In vivo **試験**：マウス骨髄小核試験（3 試験の内 2 試験は 6-7 週齢のマウス）では高用量かつ 5 日間の投与においても陰性（po および ip）であった．幼若ラット（4 週齢）の骨髄小核試験（po および ip）および幼若ラットの末梢血液小核試験（po）はいずれも陽性であった（1 あるいは 2 日間投与）．我々コンサルタントはこれらの試験において幼若動物を使用していることに疑問（代謝と排泄のバランス）を持った．マウスとラット間の結果の違いは，本当に種差によるものなのか，あるいは投与動物の週齢によるものなのかに関しても確信が持てない．**推奨事項：ラットを用いる骨髄あるいは末梢血液での小核試験を再度実施すべきである．それでもなおかつ陽性であるならば，種間の相違を理解する一助としてラットならびにマウスでの ADME の試験を実施することを勧める．**

ラットを用いた肝臓小核試験は陰性，肝部分切除後のマウスを用いた肝臓小核試験では陽性となっている．肝臓において骨髄と正反対の結果であるが，種間の差を反映しているのか，あるいはプロトコールの相違に起因しているのか，我々としても確信が持てなかった．

ラット肝を用いた UDS 試験では陰性結果が得られている．

マウスを用いた優性致死試験でも陰性結果が得られている．

雄および雌を用いた *lacZ* の突然変異試験（Muta™Mouse）が独立して行われており，いずれの試験においても肝臓では陰性であった．

ショウジョウバエを用いた翅毛スポット試験では陰性結果が得られている．

ラット甲状腺での DNA 付加体試験では陰性との報告があった．

マウス甲状腺細胞，肝臓細胞を用いたコメット試験では共に陰性の報告がある(ただし，文献は入手不可能であった)．一方，佐々木らは強制経口投与，および混餌投与したマウス肝臓から核を単離し，コメット試験を行い，高用量において陽性を得たと報告している．我々としてはこれら方法の違いが結果に影響を及ぼしているか判断できなかった．
推奨事項：細胞と核を使ってコメット試験を行い，比較すべきである．また，コメット試験においては，テール長さよりも，テールモーメントを用いて評価すべきである．

ラット肝臓で DNA 付加体を観察することが提案されている．このことは，ラット肝臓での GST-P 巣を説明するためには有用であると思われるが，マウスにおいても調べるべきである．

推奨事項: ^{14}C ラベル化合物を用いてマウス肝臓での DNA 付加体量を測定すべきである．（付加体は小さいと思われるため，^{32}P ポストラベル法は不適当かもしれない．細菌での ^{32}P ポストラベル法を行うことによってチェック可能であろう．)

酸化的損傷

コウジ酸による影響が酸化的損傷によるものかどうかについても研究がなされている．ここでとりあげた研究は，光毒性，UV による影響等を考慮したものではない．コウジ酸による酸化損傷の証拠ははっきりせず，また，酸化損傷が遺伝毒性の原因となっているかについても明らかな証拠はない．Ames 試験においていくつかの株で突然変異の誘発が見られたが，主として酸化損傷を示すような物質で引き起こされる特徴ではなかった．コウジ酸の Ames 試験での突然変異スペクトルは，過酸化水素でのそれとは異なるものであった．CHO 細胞でのコメット試験において，通常の方法ではコメットの誘発は見られず，FPG 酵素の添加によってもコメットの増強は見られなかった．このことは，FPG は DNA の酸化部位を認識し，切断する作用を持つので，酸化的損傷部位が増加していなかったことを示している．コウジ酸をマウスに混餌投与すると，28 日後に肝臓で 8-OHdG の増加が見られるとの報告があるが，この増加はデータのばらつきを考慮すると顕著ではない．さらに，この検出方法は技術的な困難さを含んでおり，評価は慎重にすべきである．

発がん性

マウス発がん性試験

コウジ酸を慢性経口投与されたマウスでは甲状腺腺腫および肝腫瘍が発生する．肝腫瘍が全て良性の腺腫か腺腫と悪性癌腫との混合かは不明である．対照群の肝腫瘍発生動物数は全ての腫瘍が癌腫であるとするには高すぎる数値であった．
推奨事項：マウス発がん性試験の生データや病理報告書を確認し，肝腫瘍の性質(良性か悪性か)を確認する必要がある．

マウス発がん性試験に関しては論文に書かれているように，甲状腺腫瘍が非遺伝毒性的なメカニズムで発生したという解釈で間違いないように思われる．コウジ酸を投与された動物ではヨードの取り込み阻害が認められている．このため，血清中の T3/T4 レベルが低下して代償性に脳下垂体から TSH の分泌が亢進する．その結果，甲状腺の細胞増殖が刺激される．また，T3/T4 レベルと TSH レベルはコウジ酸の投与を中止すると 48 時間以内に正常値に戻ることが知られている．このメカニズムはヒトでは起こらないと思われる．何故なら，ヒトではマウスに較べて甲状腺ホルモンレベルが変動しにくく，また，コウジ酸の

ヒト暴露量もこの実験に較べてはるかに低いからである．

しかしながら，我々は肝腫瘍の発生も甲状腺と同様に考えることが可能性であると感じた．コウジ酸は肝臓の酵素を誘導するかもしれず(このことは慢性毒性試験における肝重量の増加と一致している)，それゆえに甲状腺における間接的な影響を誘発するかもしれない．肝臓と甲状腺における病理組織学的な変化は，視床下部－脳下垂体－甲状腺の流れを示唆している．肝重量の増加と肝細胞の肥大はミクロゾーム酵素の誘導の結果生じたものかもしれず，このことは肝臓におけるチロキシン(T4)のクリアランスを増大させ，引き続いて血清中の T4 レベルを低下させる．この変化は脳下垂体からの TSH 分泌を上昇させ，甲状腺の濾胞細胞を肥大・活性化させるだろう．T4 の血漿中半減期が短いために，ラットはヒトに較べてこの種の変化に対して特に感受性が強い．上述したように，このメカニズムはヒトでは起こりそうにない．甲状腺毒性物質に対する肝臓の病理組織学的変化は，一般的に目立つもの(肝肥大や肝重量増加)ではなく，常にアポトーシスの増加や細胞分裂活性の増加と関連するものでもないが，その変化は前腫瘍病変(巣)や最終的には腫瘍に対して促進的に作用するかもしれない．このことは特に高用量で重要であり，明らかな毒性関連所見(壊死，増大した酸化損傷に関連した影響)が付加的な危険要因の構成要素となっている(参考文献：Capen C. C., *et al*, 1991, Endocrine System, in Handbook of Toxicologic Pathology, Academic Press, pp 675-760; Gopinath C., *et al*, 1987, The Endocrine Glands, in Atlas of Experimental Toxicological Pathology, MTP Press Limited, pp 104-121)．これらの作用メカニズムの面においては，病理組織像のはっきりとした解析，その経時的変化および代謝と排泄経路が大変重要であるが，これらの点は我々には明らかにすることはできなかった．

推奨事項：我々は，コウジ酸による肝腫瘍の誘発に関しても，甲状腺ホルモンと TSH の変化による非遺伝毒性的な解釈の可能性を提案する．この作用メカニズムを解明するためには，病理組織像のはっきりとした解析，その経時的変化，代謝(甲状腺ホルモン関連代謝酵素の誘導を含む)，排泄経路および肝肥大の説明が非常に重要である．この分野の専門家を招いて，更なる助言の提供とデータ解釈の手助けを求めるべきである．

<u>p53 ノックアウトマウスを用いた試験</u>

試験は p53 ヘテロ欠損マウスとその野生型を使用して実施され，最高 3%のコウジ酸を含む飼料を 26 週間摂取させた．遺伝子改変マウスおよび野生型マウスではともに，同程度の甲状腺の変化が見られた(腫瘍は見られず)．肝臓でも腺腫を含めた変化が両系統で見られた．腺腫は遺伝子改変動物においてコウジ酸 1.5%および 3%群で見られたが，野生型では 3%群のみで見られた．しかしながら我々は，この試験における 1 群の動物数が少ない(20-25 匹/群必要と思われるのに対して 10 匹/群であった)ために，この試験の正当性に関して確信が

もてなかった．また，炎症細胞の大量の壊死／浸潤が見られ，これは本試験において高頻度で感染が起こっていたことの可能性を否定できなかった．このことは本試験の結果の解釈において重要な問題点であり，試験を無効とせざるを得ないかもしれない．

推奨事項：新たな p53 マウス試験を適切な群サイズ(20-25 匹)で実施する．この際，全ての主要組織の組織検査をすることと野生型の対照群を設けること，被験物質は高純度であり，かつマイコトキシンの汚染のないものを使用すべきである．トキシコキネティック試験も併せて実施すべきである．

ラット試験

生涯投与のラット試験は実施されていない．三森らの試験では，コウジ酸の 20 週間の高濃度暴露後に肝臓において GST-P 巣(数と面積)の増加が認められた(2.0%で増加したが，0.5%では増加せず)．フェノバルビトンによるプロモーションの有無に関わらず，2%コウジ酸を 6 週間ラットに混餌投与したときに GST-P 巣に影響はなかった．コウジ酸の混餌投与後にラット肝臓において複製 DNA 合成が誘発されることも報告されている．

推奨事項：理想的に言えば，ラットの生涯投与発がん性試験を特性の明らかな高純度のコウジ酸を用いて実施すべきである．

結　論

コンサルタントの多くは，コウジ酸の p53 マウス試験での陰性結果（腫瘍なし）とマウス肝臓において付加体形成の見られないことが確認されれば，コウジ酸の肝発がん性が非遺伝毒性的なメカニズムによるものであるという結論を導き出せる可能性があるものと考えた．

コウジ酸の評価に引き続き，我々は食品および食品関連の遺伝毒性に関しての戦略を策定するために考慮する必要のある要因を考察した．

閾　値

遺伝毒性において陽性結果を導く多くの異なった“間接的”メカニズムが存在し，これらは，ある用量／濃度以下では遺伝毒性を誘発しない，すなわち閾値を有する．これらのメカニズムのいくつかは，飽和状態となるまでの代謝的解毒システムに関連しており，いくつかは，例えば，紡錘体分裂装置などの DNA 以外を対象としたものである．ある種の直

接的なDNA反応性の遺伝毒性物質についてさえ，DNA修復機構が飽和していない状態では，閾値を設定することが可能である．これらの点に関し，いくつかの化学物質に関しての報告があるが，公表されたデータは質的に均一でなく，国際的に承認された最小限の試験バッテリーの標準的範囲の観点からも完全なものばかりとは言えない．我々は，明確な閾値をもつ遺伝毒物および避けることのできない遺伝毒物については，EU CPMP安全性作業部会による，医薬品における遺伝毒性不純物に関する最近のポジションペーパー案において提案された“毒性事象の閾値”概念が，新たな知見が得られるまでの妥当な当面の措置として，“安全”限界を設定するのに有用であると考える．

議論をとおし，閾値は用量反応曲線の外挿だけでは設定できず，遺伝毒性メカニズムを理解することの必要性が判明した．また，ある種の細胞で設定された閾値は必ずしも他の全ての細胞種に拡大適用できるわけではなく，許容限界を提言するには追加調整が必要であることも認識された．たとえ遺伝毒性メカニズムが理解されていても閾値用量／濃度を正確に設定するには，細かく区切られた用量／濃度を用いた大々的な実験が，必要である．必要とされる検出力を有する実験の規模やデザインを決定するには，統計学的アドバイスが重要である．

“強さ（効力）”

強力な遺伝毒性を示すが発がん性は弱い物質，また遺伝毒性は弱いが発がん性は比較的強い物質が存在するため，遺伝毒性試験と発がん性試験間の強さの相関性は低いものと考えられてきた．しかしながら，*in vitro* および *in vivo* の遺伝毒性試験のバッテリーにおいて強力な遺伝毒性が認められる化合物は，多臓器ならびに複数の動物種におけるがん原物質である傾向があることも事実である．さらに，“強さ”は，共通の化学構造を持つ化合物群においては，その順位付けに有用と考えられ，ある化合物群では発がん性との相関性があることがすでに知られている．

異なった試験/フォローアップ試験の重み付け

Ames試験において明らかに強い陽性化合物は，フォローアップ試験によって結論を変えることは困難である．このことは，合理性のない“陽性”結果を与える *in vitro* 特異的なメカニズムがより多く存在するほ乳類細胞を用いた試験には必ずしも当てはまらない．一般に，1つの *in vitro* 試験での陽性化合物のフォローアップ試験には，2つの *in vivo* 試験が必要と考えられている．*In vivo* 試験の選択には，指標と試験の妥当性を考慮することが重要である．これまでは，最初に選択される *in vivo* 試験は骨髄を用いる小核試験であった．小核試験で陽性ならば，さらなる試験は必要ないと考えられる（しかし，現在では，生理学的あるいは薬理学的変化（例えば，体温の低下）によって“偽陽性”結果を与えるいくつ

かのメカニズムが知られている).

結　論

コウジ酸をモデルとしたデータ解析は，今後の食品添加物および食品関連物質中の遺伝毒性物質のコントロール（規制）のための戦略（ストラテジー）作りに向けてのアドバイスに有益であった．明確な戦略が必要であり，国際的に容認されたプロトコールに基づき実施され試験は，評価を下すために必要な骨格となる情報を提供する．不適切な試験や十分に検討されていないプロトコールを用いて実施された試験，標準化されておらずバリデートされていない様々な試験を加えることは，決定を下す際に必ずしも有用なデータを提供しない．

閾値と“強さ”を考慮することは，避けることのできない遺伝毒性物質の規制に意味がある．ある試験は他の試験よりもより重みがある．最終的なリスク評価を可能にするには，*in vitro* で陽性結果を示した化合物のフォローアップ *in vivo* 試験を注意深く選択することが重要である．

別 添 3

Report of the Kamakura Workshop on the evaluation of the risk of genotoxicity from foods and food related substances

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Summary

The workshop began by consideration of a large set of experimental data on the fermentation product kojic acid present naturally in some foods consumed in Japan and also in the past used as a food additive to prevent enzymatic browning of crustaceans. This exercise was used as a test case to help formulate the future strategy on the evaluation of risks posed by food and food-related mutagens. Kojic acid has given variable results in a variety of *in vitro* and *in vivo* genotoxicity tests, but was banned as a food additive based on weight of evidence that it is a genotoxin of concern, and the fact that it is no longer used as a food additive in Japan. Mice given high doses of kojic acid in the diet develop tumours of the thyroid and liver. Considering the positive results of kojic acid in genotoxicity tests, it was deemed probable that genotoxicity was involved in tumour initiation.

The workshop participants considered that although a large set of data from genotoxicity testing is available, there are significant gaps in the data that preclude a firm conclusion that kojic acid is a genotoxic carcinogen. Recommendations include:

i) Investigating mechanisms of genotoxicity *in vitro* and *in vivo* including DNA adduct studies

ii) Investigate ADME and toxicokinetic parameters *in vivo* to help interpret *in vivo* results, in particular to understand possible inter-species differences, the role of metabolites and to determine exposure of target tissues to kojic acid and its metabolites.

iii) Positive *in vivo* data from bone marrow micronucleus tests and the comet assay were key to the decision to label kojic acid as a genotoxin of concern, however the participants had concerns that some of the experimental protocols used may have generated conflicting results that are difficult to interpret. It was recommended that the rat micronucleus study should be repeated in adult animals and that the comet assay be repeated using whole cells rather than isolated nucleoid bodies. It was recommended that tail moment should be measured rather than tail length.

iv) Participants felt that the original p53 mouse study was compromised by low group sizes and high inflammation rates (possibly indicating infection) in the study animals. It was recommended that a new study should be carried out using the currently accepted protocol.

v) All new studies must be conducted with high purity material devoid of mycotoxin contaminants.

vi) There is a plausible non-genotoxic explanation for the induction of thyroid tumours in mice by kojic acid that can also explain the induction of liver tumours. It is unclear if the liver tumours observed included malignant carcinomas. It is recommended that the raw data, pathologists reports etc are reviewed (possibly the slides re-scored) to determine if malignant tumours were induced.

vii) Ideally a lifetime rat carcinogenicity should be completed.

It was the view of the workshop participants that if DNA adducts cannot be demonstrated in target tissues and the new p53 study is negative (i.e. no difference between transgenic and wild-type animals), then it is unlikely that carcinogenicity is linked to genotoxicity.

The lessons from gained from the review of kojic acid data were that material must be of defined quality (a challenge for food mutagens); the use of non-standard protocols lead to difficulties in interpretation due to lack of validation; investigation and subsequent understanding of mechanisms is critical; ADME studies can be critical to understanding *in vitro/in vivo* and inter-species differences; clarity is needed on the pathological interpretation of tumour studies and finally even large volumes of test data do not necessarily add to clarity of interpretation if the tests chosen do not yield usable information or address key questions.

The workshop participants went on to consider genotoxins with thresholds of action i.e. can levels of exposure be defined where exposure to genotoxins (especially those with an 'indirect' mechanism of action) does not present a concern to humans, and the impact of potency in genotoxicity tests on risk assessment.

Objectives

The JMHLW/JEMS has requested consultation to help develop a strategy in order to evaluate the risk of genotoxicity from food and related substances. The consultants were requested to identify any experiments that need to be done or to identify any databases that need to be identified to help formulate the strategy.

Using the data on the fermentation product kojic acid as an example, the consultants were asked:

i) Which tests provided useful information?

ii) Which tests did not provide useful information?

iii) Can any quantitative assessment (e.g. potency) be made from any studies? If so which?

iv) Are any mechanistic studies needed in order to further understand the genotoxicity of kojic acid? If so what type of studies?

Kojic acid

Comments on the test data provided:

Genotoxicity studies

Bacterial tests: Ames tests were reproducibly positive, both with and without S9 metabolic activation. Some evidence was provided that positive results were due to kojic acid itself rather than any contaminants or metabolites. Mutation spectrum results showed that base-pair transversions were induced. The possibility that the positive results obtained could have been induced as a result of feeding effects due to carry over of amino acids/proteins was raised, although it was noted that kojic acid was also positive in the *Rec* assay which would not be affected by feeding effects.

Photo plasmid-relaxation assay: Positive results obtained in the presence of UVA. Results suggest that superoxide radicals and hydrogen peroxide produced under these conditions. **Consultants were unsure of the validation of this assay.** In addition, this hypothesis was not supported when there was no enhancement by UV of mutation in TA102.

***In vitro* mammalian assays:** Variable results obtained. Negative for *hprt* mutation in V79 cells and *tk* mutation in L5178Y cells but positive in TK6 and WTK1 gene mutation systems. Positive in the majority of chromosome aberration assays at high concentrations (≥1000 μg/ml), but negative in others, including assays in the same cell systems giving positives. Increases in chromosome aberrations could not be attributed to high osmolality, but it was suggested they might be associated with high cytotoxicity. Insufficient data were provided to evaluate this. The chromosomal damage in CHO cells could not be attributed to oxidative damage when assessed using the FPG-Comet assay. *In vitro* micronucleus assays were considered negative

in human keratinocytes, inconclusive in CHL cells (positive after 72 hr treatment but negative after 6 hr treatment), positive in TK6 and WTK1 cells, and positive in HepG2 cells at a very high, toxic concentration.

***In vivo* assays:** Bone marrow micronucleus tests in the mouse (three studies, two in 6-7-week old mice) were negative (po and ip) despite high doses and up to 5 daily administrations. A bone marrow micronucleus test in young (4-week old) rats (po and ip) and a peripheral blood micronucleus test in young rats (po) were both positive 1 or 2 daily treatments). Consultants had some reservations about the use of young rats (metabolic capability and clearance compromised?) for these studies. Also unsure if the differences between mice and rats were due to true species differences or due to the age of the treated animals. **Recommendation: A bone marrow or peripheral blood micronucleus test should be repeated in older rats. If still positive recommend that ADME studies are done in rats and mice to help understand the inter-species differences.**

A liver micronucleus test in the rat was negative. A liver micronucleus test in the mouse after partial hepatectomy was positive. Consultants again were unsure if this reflected inter-species differences in the opposite direction to the micronucleus tests or if the differences in protocol were responsible.

A UDS test in rat liver was negative.

A dominant lethal test in the mouse was also negative.

Two *LacZ* mutation assays (in Muta™Mouse), one in male and the other in female liver, were negative.

A *Drosophila* wing spot mutation assay was negative.

A DNA adduct assay in rat thyroid was reportedly negative.

Comet assays using cells from mouse thyroid and liver were both negative (but the reference was not available). Comet assays (three) using the Sasaki method with nucleoids obtained from mouse liver were positive (po and feeding) after high doses. Consultants were unsure if the methodology used has affected the results. **Recommendation: Comet assays should be repeated comparing the whole cell method with the nucleoid method. Also recommended**

that DNA breakage should be estimated using tail moment rather than tail length.

It has been proposed that DNA adducts be investigated in rat liver. This may still be useful (in light of GST-P foci – see later) but should also be done in the mouse. **Recommendation: DNA adducts should be measured in mouse liver using C14 labelled compound (as adducts are likely to be small, P32 post-labelling may be inappropriate, could be checked by running P32 post-labelling studies in bacteria).**

Oxidative damage

Several studies investigated whether effects of kojic acid could be related to oxidative damage. The studies discussed here are those not concerned with phototoxicity and UV interactions. The evidence is mixed, and not strongly in favour of oxidative damage as a cause of genotoxicity. Several strains in the Ames test showed increases in mutation, which is not typical of an agent that acts primarily through oxidative mechanisms. The mutation spectrum in the Ames assays was also not comparable with that of hydrogen peroxide. There was no induction of comets in CHO cells or enhancement of this by FPG enzyme that recognises and cuts DNA at the sites of oxidative lesions. An increase in 8-OHdG was reported in mouse liver after 28 days of administration of kojic acid in the diet, but the increase may not be significant when viewed in light of the variability of data presented. In addition, this technique is fraught with technical difficulties, and therefore data need careful evaluation.

Carcinogenicity studies

Lifetime mouse study

Mice exposed to chronic oral administration of kojic acid develop thyroid adenomas and liver tumours. Consultants were unsure if the liver tumours were all adenomas i.e. benign or a mixture of adenomas and malignant carcinomas. The numbers quoted for control incidence were too high for the tumours all to be carcinomas. **Recommendation : the raw data and pathology reports from the study should be checked to determine the nature of the liver tumours.**

As stated in the report from the study there is a very plausible non-genotoxic explanation for the thyroid tumours. In kojic acid treated animals there is an inhibition of iodine uptake. This could lead to a subsequent decrease in serum T3/T4 levels and a compensatory increase in TSH

released from the pituitary, resulting in stimulation of thyroid cell proliferation. In addition it is known that T3/T4 levels and TSH levels return to normal within 48 h of withdrawal of kojic acid. This mechanism is unlikely to occur in humans due to a much better buffered thyroid hormone level, and the much lower exposures of people to kojic acid.

However, the consultants felt that it is possible that the liver tumours observed may also bound up with the effects in the thyroid. Kojic acid may induce liver enzymes (this is consistent with increased liver weight in the chronic toxicity studies), and hence indirect effects in the thyroid. Histopathological changes in the liver and thyroid suggest stimulation of the hypothalamic-pituitary-thyroid axis. Liver weight increase and hepatocellular hypertrophy may result from microsomal enzyme induction, which would increase the hepatic clearance of thyroxine (T4), with a subsequent lowering of T4 levels in the serum. This change would then cause an elevation of TSH secreted from the pituitary, and hypertrophy/activation of the follicular cells in the thyroid. The rat is particularly susceptible to this type of change in comparison to Man because of the shorter plasma half-life of thyroxine. As above, this mechanism is unlikely to occur in humans. While the histopathological changes in the liver for thyroid toxicants are generally not striking (hypertrophy and increased liver weight) and are not always associated with an increased level of apoptosis, increased mitotic activity, the changes may be promotable to pre-tumorigenic lesions (foci) and ultimately tumors. This is particularly important at high doses, where frank toxicity-related findings (necrosis, increased oxidative damage-related effects) constitute an additional risk factor. (For reference see Capen C C *et al*, 1991, Endocrine System, in Handbook of Toxicologic Pathology, Academic Press, pp 675-760; Gopinath C *et al*, 1987, The Endocrine Glands, in Atlas of Experimental Toxicological Pathology, MTP Press Limited, pp 104-121). In the context of these mechanisms of action, a clear analysis of the histopathological picture, its time course and the metabolism and excretion pathways is highly important. These aspect were not clear to the consultants.
Recommendation : consultants have now provided a possible non-genotoxic explanation of the liver tumours induced by kojic acid through changes in thyroid hormones and TSH. To investigate the proposed mechanism of action, a clear analysis of the histopathological picture, its time course, metabolism (including induction of thyroid hormone related metabolic enzymes), excretion pathways and liver hypertrophy is highly important. An expert in this area should be invited to provide further advice and help interpret the data.

<u>P53 knockout mice study</u>

A study has been carried out in which mice, P53 heterozygotes and isogenic wild type, were fed

diet containing up to 3% kojic acid for 26 weeks. Equivalent thyroid changes (but no tumours) were seen in both the transgenic and wild type animals. Hepatic changes were also seen in both strains, including adenomas. Adenomas were seen in both kojic acid groups (1.5% and 3%) in the transgenic strain, but just in the 3% group for the wild-type. However, the consultants were unsure of the significance of this study as the group sizes were smaller than in accepted protocols (only 10 per group rather than 20-25). Also there was massive necrosis/infiltration of inflammatory cells in each group of this study which may well signify a high incidence of infection in the study. This could compromise the study and invalidate the result.

Recommendation : A new P53 +/- study to be carried out with adequate group sizes (20-25), histology of all major tissues, plus a wild type control. Test material should be of defined high quality and be mycotoxin free. Toxicokinetic evaluation should be included.

Rat studies

No lifetime rat study is available. Studies by Mitsumori et al have shown an increase in GST-P foci (numbers and area) in liver after high exposures to kojic acid (2.0%, but not 0.5%).for 20 weeks. There was no effect on GST-P foci when 2% kojic acid was fed to rats for 6 weeks with or without promotion by phenobarbitone. There was also a reported increase in replicative DNA synthesis in rat liver after feeding kojic acid.

Recommendation : Ideally a rat lifetime carcinogenicity study should be completed with defined, high purity kojic acid.

Conclusion

Most workshop participants felt that a negative (no tumours) mouse P53 +/-study of kojic acid and lack of adducts in mouse liver may allow the conclusion that carcinogenicity is due to non-genotoxic mechanisms and not linked to mutagenicity.

Following the assessment of kojic acid the consultants considered some generic factors that need to be considered in setting future strategies on the control of food and food-related mutagens.

Thresholds

There are many different 'indirect' mechanisms that can result in positive genotoxicity results, which have a threshold dose/concentration below which genotoxicity is not induced. Some of these mechanisms relate to saturatable metabolic detoxification systems, others to non-DNA targets such as the spindle apparatus etc. It is even possible to define (low) thresholds for certain direct, DNA reactive genotoxins, before DNA repair mechanisms become saturated. It is often the case that published data on specific compounds are of variable quality and studies are not always complete in terms of the standard range of internationally accepted minimum battery of tests. The consultants felt that for genotoxins with a clear threshold and for unavoidable genotoxins, the 'Threshold of Toxicological Concern' concept recently recommended by the EU CPMP Safety Working Party in a draft new Position Paper on genotoxic impurities in drugs, could be useful for defining 'safe' limits, possibly as an interim measure, whilst new acceptable data are generated.

In discussion it was understood that thresholds cannot be defined solely by extrapolations from dose response curves, but the genotoxic mechanism needs to be understood. It was also recognised that thresholds defined in one cell type cannot necessarily be extended to all other cell types, so additional adjustments would be needed to recommend acceptable limits. Even if the genotoxic mechanism is understood, large experiments with closely spaced doses/concentrations are needed to accurately identify threshold doses/concentrations. Statistical advice is required to determine size and design of experiments with the desired power.

Potency

It has long been recognised that correlations of potency between genotoxicity tests and carcinogenicity assays are fraught with difficulty as there are potent genotoxins that are weak carcinogens and weak genotoxins that are relatively potent carcinogens. However compounds that are potent genotoxins in a battery of genotoxicity tests *in vitro* and *in vivo* tend to be potent, multi -tissue, multi-species carcinogens. In addition potency may be of value in ranking compounds in a chemically related series of compounds, where there is already a known correlation with carcinogenicity of some members of the series.

Weight given to different tests/follow-up tests.

Compounds that are unequivocally positive and reasonably potent in Ames tests are difficult to over rule by follow up testing. This is not necessarily the case for mammalian tests where there are more non-relevant, *in vitro* specific mechanisms that can give 'positive' results. Generally follow-up testing of *in vitro* positive compounds needs two *in vivo* tests. The choice of *in vivo*

test needs careful consideration of endpoint and relevance. The first test in the past has been an *in vivo* bone marrow micronucleus test. If positive, no further testing is required (although some mechanisms that generate 'false positive' results due to physiological or pharmacological changes are now recognised e.g. hypothermia).

Conclusion

The analysis of the kojic acid data has been informative regarding advice for future strategies for control of food mutagens and related materials. A clear strategy is needed in which internationally recognised tests carried out to internationally recognised protocols provide the backbone of the information required to make decisions. Adding inadequate tests and/or a variety of non-standard, non-validated tests carried out using untested protocols does not necessarily add data useful in decision making.

Consideration of thresholds and potency can be of value in attempting control of unavoidable genotoxins. Some tests carry more weight than others do and careful selection of follow-up *in vivo* tests of compounds giving positive results *in vitro* is needed to allow a final assessment of risk to be made.

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October 15, 2004

Dear consultants:

Japanese members appreciate very much constructive comments of consultants on our studies of Kojic acid for establishment of strategy to evaluate risk of food and food-related mutagens.

We are working on Kojic acid (KA) to get comprehensive data that are required to clarify its genotoxic-carcinogenicity, according to the comments raised by consultants.

At the Kamakura meeting, we presented a lot of data. We hope you understood the reason why we carried out so many experiments which were in the same category as previously done by some organization. Crucially important information about genotoxicity for risk assessment is *in vivo* genotoxicity. To clarify *in vivo* genotoxicity, *in vitro* genotoxicity should be first clarified. However, available data indicted that some were negative and some were positive even in *S. typhimurium*. Thus, we confirmed its genotoxicity in bacteria, and then using the same guaranteed KA, we examined the effects of KA in mammalian cells and in mammals.

***In vitro* assay**

1. We first confirmed that KA itself is mutagenic, using HPLC separation, and also by mutation spectra. Based on these data, we evaluated that KA is mutagenic in bacteria.
2. Next, we confirmed genotoxicity in mammalian cells. For this study, we used *TK* as a marker gene because so far no study examined effect of KA on the *tk* gene even in mouse lymphoma assay (the *Hprt* gene was used). It is considered that Hprt (or HPRT) is not sensitive enough to detect large deletion, and TK is a sensitive marker for this. We used human cells that can be considered more appropriate than rodent cells for evaluation of risks to humans. TK assay and MN assay were positive, even after paying attention to the cytotoxicity. *In vitro* comet assay was performed to confirm technical problems, because comet assay is not validated yet. In this study, TK-6 and WTK-1 cells gave positive responses at the same concentrations of KA in TK assay, MN assay and comet assay. Thus, we could confirm that KA is genotoxic in human cells and the technique of comet assay would be fine at least *in vitro*.

   We think these studies are necessary to confirm genotoxicity of KA in mammalian cells *in vitro*.

***In vivo* assay**

1. As for *in vivo* assay, as pointed out by the consultants, we used different ages of animals for rats and mice, and obtained different results between rat and mice. We performed MN assay using adult mice and rats after the Kamakura meeting. We confirmed that MN in the liver was positive in mice but negative in rats. In these assays using adult animals, partial hepatectomy was performed after administration of KA. MN in peripheral blood was negative in mice and positive in rats. Thus, we will perform ADME study in rats and mice.
2. Comet assay:
    1) Validation study of the methods using, so called, isolated nuclei and intact cells is being planned at the international level.
    2) Could you please let us know the differences between evaluation data for tail length and tail moment?
3. DNA adduct assay. We consider that detection of DNA adduct is important to clarify the mechanism of genotoxicity. Although size of DNA adduct produced by KA can be estimated to be small, the specific radioactivity of ^{14}C-KA may not be sufficient to detect DNA binding. Thus we will perform ^{32}P–postlabelling analysis using DNA from KA-treated bacteria as a positive control.

Carcinogenicity

1. Unfortunately it is impossible to re-examine the histology of liver tumors induced by KA in the study by Fujimoto et al. (1998). We agree that histological diagnosis should be accurate. However, adenoma or carcinoma is not the matter for risk evaluation of carcinogenicity. Even if all tumors induced by KA were adenomas, carcinogenicity is evaluated as positive. If genotoxicity of KA is involved in the liver adenoma development, KA is assessed as genotoxic carcinogen. We would like to hear from consultants if they agree this point or not. If so, could you please explain why you insist diagnosis of carcinoma or adenoma?
2. As for carcinogenicity study using p53+/- mice for genotoxic carcinogens, we have informed that this system does not necessarily work in liver carcinogenesis. Strong genotoxic liver carcinogen, IQ or aminophenylnorharman was not positive in $p53^{+/-}$: $p53^{+/+}$ studies. If you have any solid data indicating usefulness of $p53^{+/-}$: $p53^{+/+}$ study for liver carcinogenesis, please let us know.

Photogenotoxicity of KA

1. Recently, phototoxicity and photogenotoxicity becomes big concern in the safety evaluation for pharmaceutical drugs and cosmetic ingredients, although test methods, however, have not been well validated. Study of effect of UV looks not necessary to establish strategy for

food and food-related mutagens at the moment, therefore, this topic might be discussed separately. We, however, studied photogenotoxicity and included the data for consultation data set. We evaluated photogenotoxicity of KA by DNA plasmid-relaxation assay, bacterial gene mutation assay using TA102, and in vitro MN assay using TK-6 human lymphoid cells. We used solar simulated lamp (UV-A, B and visible light) was used for DNA plasmid-relaxation assay and *in vitro* MN assay and positive response was observed in the both assays. But, a black light (UV-A) was used for the gene mutation assay and photogenotoxic effect was not observed. The reason to explain this discrepancy could be the light source used, because KA has absorption peak 270 nm and the wavelength of UV-A ranged from 320 to 380 nm. We will confirm the effect of UVB in the bacterial mutagenicity test system.

2. Although consultants were not familiar with DNA plasmid-relaxation assay, the method is widely-used as a standard method to detect DNA strand scission. Nakagawa et al. evaluated this method using 26 chemicals that had been evaluated mainly by *in vivo* photo-skin-irritation assay. Among them, 17 chemicals were known as photo-toxins and 9 were non-photo-toxins, and DNA plasmid-relaxation assay showed good concordance (94% photo-toxins were positive and 89% non-photo-toxins were negative) (Environ. Mutagen Research 23, 107-118, 2001).

Others

1. As for hypothalamic-pituitary-thyroid axis for liver and thyroid hypertrophy, could you please kindly provide us the following references?
    1. Capen CC. in Handbool of Toxicologic Pathology, pp675-760 1991.
    2. Gopinath C, Atlas of Toxicogical Pathoology, pp104-121, 1987.

別　添　5

第31回日本トキシコロジー学会学術年回
平成16年7月6日～8日　大阪

シンポジウム1－低用量と閾値問題
シンポジウムを企画するにあったって

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Introduction to the symposium I: Assessment of low dose effect and threshold
Organizer: Makoto Hayashi (Division of Genetics and Mutagenesis, Biological Safety Research Center, National Institute of Health Sciences); Tetsuya Ono (Medical Sciences, Graduate School of Medicine, Tohoku University)

本シンポジウムでは，化学物質の安全性を評価する上で，低用量域での現象に焦点を当て，安全域をどのように設定することが可能かを考えてみたい．一般毒性では，実験動物における無作用料をもとに，ヒトへの外挿のための安全係数（不確実係数）として種差10倍，個体差10倍の合計100倍で補正することが一般的に行われている．ただし，DNA を直接標的とする遺伝毒性や，遺伝毒性を発生のメカニズムとするがん原性，すなわち遺伝毒性がん原物質（genotoxic carcinogen）には閾値は存在せず，用量をいかに下げてもこれらの発現が確率的に 0 となることはない，と考えられている．しかし，DNA についた傷も多くは修復され遺伝毒性の発現に至らないことが知られているし，がんの発現についても多くのステップが必要であり，遺伝毒性が生体内で誘発したとしても，がんを誘発する確率がいずれの場合にも高いものとは考えられない．従って，遺伝毒性や遺伝毒性がん原性についても，暴露量を下げることによりあるレベル以下の確率に抑えることは可能と考える．

遺伝毒性等の閾値の問題は放射線遺伝学の知識に基づくところが大きい．また，確率論的な取り扱い，考え方についてもっと深く理解する必要がある．本シンポジウムではこの2つのテーマを基礎に，一般毒性，遺伝毒性，がん原性の実際のデータに基づいた低用量域での反応，閾値に関する考察をするとともにリスク評価の考え方について議論を深めたい．