Hepatic Processing of Transforming Growth Factor β in the Rat

Uptake, Metabolism, and Biliary Excretion

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Abstract

Transforming growth factor beta (TGFβ), a recently discovered polypeptide, modulates growth of normal and neoplastic cells. Since little is known concerning in vivo disposition of TGFβ, we performed studies to examine the hepatic processing of biologically active ^{125}I-TGFβ in the rat. After intravenous injection, ^{125}I-TGFβ disappeared from the plasma with an initial $t_{1/2}$ of 2.2 min; partial hepatectomy delayed the plasma disappearance of ^{125}I-TGFβ by 80%. 60 min after intrafemoral injection, 63% of the recovered label was present in liver and/or bile; by 90 min, most of the label removed by the liver (83%) had been slowly excreted into bile. Nearly all the label in bile (96%) was soluble in trichloracetic acid and not immunoprecipitable by specific antiserum. Colchicine and vinblastine inhibited cumulative biliary excretion of label by 28 and 37%, respectively; chloroquine and leupeptin each increased the amount of label in bile that was precipitable by trichloracetic acid and that coeluted with authentic ^{125}I-TGFβ on molecular sieve chromatography. There was efficient first-pass hepatic extraction of ^{125}I-TGFβ (36%) in the isolated perfused rat liver, which was inhibited by unlabeled TGFβ (but not by epidermal growth factor, EGF) and by lectins in a dose-dependent manner; prolonged fasting also decreased clearance (26%). After fractionation of liver by differential or isopycnic centrifugation, radiolabel codistributed with marker enzymes for lysosomes. The results indicate rapid, extensive, inhibitable, and organ-selective extraction of TGFβ by the liver. After extraction, TGFβ undergoes efficient transhepatic transport, extensive intracellular metabolism, and slow but complete biliary excretion of its metabolites. Liver fractionation studies and pharmacologic manipulations suggest that these processes are associated with organelles that include microtubules and lysosomes. The data suggest that the liver is a major target tissue or site of metabolism for biologically active TGFβ.

Introduction

Transforming growth factor β (TGFβ)[1] is 25 kD homodimer with two 112 amino acid chains linked by disulfide bonds (1). TGFβ causes reversible morphologic transformation and growth in soft agar of mesenchymally derived mouse AKR-2B and rat NRK cells (2, 3). AKR-2B cells are stimulated to divide in monolayer by the addition of TGFβ but only after a prolonged prereplicative phase compared to other growth factors such as epidermal growth factor (EGF) (4). Also, TGFβ causes the early induction of c-sis followed by release of platelet derived growth factor (PDGF)-like material and the induction of PDGF-inducible genes (e.g., c-fos c-myc), but with delayed kinetics relative to induction by PDGF (5). Thus, the mitogenic effect of TGFβ on AKR-2B cells appears to be indirect through the induction of c-sis and subsequent autocrine stimulation by PDGF-like material. In contrast to its growth stimulatory effects on mesenchymal cells, TGFβ inhibits the monolayer growth of a number of normal epithelial cells, including rat hepatocytes (6) and human keratinocytes (7), as well as inhibiting the soft agar growth of a number of carcinoma cell lines (8). In fact, TGFβ is similar if not identical to the growth inhibitor isolated by Holley and co-workers from medium conditioned by African green monkey kidney cells (9). TGFβ appears to be an important regulator of cell proliferation for which many cell types have specific, high affinity, membrane receptors (10).

TGFβ is ubiquitous; it has been detected in normal liver, lung, kidney, submaxillary gland, brain, placenta and heart tissue, as well as in embryos (11, 12). Platelets have been shown to be a rich source of TGFβ and many laboratories purify TGFβ from outdated platelets (1). The molecule is released from cells in a latent, high molecular weight form; activation in vitro can be achieved by extremes of pH (13) and by plasmin, a cell-associated protease (Lyons et al., unpublished observations). Whether this high molecular weight form represents a precursor molecule and/or attachment to a binding protein, and whether in vivo conversion of latent to active form occurs in the plasma or elsewhere is presently unknown.

The gene for TGFβ has been cloned by Derynck and coworkers from a human genomic library and from cDNA libraries derived from human term placenta and the human fibrosarcoma line HT 1080 (14). It is transcribed into a 2.5-kb

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1. *Abbreviations used in this paper:* EGF, epidermal growth factor; IPRL, isolated perfused rat liver; ML, mitochondrial lysosomal; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor; TGFβ, transforming growth factor beta.

mRNA present in a wide variety of normal and transformed cells. Of interest, mRNA for TGFβ has not been detected in normal liver (14). Indeed, the liver may not synthesize TGFβ, but may play a role in clearing this polypeptide from the plasma. As there is ample precedent for the hepatic extraction and biliary excretion of a number of circulating macromolecules (15–17), including growth-active polypeptides such as EGF (18, 19), we examined the hepatic processing of TGFβ. Our results strongly suggest that the liver plays a major role in the clearance and metabolism of biologically active TGFβ.

Methods

Peptides and reagents. TGFβ was purified from human platelets by the method of Assoian et al. (1) with the addition of a final purification step consisting of reverse phase C-18 high performance liquid chromatography (HPLC) with a 45% to 60% acetonitrile gradient in water with 0.1% trifluoroacetic acid (10). This platelet-derived TGFβ has been shown to be homogeneous on gel electrophoresis under denaturing and reducing conditions (8).

EGF was purified from adult male mouse submaxillary glands by the method of Savage and Cohen (20); gel electrophoresis under denaturing conditions revealed a single polypeptide band.

Colchicine and leupeptin were purchased from Sigma Chemical Company (St. Louis, MO); vinblastine was from Eli Lilly & Co. (Indianapolis, IN); chloroquine was from Winthrop Laboratories (New York); and concanavalin A and wheat-germ agglutinin were from Calbiochem Corp. (La Jolla, CA).

Preparation of radiolabeled compounds. Purified TGFβ was radioiodinated with ^{125}I-labeled Bolton-Hunter reagent from Amersham Corp. (Arlington, IL) as previously described (10). Biological activity was determined by demonstrating growth-stimulating activity for AKR-2B cells in a soft agar assay and showing competition in a radioreceptor assay using AKR-2B (clone 84A) cells (10). Specific activity was determined to be ~ 80–100 μCi/μg.

Purified EGF was iodinated with Na^{125}I (Amersham Radiochemicals, Arlington Heights, IL) by a modification of the chloramine T reaction (21). Biologic activity was determined in a mitogenic assay with AKR-2B cells (21). The labeled EGF had a specific activity of 100-150 μCi/μg.

Experimental models. All experiments were conducted with male Sprague-Dawley rats weighing between 225 and 320 g. For in vivo experiments, we used rats anesthetized with intraperitoneal pentobarbital (40 mg/kg) and prepared with bile fistulae as previously described (22). In some of these experiments, we performed a hepatectomy (70%) or sham operation and took serial blood samples from the femoral artery after administration of the labeled peptide (23). For in vitro experiments, we used an isolated rat liver model perfused in a nonrecirculating fashion with a nonhemoglobin perfusate as previously described in detail (24). In some of these experiments, rats were subjected to prolonged fasting (72 h) before isolation of livers.

To assess the distribution of ^{125}I-TGFβ within the liver cell, we performed three types of tissue fractionation studies after homogenization of liver with a Potter-Elveheim homogenizer as previously described (25). First, to provide an estimate of the amount of label associated with organelles, we prepared a postnuclear supernatant containing both organelles and cell sap (extract or E fraction) by differential centrifugation and subjected it to ultracentrifugation to separate a high-speed pellet (75,000 $g \times 40$ min) or particulate fraction from a supernatant devoid of organelles. In some of these experiments, we added a detergent, Triton X-100 (1% vol:vol), to the E fraction before ultracentrifugation. In a second set of experiments, the E fraction was further separated into four additional fractions by differential centrifugation as described (25). These fractions were enriched in mitochondria (heavy mitochondrial or M fraction), lysosomes (light mitochondrial or L fraction), endoplasmic reticulum (microsomal or P fraction), and cytosol (final supernatant or S fraction). In a third set of experiments, we performed isopycnic centrifugation of ML fractions on linear sucrose gradients (10 to 60%) as described (25).

In separate experiments, we mixed lectins, unlabeled TGFβ or EGF in the perfusate of isolated livers to provide a steady-state environment to affect the hepatic extraction of the labeled peptide.

In another series of experiments in rats with bile fistulae, we injected colchicine (5 μmol/kg) or vinblastine (10 mg/kg) into the femoral vein 2 h before administration of labeled peptide. The dose of colchicine was similar to that used in assessing the effect of this microtubule binding agent on the hepatic transport of IgA (26), while the dose of vinblastine was similar to that used in assessing the effect of this microtubule binding agent on the biliary excretion of lysosomal enzymes (22). In those experiments aimed at studying the effects of lysosomotropic agents on the metabolism of ^{125}I-TGFβ, we injected leupeptin (5 mg) or chloroquine (50 mg/kg) intravenously or intraperitoneally, respectively, into rats with bile fistulae 1 h before injection of labeled peptide. These doses were similar to those used by others describing the inhibitory effect of leupeptin on the hepatic metabolism of asialoglycoproteins (27) and the inhibitory effect of chloroquine on hepatic metabolism of EGF (19).

Characterization of radiolabel in bile was performed by three separate techniques. TCA precipitation was performed with 2 ml of 10% TCA after addition of 1% BSA to 100 μl of bile. Immunoprecipitation was performed using a polyclonal antibody directed against native TGFβ, which was raised in rabbits (Keski-Oja et al., manuscript in preparation). Antibody-antigen complexes were precipitated with 10% (wt/vol) Staphylococcus A (Pansorbin, Calbiochem) and the precipitated radioactivity analyzed by polyacrylamide gel electrophoresis (PAGE) using 0.1% sodium dodecyl sulfate (SDS) or in a gamma counter. Molecular sieve chromatography was performed on a Sephadex G-50 column (1.0 × 117 cm) with a running buffer of 1.0 M acetic acid, 0.05% Triton X-100, and 0.1% BSA. The void and total column volumes were determined with dextran blue and potassium iodide, respectively.

Counting of tissue, blood, and bile samples for ^{125}I was done by gamma scintillation. Statistical analyses were done using a two-tailed Student's *t* test.

Results

Hepatic uptake of ^{125}I-TGFβ. The rate of disappearance of TGFβ from plasma was studied by injecting ^{125}I-TGFβ into the rat femoral vein, taking serial samples of blood, and determining the disappearance of label from the plasma. Fig. 1 shows that the plasma disappearance of radioactivity was rapid after systemic administration of ^{125}I-TGFβ (initial $t_{1/2}$ of 2.2±0.1 min) and that the disappearance was prolonged nearly twofold ($P < 0.03$) after 70% hepatectomy (initial $t_{1/2}$ of 4.0±0.4 min). These determinations were made after a 5-minute equilibration period following hepatectomy.

The uptake of ^{125}I-TGFβ in various organs was also determined. Fig. 2 shows that 43% of the injected radioactivity was found in liver and bile 60 min after intravenous administration of ^{125}I-TGFβ; this amount was twofold greater than all other organs combined. The hepatic extraction after systemic administration of ^{125}I-TGFβ and ^{125}I-EGF was then compared. Fig. 3 again shows that the majority of ^{125}I-TGFβ radioactivity (63%) was found in liver and bile with lesser amounts in kidney (20%) and blood (15%); in contrast to Fig. 2, the data are expressed as the percentage of recovered radioactivity. In comparison to ^{125}I-TGFβ, after systemic administration of ^{125}I-EGF, a lesser percentage of recovered radioactivity (41%) was found in liver and bile and a greater percentage in other organs. At 60 min, we could account for 70% of injected ^{125}I-

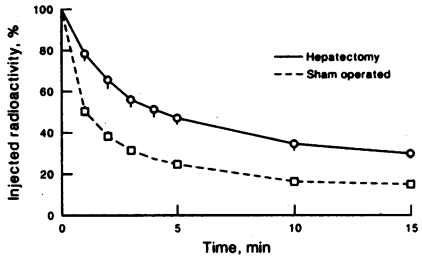

Figure 1. Plasma disappearance of ^{125}I-TGFβ in bile fistula rats after sham operation or 70% hepatectomy. Results (mean±SEM) are from three rats in each group.

TGFβ radioactivity and 31% of injected ^{125}I-EGF radioactivity in all the organs examined.

Fig. 4 compares the hepatic extraction (hepatic uptake and biliary excretion) of radioactivity after administration of radiolabeled TGFβ and EGF in both the isolated perfused rat liver (IPRL) model and in the intact animal after either intraportal or intrafemoral delivery. Results from the isolated perfused model demonstrate a greater ($P < 0.001$) first-pass hepatic clearance for radiolabeled EGF than for TGFβ (82.5±1.9% vs. 36±4.3%). The extraction percentage of ^{125}I-TGFβ in the IPRL was constant over the range of 6.4 to 128 pM. In vivo, little difference exists in the amount of ^{125}I-TGFβ extracted by the liver after portal vein versus femoral vein injection (52.5±1.5% vs. 49.6±0.5%). In contrast, more ($P < 0.001$) ^{125}I-EGF is extracted by the liver after intraportal injection compared to intrafemoral injection (70±0.7% vs. 44.8±4.1%).

Modulation of hepatic extraction. We determined the effect of fasting on the uptake process in order to provide initial insight into the factors that regulate the hepatic extraction of TGFβ. We fasted rats from solid food for 72 h, prepared isolated livers from fasted and nonfasted controls ($n = 5$), and measured first-pass extraction of ^{125}I-TGFβ. A significant decrease ($P < 0.03$) in first-pass clearance occurred after prolonged fasting, from 35.8±2.4% to 26.4±2.8% of the injected dose.

To assess the specificity of hepatic clearance of ^{125}I-TGFβ, we tested the effect of unlabeled TGFβ and of unlabeled EGF on the first-pass extraction of ^{125}I-TGFβ in the IPRL. As

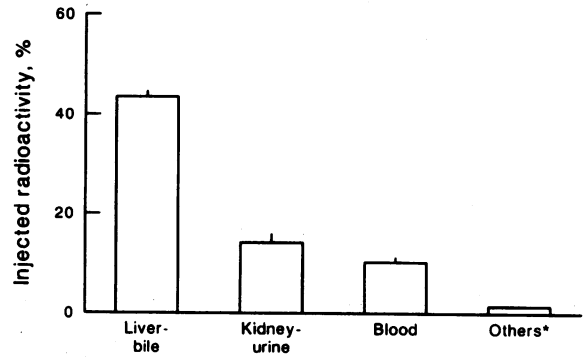

Figure 2. Organ distribution of radioactivity 60 minutes after intrafemoral injection of ^{125}I-TGFβ in bile fistula rats. Results (mean±SEM) are from three rats and are expressed as percent injected radioactivity. *Spleen, adrenals, stomach, and duodenum.

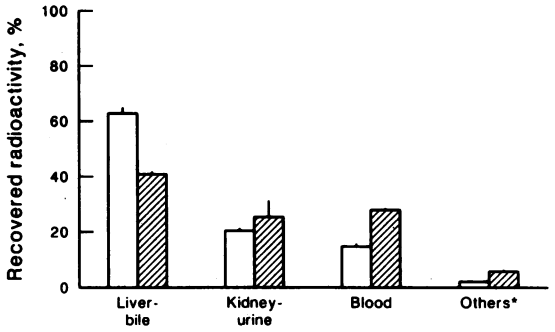

Figure 3. Organ distribution of radioactivity 60 min after intrafemoral administration of ^{125}I-TGFβ (□) and ^{125}I-EGF (■) in bile fistula rats. Results (mean±SEM) are from three rats in each group and are expressed as percent recovered radioactivity: *Spleen, adrenals, stomach, and duodenum.

shown in Fig. 5, unlabeled TGFβ, added to the perfusate in concentrations ranging from 1 to 20 nM, inhibited hepatic extraction of ^{125}I-TGFβ in a dose-dependent manner up to 73.9% of control. In contrast, cold EGF, at a concentration of 20 nM (an amount comparable to the concentration of cold TGFβ that achieved maximal inhibition in these experiments) had no effect on first-pass hepatic extraction of ^{125}I-TGFβ.

Lectins with different carbohydrate specificities were then tested to determine their effects on hepatic extraction of TGFβ (Fig. 5). Concanavalin A, which binds to mannose residues, yielded first-pass hepatic extractions of 34.9±3.3%, 25.3±2.7%, 24.4±2.3%, 17.7±2.6%, and 13.4±2.3% of the injected dose when premixed into the perfusate at concentrations of 5, 50, 100, 250, and 500 μg/ml, respectively. Wheatgerm agglutinin, which binds to *N*-acetylglucosamine and sialic acid residues, also decreased hepatic extraction. Concentrations of 0.5, 5, and 50 μg/ml resulted in extraction of 31.7±4.7%, 27.3±1.2%, and 19.4±2.6% of the injected dose, respectively.

Biliary excretion of ^{125}I from TGFβ. We examined the biliary excretion of radiolabel in order to determine the fate of

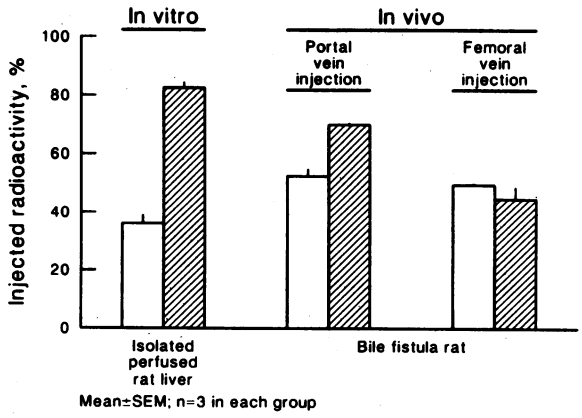

Figure 4. Hepatic extraction of radioactivity at 20 min after administration of ^{125}I-TGFβ (□) and ^{125}I-EGF (■) in vitro (isolated perfused rat liver model) and in vivo (via portal and femoral vein bile fistula rats). In the in vitro experiments, results represent first-pass hepatic clearance. Results (mean±SEM) are for at least three rats in each group and are expressed as percentage of injected radioactivity.

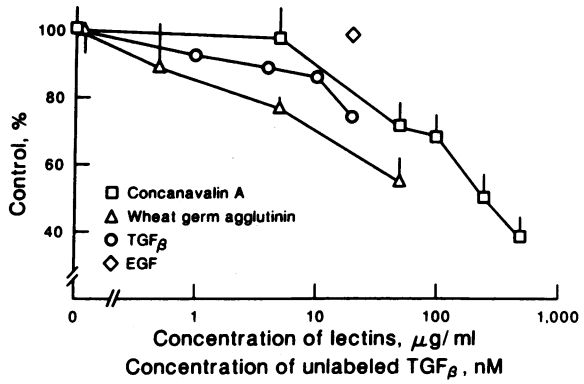

Figure 5. Effects of lectins, unlabeled TGFβ, and unlabeled EGF, on the first-pass hepatic extraction of ^{125}I-TGFβ in the isolated perfused rat liver. Results are expressed as percent of control and are mean data (±SEM) for three rats each with concanavalin A or wheat germ agglutinin and mean data for two rats each with unlabeled TGFβ and unlabeled EGF.

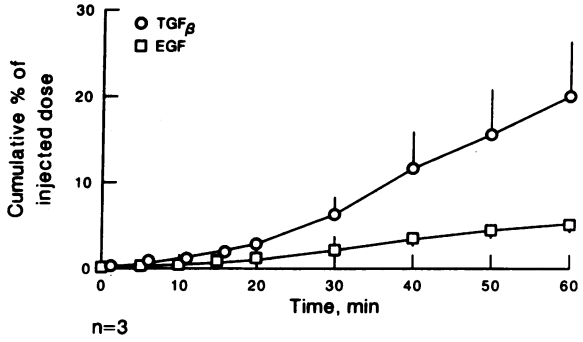

Figure 7. Cumulative biliary excretion of radioactivity after intrafemoral injection of ^{125}I-TGFβ or ^{125}I-EGF in bile fistula rats. Results (mean±SEM) are for three rats in each group.

^{125}I-TGFβ taken up by the liver. Fig. 6 shows the distribution of radioactivity in liver and bile collected over three separate time periods after systemic administration of ^{125}I-TGFβ. The overall hepatic extraction (hepatic uptake and biliary excretion) at 20, 60, and 90 min was similar (49.9±0.2%, 45.3±1.0%, and 48.1±2.0%, respectively). However, increasing amounts of radioactivity appeared in bile over time, comprising 7.0±1.2% of hepatic extraction at 20 min, 68.2±0.6% at 60 min, 84.0±0.9% at 90 min.

The kinetics of appearance of radioactivity from ^{125}I-TGFβ in bile was examined. Fig. 6 shows that after systemic administration of ^{125}I-TGFβ, the appearance of radioactivity in bile increased rapidly, peaked at 45 min, and gradually declined over 90 min. After 60 min, 20.1±6.4% of the injected radioactivity appears in bile (Fig. 7). This is significantly greater ($P < 0.05$) than the cumulative amount of the percentage of injected ^{125}I-EGF, which appears in bile (5.1±0.3%) over the same time (Fig. 7).

Characterization of ^{125}I from TGFβ in bile. To define the nature of the radiolabel in bile after in vivo administration of ^{125}I-TGFβ, we examined bile containing ^{125}I by TCA precipitation, immunoprecipitation, and molecular sieve chromatography. When bile collected serially after systemic administration of ^{125}I-TGFβ was exposed to TCA, a modest amount was TCA-precipitable (27.7±0.8% at 10 min); this percentage declined rapidly (6.4±0.6% at 25 min) and remained < 4% for the entire 90-min collection period. In contrast, 94.9±0.7% of radioactivity was TCA-precipitable when ^{125}I-TGFβ was added directly to bile ("spiked" bile) collected from rats ($n = 3$) to whom ^{125}I-TGFβ had not been administered. Only 28.3±0.5% of radioactivity in spiked bile could be immunoprecipitated using specific polyclonal antiserum to TGFβ, possibly due to the specificity and affinity of TGFβ antibodies and/or the complex nature of bile. Analysis of immunoprecipitated material after electrophoresis on 7.5–15% polyacrylamide gradient gels containing 0.1% SDS followed by autoradiography demonstrated a single band that migrated identically to ^{125}I-TGFβ (data not shown). In contrast, we were unable to demonstrate any immunoprecipitable radioactivity in bile collected after systemic administration of ^{125}I-TGFβ. Finally, Fig. 8 shows results of analysis of radioactivity in bile using molecular sieve chromatography. Bile that had been spiked with ^{125}I-TGFβ exhibited a single peak of radioactivity eluting near the void volume presumably representing intact

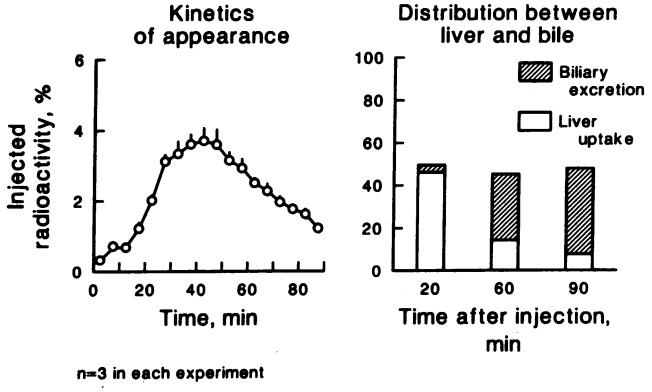

Figure 6. Kinetics of biliary excretion of radioactivity after intrafemoral injection of ^{125}I-TGFβ to bile fistula rats. Results (mean±SEM) are for three rats. Cumulative hepatic uptake and biliary excretion of radioactivity expressed as percentage of injected radioactivity for three separate time periods after intrafemoral injection of ^{125}I-TGFβ to bile fistula rats. Results (mean) are from three rats at each time.

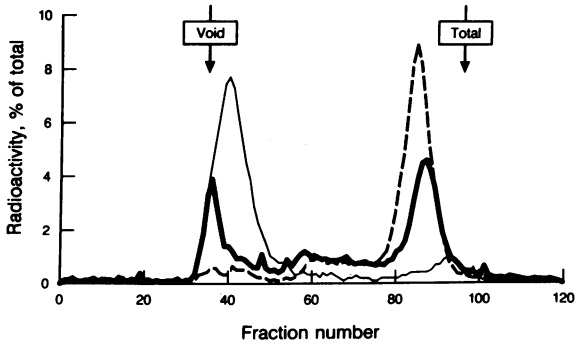

Figure 8. Molecular sieve chromatography of ^{125}I-TGFβ in bile on Sephadex G50. Chromatography was performed on rat bile to which ^{125}I-TGFβ had been added directly (——) or that had been collected from bile fistulae for 30 min after the intrafemoral administration of ^{125}I-TGFβ to rats pretreated with saline (– – –) or the lysosomotropic agent, chloroquine (▬▬).

TGFβ. In contrast, after systemic administration of ^{125}I-TGFβ, molecular sieve chromatography demonstrated a single peak of radioactivity eluting just before the total volume of the column, representing smaller molecular weight, degradation products of ^{125}I-TGFβ. These combined studies (TCA precipitation, immunoprecipitation, and molecular sieve chromatography) suggest that after systemic injection, the TGFβ that appears in bile is largely degraded.

Effects of pharmacologic agents on biliary excretion of ^{125}I from TGFβ. Since microtubule binding agents and lysosomotropic agents are known to affect the hepatic transport and biliary excretion of a number of macromolecules, their effect on the hepatic processing of ^{125}I-TGFβ was examined. Table I summarizes results of experiments with administration of microtubule binding agents (vinblastine, colchicine) and lysosomotropic agents (chloroquine, leupeptin) to rats with bile fistulae. There was no significant change in hepatic extraction of radioactivity (hepatic uptake and biliary excretion) at 60 min after administration of ^{125}I-TGFβ with both groups of agents. However, both groups of agents significantly reduced the radioactivity in bile over this same time interval with a corresponding increase in radioactivity remaining within the liver (Table I).

Of particular note, pretreatment with chloroquine and, to a lesser extent, leupeptin increased the percentage of radioactivity in bile that was TCA-precipitable (Table I). Although only 0.4% of radioactivity in bile was immunoprecipitable after pretreatment with chloroquine, this immunoprecipitable material migrated as a single band identical to authentic ^{125}I-TGFβ on SDS-PAGE followed by autoradiography (data not shown). Also, chloroquine pretreatment of rats given ^{125}I-TGFβ systemically altered the elution profile of radioactivity in bile subjected to molecular sieve chromatography (Fig. 8); two peaks, one coeluting with intact ^{125}I-TGFβ near the void volume, and the other eluting near the total volume (representing smaller molecular weight degradation products) became apparent.

Intracellular compartmentalization of ^{125}I-TGFβ. We examined the distribution of ^{125}I between a high-speed pellet and a supernatant of homogenized rat liver 20 min after intrafemoral administration of ^{125}I-TGFβ to provide initial insight into the subcellular localization of ^{125}I-TGFβ within the liver (Table II). In these experiments the majority of ^{125}I was associated with the particulate fraction. After exposure of the postnuclear supernatant to Triton X-100, approximately two-thirds of the radioactivity previously associated with the particulate fraction was released into the supernatant.

To extend our initial studies that suggested the association of ^{125}I-TGFβ with specific organelles, we next determined the subcellular distribution pattern of ^{125}I-TGFβ among liver fractions enriched in various organelles which were prepared by differential centrifugation. As shown in Fig. 9, which compared distribution patterns of ^{125}I and marker enzymes for various organelles, the distribution of ^{125}I in rat liver 20 min after intrafemoral injection of ^{125}I-TGFβ was most similar to the distribution of *N*-acetyl-β-glucosaminidase, a lysosomal enzyme, and different from the distribution patterns for enzymes associated with other organelles. In Fig. 10, we plot the distribution of ^{125}I and marker enzymes for lysosomes and mitochondria after density equilibration in a sucrose gradient of a fraction enriched in these two organelles (i.e., ML fraction) under two circumstances: first, ^{125}I-TGFβ was injected into rats 20 min before the preparation of the ML fraction, which was then placed on the gradient; second, ^{125}I-TGFβ was added directly to the ML fraction prior to placing the fraction on the gradient. When ^{125}I-TGFβ was injected into the rat and extracted by the liver before preparation of the ML fraction, the radioactivity entered the gradient and displayed a distribution pattern similar to those for *N*-acetyl-β-glucosaminidase, a lysosomal enzyme, and malate dehydrogenase, a marker enzyme for mitochondria (Fig. 10). In contrast, when labeled TGFβ was added directly to the ML fraction, the radioactivity remained at top of the gradient (Fig. 10).

Discussion

We have demonstrated that biologically active, ^{125}I-TGFβ is taken up by the liver (36% first-pass hepatic clearance) in a relatively organ-specific manner. This efficient clearance is decreased by fasting and inhibited by lectins and by TGFβ in a concentration-dependent manner; it is not affected by EGF. After extraction, radioactivity is excreted into bile slowly but efficiently, with 80% of the extracted radioactivity present in bile after 90 min; virtually all of the radioactivity in bile is TCA-soluble, not precipitable with specific antiserum to TGFβ, and demonstrates an elution profile by gel filtration different from that of authentic ^{125}I-TGFβ. All of these observations suggest extensive metabolism of the peptide. The amount of radioactivity excreted into bile is reduced by microtubule binding and lysosomotropic agents, an observation compatible with vesicular transport. The majority of the radioactivity in the liver associates with a particulate fraction and can be released by exposure to a detergent, an observation also compatible with sequestration of label in vesicles. Lysosomes

Table I. Effect of Pharmacologic Agents on the Hepatic Extraction, Biliary Excretion, and Metabolism of ^{125}I-TGFβ‡

	Percent hepatic extraction	Percent radioactivity in bile	Percent radioactivity in liver	Percent trichloroacetic acid-precipitable radioactivity in bile
Control	45.09 (±1.86)	30.84 (±1.70)	14.25 (±0.36)	7.43 (±1.68)
Vinblastine	41.14 (±0.91)	19.41 (±2.39)*	21.73 (±1.49)*	8.28 (±0.95)
Colchicine	43.82 (±0.27)	22.19 (±1.14)*	21.62 (±1.40)*	7.39 (±0.66)
Chloroquine	39.41 (±3.68)	19.01 (±0.93)*	20.40 (±2.77)	15.36 (±1.50)*
Leupeptin	42.52 (±0.39)	15.45 (±0.21)*	27.07 (±0.59)*	13.37 (±2.30)

‡ Data represent mean±SEM from at least three rats with bile fistulae in each group and are expressed as a percentage of the injected dose at 60 min after injection. Statistical analysis was performed by a two-tailed Student's *t* test. All comparisons were made relative to the corresponding control data and *P* values ≤ 0.02 are indicated by asterisks.

Table II. Percentage of Radiolabel Recovered in Supernatants and Pellets of Homogenized Rat Liver in Absence and Presence of Detergent

	Distribution of radioactivity in absence of detergent	*Distribution of radioactivity in presence of detergent*
	%	%
Supernatant	40.3 (±6.3)	79.5 (±3.0)
Pellet	59.7 (±6.3)	20.5 (±3.0)

^{125}I-TGFβ was injected into the femoral vein of rats (n = 3) and the liver removed 20 min later. The liver was homogenized and the amount of radioactivity in the supernatant and pellet after ultracentrifugation determined. In separate experiments (n = 3), Triton X-100 was added to the homogenate before centrifugation. Data are mean±SEM.

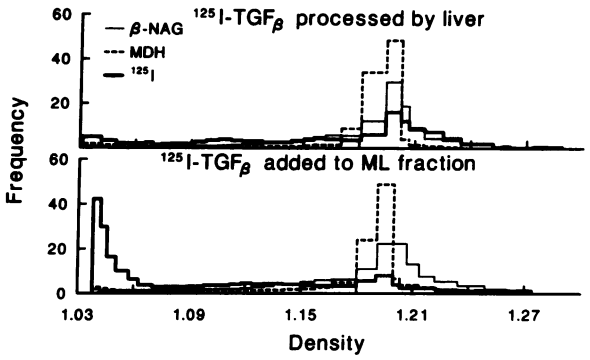

Figure 10. Distribution patterns of constituents after isopycnic centrifugation of an ML fraction of rat liver through a linear sucrose gradient (10–60%) 20 min after intrafemoral administration of ^{125}I-TGFβ (*top*) or after the addition of ^{125}I-TGFβ directly to a fraction prepared from rat liver (*bottom*). The average frequency of the components is calculated for each fraction as previously described (24) β-NAG, *N*-acetyl-β-glucosaminidase, a lysosomal enzyme; MDH, malate dehydrogenase, a largely mitochondrial enzyme.

appear to constitute one subset of vesicles that sequester ^{125}I-TGFβ, as suggested by the findings that lysosomotropic agents inhibit the degradation of this molecule during its transhepatic transport and that radioactivity and marker enzymes for lysosomes codistribute after tissue fractionation and differential or isopycnic centrifugation. Overall, we interpret these results as indicating that the liver is the major organ involved in the handling of biologically active TGFβ.

Because the hepatic processing of another growth active polypeptide, EGF, had been extensively studied (18, 19), we compared the hepatic handling of TGFβ to that of EGF. In vitro, first-pass hepatic clearance of EGF is greater than for TGFβ. In vivo, there is a significant decrease in the hepatic extraction of EGF when it is administered systemically rather than into the portal vein; such a decrease does not occur with TGFβ. These observations suggest that, while the liver has a high capacity for extracting EGF, other organs, if presented with EGF first, will also avidly take up this polypeptide. In contrast, while the liver has a lower capacity for extracting TGFβ, the uptake is more liver specific in that other organs do not avidly extract TGFβ. Differences also exist in the biliary excretion of TGFβ and EGF. Quantitatively, the liver excretes TGFβ more efficiently into bile than EGF. Nearly four times as much radioactivity from labeled TGFβ is excreted into bile as compared with labeled EGF during a comparable time period. This difference may also relate in part to the different labeling procedures we employed for EGF (i.e., chloramine T reaction) and TGFβ (i.e., Bolton-Hunter reagent). When ^{125}I is incorporated into a molecule by the former, but not by the latter technique, the ^{125}I appears to be sensitive to cellular iodinases (28); therefore, ^{125}I may diffuse out of the cell and not be excreted into bile.

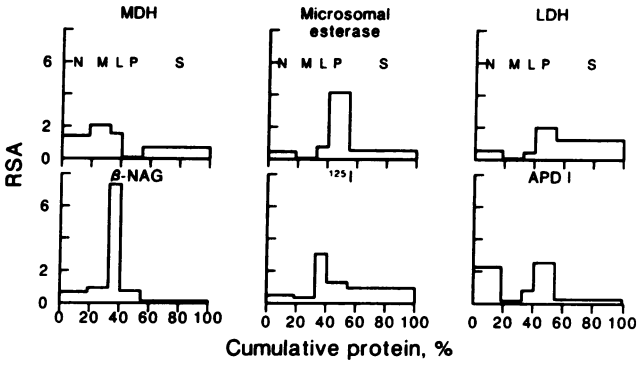

Figure 9. Distribution patterns of constituents after fractionation of a homogenate of rat liver by differential centrifugation 20 min after intrafemoral injection ^{125}I-TGFβ. Fractions enriched in nuclei (*N*), mitochondria (*M*), lysosomes (*L*), microsomes (*P*), and cytosol (*S*) are plotted from left to right in order of the average coefficient of sedimentation of their subcellular components. Each fraction is represented separately on the ordinate by the relative specific activity of the constituent (percentage of total activity recovered/percentage of total protein recovered) and cumulatively on the abscissa by its percentage protein. β-NAG (*N*-acetyl-β-glucosaminidase), a lysosomal enzyme; APDI (alkaline phosphodiesterase I), a plasma membrane enzyme; MDH (malate dehydrogenase), a largely mitochondrial enzyme. Esterase, when measured using *O*-nitrophenyl acetate, is a microsomal enzyme; LDH (lactate dehydrogenase), a cytosolic enzyme. Recovery of enzymes is calculated by summing the activities in the five fractions for a given enzyme and dividing this number by the total activity for the same enzyme in the homogenate. Results represent mean data from three experiments; recovery of constituents averaged 96% and ranged from 87 to 102% of starting material.

Fasting caused a significant reduction in the hepatic clearance of ^{125}I-TGFβ, an observation analogous to the decreased binding of ^{125}I-EGF to liver plasma membranes from fasted rats (29). This observation suggests that hepatic extraction of TGFβ is regulatable and can respond to physiologic stimuli. Also, the concentration-dependent inhibitory effect of lectins on the hepatic extraction of TGFβ is consistent with the possibility that a glycoprotein is involved in the uptake process. Along these lines, inhibition of insulin binding to its glycoprotein receptor on hepatocytes has been observed with comparable concentrations of concanavalin A (30). Moreover, results using affinity labeling techniques suggest that the receptor for TGFβ is a glycoprotein (31) and hepatocytes in culture have been demonstrated to have specific receptors for TGFβ (6). Finally, unlabeled TGFβ, but not cold EGF, inhibited the first-pass extraction of ^{125}I-TGFβ by the IPRL. All these data are consistent with the possibility that the hepatic extraction of TGFβ from the plasma compartment occurs by endocytosis

via a glycoprotein receptor on the sinusoidal pole of the hepatocyte, a receptor that is different from the receptor for EGF.

In this context, our data also suggest that the transhepatic transport of TGF occurs across the hepatocyte rather than between cells, a distinction that is important since a paracellular route for the biliary excretion of some circulating polypeptides has been proposed (32). The slow kinetics of biliary excretion and the extensive metabolism of the ligand are both consistent with a transcellular route of transport. In addition, preliminary data from our laboratory using light microscopic autoradiography and isolated liver cells suggest that hepatocytes but not nonparenchymal cells (e.g., Kupffer cells and endothelial cells) can take up ^{125}I-TGFβ.

Significant inhibition of biliary excretion of TGFβ by microtubule binding agents (vinblastine, colchicine) was demonstrated, implying involvement of microtubules and vesicles in the transport and excretory processes. The association of ^{125}I-TGFβ with a particulate fraction of a liver homogenate after high-speed centrifugation, and the release of the label from this fraction after exposure to a detergent, are also compatible with sequestration of ^{125}I-TGFβ in intracellular vesicles (33). Lysosomotropic agents (chloroquine, leupeptin) inhibited degradation of ^{125}I-TGFβ, as evidenced by the presence in bile of (*a*) significantly more TCA-precipitable radioactivity, (*b*) immunoprecipitable radioactivity, and (*c*) a peak of radioactivity coeluting with authentic ^{125}I-TGFβ on molecular sieve chromatography after administration of chloroquine. This observation suggests that lysosomes are one subclass of vesicles involved in the hepatic processing of TGFβ. Additional support for the role of hepatocyte lysosomes in the intracellular metabolism and biliary secretion of TGFβ comes from our studies demonstrating codistribution of ^{125}I-TGFβ with marker enzymes for lysosomes after differential or isopycnic centrifugation. We have previously described the existence of a lysosome-to-bile hepatic excretory pathway that may be a final common pathway whereby the hepatocyte disassembles some macromolecules and releases into bile some end-products of partial or complete lysosomal hydrolysis (34–36). Based on the data presented here, this pathway would appear to constitute the most likely route for the transhepatic transport and biliary excretion of TGFβ. Experimental approaches other than the biochemical one taken in these experiments, such as electron microscopic autoradiography, would be useful in further defining the pathway of transhepatic transport of TGFβ.

The physiological significance of our observations remains to be determined. As suggested earlier, the data are consistent with the liver being either a major target organ and/or a site of metabolism of biologically active TGFβ. TGFβ has been shown to inhibit EGF-stimulated DNA synthesis of normal rat hepatocytes (6). It is provocative to attribute control of hepatocyte growth and differentiation to the modulating influences of stimulatory (e.g., EGF) and inhibitory (e.g., TGFβ) growth factors (37). Also, serum contains detectable amounts of TGFβ (80–200 ng/ml) that is derived from platelets (38). Current data suggest that the TGFβ released by platelets in vivo is in a latent form of higher molecular weight than biologically active TFGβ (13). While it is known that this latent form of TGFβ can be activated in vitro by extremes of pH (13) and plasmin (Lyons et al., unpublished observations), the mechanism by which latent TGFβ is activated in vivo is unclear. One possibility is that activation occurs by proteolytic cleavage either in the plasma compartment or at sites of vascular injury after release of latent TGFβ from platelets. Removal of the resulting biologically active form of TGFβ from plasma by efficient hepatic clearance and subsequent lysosomal degradation would then be important to prevent unwanted biological effects of the peptide at distant sites. The data presented here, coupled with information regarding low plasma levels of TGFβ, suggest that the liver may play a major role in regulating levels of circulating, biologically active TGFβ.

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