Heme Metabolism and Turnover of Cytochrome P-450 in Tumor-Bearing Mouse Livers

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Abstract : Hepatic heme metabolism and *in vitro* translation of poly(A)⁺ RNA from livers were studied to elucidate the mechanism of the reduction of microsomal cytochrome P-450 in tumor-bearing mouse livers. Hepatic δ -aminolevulinic acid synthase activity in male C57BL/6N mice (0.203 nmol/mg protein/h) at 8 days after the transplantation of Ehrlich ascites tumor cells was the same level as that of normal livers (0.206 nmol/mg protein/h). On the other hand, hepatic heme oxygenase activity of tumor-bearing mice (0.482 nmol/mg protein/10 min) had increased 8 days following i. p. transplantation of tumor cells when compared with that of normal mouse livers (0.296 nmol/mg protein/10 min). SDS-gel electrophoresis of *in vitro* translation products of poly(A)⁺ RNA extracted from membrane-bound polysomes of the livers from tumor-bearing mice showed no significant differences from that of normal controls in the region of cytochrome P-450, that is, the molecular weight region of Mr=47,000–60,000, although microsomal protein content in this region estimated by Coomassie-blue staining was reduced. These results suggest that the decrease of heme biosynthesis or the translation of cytochrome P-450 mRNA played a very small part in the reduction of microsomal cytochrome P-450 in tumor-bearing mice.

Key words : cytochrome P-450, heme metabolism, poly (A)⁺ RNA, tumor-bearing mouse liver.

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Introduction

The reduction of cytochrome P-450 in the microsomes of tumor-bearing animal livers have been reported by several investigators (Kato *et al.*, 1968; Schacter & Kurz, 1982; Higashi & Ikeuchi, 1983; Raw, 1983). Microsomal cytochrome P-450 is composed of an undetermined number of monooxygenases with molecular weights between 47,000 and 60,000 (Haugen *et al.*, 1976). Selective inductions of these isozymes by various xenobiotics have been frequently employed for the purification and characterization of specified forms of cytochrome P-450 in animal tissues (Haugen *et al.*, 1976; Huang *et al.*, 1976). On the other hand, the importance of microsomal cytochrome P-450 in the tissues of untreated animals have been noticed recently because these constitutive cytochrome P-450 must have their physiological substrates such as steroids and fatty acids. The reduction of constitutive cytochrome P-450 in the liver might contribute to the occurrence of cancerous cachexia.

To elucidate the mechanism of the reduction of microsomal cytochrome P-450, we studied heme metabolism and *in vitro* translation of poly(A)⁺ RNA from livers of both normal and tumor-bearing mice. Previous reports by Schacter & Kurz (1984) and Beck *et al.* (1982) suggested that decreased hepatic microsomal cytochrome P-450 content in tumor-bearing animals was due to reduced synthesis of this hemoprotein. However, direct determination of the mRNA content of constitutive cytochrome P-450 is not feasible at the present time because of lack of cDNA probes for all forms of constitutive cytochrome P-450. We prepared poly(A)⁺ RNA from membrane-bound polysomes because the endoplasmic reticulum is the principal site of synthesis for the integral microsomal proteins such as cytochrome P-450 (Chen & Negishi, 1982). Our *in vitro* translation experiment suggested no significant decrease in the translation of cytochrome P-450 mRNA. We also observed enhanced heme oxidation activity in the livers of tumor-bearing mice.

Materials and Methods

Animals and tumor transplantation: Male C57BL/6N mice, 5 to 6 weeks old, were purchased from Kyushu Experimental Animals Co., Kitakyushu. The mice were inoculated i.p. with Ehrlich ascites tumor cells (approx. 3×10^7 cells). Animals were starved overnight before sacrifice and livers were perfused with cold 0.9% NaCl solution through the portal vein.

Delta-aminolevulinic acid synthase assay: Livers were homogenized in 1.15% KCl containing 0.1 mM EDTA, centrifuged at $8,000 \times g$ for 15 min and supernatants were discarded. Pellets were frozen and thawed to disrupt subcellular organelles and resuspended in 37.5 mM Tris-HCl, pH 7.4 (0.25 g of original weight/ml). A reaction mixture contained 75 mM citrate, 37.5 mM sodium phosphate buffer, pH 7.4, 15 mM MgCl₂, 7.5 mM EDTA, and 2.2 mM pyridoxal 5'-phosphate and enzyme preparation. Duplicates of 300 μ l aliquots were incubated at 37°C for 1 h. The δ -aminolevulinic acid (ALA) that formed was determined after conversion to 2-methyl-3-acetyl-4-(3-propionic acid) pyrole as described by Sassa *et al.* (1979). The concentration of ALA was determined based on the difference at 553 nm and 650 nm using an extinction coefficient at $58 \text{ mM}^{-1}\text{cm}^{-1}$.

Heme oxygenase assay: This enzyme activity was measured essentially according to the method described by Maines & Kappas (1975). The incubation mixture (6.0 ml) contained liver microsomes (12 to 30 mg protein), liver cytosol (10 mg protein), heme (17 μ M), NADPH generating system (0.85 mM glucose-6-phosphate, 3 units of glucose-6-phosphate dehydrogenase), MgCl₂ (2 mM), and phosphate buffer (0.09 M, pH 7.4). The mixture was preincubated for 5 min at 37°C and then divided into two aliquots. To the test aliquot, NADP (final concentration, 0.8 mM) in 1% NaHCO₃ was added and to the reference incubation mixture only 1% NaHCO₃ was added. After 10 min incubation, the incubation mixture was scanned between 350 to 650 nm. Bilirubin

formation was calculated using an extinction coefficient of $40 \text{ mM}^{-1}\text{cm}^{-1}$ between 464 to 530 nm.

Preparation of poly(A)⁺ RNA: Five mice livers were homogenized in 50 ml of 0.25 M sucrose containing 25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 5 mM MgCl₂ and 500 $\mu\text{g/ml}$ heparin. The homogenates were centrifuged at 15,500 rpm ($40,000 \times g$) for 40 min in a 70Ti rotor (Beckman). The pellets containing membrane-bound polysomes were homogenized in 100 ml of 0.1 M Tris-HCl buffer, pH 9.0 containing 0.1 M NaCl, 2 mM Na₂EDTA, 2 mM MgCl₂, 500 $\mu\text{g/ml}$ heparin, and 1% SDS and then mixed with 100 ml of phenol/chloroform/isoamyl alcohol [25/24/1, (v/v/v)]. The aqueous phase was washed twice with phenol/chloroform/isoamyl alcohol mixture. RNA was precipitated by ethanol, washed twice with 70% ethanol, and dissolved in distilled water. The RNA was reprecipitated in 2 M LiCl at -20°C overnight to remove heparin. After reprecipitation of RNA with ethanol, the poly(A)⁺ RNA was obtained by oligo(dT)-cellulose (Bethesda Res. Lab. Inc.) column as described by Aviv & Leder (1972).

Cell-free translation and analysis of its products: Reticulocyte lysate was purchased from New England Nuclear. The reaction mixture (25 μl) was incubated according to the assay protocol with 0.25 μg of poly(A)⁺ RNA from either normal or tumor-bearing mouse liver and 12.5 μCi of L-[³⁵S]-methionine. The translation reaction was carried out at 37°C for 1 h and mixed with an equal volume of Laemmli's sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue) and immersed for 1.5 min in boiling water. Electrophoresis of the translation products was carried out as described by Laemmli (1970) with a slight modification, that is, separation gel (7.5%) contained 0.5% of polymer of acrylamide to prevent cracking during the following procedures. After electrophoresis, the gel was immersed in 1 M sodium salicylate (Chamberlain, 1979) containing 3% glycerol(v/v) for 1 h, dried under a gel drier and exposed to Kodak RP/R X-ray film for 4–5 days at -80°C .

Other assays: Cytochrome P-450 content in the microsomes was determined by the method of Omura & Sato (1964). Protein was determined by the procedure of Lowrey *et al.* (1951) with bovine serum albumin as a standard.

Results

Changes of microsomal proteins and cytochrome P-450 contents:

The decrease of hepatic microsomal cytochrome P-450 was consistently observed from around 5 to 6 days after the tumor-inoculation (Table 1). The reduction of microsomal cytochrome P-450 was time-dependent, while the decrease of microsomal proteins was less significant. The cytochrome P-450 content at the 8th day after inoculation was approximately two-thirds of control value. For the following experiments, we decided to use livers from tumor-bearing mice at 8 days after tumor transplantation, because it is likely that the regulatory mechanism for both heme metabolism and turnover of microsomal

Table 1. Changes of microsomal proteins and cytochrome P-450 contents.

Animal	Numbers used	Microsomal proteins ^a (mg/g wet wt.)	Cytochrome P-450	
			(nmol/mg prot.)	(nmol/g wet wt.)
Normal	28	25.2±0.9 ^b (100%) ^c	1.13±0.05 ^b (100%)	27.8±0.7 ^b (100%)
Tumor-bearing				
5–6 days	12	24.2±1.8 (96.0%)	0.86±0.05 (76.1%)	21.1±1.9 (75.9%)
7–8 days	9	23.1±1.3 (91.7%)	0.82±0.08 (72.6%)	18.6±0.6 (66.9%)
9–10 days	9	23.3±1.8 (92.5%)	0.59±0.03 (52.2%)	13.5±0.8 (48.6%)
11–15 days	12	21.6±1.0 (85.7%)	0.55±0.06 (48.7%)	11.1±1.2 (39.9%)

a: Microsomal proteins recovered from original liver weight(g) b: standard error

c: per cent of control

Table 2. Hepatic ALA synthase activity in normal and tumor-bearing mice

Animal	Number of determinations	ALA synthase activity	
		nmol/mg prot./h	nmol/g liver/h
Normal	11	0.203±0.034 ^a	1.53±0.26 ^a
Tumor-bearing	17	0.206±0.018	1.61±0.12

a: standard error

Table 3. Hepatic heme oxygenase activity in normal and tumor-bearing mice

Animal	Number of determinations	Heme oxygenase activity	
		nmol/mg prot./10min	nmol/g liver/10min
Normal	12	0.296±0.030 ^a	2.16±0.24 ^a
Tumor-bearing	9	0.482±0.039 ^b	3.28±0.12 ^b

a: standard errors

b: value statistically different from the control value ($P<0.001$)

cytochrome P-450 would not be impaired severely at this time point (Nakata *et al.*, 1964). Tumor-bearing mice usually survive for about 3 weeks after the inoculation of ascites tumor cells.

Effect of tumor-bearing on hepatic δ -aminolevulinic acid(ALA) synthase activity:

ALA synthase (EC 2.3.1.37), which is the rate-limiting enzyme in heme biosynthesis, was determined in the livers from either tumor-bearing mice or normal controls. Hepatic ALA synthase activity at 8 days after tumor-inoculation was the same level as that of control livers (Table 2). The specific values expressed as nmol of ALA synthesized per either mg protein or original liver weight between tumor-bearing and control mice were similar at least under the conditions examined.

Effect of tumor-bearing on hepatic heme oxygenase activity:

In the assay of heme oxygenase (EC 1.14.99.3) activity, the cytosol fraction was utilized as the source of biliverdin reductase (EC 1.3.1.24) to convert biliverdin to bilirubin. According to the method described by Maines & Kappas (1975), liver microsomes were once prepared by centrifugation and the resulting pellets were resuspended in buffer. This microsomal preparation and an aliquot of cytosol were used as enzyme sources as described in Materials and Methods. The average values of microsomal proteins recovered from the original liver weight was about 25 mg/g and that of cytosol was about 80 mg/g, respectively.

Hepatic heme oxygenase activity was determined in the livers of normal mice and of tumor-bearing mice at 8 days after implantation of ascites tumor cells. This enzyme is also rate-limiting for heme degradation, as well as ALA synthase for heme biosynthesis. Contrary to ALA synthase, the hepatic heme oxygenase activity of livers from tumor-bearing mice was increased to 0.482 nmol of bilirubin formed/mg microsomal protein/10 min as compared with that of normal mouse livers, that is, 0.296 nmol/mg protein/10 min (Table 3).

Comparison of in vitro translation products of poly(A)⁺ RNA:

Since microsomal cytochrome P-450 is synthesized on membrane-bound polysomes (Chen & Negishi, 1982), we prepared poly(A)⁺ RNA from these fractions. The membrane-bound polysomes were isolated from livers of either normal or tumor-bearing mice. Poly(A)⁺ RNA obtained from polysomes was used in a cell-free translation system of rabbit reticulocyte lysate in the presence of [³⁵S]-methionine. The products were analyzed on SDS-gel electrophoresis. Densitometric scanning after the fluorography of the dried gels showed that the general patterns of [³⁵S]-labeled microsomal proteins of livers from normal and tumor-bearing mice were essentially similar. Furthermore, no significant reduction or even slight increase of [³⁵S]-radioactive materials were observed in the microsomal proteins around the cytochrome P-450 region (Mr=47,000 to 60,000 daltons) of tumor-bearing animals as compared with those of normal controls (Fig. 1). This was an unexpected feature of tumor-bearing mouse liver, because both the total content of microsomal cytochrome P-450 (Table 1) and color-intensities of microsomal proteins stained by Coomassie brilliant blue in this region showed reproducible reduction in the livers obtained at 8 days after tumor transplantation (Fig. 2).

Discussion

The turnover of different forms of cytochrome P-450 might be regulated selectively in animal tissues. Previously, we (Higashi & Ikeuchi, 1983) and other investigators (Kato *et al.*, 1968; Schacter & Kurz, 1982; Raw, 1983) have shown the reduction of hepatic microsomal cytochrome P-450 when animals have tumors in the extrahepatic regions. Our enzymatic analysis (Higashi & Ikeuchi, 1983) on drug-metabolizing en-

Fig. 1. Densitometric scanning of fluorography. *In vitro* translation products were analyzed on SDS-gel electrophoresis as described in Materials and Methods. Electrophoresis was from left to right. Bracket shows cytochrome P-450 region.

Fig. 2. Densitometric scanning (550 nm) of an SDS-polyacrylamide gel after electrophoresis of liver microsomes from control and tumor-bearing mice. (A) control, (B) tumor-bearing (8 days) and (C) tumor-bearing (12 days) mice. The gel was stained with Coomassie brilliant blue. Peaks numbering from 1 to 5 were assumably representative of multiple forms of cytochrome P-450. Electrophoresis was from left to right. Only cytochrome P-450 regions are shown in this figure.

zymes in microsomes of livers from tumor-bearing animals suggest that the reduction of constitutive forms of microsomal cytochrome P-450 occurred selectively but not at random. We were interested in the mechanism of the turnover of cytochrome P-450 under physiological conditions. So far, a number of studies have been done on the biosynthesis of microsomal cytochrome P-450 after treatment with xenobiotics, such as phenobarbital (Rajamanickam *et al.*, 1975) and methylcholanthrene (Chen & Negishi, 1982). Heme metabolism in livers has been also investigated in many laboratories during induction of microsomal cytochrome P-450 by xenobiotics (DeMatteis & Gibbs, 1972; Rajamanickam *et al.*, 1975). On the other hand, much less attention has been paid to the turnover of constitutive forms of cytochrome P-450.

As described in the text, hepatic microsomal cytochrome P-450 content at 8 days after tumor implantation decreased to approximately two-thirds of normal controls. We observed no decrease of ALA synthase activity in the livers of tumor-bearing mice, while heme degradation was stimulated under these conditions. Previously, Beck *et al.* (1982) reported on the reduced incorporation of δ -[^{14}C]-ALA into both total microsomal proteins and in the cytochrome P-450 region of SDS-gel in the tumor-bearing mouse liver. Direct determination of mRNA of constitutive forms of cytochrome P-450 is impossible due to the lack of cDNA-probes for these hemoproteins. There have been many reports on the significant changes of microsomal protein contents in the cytochrome P-450 region ($\text{Mr}=47,000$ to $60,000$) after the induction of these hemoproteins with xenobiotics (Guenther & Nebert, 1978; Peppriell, 1980; Ryan, *et al.*, 1979). We observed the reduction of staining intensity of microsomal proteins in this cytochrome P-450 region even at 8 days after tumor-inoculation. However, the amounts of [^{35}S]-labeled products of poly(A)⁺ RNA associated with membrane-bound polysomes did not reduce in tumor-bearing animal livers as compared with those of normal controls. Although we did not determine the distribution of mRNA for cytochrome P-450 between the free form in cytosol and the translation-engaged form in polysomes (Chen & Negishi, 1982), the transcriptions of cytochrome P-450 genes might not be reduced in the livers of tumor-bearing animals.

One possible explanation for the present results is that the cytochrome P-450 apoproteins not associated with heme might be readily degraded by proteolytic enzymes as compared with holoenzymes (Fig. 3). It was reported that the turnover of heme moiety of cytochrome P-450 was faster than that of cytochrome P-450 apoprotein (Rajamanickam

Fig. 3. Hypothesis concerning the reduction of microsomal cytochrome P-450. ALAS: δ -aminolevulinic acid synthase, HO: heme oxygenase.

et al., 1975). Exchanges of heme moieties between holoenzymes and free heme pool actually occur under physiological conditions (Sadano & Omura, 1983). In tumor-bearing mouse livers, heme released from cytochrome P-450 is metabolized rapidly by elevated heme oxidation activity. As a result of the degradation of heme, less association of free heme and cytochrome P-450 apoprotein occurred in tumor-bearing mouse livers. The reduction of δ -[^{14}C]-ALA incorporation into microsomal proteins as reported by Beck *et al.* (1982) was probably due to the rapid degradation of [^{14}C]-labeled heme in tumor-bearing mouse liver. It is, however, unknown whether the rate of association between heme and cytochrome P-450 apoprotein is a rate-limiting step of the turnover of cytochrome P-450.

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担癌マウス肝におけるヘム代謝とチトクローム P-450 の代謝回転

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要 旨: 担癌マウス肝ミクロソームのチトクローム P-450 含量の減少の機構を明らかにするために肝におけるヘム代謝, およびチトクローム P-450 の生合成を調べた。ヘム合成の律速段階である δ -アミノレヴリン酸合成酵素は, 担癌肝と正常肝とでは, ほぼ同程度の活性を示したが, ヘム分解を律速する酵素として知られるヘムオキシゲナーゼは, 担癌肝で促進していた。次に, 肝の膜結合型ポリソームより得たポリ (A)⁺RNA を, *in vitro* の蛋白質合成系に加え, [³⁵S]-メチオニンでラベルされた産物を SDS-ゲル電気泳動法で分析すると担癌肝より抽出したポリ (A)⁺RNA の中で, チトクローム P-450 の mRNA が減少していることを示唆するような結果は得られなかった。これらの結果は, ヘム合成の低下や, チトクローム P-450 の mRNA の減少が, 担癌マウス肝におけるミクロソームのチトクローム P-450 の直接の原因でないことを示唆している。

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