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〔一般論文〕

Preliminary Observation on Metabolic Activation of Ifosfamide by Rat Liver in Salmonella Reversion Aassay

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Summary

To determine whether cytochrome P-450 metabolizes ifosfamide to active form, studies were done using the 9,000 x g supernatant (S-9) from phenobarbital- or 3-methylcholanthrene-treated rat liver together with *Salmonella typhimurium* TA-100 by a modified Ames Salmonella reversion assay. The number of revertants increased with the increment in the amount of S-9 fraction added. This suggested that most if not all of the ability of the S-9 fraction to convert ifosfamide to active form resided in the microsomes. Activation of ifosfamide to a mutagen required the NADPH-generating system as a cofactor and the activating reaction was inhibited by carbon monoxide and SKF-525A. Phenobarbital and 3-methylcholanthrene significantly raised the cytochrome P-450 content, and the treatment of rats with phenobarbital resulted in a marked increase in the ability of the microsomes to activate ifosfamide by 3-methylcholanthrene but did not increase the mutagenicity. The phenobarbital-induced activating reaction was inhibited by SKF-525A and metyrapone. The characteristics of inhibition and induction of the activation of ifosfamide to a mutagenic metabolite are concordant with the conclusion that this activation is catalyzed primarily or solely by phenobarbital-inducible cytochrome P-450.

Introduction

The alkylating agent ifosfamide, an analog of cyclophosphamide, is used widely as an antitumor agent for the treatment of various cancers. This agent differs from cyclophosphamide in the placement of its chloroethyl group ; ifosfamide has one of the alkylating side chains present on the cyclic nitrogen. For an analog to have clinical utility, it must demonstrate a different spectrum of activity, greater efficacy, or decreased toxicity relative to its related compound[1]. *In vitro*, it is the active metabolite of ifosfamide, rather than the parent compound, that has alkylating activity and causes cytotoxicity, oncogenic transforma-

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tion and chromosomal aberrations. This activation of ifosfamide occures primarily in the liver and is mediated by a mixed-function oxygenase system[1,2].

It is well established that there are multiple cytochrome P-450s, each differing in substrate specificity, carbon monoxide difference spectrum, response to a variety of inducing agents, and sensitivity to different inhibitors[3]. The induction of sister chromatid exchange by ifosfamide has been reported to be associated with metabolism by the phenobarbital-induced cytochrome P-450 rather than by the polycyclic aromatic hydrocarbon-induced cytochrome P-448[1,4]. Sladek demonstrated a greater increase in the alkylating activity of ifosfamide in vitro after incubation with phenobarbital-induced liver fractions than with control or polycyclic aromatic hydrocarbon-induced liver fractions[5].

In this study, the *in vitro* activation of ifosfamide, by male rat liver, to metabolites that are mutagenic to *Salmonella typhimurium* TA-100 is characterized. Inducers and inhibitors of mono-oxygenase activity have been used in an attempt to determine which form(s) of cytochrome P-450 is(are) involved in activating ifosfamide to a mutagen or mutagens.

Materials and Methods

Chemicals Ifosfamide was purchased from Shionogi Pharmaceutical, Co. Ltd., Tokyo, Japan. NADPH, NADH and glucose 6-phosphate and ATP were purchased from Oriental Yeast Co., Tokyo, Japan, and phenobarbital and α-naphthoflavone and agar were purchased from Nakalai Tesque Inc., Kyoto, Japan. L-Histidine, D-biotin and glucose were purchased from Wako Pure Chemicals, Osaka, Japan ; ATP was purchased from Sigma Chemical Co., St. Louis, Mo., USA. 2-diethylaminoethyl-2, 2-diphenylvalerate hydrochloride (SKF-525A) was a generous gift from Smith Kline Beecham Pharmaceuticals, King of Prussia, PA, USA. All other chemicals were of analytical grade.

Preparation of 9,000 x g supernatant fraction. Male Wistar rats (150-170 g) were obtained from Japan SLC (Hamamatsu, Japan). Animals were maintained on a commerical food (Crea Japan Laboratory chow CE-2) and water ad libitum in a temperature-humidity controlled laboratory with a 12 hrlight/dark cycle. Sodium phenobarbiatal dissolved in 0.9% NaCl solution and β-napthoflavone in corn oil were given *i.p.* in doses of 80 and 40 mg/kg, respectivaely, for 2 consecutive days, and the animals were killed 24 hr after the last administration. Control animals received normal saline (5 ml/kg, *i.p.*). Corn oil (5 ml/kg, *i.p.*) had no effect on the hepatic mixed-function oxidase system or mutagenic activity. The liver was rapidly excised and then perfused with 1.15% KCl solution. The minced liver was homogenized in 3 volumes of cold 1.15% KCl solution using a Potter-Elvehjem homogenizer with a Teflon pestle. The 9,000 x g liver supernatant fraction (S-9) was obtained by centrifugation of the homogenates at 9,000 x g for 20 min. The S-9 was distributed in 3 ml portions in small sterile polypropylene tubes, frozen in dry-ice-acetone, and stored at $-80°C$. The determinations of protein and cytochrome P-450 were carried out according to the methods of Bradford[6] and Omura and Sato[7], respectively.

Mutagenicity assay. This assay was performed according to the method of Ames et al.[8] with minor modifications. Salmonella typhimurium strains TA-100 (provided by Institute for

fermentation, Osaka, Japan) was used to assay for mutagenic activity. The culture (100 μl) of his$^-$ strain of *S.typhimurimum* TA-100 was added to 50 μl of sodium phosphate (pH 7.4), 4 μmol of $MgCl_2$, 16.5 μmol of KCl, 2.5 μmol of glucose 6-phosphate, 2 μmol of NADPH, 2 μmol of NADH, 2.5 μmol of ATP, and various amounts of S-9 in a total volume of 500 μl. The reaction was started by the addition of ifosfamide (600 μg) dissolved in 100 μl of 0.1 M sodium phosphate buffer (pH 7.4). After a 20 min incubation at 37°C, 2 ml of molten soft agar were added, and the resulting mixture was poured onto a minimal agar plate containing 0.1 μmol of L-histidine and 0.1 μmol of D-biotin. The number of his$^+$ revertant colonies was counted after in cubation for 2 days at 37°C.

Results

A wide variety of drugs, carcinogens, and other xenobiotics are metabolized by microsomal cytochrome P-450. Since the activities of microsomes required to produce mutagenic metabolites were comparable to those of S-9 and since typical inducers of cytochrome P-450, such as phenobarbital and β-naphthoflavone, induced the activities, it seemed reasonable to assume that microsomal cytochrome P-450 was involved in the metabolic activation. To support this view, the effects of inhibitors of cytochrome P-450 were examined. The spontaneous reversion rate (no ifosfamide) was similar to that reported by Ames et al.[8] for TA-1535 (Fig.1). The inclusion of enzyme fractions from liver of male rats did not alter this

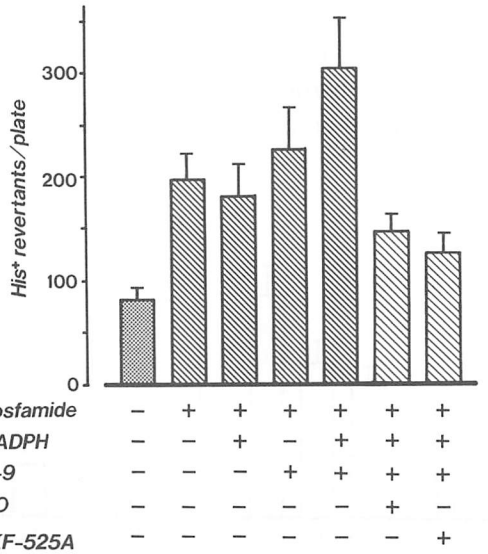

Fig. 1 Requirement of Cofactor and Effect of Inhibitors on the Enzymatic Activation of Ifosfamide to Mutagenic Metabolites in Salmonella Test System

The mutation assay was performed according to the method of Ames *et al.*[8] with minor modifications as described in Materilals and Methods. In requirement experiments, the cofactor was omitted from the incubation mixture and PBS was added. In the effect of inhibitors experiments, SKF-525 A (10 μmol) was added, and carbon monoxide (CO) was bubbled for 1 min. The reaction was started by the addition of ifosfamide (600 μg) dissolved in 100 μl of PBS. After a 20 min in cubation at 37°C, 2 ml of molten soft agar was added, and the resulting mixture was poured onto a minimal agar plate containing 0.1 μmol L-histidine and 0.1 μmol of D-biotin. The number of *his*$^+$ revertant colonies was counted after incubation for 2 days at 37°C. The assay was done in triplicate and values are means ± S.E..

spontaneous reversion rate. Ifosfamide alone (600 μg/plate) induced mutations. This mutagenic response was not changed by the addition of either the NADPH-generating system alone or S-9 fraction alone ; however, the number of revertants per plate was significantly increased by the addition of both the NADPH-generating system and S-9 fraction from the control male rat liver. As expected, the S-9 fraction and NADPH-generating system were a necessary cofactor, and the NADPH-supported activities were inhibited by carbon monoxide and SKF-525A. These findings indicate that the metabolic activationis catalyzed by microsomal cytochrome P-450. The mutagenic response in TA-100 for mutations induced by ifosfamide in the absence of an enzyme fraction has been subtracted in subsequent experiments, thus allowing the measurement of the enzymatic activation of ifosfamide.

The mutagenicity assay was linear with both ifosfamide and S-9 fraction concentration (Fig.2). In the presence of S-9 fraction, increasing doses of ifosfamide produced proportional increases in the mutation rate in *S.typhimurium* TA-100. The ability of S-9 fraction to activate ifosfamide to a mutagen was localized in the microsomes. At an ifosfamide concentration of 600 μg/plate, an increasing amount of liver S-9 fraction caused a linear increase in the number of revertant colonies per plate.

To characterize the enzyme system activating ifosfamide to a bacterial mutagen, the liver S-9 fraction from control male rats was compared to the S-9 fraction prepared from rats pretreated *in vivo* with phenobarbital and β-naphthoflavone that induce hepatic monooxygenase activity. Phenobarbital induces predominantly cytochrome P-450, whereas β-naphthoflavone induces cytochrome P-448[3]. As shown in Figs.3 and 4, the activities were induced to a higher extent by phenobarbital followed by β-napthoflavone and saline (control) when the activities were calculated on the basis of μg of S-9 fraction. Unlike the saline or

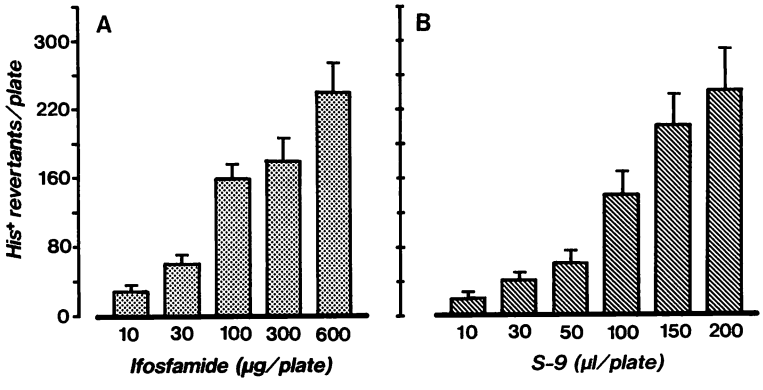

Fig. 2 Dependence of Mutagenicity on Ifosfamide and 9,000 x g Supernatant Concentration in Salmonella Test System

Panel A ; S.typhimurium TA-100 (0.1 ml), 9,000 x g supernatant fraction (S-9) from control male rat liver (0.1 ml) and an NADPH-generation system were mixed rapidly with 0.1 ml of ifosfamide solution.
Panel B ; Experiments with various amounts of control male rat liver S-9/plate were done at 600 μg/plate of ifosfamide. The mutation assay was as described in Materials and Methods. The number of spontaneous revertants was subtracted. Values are means ± S.E. for three separate experments performed in duplicate.

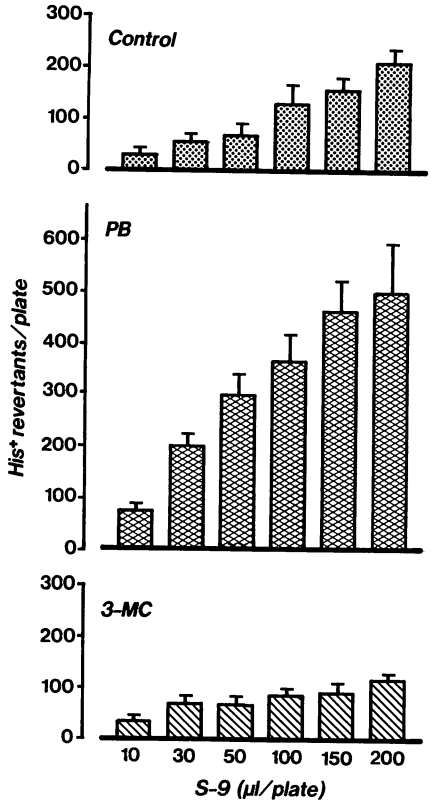

Fig. 3 Comparison of the Activation of Ifosfamide to a Mutagen by Control, Phenobarbital- and 3-Methylcholanthrene-pretreated Rat Liver 9,000 x g Supernatant Fraction

Each group of animals consisted of 3-4 rats. Rats for induction experiment were treated with phenobarbital or 3-methylcholathrene according to established procedures for induction of the hepatic monooxygense system. Sodium phenobarbital in 0.9 % NaCl solution and 3-methylcholanthrene in corn oil given i.p. at doses of 80 and 20 mg/kg, respectively, for 2 days, and the animals were killed 24 hr after the last administration. Control rats were not treated because of corn oil (5 ml/kg, i.p.) had no effect on the hepatic mixed-function oxidase system. *S.typhimurium* TA-100 (0.1 ml), NADPH -generating system and various amounts of resuspended 9,000 x g supernatant from control (dotted column), or phenobarbital (netted column)- and 3-methylcholanthrene (shaded column)- induced rat liver were mixed rapidly with ifosfamide (600 μg in 0.1 ml). The mutation assay was as described in Materials and Methods. The number of spontaneous revertants was subtracted. Values are means ± S.E. for three separate experiments performed in duplicate.

phenobarbital S-9 fraction, the β-naphthoflavone-induced activation of ifosfamide to a mutagenic metabolite was not linear with the S-9 fraction concentration.

The results of the induction studies suggest that activation of ifosfamide to a mutagen is catalyzed by a phenobarbital-responsive form of cytochrome P-450. For this reason, the liver S-9 fraction from phenobarbital-pretreated rats was used to test the effect of SKF-525A, metyrapone and α-naphthoflavone on the activation of ifosfamide to a mutagen. Inhibition by SKF-525A or metyrapone has been interpreted as evidence for the involvement of a phenobarbital-inducible cytochrome P-450[9~11]. Conversely, inhibition by low concentrations of α-naphthoflavone has been interpreted as evidence for the involvement of a cytochrome P-448 monooxygenase[11~13]. Activation of ifosfamide to a mutagen could be inhibited

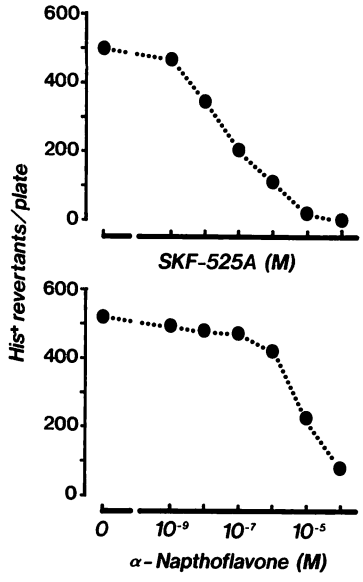

Fig. 4 The Effect of SKF-525 A, metyrapone and α-Naphthoflavone on the Activation of Ifosfamide to a Mutagen by Phenobarbital-induced Rat Liver

S.typhimurium (0.1 ml), inhibitor SKF-525 A, metyrapone and α-Naphthoflavone (0.1 ml) and phenobarbital-induced 9,000 x g supernatant fraction (100 μl) were mixed rapidly with ifosfamide (600 μg in 0.1 ml). The experimental condition for phenobarbital-induced rat is as described in Fig. 3. The mutation assay was as described in Materials and Methods. The number of spontaneous revertants was subtracted. Values are means ± S.E. for three separate experments performed in duplicate.

by SKF-525A, metyrapone or α-naphthoflavone ; however, SKF-525A was an extremely strong inhibitor with an IC_{50} of 4 x 10^{-7} M. In the absence of ifosfamide, none of these inhibitors altered the spontaneous reversion rate of *S.typhimurium* TA-100.

Discussion

The mixed-function oxidase of hepatic microsomes oxidizes a variety of xenobiotics in the presence of oxygen and NADPH-generating system. Many xenobiotics existing in the environment require metabolic activation to exert their mutagenic and carcinogenic actions[4, 14]. Active metabolites of ifosfamide, rather than the parent compound, have alkylating and mutagenic activities and cause cytotoxic reaction, mutations, chromosomal aberrations and oncogenic transformations[1, 2, 4]. The activation of ifosfamide to therapeutic and toxic metabolites is catalyzed by a cytochrome P-450 monooxygenase system[1]. It is now well established that there are multiple cytochrome P-450, each differing with respect to substrate specificity, carbon monoxide difference spectrum, response to a variety of inducing agents, and sensitivity to different inhibitors[3]. One interpretation of the lack of correlation between in vivo metabolism of ifosfamide to alkylating metabolites and hepatic in vitro metabolism is that ifosfamide is also converted to alkylating metabolites in extrahepatic tissues. Our work reported herein shows that an enzyme in the S-9 fractions is responsible for the metabolic activation of ifosfamide. This enzyme required NADPH as a cofactor to metabolize ifosfamide to mutagenic metabolites, and the NADPH-supported activities were

inhibited by carbon monoxide and SKF-525A (Fig.1). These results indicate that microsomal cytochrome P-450 is involved in the metabolic activation.

Moreover, as shown in Figs.3 and 4, the mutagen-producing activities were rather specifically induced by phenobarbital when calculated on the basis of μl volume of S-9 fraction. Phenobarbital and β-naphthoflavone induce different species of cytochrome P-450[3]. Previous studies have demonstrated that, except for acrolein, all oxazaphosphorine metabolites were mutagenic[4]. Thus, the mutagenic activity of ifosfamide generated by biotransformation probably represnents the cumulative effect of a variety of metabolites involving different metabolic reactions. An increase in ifosfamide mutagenicity is not observed in the presence of the β-naphthoflavone-induced S-9 fraction. The lack of increase in the alkylating activity of metabolites of ifosfamide has also been reported when β-naphthoflavone-induced microsomes were compared with the control level in rats[1,2]. This lack of increase in both mutagenicity and alkylating activity suggested the existence of alternate metabolic pathways, mediated preferentially by cytochrome P-448 that compete with the mutagenic metabolite producing the cytochrome P-450 pathway. This hypothesis can be tested because a cytochrome P-448-dependent pathway should be sensitive to inhibition by low concentrations of α-naphthoflavone ($10^{-9}-10^{-8}$ M)[11~13]. However, 10^{-7} Mα-naphthoflavone does not alter the activation of ifosfamide by the phenobarbital-induced S-9 fraction. Thus, a cytochrome P-450 species, probably phenobarbital-innducible cytochrome P-450, is capable of metabolizing more efficiently these substrates to mutagenic metabolites than is a cytochrome P-450 induced by β-naphthoflavone. In accordance with this idea, SKF-525A and metyrapone, a specific inhibitor of phenobarbital-inducible cytochrome P-450[9~11], inhibited the activities to a higher extent than did α-naphthoflavone which is known to inhibit β-naphthoflavone-induced cytochrome P-450.

The characteristics of inhibition and induction of the activation of ifosfamide to a mutagenic metabolites are concordant with the conclusion that this activation is catalyzed or solely by a phenobarbital-inducible cytochrome P-450. Phenobarbital-inducible cytochrome P-450 is also involved in the activation of ifosfamide to metabolites promoting alkylation[1,2] and sister chromatid exchange[4]. It would thus appear that the desirable effects of this drug as cancer chemotherapeutic agents (alkylating activity) are not dissociable from its undesirable side-effects (mutagenicity and promotion of sister chromatid exchange).

In summary, ifosfamide exerts its mutagenicity after undergoing metabolic activation by an enzyme in the S-9 fraction. The treatment of rats with phenobarbital resulted in a marked increase in the ability of S-9 fraction to activate ifosfamide, and the activation reaction required the NADPH-generating system as cofactor and was inhibited by carbon monoxide and SKF-525A.

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