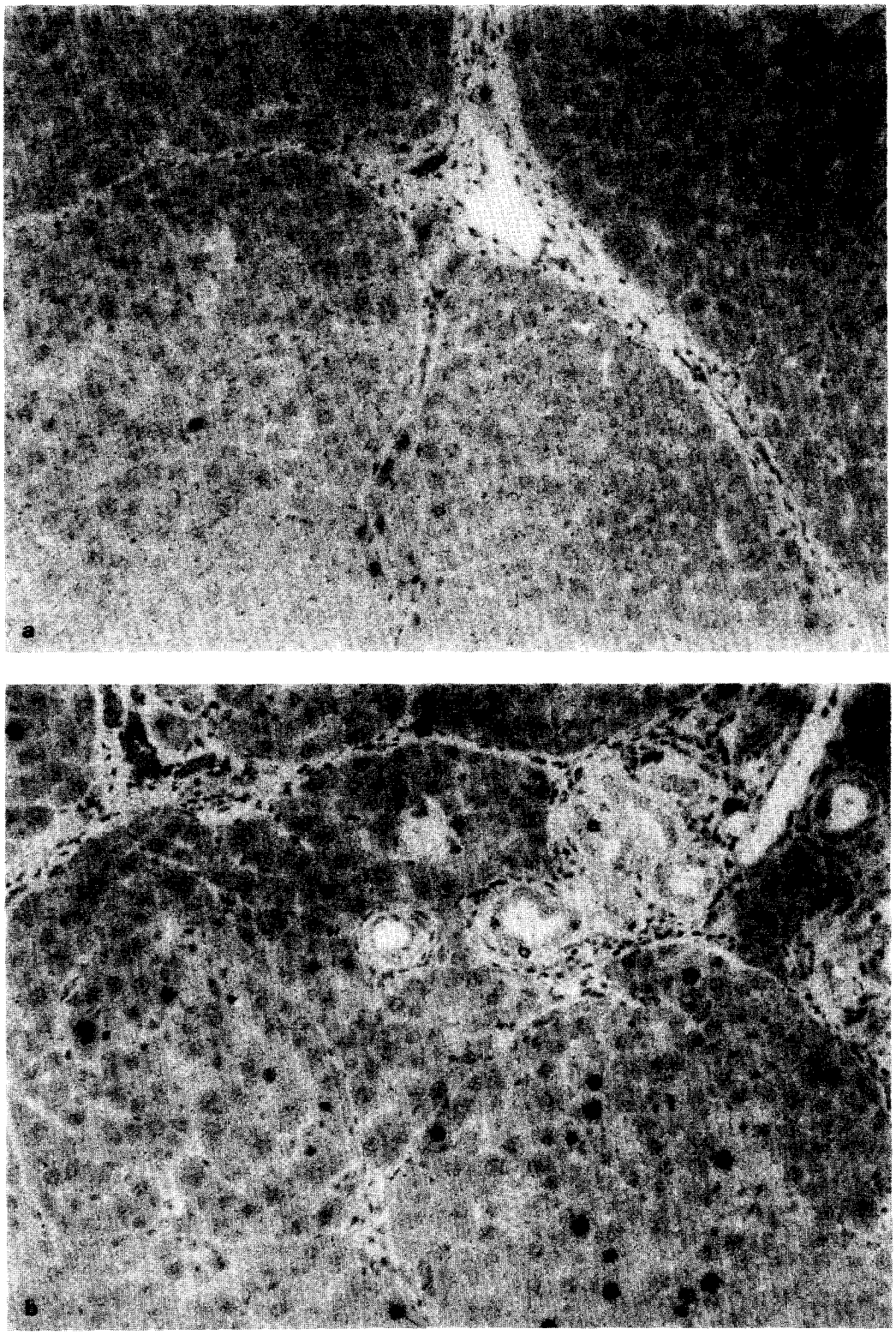

FIG. 4. Autoradiographic appearance of cirrhotic liver from rats undergoing sham or 70% hepatectomy. (a) Sham operation, (b) 70% hepatectomy, (c) 70% hepatectomy with TGF-α treatment (Hematoxylin and eosin stain; ×160).

the difficulty in studying this phenomenon was the lack of a reliable model for cirrhosis which could be reproduced in reasonable quantity. Proctor and Chatamra introduced a relatively easy, high yield method to produce micronodular cirrhosis in rats [13]. We applied this model in the present study and produced cirrhotic rats, which have been randomly distributed to each experimental group to avoid variations in hepatic functional

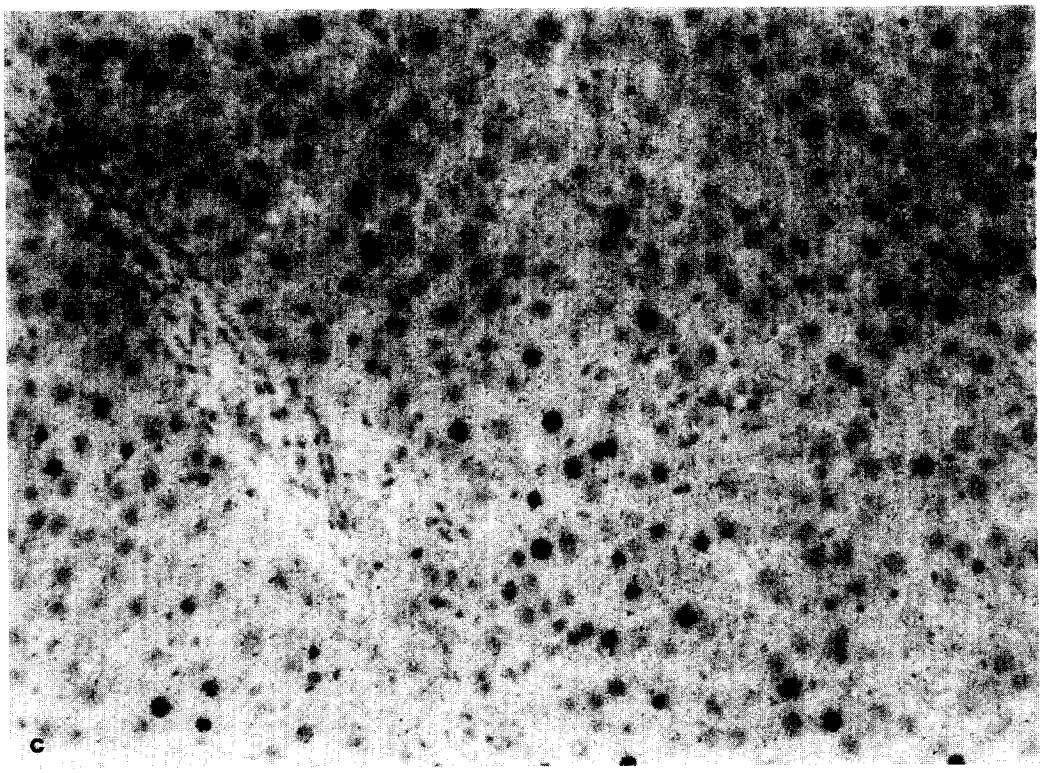

FIG. 4.—*Continued*

reserve among the rats. Treatment with carbon tetrachloride and phenobarbital was withheld at least 14 days before the hepatectomy, a time sufficient for the effect of both carbon tetrachloride (acute toxicity) and phenobarbital to disappear [25, 28, 29]. Interestingly, Rozga and colleagues from Sweden have reported that phenobarbital-treated rats do not regenerate despite the absence of histologic cirrhosis [30]. The authors reported changes in liver weight, but provided no data on rates of DNA synthesis. Using isolated hepatocytes, Jirtle and Meyer have recently documented a biphasic inhibition of epidermal growth factor-stimulated proliferation in phenobarbital rats [31]. Our data confirm a decreased liver weight in phenobarbital-treated rats (Table 1). Taken together, the data suggest that phenobarbital-treated rats may not be the best negative controls for *in vivo* studies on liver regeneration.

[^{3}H]Thymidine incorporation into DNA measured *in vivo* appears to be a simple and quantitative method for evaluating liver regeneration [18, 32]. There is an excellent correlation between [^{3}H]thymidine incorporation and a [^{3}H]thymidine labeling index as determined by autoradiography [33]. We thus applied both methods to estimate hepatic DNA synthesis after hepatectomy. [^{3}H]Thymidine incorporation was measured 24 hr after hepatectomy, when DNA synthesis and cell division are reported to be maximal [34]. The use of two independent methods of quantitating hepatocyte proliferation, [^{3}H]thymidine incorporation, and the autoradiographic nuclear labeling index complement each other and clearly document increased DNA synthesis in TGF-α-treated cirrhotic rats subjected to partial hepatectomy (Figs. 4a, 4b, and 4c).

Hepatic DNA synthesis after partial hepatectomy in cirrhotic rats was significantly lower than in normal and PB-treated controls (Table 2). Although the etiology of cirrhosis is different, this finding is consistent with the impaired liver regeneration seen in cirrhotic patients [35].

There have been a number of humoral factors which promote liver growth, including insulin, glucagon, EGF, TGF-α, and hepatocyte growth factor (HGF) [8, 36]. Of these, only a few have been shown to stimulate liver regeneration *in vivo* using normal rats [17]. Although TGF-α is known to stimulate DNA synthesis in cultured hepatocytes [8], to our knowledge, no data are available as to the effect of TGF-α on liver regeneration *in vivo*. A dose of 30 nmole/kg of TGF-α was chosen based on previous *in vivo* studies with EGF [17]. It is possible that even more exaggerated effects could be seen at higher doses. Figure 3a shows that exogenous TGF-α improved impaired DNA synthesis after hepatectomy in cirrhotic rats, whereas no effect was seen in normal rats. Since studies of TGF-α have typically measured mRNA levels or receptor binding activity, the amount, form (TGF-α precursors in larger forms are speculated to have some functional significance), and location of the TGF-α gene product are not completely known [37–39].

Measurement of [^{14}C]leucine incorporation was done to provide an estimate of protein synthesis in normal

and cirrhotic liver after sham or 70% hepatectomy. The differences in [^{14}C]leucine incorporation were not significantly different in normal or cirrhotic rats (Fig. 3b). The [^{14}C]leucine data present problems in interpretation due to the fact that in normal liver, some proteins (thymidine kinase, aspartate amino transferase, and α-fetoprotein) increase after hepatectomy while other proteins decrease (glucose-6-phosphatase, albumin, and transthyretin) [40–42]. Essentially nothing is known about altered protein synthesis in cirrhosis. Table 1 shows decreased plasma albumin in cirrhotic rats suggesting that decreased albumin synthesis is present. Cirrhotic liver appears able to increase protein synthesis after hepatectomy (Fig. 3b).

The reason why exogenous TGF-α was effective in cirrhotic liver may be because intrinsic TGF-α, which may be important for promoting liver regeneration [8], is not fully supplied by damaged hepatocytes in cirrhosis. This would imply a basic, fundamental defect in TGF-α gene expression. An alternative explanation would be an impairment in binding affinity of TGF-α for its receptor, which is overcome by increased circulating levels of peptide. In contrast, regenerating normal (i.e., non-cirrhotic) liver may be fully or maximally stimulated by intrinsic TGF-α so that exogenous TGF-α could not produce a significant incremental effect. Another concern, not answered by experiments such as these, is the possibility that TGF-α might stimulate growth of tumor cells in the clinical setting of surgical resection for metastatic or primary liver cancer. Prior to actual clinical trials, appropriate preclinical experiments should be devised to ensure such tumor stimulation does not occur.

In conclusion, impaired DNA synthesis after partial hepatectomy in cirrhotic rats was improved by exogenous TGF-α. Although the significance of TGF-α in liver regeneration in cirrhosis has yet to be assessed, this model may be useful for the study of the mechanisms of hepatocyte growth.

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