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研究成果の刊行物・別刷

総　説

組織病理学用の切片を動物細胞の培養担体に利用した先端研究

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Advanced research utilizing histopathological sections as a culture substratum of animal cells

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Various culture substrata reflecting the microenvironment of cells *in vivo* have been developed to regulate multicellular structure and function. The substratum made of tissue/organ sections for histopathology (TOSHI), conserving microarchitecture and components *in vivo*, possessed the potential for inducing cell behavior in response to its microenvironmental signaling cues. Taking advantage of the TOSHI-substratum, we recently succeeded in the following two studies. Mouse embryonic stem cells were efficiently differentiated into hepatocyte-like cells when cultured on the TOSHI-substratum prepared from regenerating mouse livers after carbon tetrachloride intoxication. Also, the time-course cell behavior of two different cell lines on various TOSHI-substrata prepared from rat mature organs was converted into a three-dimensional graph chart, i.e. a mathematical model. Therefore, we hope that the former could be applied to the preparation of tailor-made hepatocytes *in vitro* and the latter will contribute to investigate the unknown characteristics of cells and/or tissues, i.e. cellomics and/or histomics, respectively. This review also describes visions for the future application of TOSHI-substrata to medical and pharmaceutical researches.

【キーワード】
細胞挙動，創薬，再生医療，セロミクス，ヒストミクス

1．はじめに

生体内の組織を構成する細胞は，経時的に変化している組織特異的な微小環境からのシグナ

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ルを認識して増殖や分化をはじめとする細胞挙動を決定している。この微小環境は，「多細胞間の間隙を流れている細胞外液」と「接着依存性細胞の足場となる細胞外マトリックス」である。これまでに，この微小環境の役割を模倣して様々な培養担体が開発され，多くの細胞培養システムが誕生してきた。例えば，前者の役割を再現する目的で開発された限外濾過膜製ホロファイバー（中空糸）束から成る担体は，生理活性物質の生産などに有用な細胞の高密度大量培養を達成できる通液培養システムを創出した。また，後者の役割を再現するために開発された各種細胞外マトリックス成分を加工した担体，真皮や小腸粘膜より細胞を除去して調製した無細胞性組織の担体，あるいは生体親和性の人工材料を加工した担体は，組織の再構築や薬剤に対する細胞応答の解析などに有用な数々の三次元培養システムを創出してきた[1]。

本稿では，生体組織の微細構造と構成成分が部域特異的に残存している組織病理学用の切片に着眼して開発した切片担体と，その担体を利用した培養システムについて紹介する。さらに，切片担体に用いる組織と切片担体上で培養する細胞の組合せ方を工夫することで創薬あるいは再生医療などへの応用が期待できる研究構想についても言及する。

2．切片担体の開発

細胞挙動を制御する様々な培養担体が開発されてきたが，生体組織の複雑な構造と成分の双方を反映した培養担体は未開発であった[2-4]。そこで，組織病理学の分野では染色による形態観察を目的として日常的に作製される「組織を薄切した切片」に着眼した。何故なら，切片には生体組織の微細構造のみならず抗体や核酸プローブで検出されるように様々な生体分子が部域特異的に残存しているので，細胞の培養担体に応用することで切片に介在している部域特異的な微細構造や生体分子に依存したシグナルを培養細胞に伝達できるのではないかと考えた。このような観点から，切片を動物細胞の培養担体に活用する培養新技術を開発し，原著論文では新しい概念で作製した「組織病理学用の組織／器官切片（Tissue/Organ Sections for HIstopathology）」担体なのでTOSHI担体と命名した[5,6]。

動物組織を薄切した切片担体は，ヒトを含む様々な動物のあらゆる組織より作製できる。具体的には，先ず組織病理学の実験手技に従って凍結組織，パラフィン包埋組織，あるいは樹脂包埋組織をミクロトームで薄切した切片をスライドグラス上に伸展し乾燥する。次に，前二者については凍結包埋剤あるいはパラフィンを除去した後（樹脂切片は脱樹脂できないのでそのまま），細胞培養に利用するため抗生物質を添加したＰＢＳあるいは70%エタノール等の処理により滅菌してから細胞培養液で平衡化して培養担体として使用する。以上が切片担体を作製する概略であるが，生体組織由来の構造や成分を切片担体にどのように取り入れるかは目的次第でいろいろと工夫する必要がある。例えば，新鮮な組織は未固定のまま凍結するのかホルマリン等で固定するのか，あるいは培養担体として使用する組織切片に対してタンパク変性を目的として熱や紫外線などで物理学的処理を施すのか，脱細胞化や脱脂を目的として界面活性剤や有機溶媒などで化学的処理を施すのか，または特定の抗原の露出やブロッキングを目的として酵素や抗体などで生物学的処理を施すのか，等々である。このようにして作製した切片担体上に細胞を播種すると，培養細胞は組織切片に介在する部域特異的なシグナルを認識して経時的に細胞挙動が誘導されるので，目的に応じた解析を進めることができる（図１）。

3．切片担体を利用した培養システム

当初の研究では，ウシ胎盤の凍結組織より作製した切片担体上で，異なる４種類の細胞（ヒト絨毛癌細胞株であるBeWo細胞，ウシ肺動脈血管内皮細胞株であるCPAE細胞，正常ヒト新生児包皮皮膚線維芽細胞であるNHDF細胞，およびラット褐色細胞腫であるPC-12細胞）を培養した。その結果，胎児側胎盤領域ではBeWo細胞のスフェロ

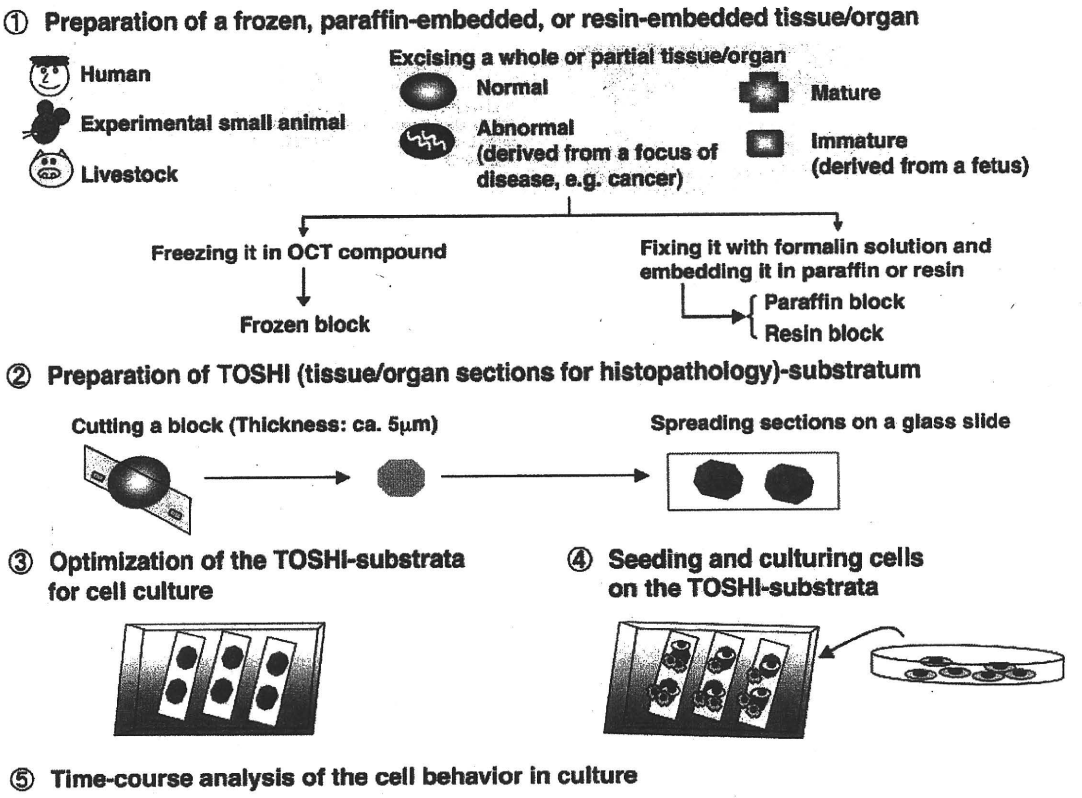

Fig. 1

Schematic procedure for preparing TOSHI (tissue/organ sections for histopathology) -substratum and culturing cells on it. Reproduced and partially-modified from Ref. 7 with permission of the publisher.

イド（多細胞性球状凝集塊），CPAE細胞の毛細血管網様構造，およびPC-12細胞の神経網様構造を形成する細胞の分化誘導が観察された。また切片担体には，無血清培養で誘導されるPC-12細胞のアポトーシスを阻害して細胞生存率を維持する活性があった。さらに，ドデシル硫酸ナトリウム（SDS）で脱細胞化処理した切片担体上にNHDF細胞を培養して，担体由来の細胞外マトリックス成分を巻き込んだ3次元組織を再構築する技術を確立した[5-8]。以上の結果は，1つの組織に由来する切片担体上で様々な細胞株の挙動を網羅的に解析するセロミクス研究が展開できることを示唆する[9]。

一方，四塩化炭素で軽度の肝障害を惹起した後の肝再生状態にあるマウスに，尾静脈よりマウス胚性幹細胞（以下，ES細胞）を投与すると，ES細胞は肝臓に移行した後に生着して肝細胞に分化することが報告されている[10]。そこで，この四塩化炭素を投与した後の障害あるいは再生の様々なステージにある肝組織より切片担体を作製した後，各切片担体上でES細胞を培養して接着，増殖および肝細胞への分化に関する細胞挙動を解析した。その結果，ES細胞の経時的な挙動は，切片担体に用いた肝組織の状態により異なることが分かった。具体的には，障害進行過程の肝組織より作製した切片担体上で培養したES細胞は丸い形態を示し，接着率と分化効率は共に低かった。これに対して，再生進行過程の肝組織より作製した切片担体上で培養したES細胞は敷石状に伸展し，接着率が高かった。特に，再生活性の強い四

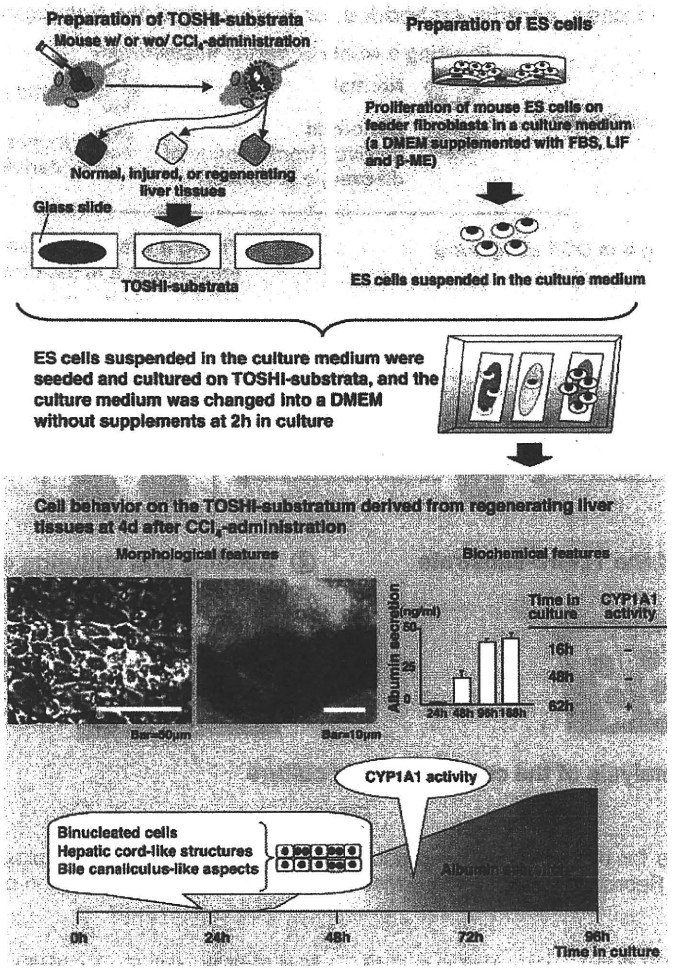

Fig. 2

Outline of the experiment for analyzing cell behavior of mouse embryonic stem (ES) cells cultured on the TOSHI-substrata derived from normal, injured, or regenerating mouse liver tissues.

塩化炭素投与後４日目の切片担体上では，培養24時間目までに約４倍に増殖する細胞集団が認められ，索状構造を形成した細胞集団や二核細胞も存在した。また，この４日目の切片担体上では，培養24時間目までに約70%の接着細胞がアルブミンを発現し，培養48時間目には培養液中へのアルブミン分泌，さらに培養62時間目にはCYP１A１活性も確認された（図２）。つまり，再生過程の肝組織より作製した切片担体を利用することで，ES細胞を短時間で効率よく肝細胞様細胞へ分化誘導できることが明らかとなった[7,8,11]。以上の結果は，様々な組織に由来する切片担体上で１つの細胞株の挙動を網羅的に解析するヒストミクス研究が展開できることを示唆する[9]。

上述の研究成果をもとに，切片担体と培養細胞の組み合わせは多種多様であるので，将来的には相互作用の解析結果を集積したデータベースを構築することが重要になると考えた[9]。そこで，最近の研究では，切片－細胞間の相互作用データベースを構築する第一段階として，数種類の臓器よ

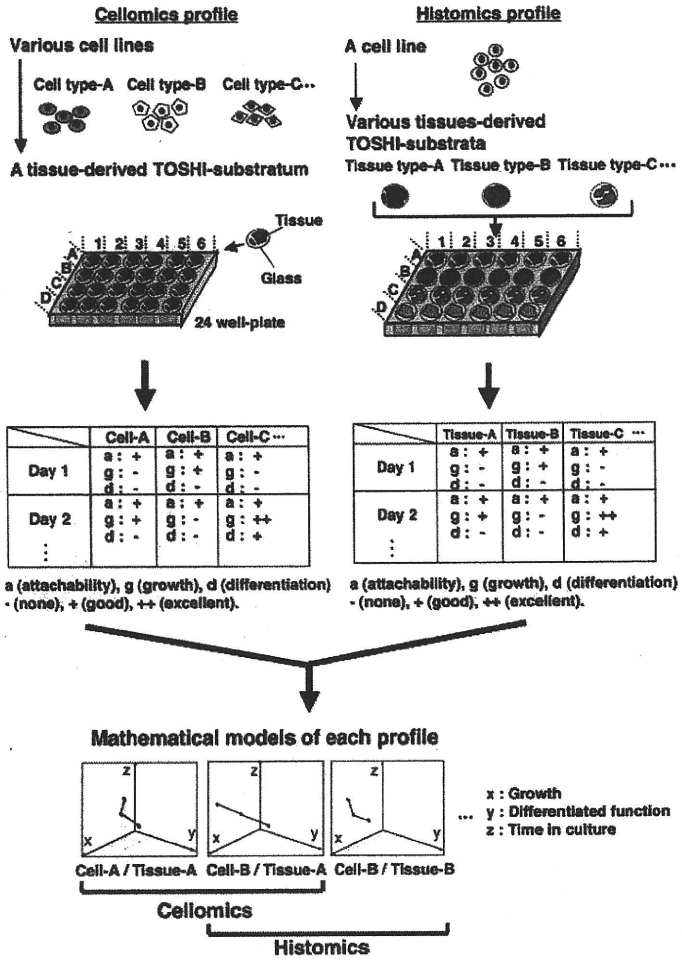

Fig. 3

Novel research concept for cellomics and histomics. Reproduced and partially-modified from Ref. 7 with permission of the publisher.

り作製した切片担体上で異なる2種類の細胞の挙動を解析した後に，解析データの数理モデル化を試みた。具体的には，ラットより摘出した各臓器（大脳，胸腺，心臓，腎臓，精巣，肝臓，など）より切片担体を作製した後，ラットインスリノーマ細胞株であるRIN5F細胞およびヒト肝癌細胞株であるHepG2細胞を培養した。それぞれの切片担体と培養細胞の組み合わせごとに経時的な細胞増殖および分化機能（培養液中に分泌されるインスリンまたはアルブミン量）を解析し，得られたデータを3次元グラフとして表記した。その結果，数理モデル化した細胞挙動のプロファイルは，細胞が同じでも切片に用いた組織に依存して異なること，また切片に用いた組織が同じでも細胞に依存して異なることを実証した（投稿論文準備中）。このことは，1つの組織に由来する切片担体を利用して異なる多数の細胞株を網羅的に解析したセロミクスデータと，1つの細胞株を利用して異なる多数の組織に由来する切片担体を網羅的に解析したヒストミクスデータの数理モデル化が可能であることを示唆する（図3）。今後，組織切片と細胞の相互作用プロファイルを集積したデータベ

ースが構築できれば，「特性を診断したい細胞と特性既知の組織切片」または「特性を診断したい組織切片と特性既知の細胞」を組み合せた培養から得られる細胞挙動プロファイルをデータベースへフィードバックすることで新しい診断システムが創出できると考えている[7-9]。

また，最近，ヒト前立腺がん細胞株であるDU145細胞をラット各種臓器より調製した切片担体上で培養して挙動変化を観察する実験を開始した。その結果，切片担体に依存して細胞の接着増殖性が異なるのみならず，細胞形態も大きく変化することが分かってきた（図4）。

4．創薬あるいは再生医療などへの応用構想

「動物組織を薄切した切片担体」は，培養細胞と切片担体の組み合わせ方を工夫することで，生命科学の基盤研究のみならず創薬や再生医療への応用研究が展開できると考えている（図5）。

創薬への応用研究としては，生理活性物質の探索，あるいは薬効および毒性の評価などが展開できると考えている。生理活性物質の探索では，特定の細胞が切片担体の特定部域で接着，増殖（阻害），分化などの細胞応答特性を示す培養モデルの特徴を利用して，特定部域からリガンド，増殖（阻害）因子，分化誘導因子などの生理活性物質を単離する研究が展開できる。また，薬効および毒性の評価では，動物実験代替法として有用な培養モデルを以下のように構築できる。通常用いる切片担体は厚みが5μmなので，厚みが10mmの組織であれば2,000枚の切片担体を作製することができる。また，実験動物一個体に化学物質を投与すれば，化学物質の薬効あるいは毒性を反映した切片担体は標的器官のみならず全身の諸器官からも作製することができる。従って，実験動物一個体を有効に活用して多数の切片担体を作製し，切片担体に介在する薬効あるいは毒性を様々な培養細胞の挙動で解析する新しい組織培養システムの構築が期待できる。ひいては特定の化学物質を投与した同じ実験動物に由来する切片担体を，複数の異なる研究機関でバリデーションに利用することも期待できる。さらに，切片担体は実験動物のみならずヒトの様々な生検材料からも作製できる。つまり，切片担体を利用した薬効および毒性の試験モデルは，実験動物の削減，あるいは置き換えの観点から有用な代替法になると考えられる[4,7]。

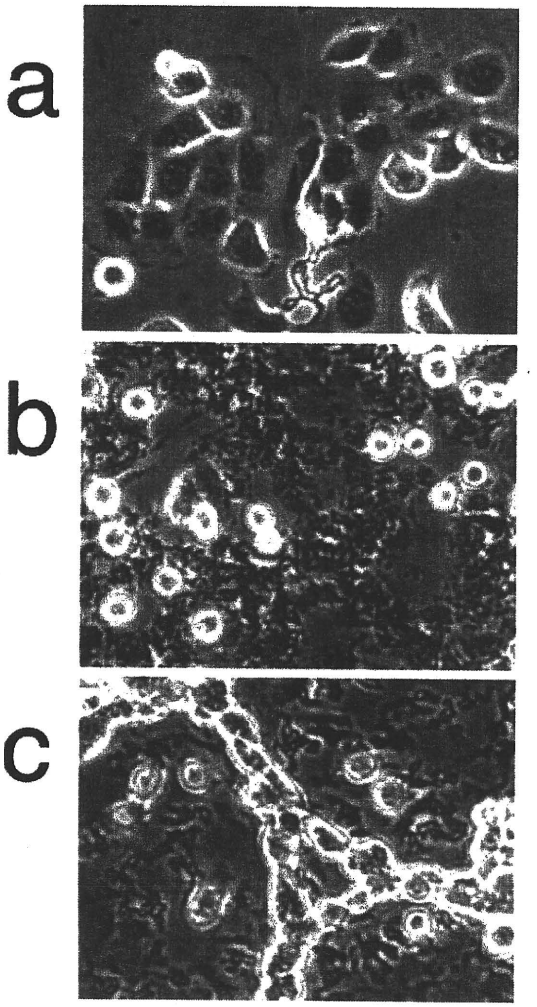

Fig. 4

Phase-contrast microphotographs of DU145 cells cultured for 2days on plastic (a), TOSHI-substrata derived from rat cerebellum (b) and kidney (C).

また，再生医療への応用研究としては，オーダーメイド成熟細胞の調製や自家移植用組織の再構

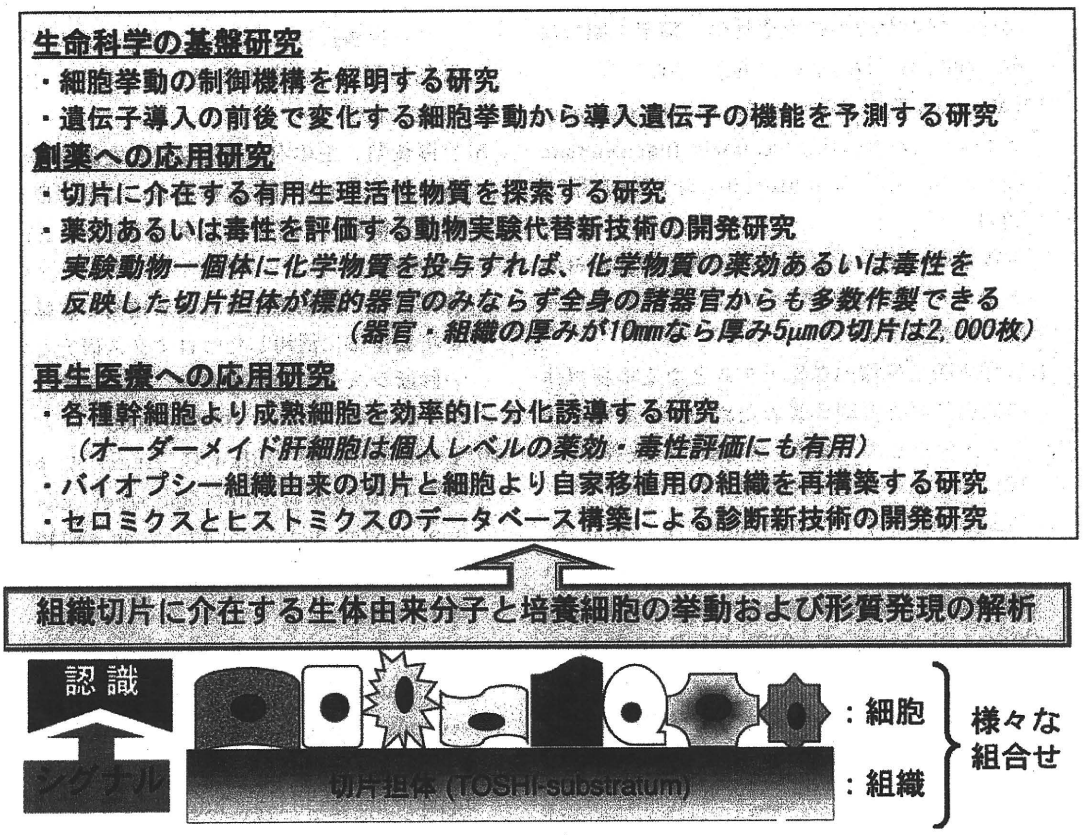

Fig. 5

Advantages of the culture system utilizing TOSHI-substratum.

築などに展開できると考えている。その他，上述のセロミクスおよびヒストミクスのデータベースを構築する研究が発展すれば，患者より分離した細胞や組織を診断する全く新しい技術を創出できると考えている[6,8,9]。

5．おわりに

創薬あるいは再生医療の研究発展には，今後どのような培養システムの開発が重要となってくるのであろうか。例えば，細胞挙動の制御，生理活性物質の探索，あるいは動物実験の代替に有用な培養システムの開発ではないかと考えている。具体的には，幹細胞の分化系譜を制御するとともに安全性を評価できるような培養システム，今までに注目されていなかった資源の宝庫から生理活性物質を探索できるような培養システム，あるいは薬効・毒性を評価するための動物実験を削減できるような培養システムである。このような培養システムの開発には，本稿で紹介した切片担体の活用構想が役立つと期待している。

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Carcinogenic risk of heterocyclic amines in combination – Assessment with a liver initiation model

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Abstract

Carcinogenic potential of heterocyclic amines (HCAs) was investigated using an *in vivo* 5-week initiation assay with quantitative evaluation of glutathione *S*-transferase placental form (GST-P) positive foci in rat liver. Numbers of GST-P positive foci were significantly increased with individual administration of six different HCAs, indicating utility of the assay. It was therefore applied to investigate risk with multiple HCAs in combination. Unexpectedly, concomitant treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) did not result in any additive carcinogenicity. In the rats taking MeIQx prior to PhIP the value was almost equal to the sum total of individual data, indicating additive initiation activities. In contrast, simultaneous or prior administration of PhIP rather exerted inhibitory effects on the carcinogenic potential of MeIQx. Moreover, microarray and quantitative RT-PCR assessment revealed that PhIP induced cytochrome P450 1A1, responsible for both activation and detoxification of HCAs, more strongly than MeIQx. It is noteworthy that complex exposure to multiple HCAs is not necessarily associated with increased risk of carcinogenesis because they are simultaneously and continuously ingested under normal circumstances.

Keywords: Heterocyclic amine; Mixture; Initiation; Cytochrome P-450; Glutathione *S*-transferase placental form

1. Introduction

More than 10 mutagenic and carcinogenic heterocyclic amines (HCAs) are produced by cooking or heating of meat or fish and these are now generally considered as important environmental risk factors for human carcinogenesis (Sugimura et al., 2004). Since humans are presumed to be simultaneously and continuously exposed to several HCAs in their diets, assessment of risks of these agents in combination is particularly important. In animal experiments, additive or synergistic effects of 5 or 10 different HCAs were indicated in the post-initiation phase using a medium-term liver bioassay (Hasegawa et al., 1991,1994).

Abbreviations: 2-AAF, 2-acethylaminofluorene; b.w., body weight; CCl_4, carbon tetrachloride; CYP, cytochrome P450; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; Glu-P-2, 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole; GST-P, glutathione *S*-transferase placental form; HCA, heterocyclic amine; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PH, partial hepatectomy; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole.

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On the other hand, Tsuda et al. indicated that mixtures of HCAs do not always act additively and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) rather tended to inhibit the carcinogenic potential of other HCAs (Tsuda et al., 1999).

Difficulties may be experienced in conducting long-term animal experiments for assessment of combined risks since they require large numbers of animals and massive investment in facilities. One way to overcome the problems is to adopt an *in vivo* 5-week initiation assay system. This is based on the two stage hypothesis of carcinogenesis and has already been employed for assessment of initiation activities of chemicals by quantitative evaluation of glutathione *S*-transferase placental form (GST-P) positive foci in rat liver (Sakai et al., 2002b). These preneoplastic lesions are considered reliable surrogates for liver tumors (Tatematsu et al., 1987). The 5-week initiation assay consists of induction of cell proliferation by two-thirds partial hepatectomy (PH) and subsequent administration of test chemicals to rats. If initiated cells are present in a liver, they rapidly grow to form GST-P positive foci with use of selection pressure, achieved with exposure to 2-acethylaminofluorene (2-AAF) and carbon tetrachloride (CCl_4). In this assay non-hepatocarcinogens can also induce GST-P positive foci regardless of their normal target organs (Sakai et al., 2002b), presumably due to the proliferating status of hepatocytes during exposure. Importantly, we earlier found that initiation activities of different carcinogens were summated with multiple chemical administration in this assay (Sakai et al., 2002a).

In the present study, the same *in vivo* 5-week initiation assay model was employed to evaluate the initiation activities of six HCAs individually and in combination, with a special focus on PhIP and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx). Moreover, the expression of metabolic enzymes after administration of PhIP and/or MeIQx was investigated with cDNA microarrays and quantitative RT-PCR analysis to cast light on mechanisms of interaction of these two carcinogens.

2. Materials and methods

2.1. Animals

Six weeks old male F344 rats were purchased from Charles River Japan Inc. (Atsugi, Japan) and housed in plastic cages on wood chips for bedding under constant conditions (12 h light/dark cycle, 60% humidity at 22 ± 2 °C). They were fed Oriental NMF diet (Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum* and allowed to acclimatize for 1 week before the start of experiment, at which time they were 7 weeks old. All animals were handled in accordance with the guidelines for animal experimentation of Aichi Cancer Center Research Institute.

2.2. Chemicals

Six different HCAs, PhIP, MeIQx, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) were purchased from the Nard Institute (Osaka, Japan). CCl_4 was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Diet containing 0.015% of 2-AAF was purchased from Japan Clea Co. (Tokyo, Japan).

2.3. In vivo 5-week initiation assay

In Exp. I-A, we first determined initiation activities of each HCA as shown in Fig. 1 (top panel). All rats underwent PH and were intragastrically administered one HCA 12 h thereafter. HCAs were suspended in corn oil and the doses are as listed in Table 1. Subsequently, the rats were fed on basal diet for 2 weeks, followed by diet containing 0.015% 2-AAF for the next 2 weeks. Three weeks after PH, all rats received CCl_4 as a stimulus for proliferation. At the end of week 5, all the survivors were sacrificed and slices of their livers were fixed in 10% neutral buffered formalin. Immunohistochemical staining and analysis of GST-P positive foci were conducted as previously reported (Sakai et al., 2002b).

In Exp. I-B, we secondly examined carcinogenic risks with different HCAs in combination as shown in Fig. 1 (bottom panel). PhIP and MeIQx were selected for analyses because these are two of the most abundant HCAs in cooked foods (Felton et al., 1986; Sugimura et al., 2004). The doses were determined based on the results of Exp. I-A and the actual ratio of both HCAs in cooked beef (Lynch et al., 1992) in consideration of dietary exposures in human. Twelve and 30 h after PH were selected as administration time points in line with our previous report (Sakai et al., 2002a). After PH, rats were divided into eight groups. In

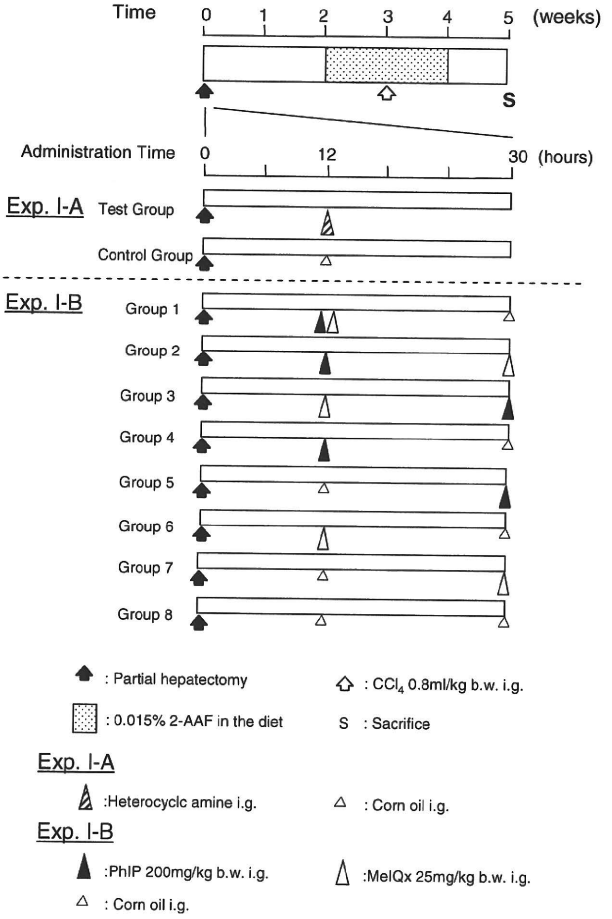

Fig. 1. Schematic representation of the protocols for Experiment I. Basic procedures were common in Experiment I-A and I-B except for administration of test compounds. The administration protocol is described in detail. Exp., experiment.

Table 1
Numbers of GST-P positive liver cell foci induced by single HCAs in Experiment I-A

Chemical	Dose (mg/kg)	Test group		Control group		*P* value	Mutagenicity[b] (revertants/μg)
		n	No. (/cm^2)[a]	*n*	No. (/cm^2)[a]		
PhIP	50	14	0.8 ± 1.2	9	1.5 ± 1.6	NS	1800
	100	12	9.9 ± 11.5	13	4.4 ± 3.1	NS	
	200	10	9.7 ± 3.7	5	3.8 ± 2.5	<0.05	
MeIQx	50	15	3.5 ± 2.3	9	1.5 ± 1.6	<0.05	145,000
IQ	50	15	19.5 ± 14.9	9	1.5 ± 1.6	<0.001	433,000
Trp-P-1	50	15	9.0 ± 4.8	9	1.5 ± 1.6	<0.001	39,000
Glu-P-1	50	15	5.1 ± 5.7	9	1.5 ± 1.6	<0.01	49,000
Glu-p-2	50	13	2.1 ± 2.4	9	1.5 ± 1.6	NS	1900
	100	13	12.7 ± 4.5	13	4.4 ± 3.1	<0.001	

NS, not significant.
[a] Data are mean ± SD values.
[b] Reported mutagenicity in *Salmonella typhimurium* TA98 with S9 mix (Sugimura et al., 2004).

group 1, animals received simultaneous administration of PhIP (200 mg/kg b.w.) plus MeIQx (25 mg/kg b.w.) at 12 h and then vehicle (corn oil) alone at 30 h. Both compounds were given separately at 12 h and then at 30 h in groups 2 (PhIP → MeIQx) and 3 (MeIQx → PhIP). Single compounds were given at 12 or 30 h to animals of groups 4–7. Control group rats were given only vehicle (group 8). Subsequent processing was as in Exp. I-A.

2.4. Gene expression analysis of metabolic enzymes

In Exp. II-A, we comprehensively investigated the expression of metabolic enzymes after combined treatment of PH and HCAs administration using cDNA microarrays. Rats were intragastrically administered a mixture of PhIP plus MeIQx (group 1), PhIP (group 2), or MeIQx (group 3) at 12 h after PH and killed 18 h thereafter (at 30 h after PH) to correspond with the second administration time in Exp. I-B. The administration doses were also in accordance with Exp. I-B (PhIP 200 mg/kg b.w. and MeIQx 25 mg/kg b.w.) and control group rats were given only corn oil (group 4). At sacrifice, right lobes of livers were immediately frozen in liquid nitrogen. Four samples from four rats per group were investigated. Total RNA was isolated from frozen liver samples according to a TRIzol Reagent protocol (Invitrogen, Carlsbad, California) with deoxyribonuclease (DNase) treatment using DNase I (Invitrogen). Microarray analysis was performed by Daiyu-kai Institute of Medical Science (DIMS) (Nagoya, Japan) using a customized PamChip® microarray (PamGene B.V., Den Bosch, The Netherlands) named RatTox ver. 1.0 provided by Olympus (Tokyo, Japan) as previously described (Hokaiwado et al., 2004). RatTox ver. 1.0 carries 28 genes in duplicate, including mainly for rat metabolizing enzymes as well as four positive control and one negative control genes. The complete gene list is given in Fig. 3. For data analysis, normalization was conducted for each gene against glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.5. Quantitative real-time RT-PCR analysis

In Exp. II-B, quantitative real-time RT-PCR of cytochrome P450 (CYP) 1A1 and 1A2 was performed with the LightCycler system (Roche Diagnostics, Mannheim, Germany), with GAPDH as an internal control. First strand cDNA was synthesized from DNase-treated total RNA with Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions and PCR was performed basically as described earlier using a QuantiTect SYBR Green PCR Kit (QIAGEN, Tokyo, Japan) (Sakai et al., 2001). Briefly, 20 μl of reaction mixture was prepared containing 1 μl of cDNA, 20 μM each of forward and reverse primers and 2X QuantiTect SYBR Green PCR Master Mix. Primers used for CYP1A1 were 5′-AAGCCCATGTTCCTGTTTA-3′ and 5′-GAGGCAACTTGGACTACACA-3′; for CYP1A2, 5′-CCCCAGGAAGAGCGAGGAG-3′ and 5′-CGCAGGACCGGAAAGAAGTC-3′; for GAPDH, 5′-CCCCCAACACTGAGCATC-3′ and 5′-TGAGGGTGCAGCGAACTTTA-3′. Cycling conditions were as follows: Initial denaturation at 95 °C for 10 min and then 50 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 15 s. SYBR Green fluorescence was measured at the end of each extension. Normalization of the data was achieved by quantitating the cycle number at an arbitrary fluorescence intensity in the linear exponential phase using the LightCycler system by calculating the ratio of cycle number of each enzyme relative to that of GAPDH cDNA.

2.6. Statistical analysis

The significance of differences between groups in the numbers of GST-P positive foci per unit area and mRNA levels was statistically evaluated using Mann–Whitney's *U*-test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Initiation activities of HCAs

Data for the numbers of GST-P positive foci induced by the six kinds of HCAs are summarized in Table 1. First, we individually administered each HCA at 50 mg/kg b.w. Induction of GST-P positive foci was significant with MeIQx, IQ, Trp-P-1, and Glu-P-1. There were no significant differences from the control with low doses of PhIP (50 and 100 mg/kg) and Glu-P-2 (50 mg/kg). However, on increasing the administration doses for PhIP to 200 mg/kg and Glu-P-2 to 100 mg/kg, significant development of GST-P positive foci was observed.

Quantitative data for GST-P positive foci in Exp. I-B are illustrated in Fig. 2. The values in the groups given PhIP and/or MeIQx (groups 1–7) were significantly higher than the control group (group 8) (groups 1, 2, and 7, $P < 0.0005$; groups 3 and 6, $P < 0.0001$; group 4, $P < 0.005$; group 5, $P < 0.05$). With multiple chemical administration groups, the value for MeIQx prior to PhIP (group 3) was also significantly higher than with PhIP or MeIQx alone at the same time points ($P < 0.05$, vs. groups 5 and 6), indicating summation effects. However, when the two HCAs were administered in reverse order (group 2), there was no significant difference between the serial

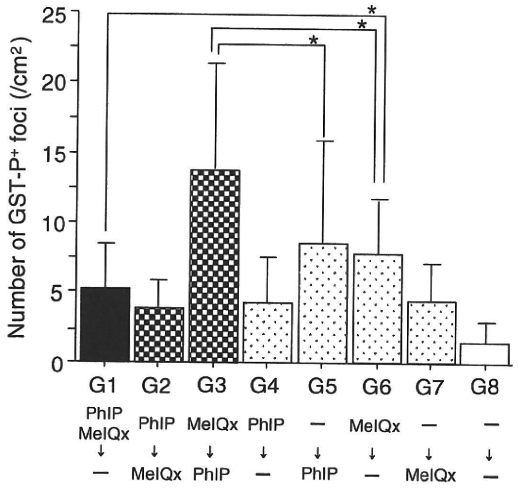

Fig. 2. Number of GST-P positive liver cell foci > 0.1 mm diameter in Experiment I-B. Data are mean ± SD values for 15–16 animals in each group. $^{*}P < 0.05$. Note the significant differences from the control group (group 8) in all cases (groups 1, 2, and 7, $P < 0.0005$; groups 3 and 6, $P < 0.0001$; group 4, $P < 0.005$; group 5, $P < 0.05$).

treatment and individual treatment with PhIP (group 4) or MeIQx (group 7). Furthermore, the number of GST-P positive foci induced with MeIQx (group 6) was significantly suppressed with the simultaneous administration of PhIP (group 1) ($P < 0.05$, vs. group 6).

3.2. Gene expression of metabolic enzymes

Data for relative-quantification of mRNAs of 23 genes in livers of hepatectomized and HCAs-administered rats on microarray analysis are illustrated in Fig. 3. CYP1A1 with administration of PhIP with/without MeIQx (groups 1 and 2) and CYP1A2 with administration of PhIP and/or MeIQx (groups 1–3) were up-regulated with statistical significance as compared with control values (group 4) ($P < 0.05$). In addition, the expression level of CYP1A2 was significantly higher in PhIP-treated than in MeIQx-treated animals ($P < 0.05$).

Gene expression levels of CYP1A1 and 1A2 examined by quantitative RT-PCR are summarized in Fig. 4. The values for CYP1A1 and 1A2 in the groups given PhIP and/or MeIQx (groups 1–3) were significantly higher than for controls (group 4) ($P < 0.05$). Levels of CYP1A1 were markedly elevated in rats treated with PhIP (group 1 and 2) as compared to MeIQx-treated rats (group 3) with statistical significance ($P < 0.05$, respectively). Inter-group differences in levels of CYP1A2 were not apparent among the HCA-treated groups (groups 1–3).

4. Discussion

Induction of GST-P positive foci was significant with each HCA, including the non-hepatocarcinogen PhIP, using the present *in vivo* 5-week initiation assay (Table 1), indicating its utility for analysis of the carcinogenic potential of this important group of environmental agents. HCAs are *N*-hydroxylated mainly by CYP 1A2 at the first step of their metabolic activation (Sugimura et al., 2004) and it has been reported that the metabolic activity of CYP 1A2 is maintained after PH (Trautwein et al., 1997). In this study, the initiation activities of HCAs reflected their reported mutagenicities in the Ames assay using *Salmonella typhimurium* TA98 (Sugimura et al., 2004). Weaker mutagens, PhIP and Glu-P-2, required higher administration doses for significant induction of GST-P positive foci and the strongest mutagen of the six HCAs, IQ, induced the most GST-P positive foci at the dose of 50 mg/kg body weight. In addition, the time taken for metabolism of each HCA might have an effect on the degree of initiation activities because cell proliferation fluctuates after PH and influences the formation of GST-P positive foci (Sakai et al., 2001).

Investigation of complex risks of multiple HCAs showed that simultaneous administration of PhIP and MeIQx (group 1) did not cause additive initiation, rather inhibitory effects of PhIP on MeIQx induction of lesions being observed. This result supports the previous finding that PhIP tends to reduce the formation of preneoplastic lesions induced by other HCAs in rats (Tsuda et al., 1999). Our data extend this interaction to the initiation stage of carcinogenesis. Furthermore, when considering actual dietary exposures in human, our data increases in importance because both HCAs are actually contained in foods in the similar ratio to our experimental doses (Lynch et al., 1992; Sugimura et al., 2004). With sequential administration, the order of exposure proved important. In the group receiving MeIQx prior to PhIP (group 3) the value was almost equal to the sum total of individual data as expected, indicating summation of initiation activities. In contrast, though the same HCAs were administered in group 2, the summation of individual values disappeared on reversing the administration order, indicating inhibitory effects of prior treatment of PhIP on MeIQx induction of lesions.

HCAs undergo a very complex metabolism involving several groups of xenobiotic metabolizing enzymes, including both phase I and II species, and induction of these enzymes modulates the carcinogenicity of HCAs (Dashwood, 2002; Sugimura et al., 2004). It is thus very possible that induction of some specific enzymes by prior treatment of HCAs affected the metabolism of sequentially administered HCAs in the present *in vivo* 5-week initiation assay. To clarify the inhibitory effects of prior treatment of PhIP, the expression of metabolic enzymes after combined treatment of PH and HCAs was comprehensively investigated. CYP1A1 and 1A2 were significantly up-regulated in regenerating rat liver by PhIP and MeIQx. Up-regulation of CYP1A2 would not be expected to cause the inhibitory effect of PhIP against MeIQx because CYP1A2 is known to be selectively involved in metabolic activation of MeIQx (Yamazoe et al., 1988). Indeed, it has been shown that

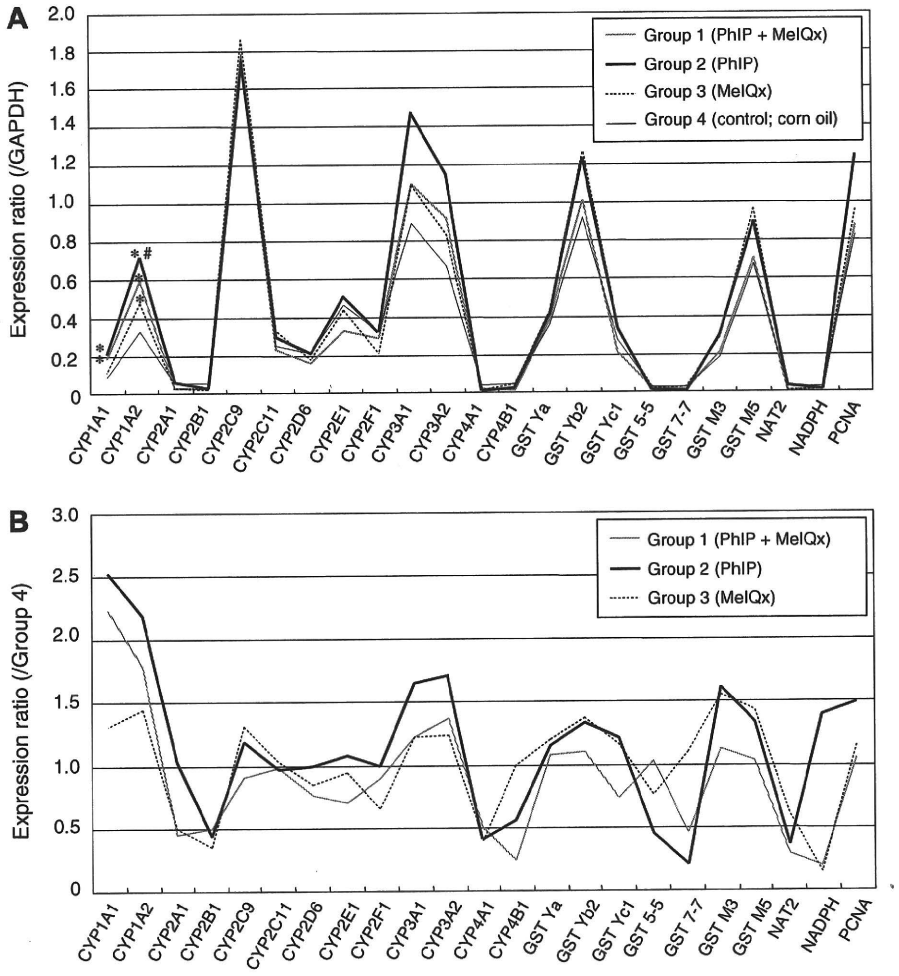

Fig. 3. Gene expression levels of mRNA after combined PH and HCA administration by microarray analysis. Data are relative values normalized to GAPDH (A) and compared with the control group (B). *Significantly different from the control group, $P < 0.05$. #Significantly different from group 3, $P < 0.05$.

enhancement of experimental carcinogenesis by MeIQx is closely associated with induction of CYP1A2 (Nishikawa et al., 2002). However, it was demonstrated using a medium-term liver bioassay that CYP 1A2 inducers, caffeine and fenbendazole, did not modify MeIQx-induced rat hepatocarcinogenesis despite up-regulation of CYP 1A2, suggesting elevated CYP1A2 expression is not sufficient to enhance MeIQx-induced carcinogenesis (Kuribayashi et al., 2006; Suzuki et al., 2002). On the other hand, CYP 1A1 might have partially contributed to the inhibitory effects of prior treatment of PhIP against MeIQx. It was much more strongly induced by PhIP than with MeIQx and can activate HCAs via *N*-hydroxylation of exocyclic amine, also catalyzing competing ring hydroxylation of HCAs followed by conjugation to sulfuric and glucuronic acid, the major pathways of detoxification. Some chemopreventive compounds, such as indole-3-carbinol, preferentially induce CYP1A1 versus CYP1A2 and suppress the carcinogenicity of HCAs by shifting the metabolism of HCAs (Dashwood, 2002). The induction of CYP1A1 was also reported in human cell lines incubated with HCAs (Hummerich et al., 2004) and small intestines of human volunteers following a meal of grilled meat diets containing large amounts of HCAs (Fontana et al., 1999). However, further study is needed to determine whether the CYP1A1 enhancement suppresses the carcinogenicity of MeIQx in humans as in rodents because the interspecies differences in the metabolism of MeIQx exist between rats and humans (Turesky et al., 2002). Nonetheless, our data would be still important indicating the possibility that some HCAs interact suppressively via the induction of their metabolic enzymes *in vivo*.

We must take into account the change of time required for the metabolism of MeIQx because the cell proliferation influences GST-P positive foci formations as mentioned above. It was possible that MeIQx was metabolized faster in group 2 (PhIP → MeIQx) as a result of the induction of metabolic enzymes after PhIP administration than in group 7 (solvent → MeIQx). We earlier revealed that the change of cell proliferation level was much smaller between