Review

Alternative Animal Models for Carcinogenicity Testing -Evaluation of Gene-engineered Models-

発がん物質の中期代替検索法

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Summary

Use of animals for the determination of carcinogenicity of compounds is still the most reliable method. The traditional approach for detecting carcinogens, the long-term (generally 2 years in rats) study in rodents, has the disadvantages of being expensive and time consuming. Furthermore, taking into account animal welfare considerations, attention has recently been concentrated on development of alternative methods to reduce testing time and animal number. For this purpose, various types of 2-stage carcinogenesis models and, more recently, examples featuring genetic-engineering have been developed. In this review, we concentrate on gene-engineered animal models (GEM) for evaluation of carcinogenicity and mutagenicity. Currently, the GEMs most commonly used are the *ras*H2, *p53*$^{+/-}$ and Tg.AC models. The *ras*H2 mouse appears to be the most appropriate for general carcinogenicity testing because of sufficient validation studies using known carcinogens. Other murine models, *p53*$^{+/-}$ and Tg.AC, need more validation studies, historic background data, unexpected tumor site, and tumor characterization. Rats with the same transgene as *ras*H2 mice are also promising because of documented induction of mammary carcinomas by a variety of carcinogens. Animal models for evaluation of mutagenesis are also more reliable than simple *in vitro* models, although examples are obviously required which allow a simplified detection method. Overall findings indicate that prediction of two-year rat bioassay outcomes with early assessment by GEMs may have the potential to increase the efficiency of strategies for identification of human carcinogens.

1．がん原性の検出のための遺伝子改変動物モデル

長期発がん試験に替わる短・中期発がん試験モデルとして、マウスでは1）ヒト型*c*-Ha-*ras*遺伝子導入マウス（*ras*H2）モデル、2）がん抑制遺伝子*p53*の片側アレルを欠損させたノックアウトマウス（*p53*$^{+/-}$）モデル、3）*v*-Ha-*ras*遺伝子導入マウス（Tg.AC）モデルおよび、4）色素性乾皮症修復遺伝子欠損マウス（XPA$^{-/-}$）モデルがある。ラットでは、1）ヒトプロト型*c*-Ha-*ras*トランスジェニックラット（H*ras*128）および2）SV40TAgトランスジェニックラットがある。このような短期・中期発がんモデルを利用した場合には、その実施に際しては試験法選択の科学的な根拠が必要である。

1-1．トランスジェニックマウス

トランスジェニック動物の作製は受精卵に外来遺伝子を人為的に組み込む方法によって行う。妊娠したドナー動物から採取した受精卵前核にマイクロキャピラリーを用いて目的とするDNAを注入する。その受精卵を、偽妊娠動物（仮親）の卵管内に移植して自然分娩させると出生仔中にDNAが組み込まれた動物が0.1～1％程度の確率で得られる。がん遺伝子が組み込まれた場合あるいはがん抑制遺伝子が欠損した場合には通常は発がん感受性の亢進がみられる。また化学物質による遺伝子変異のレポーター遺伝子を導入して*in vivo*変異原性の検出、化学物質の受容体等を導入して毒性発現の機序の解析や毒性評価の短期化を図ることも試みられている。

1-1-1．*ras*H2マウス

ヒトプロト型*c*-Ha-*ras*遺伝子を導入したマウスで、勝木ら

によって作出された[1,2]。このマウスでは、発癌物質の投与および自然発生において皮膚腫瘍、前胃腫瘍、リンパ腫、血管肉腫等が野生型より短期間に発生する（前胃腫瘍は*N*-methyl-*N*-nitrosourea 50 mg/kg腹腔内投与の場合12週で100％；自然発生は18ヵ月以内に50％）。自然発生腫瘍は肺腫瘍、前胃・皮膚扁平上皮乳頭腫、脾血管腫、肝腺腫等で6ヵ月までの発生は少ない[3,4]。使用するマウスの背景系統によって腫瘍の発生する臓器が若干異なるが、現在ではBALB/cByJ（雌）×C57BL/6J-Tg *ras*H2（雄）の交雑F1が使用されている。発生した腫瘍では導入した遺伝子に点変異が高い頻度にみられるが、内在*ras*遺伝子の変異は殆どない。短期試験代替法として26週投与の実験において、遺伝毒性・非遺伝毒性発がん物質による発がん感受性について、既知の発がん物質との整合性について検証した結果、多くの遺伝毒性発がん物質（変異原性物質）では陽性を示し、非遺伝毒性発がん物質（非変異原性物質）では83％（5/6）に陽性結果が得られた[5,6]。また非がん原性物質はすべて陰性結果であり、偽陽性のない点も注目される[3]。非遺伝毒性発がん物質では解熱剤のPhenacetin、合成ホルモンのDiethylstilbestrol等は陽性であるが、17-β-Estradiol、鎮静剤のPhenobarbital、免疫抑制剤のCyclosporin A等は陰性であった[3]。*ras*H2マウスはホモ個体が得られず、非導入近交系BALB/cByJ系雌マウスと導入遺伝子をヘテロにもつC57BL/6JJcl系雄マウスとのF1（CB6F1-Tg *ras*H2）が使用されている。

1-1-2. Tg.ACマウス

ζ（zeta)-グロビンのプロモーター下に*v*-Ha-*ras*遺伝子を導入したもので、FVB/Nマウスに戻し交配されたものが米国Taconic Farms社で維持されている[7]。導入遺伝子の持続的な発現は骨髄を除いて検出されないが、皮膚創傷、UV照射、発癌プロモーターである12-*O*-tetradecanoylphorbol-13-acetate（TPA）等の化学物質の皮膚暴露によって導入遺伝子の活性化が見られる[8~10]。TPAのみを皮膚に塗布しても扁平上皮腫瘍が発生するため、二段階皮膚発がん物質/プロモーターの検索モデルとしての有用性がある。Dimethylvinyl chloride[11]の経口投与によっても皮膚腫瘍、さらに前胃扁平上皮腫瘍が発生する。この方法によって遺伝毒性・非遺伝毒性両方の発がん物質検出モデルへ検証が試みられてきたが、経口投与で野生型FVB/Nマウスに前胃腫瘍、経皮投与で肝腫瘍を発生させるトリエタノールアミンでは、Tg.ACマウスで腫瘍の発生はなかった[12~14]。したがって、皮膚を除く臓器における腫瘍発生では既知の結果と整合性の高い結果が得られていない[15]。以上から、Tg.ACモデルは、期待されたほど十分に発がん感受性が高くなく（was not overly sensitive)、ヒト発がんリスクの補助的試験法として有用であるとされている（表1）[16]。

1-2. ノックアウトマウス

ターゲッティングによってES細胞の目標とする遺伝子またはそのプロモーター領域を欠損（あるいは変異）させ、その遺伝子が機能しないようにして作出された動物である。一般にがん抑制遺伝子が欠損した場合には発がんの亢進が観察される。ES細胞が樹立されていることが前提となるので、げっ歯類で作製されているのはマウスのみである。ラットES細胞樹立の報告[17~19]はあるが実用化には至っていない。今後、ES細胞のみならずiPS細胞[20~22]や新しい技術[23,24]を用いたノックアウト動物が作製されて発がん物質の中期検索法への利用が進展することが期待される。

1-2-1. *p53*$^{+/-}$マウス

*p53*遺伝子のExon5の欠損した*p53*$^{+/-}$C57BL6マウス[25]と、Exon2の欠損した*p53*$^{+/-}$CBAマウス[26]が中期発がん試験に用いられている。他に、*p53*のExon 2-6が欠損しているマウスが2系統作製されている[27,28]。これらのマウスは、野生型マウスに比べ、化学発がん物質に対する感受性が高い[25,29]。*p53*遺伝子はDNA傷害の修復に関与するために、ガンマ線照射でも皮

表1. Tg.ACマウスの発がん物質に対する発がん感受性[16]

	Compound	Skin	Oral
Human carcinogens	Cyclosporin A	+	±
	Diethylstilbestrol	+	−
	Ethinyl estradiol	+	−
	Phenacetin	−	−
	Melphalan	±	+
	Cyclophosphamide monohydrate	±	+
Rodent cacinogens	Sulfamethoxazole	−	−
	Di(2-ethylhexyl) phthalate	−	−
	WY-14643	−	±
	Clofibrate	+	NT
	Methapyrilene HCl	−	NT
	Reserpine	−	−
Noncarcinogen	Sulfisoxazole	−	−

+, positive; −, negative; ±, equivocal; NT, not tested

膚腫瘍の発生期間が短縮される[30]。このマウスではリンパ腫が共通して発生するが、遺伝背景を変えると発生腫瘍スペクトラムが変わり、C57BL/6背景ではリンパ腫、129/SV背景では悪性奇形腫、BALB/c背景ではLi-Fraumeni症候群に好発する乳がんが多く発生する[31~33]。したがって、*p53*$^{+/-}$マウスを中期発がん試験に用いる場合には、マウスの背景系統を十分考慮すべきである。雌C57BL/6Ntacと雄*p53*$^{-/-}$ N4マウスを交配させたB6.129N5-Tr*p53*が市販されている。自然発生腫瘍の少ない6ヵ月間が適切な試験期間である。

発がん感受性試験において、遺伝毒性物質のMelphalan、Cyclophosphamideは陽性、非遺伝毒性物質ではCyclosporin AとDiethystilbestrolは陽性であったがPhenacetin、17-β-Estradiolは陰性であった。変異原性陰性の肝発がん物質であるペルオキシゾーム増生物質のうち、ClofibrateおよびDiethylhexylphthalate（DEHP）では肝腫瘍のわずかな増加がみられた。非がん原性物質については、いずれの化合物においても陰性であった[34]。以上から、*p53*$^{+/-}$モデルは遺伝毒性発がん物質の検出において信頼性の高いモデルとされている。

1-2-2. XPAノックアウトマウス

色素性乾皮症（Xerodema pigmentosum）はDNA修復酵素の先天性異常による高発がん性を示す常染色体劣性遺伝病である。紫外線暴露によって健常人の1000～2000倍の高頻度に皮膚がんが発生する[35,36]。遺伝子異常の差異によってA～Gの相補性群とバリアントの8群があるが、日本人ではA群が多く欧米ではC群とD群が多い[37]。A群色素性乾皮症の原因遺伝子としてDNA除去修復遺伝子XPAが同定された[38]。XPAはヌクレオチド除去修復に関与すると考えられている。

XPAノックアウトマウス（XPA$^{-/-}$）[39,40]の皮膚に紫外線（UV-B）を照射するとヒトと同様に皮膚扁平上皮がんが高頻度に発生する。発がん感受性試験において遺伝毒性発がん物質ではdimethylbenz[*a*]anthraceneの頻回塗布でも皮膚乳頭腫が生じる。さらに、非遺伝毒性発がん物質のペルオキシゾーム増生物質WY-14643は発がんするがClofibrate、DEHPでは陰性であった。同様にPhenacetinはXPA$^{-/-}$で陰性、Cyclospolin AおよびDiethylstilbestrolは XPA$^{-/-}$とXPA$^{-/-}$/*p53*$^{+/-}$交配系統で陽性を示し、17-β-EstradiolはXPA$^{-/-}$では陰性、XPA$^{-/-}$/*p53*$^{+/-}$では陽性であった。非がん原性物質のMannitol、Ampicillinでは陰性であった。しかしながら、XPA$^{-/-}$およびXPA$^{-/-}$/*p53*$^{+/-}$モデルのいずれも背景データがまだ少なく、実用には至っていない[41]。

1-3. トランスジェニックラット

ラットは、マウスよりも大型であり解析に必要な組織を採取するのに有利であり、ラットを用いた化学発がん研究による前がん病変の生物学的情報が豊富である。さらにマウスと同様の手法で遺伝子導入ができる。そのために遺伝子改変による発がん高感受性系統の作出が期待されている。しかしながら、マウスに比べてトランスジェニックラットの報告は少ない。その理由は、飼育に要する費用がマウスより高額となることが挙げられる。

1-3-1. ヒトプロト型*c*-Ha-*ras*トランスジェニックラット（H*ras*128）

ヒトプロト型*c*-Ha-*ras*遺伝子を導入したトランスジェニックラットで、遺伝子は*ras*H2マウスに導入したものと同じである。発がん物質に対し非常に高い感受性を示し、10週程度の短期間に乳腺がんが発生する[42]。乳腺を標的とする物質のみならず、乳腺を標的としていない発がん物質も乳腺がんを発生させることから、乳腺がんの発生を指標として各種化学物質の発がん性を評価可能である[43]。H*ras*128ラットは乳腺発がんに加え、食道[44]、舌[45]、膀胱[46]、皮膚[47,48]においても高い発がん感受性が見られる。以上から、ヒト乳腺発がんと環境中発がんおよび発がん修飾因子の解析モデルへ応用できると考えられるが、既知の発がん物質による検証はまだ十分とは言えない（表2）。

表2. Hras128の発がん物質に対する乳腺発がん感受性

	Compound	+/−
Mammary carcinogen	MNU	+
	DMBA	+
	PhIP	+
	3-MC	+
	B[a]P	+
Non-mammary carcinogen	DHPN	+
	Anthracene	+
	Pyrene	−
	NNK	−
	IQ	+
	MeIQx	+
	AOM	+
	DEN	−
	TPA	+
	NMBA	−
	DMA	−

+,有意差有；−,有意差無（溶媒対照との比較）

1-3-2. SV40TAgトランスジェニックラット

腫瘍ウイルスSV40の初期遺伝子からは、スプライシングパターンの異なる分子量90kDの大型T抗原と17kDの二種の小型T抗原が産生される。大型T抗原（Large T antigen）は、がん抑制遺伝子である*Rb*や*p53*等と結合し、小型T抗原は、蛋白ホスファターゼ2A（PP2A）と相互作用してがん遺伝子として機能する。このSV40 T抗原をラットに導入した数種のSV40 T抗原トランスジェニックラットが作製された。

肝臓に発現するようアルブミンプロモーターを用いたSV40

T抗原トランスジェニックラットが確立されている[49]。このラットは、4～9ヵ月齢で100 %の頻度で肝細胞腺腫またはがんが発生する。

さらにPhosphoenolpyruvate carboxykinase（PEPCK）プロモーターを用いたSV40T抗原トランスジェニックラットも作製されている[50]。このラットでは、T抗原が主に膵臓・脳に発現し、膵島の過形成および膵島がんが発生する。前立腺を標的としてProbasinプロモーターを用いたSV40T抗原トランスジェニックラットでは、前立腺がんが高率に発生する[51]。このラットでは15週齢で100 %の頻度でアンドロゲン依存性の前立腺がんを発生する。これらのラットを用いた既知の発がん物質による検証はまだ十分とは言えない。

1-4. まとめ

1997年に開催された、第4回医薬品認可国際協調会議（International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human Use、ICH4）において、従来の2種のげっ歯類（ラットとマウス）での2年間長期発がん試験の代替法として、1種類のげっ歯類の長期発がん試験の実施に加えて、遺伝子改変動物を用いた短～中期発がん試験モデル（マウスについては26週間投与による中期試験）、イニシエーション・プロモーションモデルや新生児動物モデルの中から一つの試験を実施してがん原性を評価することが認められた。また国際NPOの環境保健科学研究所（Health and Environmental Sciences Institute, HESI）が主催して、1997～2001年に、50以上の日、米、欧の政府、大学、企業の研究施設が参画して、6ヵ月の統一プロトコールによる評価試験が実施された。試験動物として、*p53*$^{+/-}$マウス、*ras*H2マウス、Tg.ACマウスおよびXPAホモ型ノックアウトマウス、さらに新生仔マウス試験とハムスター胎仔細胞試験が加えられた。これら結果はToxicologic Pathology誌に特集号にまとめられている（Toxicol. Pathol., 2001, Vol. 29, No.1 suppl.）[3, 16, 34, 41]。*ras*H2マウスと*p53*$^{+/-}$マウスが「acceptable」、Tg.ACマウスについては「limited usefulness」と報告している。

以上の遺伝子改変モデルの長所・短所を表3にまとめる。

表3. ILSIの検証作業により明らかとなった問題点

モデル	問題点
*ras*H2	遺伝毒性発がん物質すべてを必ずしも検出できない ホルモンに対して陽性結果が得られているが、そのメカニズムが不明
p53$^{+/-}$	遺伝毒性発がん物質すべてを必ずしも検出できない 発がんメカニズムとして*p53*の変異・欠損が発がんに必ずしも関与していない
Tg.AC	経口投与と経皮投与での試験結果が異なる 発がん感受性が高いといわれているが、必ずしもすべてを検出できない 発がんメカニズムが明確ではない
XPA$^{-/-}$	検証試験の数が少なく、最終評価は困難 被験物質を9ヵ月まで投与しない限り発がん評価は困難（他は26週）

2. 変異原性物質の検出のための遺伝子改変動物モデル

Ames試験等では検体は生体防御機構による代謝を経ていないので、実際の動物試験と一致しないデータを得られる場合がある。そのために予め被験物質を*in vitro*でミクロソーム分画によって、あるいは一旦動物体内に入れて代謝活性化を図る方法が考案された。こうした工夫によって代謝活性化の問題はある程度解決されたが、*in vitro*では被験物質の変異原性/発がん標的臓器についての情報は得られない。被験物質の生体内における遺伝子突然変異誘発性とその標的臓器の情報が得られるように、指標遺伝子を導入したモデルが考案されている。

2-1. Big Blueマウス/ラット（*lambda*/*lac* I 遺伝子導入マウス/ラット）

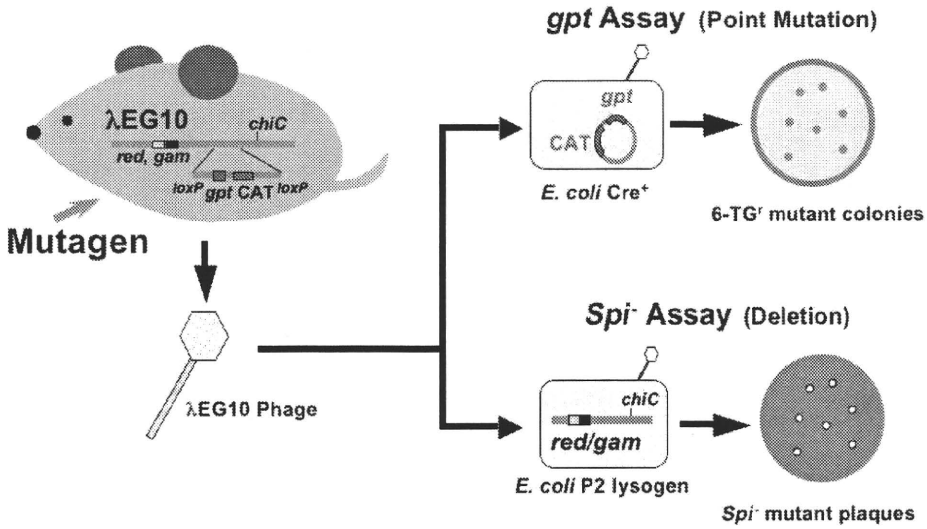
大腸菌のβ-galactosidaseの構造遺伝子*lacZ*遺伝子のリプレッサー遺伝子である*lac*Iが導入されたBig Blue Mouse が作出された[52]。変異体（*lac*I$^-$）の検出にカラー・セレクション法を用いている。この方法は組織からのDNAを*Lambda*ファージにパッケージングして、これを*E.coli*に感染させて、X-Galプレートに播いて青色の変異体プラークの数を算定する。この方法は煩雑なために、*Lambda*ファージのプラーク形成に関与する遺伝子である*cII*を用いて突然変異体をポジティブセレクションすることによって簡略化がなされている[53]。背景系統は、マウスはC57BL/6、B6C3F1、ラットはF344である。

2-2. Mutaマウス

バクテリアファージの*Lamda gt*10遺伝子に、大腸菌の*LacZ*遺伝子を組み込んだ*Lamda gt*10 *LacZ*ベクターを導入したマウスである[54]。パッケージングしたファージ溶液を、*E. coli* C（*lac*$^-$, *gal*E$^-$）培養液と混合し吸着操作を行い、変異体の選択には、基質のphenyl-β-galactosidaseを含むLB培地に突然変異した*lacZ*$^-$ファージのみがプラークを形成する方法を用いる（ポジティブ・セレクション）[55]。全プラーク数はphenyl-β-galactosidaseを含まない培地で算出する。*cII*を用いたポジティブセレクションも可能である[56]。

2-3. *gpt*（グアニンホスホリボシルトランスフェラーゼ）Δ（デルタ）トランスジェニックマウス/ラット

Big Blue Mouseでは、変異体（*lac*I$^-$）の検出に、カラー・



付図. *gpt*Δトランスジェニックマウス/ラットにおける変異体検出

セレクション法を用いているため手法が煩雑であるが、Muta Mouseは変異体（*lacZ*⁻）の検出はポジティブセレクションを用いるため手法は容易である。しかし、*lacZ*のコード領域が3 kbもあって変異部位の同定は手間がかかり、放射線などによる欠失変異を検出しにくい。この欠点を改良するために、点突然変異検出レポーター遺伝子である大腸菌*gpt*遺伝子と欠失変異検出用のレポーター遺伝子λファージ*red/gam*遺伝子を持つλEG10を組み込んだトランスジェニックマウス*gpt*Δ（C57BL6/J）が開発された[57]。マウスと同じ導入遺伝子λEG10をもつ遺伝背景の異なるSD系とF344系トランスジェニックラットも開発されている[58,59]。

この方法では、大腸菌*gpt*遺伝子をレポーターとする6-thioguanineセレクションによって点突然変異（塩基置換変異とフレームシフト）を検出し（*gpt* Assay）、λファージの*red/gam*遺伝子をレポーターとするSpi⁻セレクションでは欠失変異が検出できる（Spi⁻ Assay）。（付図）

3. まとめ

遺伝子改変動物を用いた長期発がん試験に替わる中・短期検索法、ならびに従来の*in vitro*変異原性検索法に替わる*in vivo*変異原性検索モデルについて記述した。前者では特定の遺伝子断片（DNA）を導入し作出したトランスジェニック動物（マウス、ラット）や目的とする遺伝子を不活性化や欠失させたノックアウトマウスがあり、短期に発がんする形質を利用してICH4での合意やHESIにおける検証と相俟って、代替法への応用が奨められてきた。*ras*H2と*p53*$^{+/-}$マウスではとくに前者が実際に用いられるようになった。*ras*H2マウスと同じ遺伝子をもつH*ras*128ラットは、マウスと異なり、外表面から観察できる乳腺がんの発生を判定指標としているので便利ではあるが、背景データが少ないために今後の検証データの集積が課題である。変異原性検出モデルは標的臓器が特定できるという特性があって有用性は極めて高いが、検出作業がやや煩雑であるために広く普及はしていない。これらの方法は、今後一層重要となると考えられるが、さらに利便性を考慮した簡便なモデルの作出が期待される。

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厚生労働省・変異原性試験等結果検討特別会議委員、内閣府食品安全委員会農薬専門調査会確認評価委員、国際癌研究機構（WHO International Agency for Research on Cancer）・発がん物質リスク評価モノグラフ諮問委員（1998、2003、2008）。

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Letter

Serum level of expressed in renal carcinoma (ERC)/ mesothelin in rats with mesothelial proliferative lesions induced by multi-wall carbon nanotube (MWCNT)

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ABSTRACT — Expressed in renal carcinoma (ERC)/mesothelin is a good biomarker for human mesothelioma and has been investigated for its mechanistic rationale during the mesothelioma development. Studies are thus ongoing in our laboratories to assess expression of ERC/mesothelin in sera and normal/proliferative/neoplastic mesothelial tissues of animals untreated or given potentially mesothelioma-inducible xenobiotics, by an enzyme-linked immunosorbent assay (ELISA) for N- and C-(terminal fragments of) ERC/mesothelin and immunohistochemistry for C-ERC/mesothelin. In the present paper, we intend to communicate our preliminary data, because this is the first report to show how and from what stage the ERC/mesothelin expression changes during the chemical induction of mesothelial proliferative/neoplatic lesions. Serum N-ERC/mesothelin levels were 51.4 ± 5.6 ng/ml in control male Fischer 344 rats, increased to 83.6 ± 11.2 ng/ml in rats given a single intrascrotal administration of 1 mg/kg body weight of multi-wall carbon nanotube (MWCNT) and bearing mesothelial hyperplasia 52 weeks thereafter, and further elevated to 180 ± 77 ng/ml in rats similarly treated and becoming moribund 40 weeks thereafter, or killed as scheduled at the end of week 52, bearing mesothelioma. While C-ERC/mesothelin was expressed in normal and hyperplastic mesothelia, the protein was detected only in epithelioid mesothelioma cells at the most superficial layer. It is thus suggested that ERC/mesothelin can be used as a biomarker of mesothelial proliferative lesions also in animals, and that the increase of levels may start from the early stage and be enhanced by the progression of the mesothelioma development.

Key words: Serum mesothelin, Rat, MWCNT, Mesothelial proliferative lesions

INTRODUCTION

Mesothelioma is a highly aggressive malignant tumor and developed in people previously exposed to asbestos, after a long latency period of 30-40 years. It is desired to establish a biomarker that can identify potential patients with early stage tumors or even as yet without tumors among the high-risk population.

Expressed in renal carcinoma (ERC)/mesothelin is a product of the *Erc* gene discovered in renal carcinomas of the Eker rats (Hino *et al.*, 1995) and confirmed as a homolog of the human *mesothelin/megakaryocyte poten-*

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ciating factor gene (Hino, 2004; Yamashita *et al.*, 2000). A 71-kDa glycosylphosphatidylinositol anchor-type membranous protein is produced and physiologically cleaved by a furin-like protease to yield a membrane-binding 40-kDa C-terminal (C-ERC/mesothelin) and a secreting 31-kDa N-terminal (N-ERC/mesothelin) fragments (Chang and Pastan, 1996; Maeda and Hino, 2006; Yamaguchi, *et al.*, 1994). ERC/mesothelin is a useful marker for human mesothelioma cases (Hino *et al.*, 2007; Maeda and Hino, 2006) and its specific enzyme-linked immunosorbent assay (ELISA) system has been developed (Hagiwara *et al.*, 2008; Nakaishi *et al.*, 2007) for the clinical use (Maeda and Hino, 2006; Shiomi, *et al.*, 2006, 2008; Tajima *et al.*, 2008). The most important question is as to whether ERC/mesothelin can be efficient also in the early phase of the mesothelioma development, and studies are ongoing in our laboratories to assess ERC/mesothelin levels in animals untreated or given potentially mesothelioma-inducible xenobiotics.

We preliminarily assessed ERC/mesothelin levels using the samples of our previous study demonstrating the induction of mesothelial proliferative lesions in male Fisher 344 rats given multi-wall carbon nanotube (MWCNT) (Sakamoto *et al.*, 2009) In the present paper, we intend to communicate this preliminary data, despite its very limited sample numbers, because this is the first report to show how and from what stage the ERC/mesothelin expression changes during the chemical induction of mesothelial proliferative/neoplatic lesions.

MATERIALS AND METHODS

Ethical consideration of the experiments

An experimental protocol was approved by the Experiments Regulation/Animal Experiment Committees of the Tokyo Metropolitan Institute of Public Health for its scientific and ethical appropriateness, including concern for animal welfare, with strict obedience to domestically and internationally applicable declarations, laws, guidelines and rules.

Samples

Male Fisher 344 rats were purchased at their age of 4 weeks old from Charles River Laboratories Japan Inc. (Kanagawa, Japan) and maintained in our animal room (24-25°C, 50-60% relative humidity, 10 times/hr air ventilation and 12-hr light/dark cycle) until use.

Normal rat samples

Serum samples were obtained from 3, 1 and 2 untreated rats at their ages of 11, 42 and 81 weeks old, respectively.

Vehicle/MWCNT-treated rat samples

As detailed elsewhere (Sakamoto *et al.*, 2009), rats were given a single intrascrotal administration of 1 ml/kg body weight of vehicle (2% carboxymethylcellulose) or 1 mg/kg body weight of MWCNT at the age of 12 weeks old and left untreated for up to 52 weeks. In the present study, 9 samples were used: 3 from vehicle-treated rats killed as scheduled at the end of week 52, without any mesothelial changes; 3 from MWCNT-treated rats similarly killed, with mesothelial hyperplasias but without mesotheliomas; and 3 from other MWCNT-treated animals, 1 killed as moribund at week 40 and 2 killed as scheduled at the end of week 52, with early and advanced stages mesotheliomas and hemorrhagic ascites. Serum samples and in the last case an ascites sample were obtained at the time of the autopsy, and 10% neutrally buffered formalin-fixed, paraffin-embedded, mesothelial tissue samples were routinely prepared.

ERC/mesothelinin ELISA assay

Serum and ascites ERC/mesothelin levels were analyzed using rat N- and C-ERC/mesothelin assay kits (Immuno-Biological Laboratories [IBL] Co., Ltd., Gunma, Japan) adapting from the method of Hagiwara *et al.* (2008), the detection limit being 0.1 ng/ml. A 6-µl aliquot was diluted with 234 µl of phosphate-buffered saline containing 1% bovine serum albumin and 0.05% Tween 20. Assays were conducted according to the manufacturer' instruction to measure an optical density at 450 nm. Each sample was assessed in duplicate.

Histology and C-ERC/mesothelin immunohistochemistry

Two serial, 4-µm-thick sections were prepared and deparaffinized. One was processed through a routine hematoxylin and eosin (HE) staining procedure and histgtologically examined. The other was heated in 10 mM citrate buffer, pH 6.0, treated with 3% hydrogen peroxide, incubated with a primary anti-rat C-ERC/mesothelin antibody (IBL) overnight at 4°C, washed with tris-buffered saline, and re-incubated using an Envision system (DAKO Japan Company, Limited, Tokyo, Japan). Signals were visualized by 3,3'-diaminobenzidine, and the sections were counter-stained with hematoxylin.

Statistical analysis

Statistical significance of intergroup difference for the N-ERC/mesothelin level was assessed using Student's *t*-test, and *p*-values less than 0.05 were considered significant.

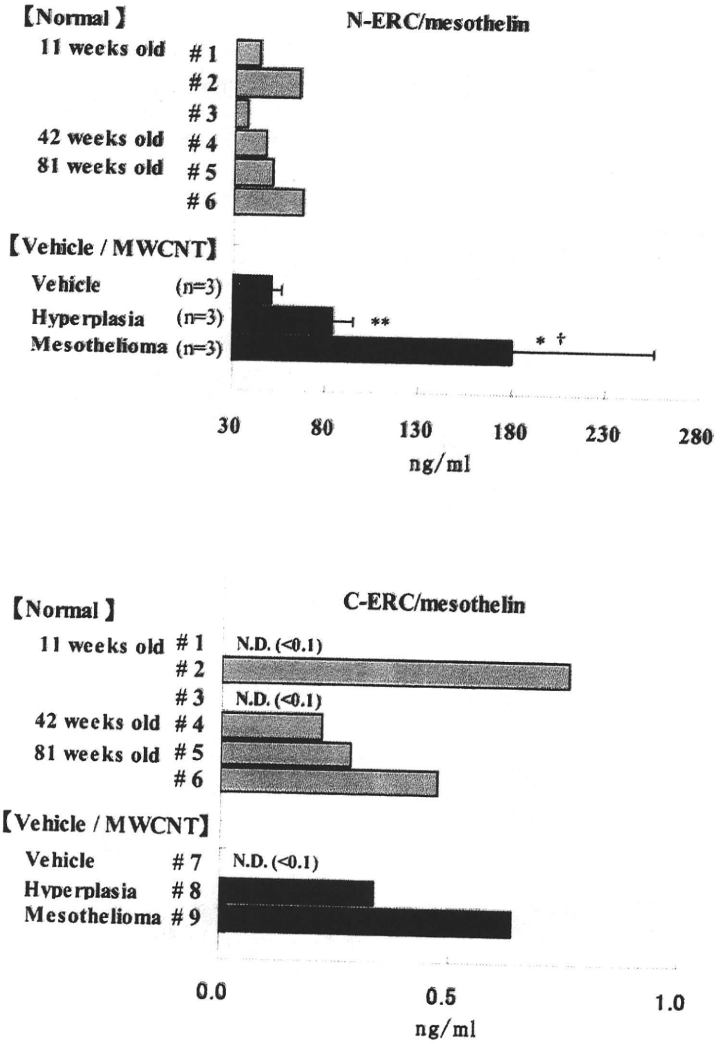

Fig. 1. Serum levels of A, N-ERC/mesothelin and B, C-ERC/mesothelin. Each data is a mean of duplicate assays. #1- #6 in Fig. A and #1- #9 in Fig. B show sample numbers. "N.D.", not detectable, indicates that the data was below the detection limit of 0.10 ng/ml. Values of N-ERC/mesothelin of samples from vehicle, hyperplasia and mesothelioma groups in Fig. B represent the means ± S.D. (n = 3).
Statistical significant difference by Student's t-test: $^{*}P < 0.05$, $^{**}P < 0.01$ as compared from the vehicle group and $^{\dagger}P < 0.05$ as compared from the hyperplastic group.

RESULTS AND DISCUSSION

Serum N-ERC/mesothelin levels of the normal rats were 43.4, 65.1 and 37.3 ng/ml, 47.1 ng/ml and 51.1 and 67.5 ng/ml; while those of C-ERC/mesothelin were < 0.1, 0.8 and < 0.1 ng/ml, 0.2 ng/ml and 0.3 and 0.5 ng/ml; for 11, 42 and 81 weeks of their ages, respectively (Fig. 1). These were respectively within the same range, and the N-ERC/mesothelin levels were substantially higher than the C-ERC/mesothelin levels. No apparent age-dependent changes were obtained for either fragment.

Serum N-(n = 3) and C-(n = 3) ERC/mesothelin levels of the vehicle-treated rat was 51.4 ± 5.6 ng/ml and < 0.1 ng/ml, respectively, within the normal ranges, whereas serum N-ERC/mesothelin levels of MWCNT-treated rats were increased by the induction of mesothelial hyperpla-

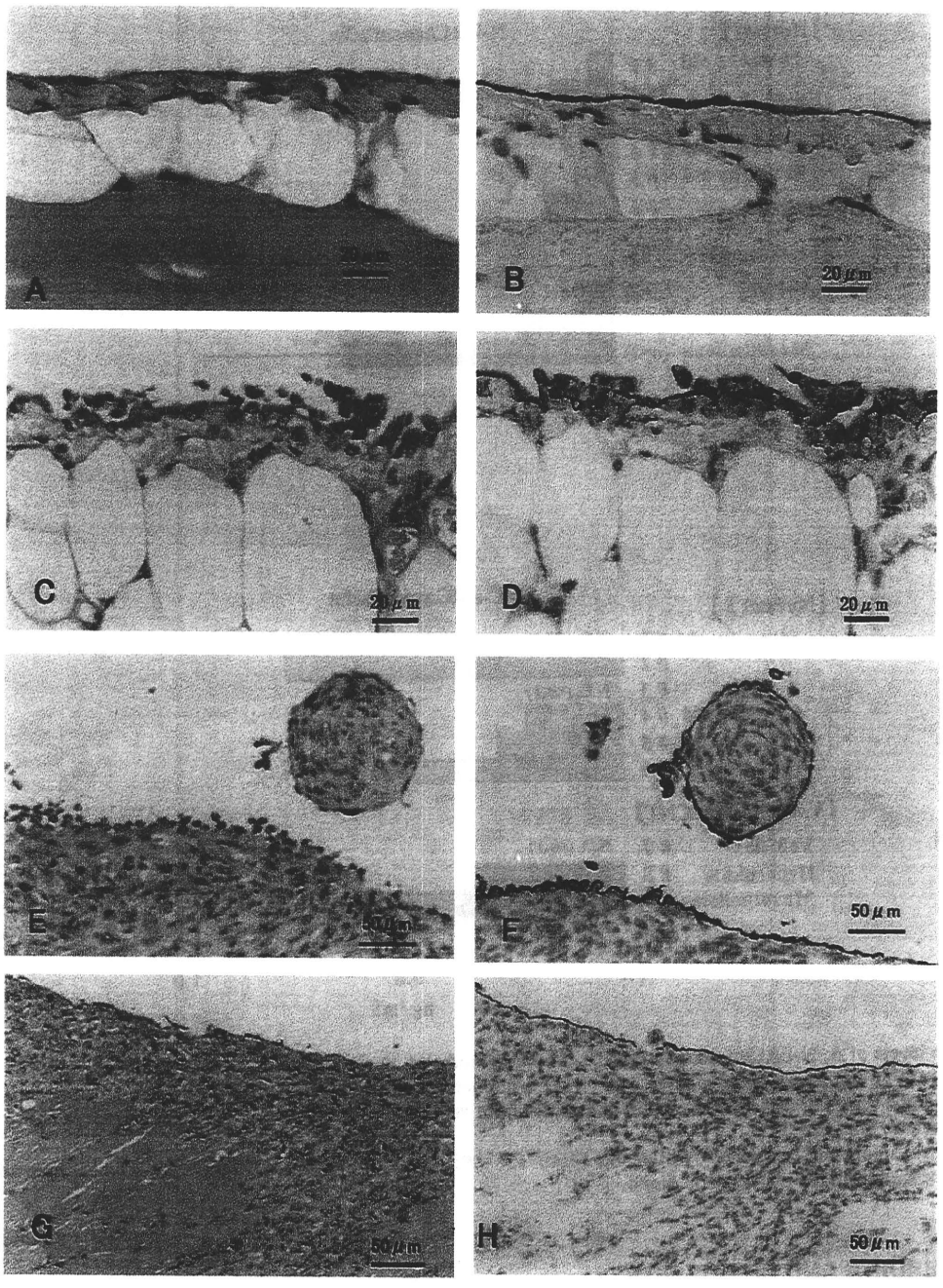

Fig. 2. Representative histology and C-ERC/mesothelin immunohistochemistry.
A, intact mesothelia in the parietal peritoneum of the vehicle-treated rat, HE; B, A's serial section, C-ERC/mesothelin; C, a mesothelial hyperplasia in the retroperotineal fat tissue of the MWCNT-treated rat, HE; D, C's serial section, C-ERC/mesothelin; E, an early-stage epithelioid/polypoid type mesothelioma in retroperotineal fat tissue of the other MWCNT-treated rat, HE; F, E's serial section, C-ERC/mesothelin; G, an advanced-stage mostly-sarcomatoid/invasive-nodular type mesothelioma in the diaphragm of the MWCNT-treated rat (the same animal as E/F), HE; H, G's serial section, C-ERC/mesothelin. Bars with their lengths are inserted to indicate magnifications.

sia (83.6 ± 11.2 ng/ml) and further by that of mesothelioma (179 ± 77 ng/ml; 3,004 ± 665 ng/ml in ascites) (Fig. 1). Serum N-ERC/mesothelin levels in experimental animals have only been assessed in Eker and Wistar rats and nude mice, untreated or transplanted with a rat mesothelioma cell line, MetEt-40 (Hagiwara *et al.*, 2008; Nakaishi *et al.*, 2007). The present data for the first time demonstrates that serum N-ERC/mesothelin level was increased already at the stage of preneoplastic, mesothelial hyperplasia and further increased by the chemical induction of mesothelioma. This may be in line with the recent findings that elevated serum mesothelin is detected before the development of grossly visible carcinoma lesions in a rat pancreatic carcinoma models (Fukamachi *et al.*, 2009). In human mesothelioma, it has been reported that N-ERC/mesothelin level increased with the stage went up of epithelioide type mesothelioma in human case (Shiomi *et al.*, 2008). It is not known, however, how N-ERC/mesothelin levels change in the stage of preneoplasia at this moment. Large-scaled, detailed investigations using the MWCNT-mesothelioma model are ongoing in our laboratories.

Serum C-ERC/mesothelin levels of MWCNT-treated rats were 0.4 and 0.6 ng/ml, within the normal/vehicle range (Fig. 1B). This is in accordance with the previous finding in nude mice transplanted with MetET-40 (Hagiwara *et al.*, 2008), and can be attributed to the membrane-binding property of C-ERC/mesothelin (Maeda and Hino, 2006). Ascites level of C-ERC/mesothelin in the mesothelioma case was slightly increased to 10.9 ng/ml (Fig. 1B). This is speculated to result from a release of C-ERC/mesothelin by phosphatidylinositol-specific phospholipase C (Chang and Pastan, 1996) or a contamination of desquamated mesothelioma cells.

Immuohistochemical C-ERC/mesothelin signals were constantly detected in cell membranes. C-ERC/mesothelin was detected in intact (Figs. 2A and B) and hyperpastic (Figs. 2C and D) mesothelia. Taken the ELISA data together, it might be possible that the ERC/mesotheliin level starts increased from the preneoplastic stage.

C-ERC/mesothelin was found only in epithelioid (mesothelioid) tumor cells present at the most superficial layer of early-stage, epithelioid/polypoid (Figs. 2E and F) and advanced-stage, mostly-sarcomatoid/invasive-nodular (Figs. 2G and H) types of mesotheliomas. In humans, epithelioid types, but not sarcomatoid types or sarcomatoid components of biphasic (mixed) types, immunohistochemically express C-ERC/mesothelin (Chang and Pastan, 1996; Shiomi *et al.*, 2008; Ordóñez, 2003). Accordingly, serum N-ERC/mesothelin levels were only slightly increased or often unchanged in sarcomatoid and biphasic types, in contrast to the epithelioid type (Hassan *et al.*, 2006; Shiomi *et al.*, 2008). The present findings suggest that the increaseof ERC/mesothelin levels in mesotheliomas may be a universal event for all types and stages, and that C-terminal fragments may become unproduced or changed its tertiary structure and/or epitope construction by the neoplastic conversion.

In conclusion, the present data suggests that ERC/mesothelin can be used as a biomarker of mesothelial proliferative lesions also in animals, and that its increase of its levels may start from the early stage and be enhanced by the progression of the mesothelioma development.

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A Medium-Term, Rapid Rat Bioassay Model for the Detection of Carcinogenic Potential of Chemicals

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A Medium-Term, Rapid Rat Bioassay Model for the Detection of Carcinogenic Potential of Chemicals

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ABSTRACT

The Ito Liver Model and the Ito Multi-organ Model are used in conjunction and constitute an efficient and rapid bioassay for the identification of both genotoxic and nongenotoxic carcinogenic chemicals. The Ito Liver Model is an 8-week bioassay system that uses the number and size of foci of hepatocytes positive for glutathione S-transferase placental form (GST-P) as the end-point marker. One hundred fifty-nine compounds were tested using the Ito Liver Model: 61 of 66 hepatocarcinogens tested positive, and 10 of 43 nonliver carcinogens were also positive. The false-positive detection of noncarcinogens was low; a single false-positive result was obtained from the 50 noncarcinogens tested. Since more than half of all known carcinogens are hepatocarcinogens in rodents, the initial 8-week bioassay is able to detect most carcinogens. The Ito Multi-organ Model is a 28-week bioassay system for the detection of carcinogens that were not identified by the Ito Liver Model. Results are evaluated by preneoplastic and neoplastic lesions in major organs. Forty-four compounds were tested using the Ito Multi-organ Model: 17 out of 17 liver carcinogens were positive, and 19 out of 22 (86%) nonliver carcinogens were positive. None of the five noncarcinogens tested positive.

Keywords: medium-term bioassay; carcinogens; liver GST-P; multi-organ.

INTRODUCTION

Identification and control of carcinogens in the environment are of prime importance to reduce cancer risk in humans. Long-term chronic administration assays for the detection of carcinogenicity and toxicity using rodents have been the standard for the evaluation of the carcinogenic potential of chemicals. The requirements call for testing in two rodent species, usually rats and mice, of each sex, at three dose levels (zero, low, middle, and high) of the test compound for 2 years.

Although this standard has long been used worldwide, 2-year carcinogenicity studies are too costly to test all the chemicals being introduced into the environment. Furthermore, there is political pressure to decrease the number of animals used for carcinogenicity testing because of animal welfare considerations (Ashby and Tennant 1991). A guideline proposed by the International Conference on Harmonization (ICH) recommends reducing long-term protocols by utilizing only one rodent species and replacing the second long-term rodent assay with an alternative bioassay (ICH Steering Committee 1997).

It is known that mutagenicity does not always correlate with carcinogenicity, and there are a variety of chemicals in use, typically represented by pesticides and herbicides, that are not mutagenic but are carcinogenic. A whole-body animal study is the only method to test the carcinogenic potential of a nongenotoxic chemical. Therefore, any alternative bioassay must be an in vivo, whole-body assay. Our laboratories have focused

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on the development of a rapid in vivo bioassay system able to detect both genotoxic and nongenotoxic carcinogens.

Since more than half of all known carcinogens are hepatocarcinogens in rodents, we initially focused on establishment of a medium-term liver bioassay system. We developed an 8-week rat liver bioassay, known as the Ito Liver Model, which is able to detect rat hepatocarcinogens with a high degree of accuracy (Shirai, Hirose, and Ito 1999). This protocol is cited in the sixth edition of *Casarett and Doull's TOXICOLOGY* as a potential alternative bioassay (Pitot and Dragan 2001). In addition, we developed a 28-week model, known as the as Ito Multi-organ Model, to detect carcinogens that are not identified by the Ito liver model.

Using the Ito Liver Model in conjunction with the Ito Multi-organ Model, most carcinogens can be identified after 8 weeks, and the remaining carcinogens can be identified after an additional 28 weeks. In the present review, the Ito Liver Model and the Ito Multi-organ Model are briefly described.

Background

In 1976, Solt and Farber developed a protocol in which foci of liver cells expressing an altered repertoire of enzymes could be induced in rats within 4 weeks. Their protocol was based on their observation that in rats treated with diethylnitrosamine (DEN) followed by the hepatocarcinogen 2-acetylaminofluorene (2-AAF), DEN-altered hepatocytes were able to respond to growth stimuli evoked by a two-thirds partial hepatectomy and form distinct foci. In contrast, normal hepatocytes were not able to respond to the growth stimuli because of the toxic effect of 2-AAF. This observation was described as a "selection process" by altered hepatocytes (Solt and Farber 1976). The application of this observation for carcinogen detection was examined by treating rats with test compounds to generate altered hepatocytes followed by feeding with 2-AAF and stimuli to induce hepatocyte proliferation, and the principle of the method was validated: treatment with representative carcinogens resulted in the formation of foci of altered hepatocytes (Tsuda, Lee, and Farber 1980).

In other studies, Peraino and associates reported a two-stage model in which hepatic tumor growth was enhanced by chemicals such as phenobarbital given after initiation with 2-AAF (Peraino et al. 1975, 1977, 1980). Their data suggested that a two-stage approach could be utilized for detection of carcinogenic responses to chemicals: either test compounds could given at the initiation stage followed by appropriate promoting agents or test compounds could be given during the promotion stage after initiation with DEN.

Based on the concepts presented above, we established an assay system to evaluate the hepatocarcinogenicity of chemicals for their promotion potential (Ito et al. 1996, 1997; Ito, Tamano, and Shirai 2003): we used the promotion potential of hepatocarcinogens because almost all carcinogens have a promotion effect when repeatedly administered (Peraino et al. 1975, 1977, 1980). The preneoplastic nature of altered hepatic foci and the usefulness of such lesions as indicators of preneoplastic development are now well accepted (Bannasch 1986; Oesterle and Deml 1990; Tatematsu et al. 1977). The phenotypic characteristics of preneoplastic lesions in the liver have been extensively studied, and immunohistochemical staining for glutathione S-transferase placental form (GST-P) was found to be the best marker for visualization of lesions and their quantitative analysis (Ogiso et al. 1985; Tsuda et al. 2003, 1985).

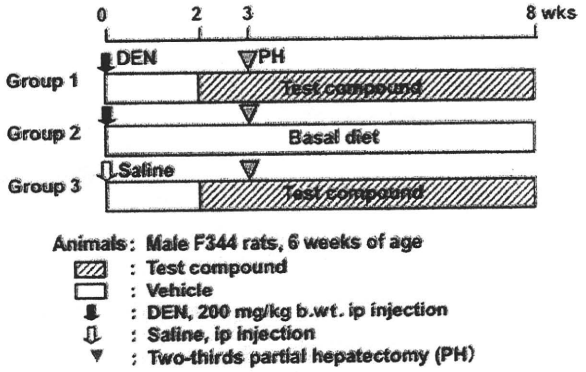

FIGURE 1.—Protocol of Ito Liver Model. Six-week-old male F344 rats are initially given a single intraperitoneal injection of diethylnitrosamine (200 mg/kg) to initiate liver carcinogenesis. Two weeks after initiation, the test compound is administered for 6 weeks. Animals are sacrificed at the end of week 8. All rats are subjected to two-thirds partial hepatectomy on week 3. The end-point marker is glutathione S-transferase placental form-positive (GST-P$^+$) liver cell foci. The numbers and sizes of GST-P$^+$ liver cell foci are analyzed using an image-analyzer and expressed as values per unit liver section (1 cm^2). When values, number, and/or area per unit area of GST-P$^+$ foci are significantly enhanced ($P < .05$) over the control value, a chemical is judged to possess carcinogenic potential for the liver.

Assay Protocol and Results

Ito Liver Model

Figure 1 shows the protocol employed in Ito's laboratory as a medium-term liver bioassay model. Male F344 male rats, 5 weeks old, are divided into three groups consisting of 15 to 20 animals each. Group 1 is given a single intraperitoneal injection of DEN, 200 mg/kg b.wt., dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks, the rats receive a test compound mixed in the basal diet or drinking water or by repeated intraperitoneal, subcutaneous or intravenous injections. The rats are subjected to two-thirds partial hepatectomy (PH) at the end of week 3. Group 2 is given DEN and PH in the same manner as for group 1, but without administration of the test compound. Group 3 is injected with saline instead of DEN and then subjected to administration of the test compound and PH as in groups 1 and 2 (Figure 1). All animals are sacrificed at the end of week 8. The liver tissues, three to four slices from the cranial and caudal lobes of the right lateral lobe and caudal and/or cranial part of the caudal lobe, are excised and fixed in ice-cold acetone or 4% paraformaldehyde solution in phosphate

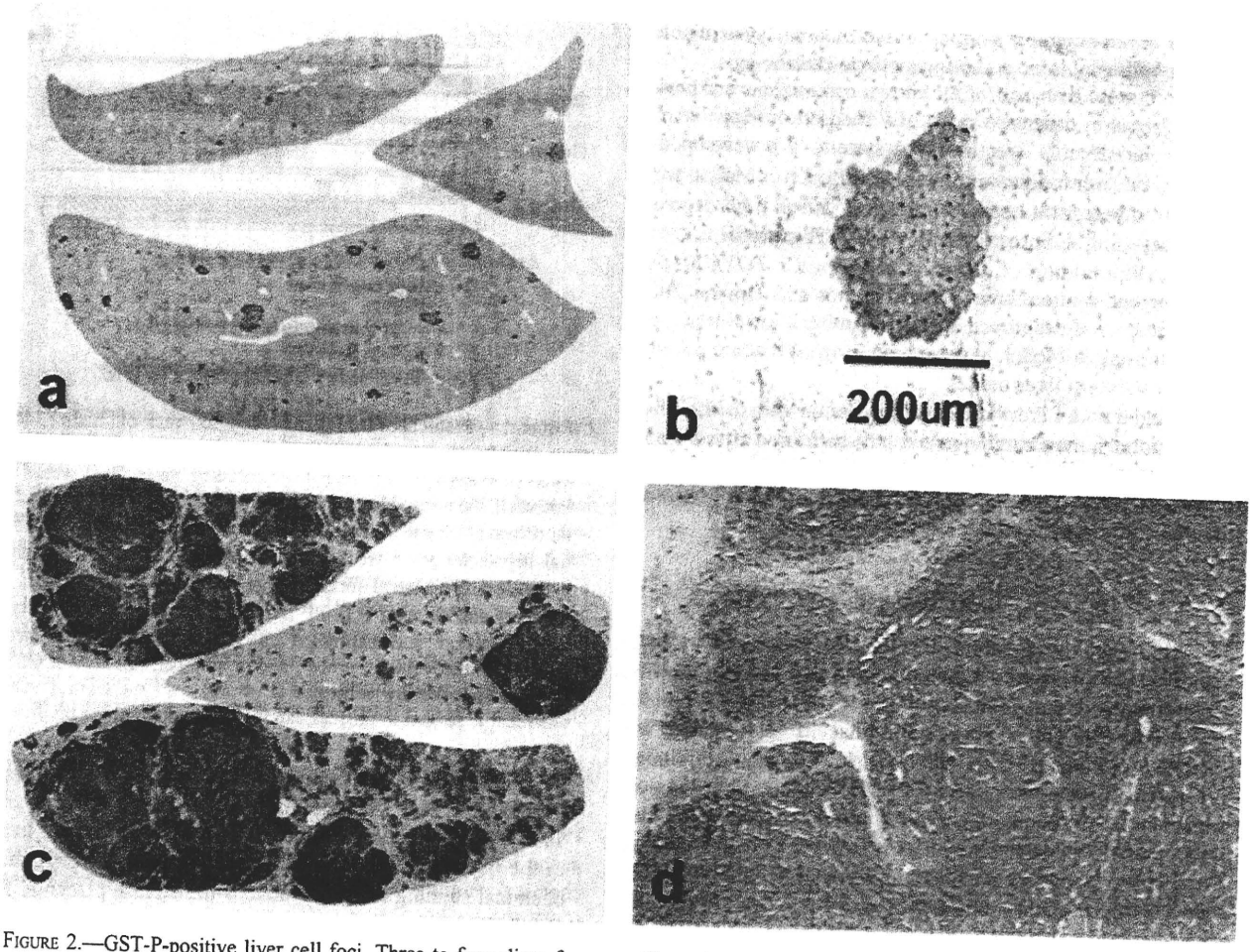

FIGURE 2.—GST-P-positive liver cell foci. Three to four slices from paraffin embedded liver (left) are immunostained with GST-P antibody. Lesions greater than 200μm (right) in diameter are included for counting. GST-P is consistently expressed from small foci to adenomas and hepatocellular carcinomas. A clear correlation between GST-P-positive foci and the incidence of hepatocellular carcinomas can be seen. (a) A low magnification view of a slide from a rat treated with phenobarbital (0.05%, in the diet). (b) Smallest focus included for counting purposes. (c) A low-magnification view of a slide from a rat treated with 2-AAF (0.02%, in the diet). (d) Higher-magnification view of hepatocellular carcinoma: the carcinoma is clearly positive for GST-P.

buffer at pH. 7.4 for subsequent paraffin embedding and immunohistochemical demonstration of GST-P-positive foci (Figure 2). Numbers and areas of GST-P-positive foci more than 0.2 mm^2 in mean diameter are included for measuring by an image processor. The results are assessed by comparing the values between group 1 (DEN-test compounds) and group 2 (DEN alone). Group 3 serves to assay the potential of the test chemicals to induce GST-P-positive foci without prior DEN exposure. Statistical analysis of differences between means is carried out using Student's or Welch's *t*-tests after application of a preliminary *F*-test for equal variance, and scoring of carcinogenicity, promotion, or inhibition is made on the basis of differences in *P*-values between groups; positive = increase at $P < .05$ in either number or area of foci.

Until the protocol was finalized, the following were extensively investigated to maximize the predictive potential of the model (Hasegawa and Ito 1992; Ito et al. 1997, 1992; Shirai 1997; Shirai, Hirose, and Ito 1999):

1. use of PH as a tool for induction of hepatocyte proliferation,
2. the most suitable end-point marker enzyme,
3. whether results with GST-P-positive foci can predict carcinoma development in a dose dependent manner, and
4. specificity of the protocol for detection of carcinogens.

Since PH was introduced by Higgins and Anderson in 1931, it has been extensively employed for investigation of cell proliferation and regeneration. After two-thirds PH, the rodent liver recovers quickly and returns to near preoperative weight

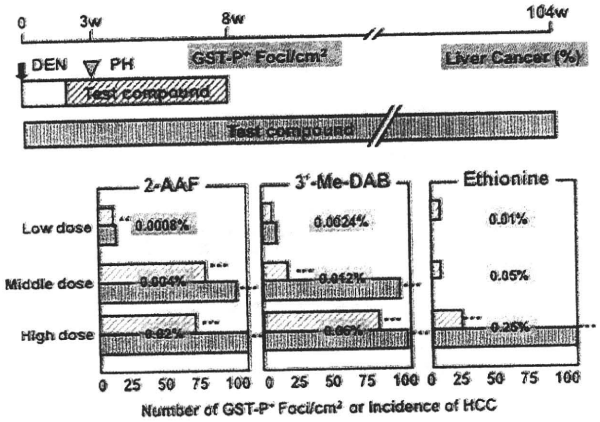

FIGURE 3.—Comparison of GST-P-positive foci and carcinoma. Results obtained from the Ito Liver Model and long-term 2-year studies are shown. Different doses of three representative hepatocarcinogens—2-acetylaminofluorene, 3′-methy-4-diaminoazobenzene, and ethionine—were administered.

TABLE 1.—Results for 159 Compounds in the Ito's Test

	No. of Positive Compounds/Examined (%)			
	Mutagenicity (Ames test)			
Test Compounds	Positive	Negative	Unknown	Total
Liver carcinogen	31/32(97)[a]	29/33(88)[b]	1/1(100)	61/66(92)
Non-liver carcinogen	7/26(27)	2/15(13)	1/2(50)	10/43(23)[d]
Not carcinogenic	0/6(0)	1/42(2)	0/2(0)	1/50(2)

[a] 4,4-Diaminodiphenylmethane gave negative results
[b] Four chemicals, Clofibrate, Di(2-ethylhexyl)adipate, Di(2-ethylhexyl)phthalate, Trichloroacetic acid, gave negative result.

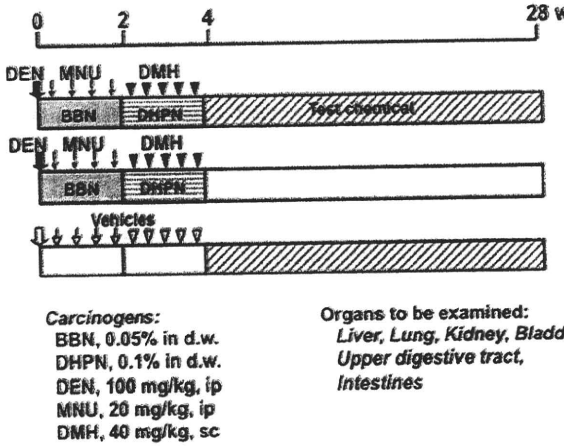

FIGURE 4.—Protocol of The Ito Multi-organ Model (DMBDD Model). Six-week-old F344 male rats are given i.p. injections of diethylnitrosamine (DEN, 100 mg/kg body wt.) and N-methylnitrosourea (MNU, 20 mg/kg body wt.), s.c. injections of 1,2-dimethylhydrazine (DMH, 40 mg/kg body wt.), and 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) and 0.1% 2,2'-dihydroxy-di-n-propylnitrosamine (DHPN), both in the drinking water, for a total initiation period of 4 weeks (DMBDD treatment). The test compound is then administered for the following 24 weeks. The rats are sacrificed at the end of week 28. The liver, lung, thyroid, kidney, bladder, upper digestive tract (esophagus and forestomach), and intestines are examined for preneoplastic and neoplastic lesions and compared with the control rats.

within 1 week with peak DNA synthesis at about 24 hr; induction of hepatocyte growth factor appears to be one mechanism by which the liver recovers from PH (Matsumoto and Nakamura 1992). Use of hepatotoxins such as carbon tetrachloride (CCl_4) or D-galactosamine is an alternative method to induce liver cell proliferation. However, neither of these agents stimulates cell proliferation equivalent to PH in our system because induction of cells to enter S-phase of the cell cycle by these chemicals is sluggish (data not shown).

We have not yet elucidated the role of cell proliferation induced by PH at week 3 in the appearance of liver cell foci. It is possible that a majority of carcinogens are toxic to hepatocytes, causing retardation of the compensatory regenerative response to PH by noninitiated hepatocytes, allowing focal expansion of initiated hepatocytes. In this regard, it is known that initiated cells reduce phase I CYP enzyme expression and increase phase II enzyme expression (Liu et al. 2005; Tsuda et al. 1996), and this altered enzyme expression enables them to escape the effects of toxic compounds.

The expression of several different enzymes is altered in liver preneoplastic lesions (Ogawa et al. 1982; Tsuda et al. 1992). We compared the use of a variety of enzyme markers to visualize liver lesions (Tsuda et al. 2003, 1984). GST-P was found to be the most appropriate for practical use and is expressed continuously from the early lesion to the appearance of hepatocellular carcinoma (Kitahara et al. 1984; Tsuda et al. 1996, 2003).

Several studies have shown the validity of using GST-P-positive foci as a surrogate end-point in predicting carcinogenic potential (Ogawa et al. 1982; Tatematsu et al. 1985; Tsuda et al. 1984, 1988). One of these studies is shown in Figure 3. There was a clear correlation between GST-P-positive foci and incidence of hepatocellular carcinomas after administration of different doses of the well-known hepatocarcinogens 2-acetylaminofluorene, 3′-methy-4-diaminoazobenzene, and ethionine (Hagiwara et al. 1993).

A total of 159 compounds were examined using the Ito Liver Model. They are classified into three categories (Table 1): (1) hepatocarcinogens; (2) carcinogens targeting organs other than the liver (nonhepatocarcinogens); and (3) compounds negative for carcinogenicity in 2-year tests in rats and mice (noncarcinogens). The compounds can also be divided into three categories according to their reported mutagenicity: mutagenic compounds, nonmutagenic compounds, and compounds with unknown mutagenic potential. Comparisons of the results obtained using the Ito Liver Model and reported *Salmonella* mutagenicity and long-term carcinogenicity testing are summarized in Table 1. It is especially noteworthy that the Ito

TABLE 2.—Results of 44 Compounds in the Medium-term Multi-organ Carcinogenesis Bioassay (DMD/DMBDD Model)

	Positive Compounds/Examined (%)			
	Mutagenicity (Ames test)			
Test compounds	Positive	Negative	Unknown	Total
Liver carcinogen	12/12(100)	5/5(100)	0/0(0)	17/17(100)
Non-liver carcinogen	10/11(91)[a]	8/10(80)[b]	1/1(100)	19/22(86)
Not carcinogenic	0/1(0)	0/4(0)	0/0(0)	0/5(0)

[a] One negative compound is Benzo[a]pyrene.
[b] Two negative compounds are Sesamol and Daminozide.

Liver Model identified 59 of 64 (92%) liver carcinogens, irrespective of their mutagenicity, leaving only 5 false negatives; 30 out of 31 (97%) mutagenic and 29 out of 33 (88%) nonmutagenic hepatocarcinogens were identified. Three out of the four nonmutagenic carcinogens that gave false negative results were carcinogenic peroxisome proliferators, known to suppress GST-P expression. It is noteworthy that the false-positive and false-negative rates are 2.1% and 3.1%, respectively. It was also noted that many chemicals positive in the Ito Liver Model were hepatotoxins (Ward et al. 1989). These results clearly demonstrate that this medium-term liver bioassay is excellent for detection of liver carcinogens (Ito, Tamano, and Shirai 2003; Shirai 1997).

A formula for the validity of carcinogen screening tests is described by Cooper, Saracci, and Cole (1979). This formula evaluates five categories: sensitivity, specificity, predictive value (positive predictivity), false-positive rate, and false-negative rate of the screening test. When the Ito Liver Model was evaluated, all five categories demonstrated excellent values (Shirai, Hirose, and Ito 1999). The Ito Liver Model was accepted as an alternative protocol to replace one of the 2-year chronic administration assays at the Fourth International Conference on Harmonization (ICH Steering Committee 1997).

Ito Multi-organ Model (DMBDD Model)

The Ito Multi-organ Model was developed for the detection of carcinogens not identified by the Ito Liver Model. F344 male rats are given i.p. injections of diethylnitrosamine (DEN, 100 mg/kg body wt.) and N-methylnitrosourea (MNU, 20 mg/kg body wt.), s.c. injections of 1,2-dimethylhydrazine (DMH, 40 mg/kg body wt.), and 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) and 0.1% 2,2'-dihydroxy-di-n-propylnitrosamine (DHPN), both in the drinking water, for a total initiation period of 4 weeks (DMBDD treatment) (Akagi et al. 1995; Ito et al. 1996). Then rats are given the test compound in the diet or drinking water or by injection for the following 24 weeks. The animals are sacrificed at the end of week 28 (Figure 4). The organs targeted by the 5 different carcinogens—the liver, lung, thyroid, kidney, bladder, upper digestive tract (esophagus and forestomach), and intestines—are histologically examined for preneoplastic and neoplastic lesion development (Fukushima et al. 1991; Ito et al. 1996).

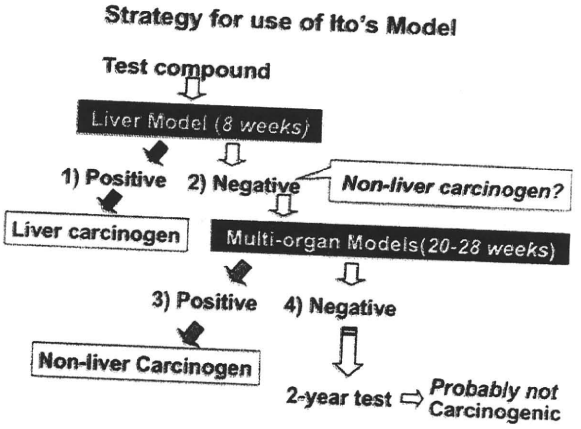

FIGURE 5.—Strategy for the Use of Ito's Model. (1) Positive compound in the liver model: is a liver carcinogen. (2) Negative compound in the liver model: test using the multi-organ model. (3) Positive compound in the multi-organ model: is a carcinogen. (4) Negative in both the liver and multi-organ models: is probably not carcinogenic.

A total of 44 compounds were examined using the Ito Multi-organ Model. A summary of the results is presented in Table 2. All 17 liver carcinogens tested positive, including peroxisome proliferators, and 19 of 22 nonliver carcinogens (86%) tested positive. The Ito Multi-organ Model was able to identify carcinogens irrespective of their mutagenicity; 22 of 23 mutagenic carcinogens and 13 of 15 nonmutagenic carcinogens were identified (Table 2).

The medium-term bioassay described here is a rapid, reliable, and practical tool for the prediction of the carcinogenic potential of chemicals. The strategy for the use of the Ito Model is presented in Figure 5.

1. Positive in liver model: carcinogen.
2. Negative in liver model: apply the multi-organ model.
3. Positive in the multi-organ model: carcinogen.
4. Negative in both the liver and multi-organ models: probably not carcinogenic.

The system is now internationally well recognized and recommended as an alternative carcinogenicity test.

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