cin C, Ara-C, colchicine, acrylamide and potassium bromate. Although there were no significant differences in MN induction among mice when 2,000 cells were analyzed, clear differences became apparent when one million cells were analyzed. It indicates that larger sample sizes give higher power of statistics and also that the sensitivity of MN assay can be improved when cells but not animals are considered as evaluation units. However, lowest doses for MN induction by potassium bromate or acrylamide were not changed even after the sample sizes were increased to one million cells per mouse. He also introduced current topics in International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) where test batteries for genotoxicity were being reorganized.

Session 2 (chaired by Samuel M. Cohen and Akiyoshi Nishikawa)

5. *in vivo* Approaches to Study Mechanism of Action of Genotoxic Carcinogens

Akiyoshi Nishikawa (National Institute of Health Sciences, Japan)

Nishikawa reported *in vivo* approaches to study mechanism of action of genotoxic carcinogens. Currently, genotoxicity and carcinogenicity of chemicals are assessed separately by genotoxicity assays, i.e., Ames test, *in vitro* chromosome aberration test (or mouse lymphoma gene mutation test) and mouse MN test, and by long-term rodent carcinogenicity test, respectively. It is uncertain, therefore, to what extent the detected genotoxic potential can contribute to the carcinogenicity. To solve the issue, he utilized *gpt* delta transgenic rats and mice carrying lambda phage EG10 as a reporter for mutations and showed these animals were powerful tools for the evaluation of both genotoxicity and carcinogenicity in the same organs. Interestingly, MX, which is a genotoxic chlorinated water by-product in Ames test, failed to exert genotoxicity or carcinogenicity *in vivo*. On the other hand, dicyclanil, a known non-genotoxic carcinogen, was genotoxic in the liver of female *gpt* delta mouse. He reported these animal models might have great potential to apply for risk assessment of genotoxic carcinogens. Understanding of the detailed mechanism of carcinogenic action would be crucial for more precise risk assessment of genotoxic carcinogens at low doses.

6. Possible Involvement of Adaptation Mechanisms in the Achievement of an Ineffective Dose Range for the Carcinogenicity of Genotoxic Carcinogens

Dai Nakae (Tokyo Metropolitan Institute of Public Health, Japan, Tokyo University of Agriculture, Japan)

Nakae reported that genotoxic carcinogens had ineffective doses for the carcinogenicity and some adaptation mechanisms might contribute to this phenomenon. To demonstrate this postulate, he and his colleagues performed large scale studies using male Fischer 344 Big Blue rats given a 16-week chronic feeding administration of 0.0001 to 1 ppm of genotoxic carcinogen, i.e., DEN. The number and area of GST-P positive foci in liver were significantly increased only at the highest dose of 1 ppm while mutant frequencies were elevated at a dose of 0.001 ppm and the above. Levels of 8-hydroxyguanine were not changed at all doses used. He suggested these findings might indicate the existence of a practical threshold or an ineffective dose range for the carcinogenicity of genotoxic carcinogens. To utilize the DNA adduct as a marker to determine a practical threshold, he concluded it needs validation of large bodies of data.

7. Possible Dose Threshold for Liver Carcinogenesis by Mutagenic Liver Carcinogens

Hiroyuki Tsuda (Nagoya City University Graduate School of Medical Sciences, Japan)

Generally, under industrial exploitation procedure, the development of new chemicals is immediately stopped when their genotoxicity is clarified. However, Tsuda and his colleagues claimed that some genotoxic substances had non-effective doses in long-term animal experiments. He proposed the existence of the biological threshold level for genotoxic liver carcinogens. Tsuda had examined seven chemicals, i.e., 1,4-dioxan, 2,4-diaminotoluene, *N*-nitrosomorpholine, 1,2-dimethylhydrazine, quinoline, 2-nitropropane and carbon tetrachloride, which were produced during manufacturing process of petroleum-related products, with two individual medium-term carcinogenesis assays (Ito-model for promotion assay and Tsuda-model for initiation assay). In these models, GST-P positive foci were used as a marker to detect preneoplastic lesions. Tsuda suggested that biological threshold levels might exist around NOAEL, but interactions between the threshold and biological defense responses were not clear, and concluded that more extensive research is required to clarify the definitive biological threshold level.

8. Thresholds in Genotoxicity and Carcinogenicity: Urinary Bladder Carcinogens

Samuel M. Cohen (University of Nebraska Medical Center, U.S.A.)

Thresholds for carcinogenic risk are a profound theme that requires extensive discussion on the mechanisms from DNA-damage to carcinogenesis. Cohen showed three bladder carcinogens in rodent models and discussed relationships among genotoxicity, cytotoxicity and carcinogenicity. A non-genotoxic substance uracil formed urinary solids which induced cytotoxicity and cancer in a threshold manner. Genotoxic substance 2-acetylaminofluorene (AAF) is DNA reactive and forms bladder DNA adducts in a dose-responsive linear manner. However, the tumor response is non-linear because cytotoxicity at high doses increases cell proliferation, a necessary component for the carcinogenesis. Arsenic is genotoxic and induces bladder cancer in animal models and humans. However, the genotoxicity occurs by indirect mechanisms, not by direct DNA reactivity. Therefore, the genotoxicity may have a threshold, occurring only at high doses. In discussion, he proposed that "DNA reactivity" was a more definitive term than "genotoxicity", because genotoxicity included mechanisms other than DNA reactivity, such as spindle poison or topoisomerase II inhibition. He claimed that it needs careful considerations to use the term of "threshold". He concluded that true threshold should be defined as the levels where zero cancer risks are expected although practical threshold may be valuable for practical purposes.

Session 3 (chaired by Kirk Kitchin and Masao Hirose)

9. Roles of the Food Safety Commission

Masao Hirose (Food Safety Commission, Japan)

Hirose introduced the Food Safety Commission (FSC) in Japan, which was established in 2003, and its major roles, i.e., risk assessment of the hazards contained in foods, risk communication and responses to emergency situations. FSC includes three risk assessment groups for chemical substances, biological materials and emerging foods. FSC has received requests related to risk assessment for more than 1000 items from the risk management organizations such as Ministry of Health, Labour and Welfare, since establishment. The assessments have been completed for about 550 items including 154 pesticides and 165 veterinary medicines. FSC conducts assessment on its own initiative (self-tasks) when the Commission considers issues needed to be evaluated from the analyses of food safety information, public opinion and similar information. FSC follows the classical concept that genotoxic carcinogens do not have threshold levels and thus ADI cannot be applied to genotoxic and carcinogenic compounds added to foods such as food additives and pesticides. From the FSC's point of view, it would not proper to establish ADI to genotoxic compounds.

10. Thresholds for Genotoxic Carcinogens: View from the National Food Safety

Takashi Kunieda (Ministry of Health, Labour, and Welfare, Japan)

Kunieda reported that regulatory approaches to genotoxic carcinogens in food have not been well established yet and thus global consensus-building in this field is needed. Regulation of carcinogens in food is one of major issues in the national food safety program, because cancer deaths account for 30 percent of all deaths in Japan and most consumers have special concerns about the carcinogenicity of substances in food. Non-genotoxic substances do not directly damage DNA, and carcinogenic thresholds are considered to exist. It is possible to ensure the safety of these substances by establishing applicable standards based on the ADI or tolerable dairy intake (TDI). On the other hand, genotoxic substances directly damage DNA, and no carcinogenic thresholds are considered to exist. As the ADI or TDI cannot be established, it is required to individually respond to ensure the food safety from these substances, according to characteristics of them. Risk management is carried out for unavoidable chemicals based on the following risk assessments approaches to reduce human exposure to ALARA: carcinogenic risk calculated by low-dose extrapolation and margin of exposure using the benchmark dose.

11. Consumers View

Kazuo Onitake (Japanese Consumers' Co-operative Union)

Onitake reported the opinion from the consumers' view as a representative of Japanese Consumers' Co-operative Union (JCCU), whose major objective is to protect the health of consumers. JCCU has been addressing many issues related to food safety, such as food additives and residues of agricultural chemicals, for a long period of time. JCCU is of the opinion that when managing risk associated with the use of chemicals or with the presence of chemicals as contaminants from the environment, risk assessments should be performed before any action is taken and other legitimate factors should be taken into consideration. JCCU agrees with the principle that genotoxic carcinogens do not have biological threshold and ADI cannot be applied to those chemicals intentionally added to foods such as food additives, pesticides and veterinary drugs. JCCU believes that this position is responding to the expectations of

consumers who are concerned about any possible risks from genotoxic carcinogens in food.

12. Theoretical and Experimental Approaches to Possible Thresholds of Response in Carcinogenicity

Kirk T. Kitchin (Environmental Protection Agency, U.S.A.)

Kitchin reported that no convincing examples of carcinogenic thresholds in humans are known, except for one theoretical approach, the two-stage clonal growth model by the Moolgavkar group. In animals, at least four good examples of carcinogenic thresholds have been observed. DNA adducts data for the five well studied chemicals were fairly linear while the foci and tumor data show supralinear, linear and threshold curves, making it difficult to generalize. Currently there is no good scientific and regulatory understanding of chemicals that act simultaneously or sequentially via both linear and nonlinear carcinogenic pathways (genotoxic and nongenotoxic). In order to elucidate the dose-response of chemicals of dual carcinogenic dose-response properties (linear and non linear), Kitchin proposes the studies for two or more such chemicals in a large scale coordinated fashion employing at least 1,000 animals, five different treatment groups, six different study parameters and 8 different scientific disciplines.

Session 4 (chaired by David Lovell and Yoshiya Shimada)

13. Modification of Threshold Dose in Radiation-induced Mouse Lymphoma Development

Yoshiya Shimada (National Institute of Radiaological Sciences, Japan)

Shimada reported the studies of radiation-induced mouse thymic lymphoma focusing on dose response of lymphoma induction and the effects of genetic factors, i.e., DNA repair capacity of mouse, and environmental factors, i.e., alkylating agents. The dose limit for radiation protection is based on the LNT hypothesis, where the carcinogenic risk is proportional to radiation dose, even at low doses. However, the results showed that the dose response relationship for mouse thymic lymphomagenesis after repeated X-irradiation has an apparent threshold at dose of around 400 mGy per fraction. DNA repair capacity for double strand breaks or mismatch of nucleotides is a critical determinant for manifestation of threshold.

14. The Progress of Trace Analytical Technique for Measurement of Chemicals in Foods

Munetomo Nakamura (Japan Food Research Laboratories)

Nakamura reported the recent progress of analytical methods using gas chromatograph/mass spectrometer (GC/MS(/MS)) and liquid chromatograph/mass spectrometer (LC/MS(/MS)). In 2006, the positive list system for agricultural chemicals was introduced in Japan. At the same time, many maximum residue limits have been established. Therefore, a lot of analytical methods for residual chemical substances had to be developed. GC/MS(/MS) or LC/MS(/MS) technique can analyze many substances at one time with good selectivity and sensitivity. Those benefits simplify purification steps too. Those methods are adopted as official methods for analysis of pesticide residues in foods, veterinary medicines and carcinogenic and genotoxic mycotoxins.

15. Statistical Consideration on the Identification of Threshold through Toxicological Experiments

Isao Yoshimura (Tokyo University of Science, Japan)

Yoshimura argued that, in principle, it is impossible to identify the threshold via hypothesis testing in the case of toxicological experiments because the probability of false negative decisions cannot be managed in this context. When a mechanism for producing a threshold is hypothesized from a toxicological (or biological) perspective and is mathematically formulated as a dose-response relationship, statistics may be helpful in evaluating the existence (or non-existence) of the threshold. It is important to select a model from a particular set of mathematical dose-response functions. The determination of a practical threshold using *in vitro* experiments may be an alternative to the identification of a "true" threshold, if an appropriate *in vitro* assay affords a large scale experiment at low doses.

16. Statistical Perspective on the Threshold Problem in Toxicological Experiments

David P. Lovell (University of Surrey, U.K.)

Lovell reported mathematical and statistical approaches which do or do not include thresholds and statistical methods which try to identify no observed effect levels (NOELs). There is an increasing appreciation of the potential to identify 'pragmatic' thresholds using experimental systems with a range of biomarkers. The accurate characterization and estimation of these dose-response relationships require careful experimental design which can improve the accuracy of the estimates of the response while avoiding the introduction of ar-

tefactual effects. Statistical approach such as Design of Experiment (DoE) methodology, which builds on the traditional factorial design, can provide efficient approaches for the description and estimation of dose-response relationships of both individual and combinations of agents.

Session 5 (chaired by Minako Nagao and Hansruedi Glatt)

17. Cells Genetically Engineered for Xenobiotic-metabolizing Enzymes: Detection of Genotoxic Effects at Extremely Low Substrate Concentrations

Hansruedi Glatt (German Institute of Human Nutrition, Germany)

Glatt developed Chinese hamster V79 cell lines expressing various human phase-I and phase-II enzymes. Using the transgenic cell lines, he investigated the genotoxicity of a lot of pro-genotoxicants. Human CYP1B1 expressed in the target cell (V79-hCYP1B1) exhibited the genotoxicity of benzo[a]pyrene (BP) at less than 10 nM, while rat liver S9-mediated assay required 7 μM to induce gene mutations. BP induced sister chromatid exchange (SCE) from 10 pM in the cells. The concentration-response curve [$y = f(x)$] for SCE–unlike for gene mutations–strongly deviated from linearity. Other promutagens required expression of CYP forms different from CYP1B1 and/or non-CYP enzymes (such as sulfotransferases or acetyltransferases) for their activation at low substrate concentrations. In general, compounds requiring expression of non-CYP enzymes in recombinant cells remained inactive in the standard V79/S9 gene mutation assay.

18. Genotoxic consequences of a single double strand break in human cells

Masamitsu Honma (National Institute of Health Sciences, Japan)

Honma mentioned that "threshold of genotoxicity" can not be established, because genotoxicity is generally recognized by experimental assays. Experimentally, thresholds are inferred from dose-reduction experiments in which dosages are decreased to the level at which adverse effects are no longer observed. This strategy demonstrates not a threshold, but rather a detection limit. Ultimately, the most straightforward evidence for a genotoxic threshold would come from examining the effect of a single DNA damage. If this causes mutation, no threshold will exist. If it does not, there will be a threshold for genotoxicity. He developed a novel system to introduce a unique double-strand break (DSB) into the genomic DNA of human cells by restriction enzyme digestion, and demonstrated that 99 % of DSB are repaired by error-prone repair resulting deletion mutations. This result suggested that there is no threshold for genotoxic compounds which cause DSB.

19. Additive Mutagenic Effects of DNA Damages Formed by Multiple Mutagens at Virtually Non-mutagenic Dose Level of Each

Toshihiro Ohta (Tokyo University of Pharmacy and Life Sciences, Japan)

Ohta reported additive mutagenic effects induced by multiple mutagens in which each mutagen did not show mutagenicity at low levels. Six mutagens (furylframide, MX, 4-nitroquinoline *N*-oxide, sodium azide, 1-nitropyrene and captan) induced base-substitution mutations much more efficiently in *Salmonella typhimurium* TA100 (*hisG46*, *rfa*, *uvrB*/pKM101), a strain deficient in nucleotide excision repair, than in TA1975P (*hisG46*, *rfa*/pKM101), a repair proficient strain. Virtually non-mutagenic dose levels were selected by looking for the doses where the chemical was apparently mutagenic to strain TA100 but not to strain TA1975P. The six mutagens were mixed at the virtually non-mutagenic dose level of each and a possible combined mutagenic effect was investigated with strain TA1975P. A significant and reproducible increase in the number of revertants in TA1975P was observed with combined mutagens. Similar investigations were performed using six heterocyclic amines.

20. Consideration on Extension of the Threshold Concept in Animals to Humans

Minako Nagao (Keio University, Japan)

Nagao reported the history of toxicology to reevaluate the presence or absence of threshold in genotoxicity or carcinogenicity. In standard animal carcinogenesis studies, its detection limit is about 10%. In *in vivo* genotoxicity studies, on the other hand, detection limits are about 2-fold of the background. Even if a significant increase in mutation frequency is not observed, mutation spectrum analyses sometimes demonstrate induction of genetic changes. Thus, impacts of the biological responses occurring under the detection limit of an assay system need to be extensively investigated. She also suggested the presence of thresholds in neoplasm induction by PhIP in the colon but not in the breast or hematopoietic system. The presence or the absence of thresholds for a particular carcinogen might be different depending on the target organs. She concluded that clarification of underlying mechanisms would be necessary to confirm presence of threshold.

21. Scientific Implications and Social Impact of Threshold Concept for Genotoxic Carcinogens

Yuzo Hayashi (Japan Health Food & Nutrition Food Association, Japan)

Hayashi discussed the classification of genotoxic and non-genotoxic carcinogens. This classification, however, can not be applied to all instances due to insufficiencies in necessary information. Therefore, the non-threshold concept was introduced exclusively for genotoxic carcinogens and has been adopted in Japan as a basis for regulatory risk assessment. Dose-response studies recently conducted with various genotoxic agents suggest the existence of a threshold. It should be emphasized, however, that a threshold is not a value which can be determined directly from dose-response data. In this context, scientific efforts in support of the adoption of a threshold should be focused on the development of appropriate mathematical models, and the establishment of toxicological concepts. A realistic step towards a paradigm shift from the non-threshold concept is to seek general consensus on the introduction of an appropriate "virtually safe dose" instead of a threshold.

22. Closing Remarks

Shoji Fukushima (Japan Bioassay Research Center)

Fukushima emphasized that evaluation of threshold in carcinogenicity of genotoxic carcinogens is a very important problem in cancer risk assessment and management. Furthermore, various services as well as consumers and industrial workers mutually desire the fast solution of this problem. In the present Symposium, the speakers did the presentations on the matter of risk assessment, risk management and risk communication for free and active discussion as well as exchanging ideas and opinions. Compared to the Symposium organized in two and half years before by Dr. M. Hayashi (NIHS, formerly) and he, in this time more people were gathered and a deeper and mutual comprehension was achieved. It is very important to evaluate the benefit and risk of chemicals on the basis of our latest scientific results and to continue discussion and argumentation on carcinogenic threshold. Furthermore, together with overall look on the problem of threshold, more and more understanding is continuously desired.

遺伝毒性物質に閾値はあるのか?

本間正充
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1 はじめに

食品の安全性に対して多くの国民が関心を寄せている今日，残留農薬や食品添加物等の食品中に含まれる微量の化学物質の安全性が問題となっている．多くの化学物質の毒性は，健康リスクを評価する場合，理論的，実証的研究から，これ以下であれば健康影響がみられないレベル，すなわち閾値がある用量反応モデルが用いられてきた．これにより1日摂取許容量(acceptable daily intake；ADI)を定めることができる．しかしながら，その化学物質の発がん性が問題となり，さらに遺伝毒性が認められるとやっかいである．他の毒性と異なり遺伝毒性には閾値がないとされているため，摂取量をゼロにしない限り健康リスクもゼロにならないとの論理からADIを設定することができない．ここに遺伝毒性発がん物質のリスク管理の問題点がある．

2 遺伝毒性とは?

遺伝毒性(genotoxicity)は遺伝子の本体であるDNAや染色体に対する毒性である．その定義は曖昧かつ広義であるが，一般には「DNAや染色体の構造的，もしくは量的変化を引き起こす性質」をいう．別の言葉として変異原性(mutagenicity)があるが，こちらは遺伝毒性に比べて狭義であり，主としてDNAや染色体に対する損傷の結果として生じる突然変異等の誘発能を示す(図1)．変異原性が最終的な遺伝的影響を示すものであり，それ以外の遺伝毒性はDNAや染色体が何らかの影響を受けたことによる一過性の変化であることが多い．遺伝毒性は他の毒性と異なり，それ自体の毒性の実態をつかむことができない．肝毒性，神経毒性，発がん性などは症状や病変として我々の体で認識できるが，遺伝毒性自体の症状や病変はない．

遺伝毒性はその結果として，がんや遺伝性疾患を引き起こす．したがって遺伝毒性とは，それら疾患を引き起こすポテンシャルの1つであり，その有無は遺伝毒性試験によって認識される．図1に一般的な遺伝毒性試験を示す．遺伝子DNAはバクテリアからほ乳類まで共通する生命の設計図であり，様々な動物種を用いた試験法が開発されている．また，そのエンドポイントはDNAの損傷，染色体の構造的，もしくは数的変化，遺伝子突然変異等，多岐にわたる．このなかで代表的な試験法としてはエームス試験，染色体異常試験，小核試験(*in vivo*)が挙げられる．これら試験は医薬品を初めとする多くの化学物質の安全性を評価する上で必須の試験として義務づけられている．

遺伝毒性試験は一般的に，遺伝毒性ハザードの有無を検出する定性的試験法であり，その結果は「陽性」もしくは「陰性」として判定される．しかしながら，毒性には本来，量的相関性

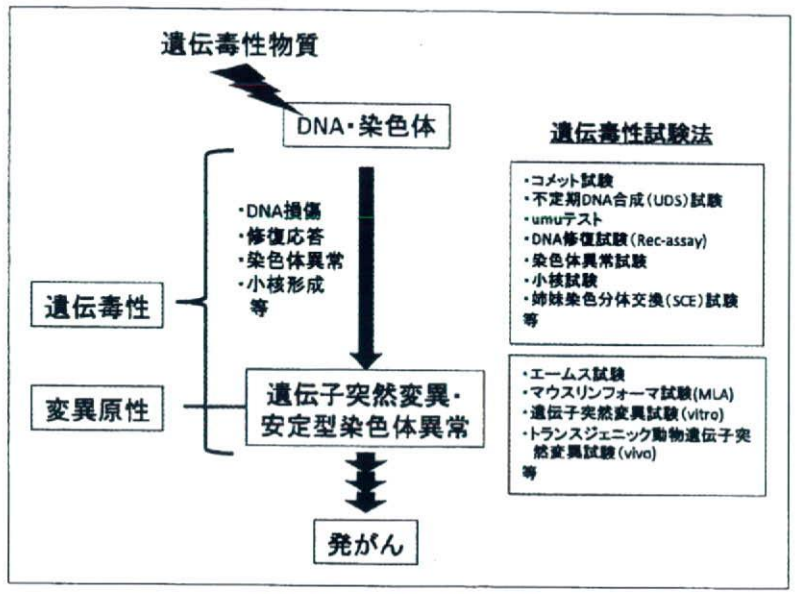

図1　遺伝毒性とその試験法

があることが常識であり，遺伝毒性のような一義的に陽性，陰性を決定することの方が特殊といえる．したがって，遺伝毒性の閾値問題はこの結果の定性的評価法に端を発するといえよう．最近になってトランスジェニック動物の開発等によって遺伝子突然変異試験等の定量的試験法が *in vivo* で実用可能となった．また，遺伝子突然変異はがんを引き起こす直接要因であることから，その試験結果は発がん性遺伝毒性物質評価の重要なエビデンスにもなりうる．

3　遺伝毒性発がん物質，非遺伝毒性発がん物質

遺伝毒性試験の目的の1つは，化学物質等の発がん可能性を調査するためのスクリーニングである．しかし，遺伝毒性試験で陽性となったからといっても必ずしも発がん性があるとは限らない．遺伝毒性試験結果とげっ歯類発がん性試験結果の相関性は，試験系によっても異なるが60～80%程度である．スクリーニングとしての目的上，できるだけ多くの発がん可能性を検出することが求められるため，感度の高い試験法が開発・利用されてきたが，それでも一部の発がん性物質に関しては陰性を示す．これらが非遺伝毒性発がん物質である．がんは遺伝子の病気であり，必ず遺伝的な変化を伴うと考えられるが，これらの物質は自然に生じたがん原細胞の増殖の亢進などを通じてがんの形成を助けるものと考えられる．ホルモン作用を持つ化学物質の一部などがこれに相当する．

遺伝毒性物質にはベンツピレン，アフラトキシンB1，N-ニトロソ化合物，アルキル化剤などの強力な発がん物質が含まれる．これら化学物質はDNAに直接作用し，切断，架橋，付加体の形成，脱塩基，酸化損傷，アルキル化等を引き起こし，その結果，高い確率で突然変異を引き起こす．一方，遺伝毒性試験で陽性であっても直接DNAに作用しないものもある．チュブリンの重合阻害剤であるコルヒチンは細胞分裂装置に影響を与え，染色体異常を引き起こす．また，DNA修復阻害，アポトーシス抑制，細胞周期停止などを引き起こす化学物質も遺伝毒性試験で陽性を示すことがある．

これら，化学物質のターゲットはDNAではなくタンパク質であり，非DNA損傷性遺伝毒

性物質と定義することができる．*In vitro* 遺伝毒性試験は陽性反応を示しやすく，その毒性メカニズムが不明であることもある．強い細胞毒性，高浸透圧，沈殿の生成，非生理的 pH などは非特異的な影響により陽性反応を示すこともあるので注意を要する．通常，1つの遺伝毒性試験の結果から遺伝毒性の有無を判定することは困難であり，複数の試験結果から試験条件や反応の程度などを考慮して判定することが多い．遺伝毒性試験で陰性を示すもの，また陽性を示しても非 DNA 損傷性であるものを総称して非遺伝毒性物質と呼ぶこともある．

4 遺伝毒性物質に閾値はあるのか？

遺伝毒性物質が DNA と反応し遺伝子突然変異をもたらす．突然変異は確率論的(stochastic)事象であり 0 になることはない．また，たった1つの遺伝子突然変異でも，その変異ががん遺伝子，がん抑制遺伝子などの細胞のがん化に重要な遺伝子に生じた場合，1つのがん原細胞が生じ，それだけで発がんに至ることがある．したがって，この発がんの確率も 0 にはならず，理論的に遺伝毒性発がん物質に閾値を設定することはできない．

一方，タンパク質に作用する非遺伝毒性発がん物質に関してはどうであろうか？ 1つの細胞中には遺伝子は多くても 2 コピーしか存在しないのに対して，タンパク質分子は数多く存在する．高濃度の化学物質が多くのタンパク質と作用すれば発がんに至る影響が表れるかもしれないが，少数であれば影響はないことは容易に想像できる．このようなことから非遺伝毒性発がん物質に関しては理論的に閾値が設定できる．また，幾つかの実験により非遺伝毒性発がん物質の閾値の存在は証明されており，多くの専門家はこの問題に関して異論はない．問題は遺伝毒性発がん物質の閾値である．

遺伝毒性発がん物質の発がん性，遺伝毒性，DNA 付加体の形成に閾値が存在するかどうかの検討が多くの研究者によって動物実験等によってなされている．アフラトキシン B1 やベンツピレンを動物に投与した場合，肝 DNA 付加体の形成は用量相関性を示す．DNA 付加体の検出は質量分析機の進歩により通常，人が曝露するレベルより 2 桁低いレベルの検出まで可能となっており，極低用量でも用量相関性が観察される．DNA 付加体の形成は化学反応であり，DNA と反応する化学物質が存在する限り形成を否定できないため閾値がないとするのが一般的である．

生物学的反応である遺伝毒性の閾値の存在の証明には，動物に投与する遺伝毒性物質の用量を段階的に下げて無作用量が存在するかどうかを，様々な遺伝毒性のエンドポイントで検出する方法がとられている．遺伝子突然変異試験の場合，無作用量とは自然誘発突然変異レベルを示す．このような実験は千～十万倍の用量域で行うため，用量を対数換算して表示することがしばしば見られる．図 2 は $y=ax+b$ の用量相関性を示す反応の 2 つのグラフを示す．これは閾値なしモデルであるが，対数表示だと閾値があるようにみえる．これは錯覚であり，このような図から閾値を論じるべきではない．同様に，低用量域ではその増加量が極くわずかで有意差がないため閾値とみなすとする論理もあるが，これも正しくない．それは試験の検出力が乏しく，自然突然変異の変動が大きいため統計的に有意にならないだけである．先に述べたように遺伝毒性は遺伝毒性試験によって認識される．すべての試験には検出限界があり，用量を段階的に下げて，無作用量が存在するかどうかをみるというストラテジーは，閾値よりもむしろ検出感度をみるに過ぎない．そもそも(閾値の)存在を，非検出をもって証明することが論理的に無理があるように思われる．

一方，極低用量域では高用量域からの一義的外挿では説明できない生物学的反応が効率的に働くため閾値を設定できるとの説もある．ここでの生物学的反応とは DNA 修復，代謝反応，

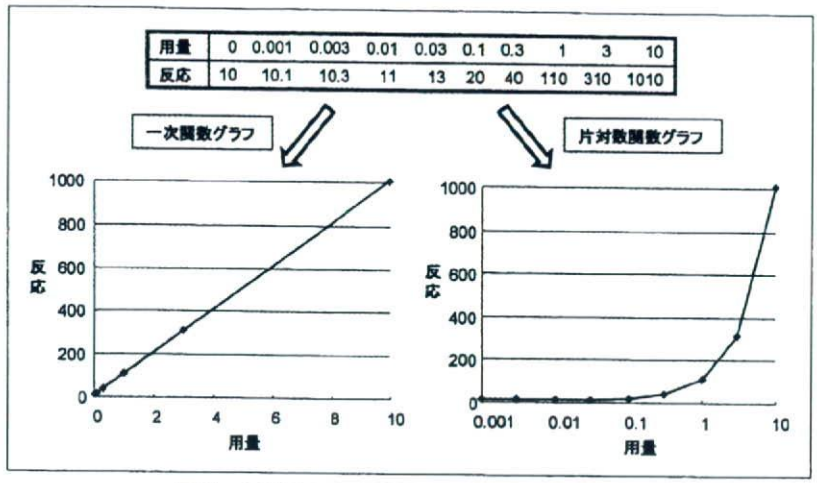

図2　閾値なし用量相関モデルのグラフ表示

スカベンジャーなどの防御機構が考えられる．しかしながら，このような防御機構は遺伝毒性の発生確率の低減化には寄与するが，閾値を作る根拠にはならない．DNA 付加体の除去には塩基除去修復機構が働き，これは一般にエラー非発生型の修復機構であるが，10^{-6} 以下の発生頻度でエラーが起き，突然変異を引き起こす．同様に，化学物質の無毒化に働く薬物代謝や，スカベンジャーも 100% の効率で働くという保証はない．

このような科学的・理論的解釈では閾値を設定できないが，現実的には極低用量の遺伝毒性反応は，自然に起きる反応と区別をすることが困難であり，閾値と見なしてもいいのではないかという考え方もある．一般に遺伝子突然変異試験の自然突然変異頻度は 10^{-6} 程度であり，一定のばらつきを持つ．この原因として酸化ストレス，老化等の内的要因や，環境中に極微量存在する試験物質以外の化学物質や放射線，紫外線などの影響が考えられる．これにより，自然に起きる遺伝毒性反応内に収まるようなレベルを「現実的閾値(practical もしくは pragmatic threshold)」とするものである．しかし，これは閾値とは別の問題である．現実的閾値の考えはある種の妥協であり，専門家の中でもこの考えに合意はできていない．したがって，発がん物質が遺伝毒性試験陽性，特にそれが発がん標的組織であれば，その発がん性に閾値を設定することはできず，ADI のような安全量を設定することはできない．

5　遺伝毒性発がん物質のリスク管理

それでは，遺伝毒性発がん物質に閾値が設定できなければそのリスク管理はできないのであろうか？ 米国においては 1958 年に「発がん性の可能性がある化学物質はいかなる低用量でも安全とみなすことはできない」という，いわゆるデラニー条項により，動物に対して発がん性を示す農薬が残留する加工食品の販売が禁止され，その後，適用範囲が着色料，動物用薬品，飼料に拡大された．しかしながら，このゼロリスク思想は現実的には多くの矛盾点があった．主な矛盾点としては，①分析技術の進歩により，微量な化学物質も検出可能となり，検出限界である安全レベルがどんどん低くなってしまうこと．②発がん性の有無だけが強調されているため，他の毒性が低くて，安全性の高い化合物ができても，わずかの発がん性のため代替できないこと．③人工化学物質のみを対象としているため，天然由来の発がん物質は無視されていること．④動物実験の発がん性試験は，必ずしも人に対する発がん性と一致しないこと，などが挙げられる．

これらのことから，1996年「食品品質保護法」の制定とともにデラニー条項は廃止された．

閾値を設定しゼロリスクを追求するのに対して，「発がん可能性がある化学物質が十分に低濃度であれば，その発がん可能性は極めて小さくなり，その程度が社会的に許容できるリスクレベルであれば実質的に安全と見なし得る」とのリスク管理の方法もある．この量を実質安全性量(virtually safety dose；VSD)といい，そのリスクレベルを「無視しうる(negligible)」，もしくは「許容できる(acceptable)」リスクとする．ここでの許容できるリスクとしてのがんの生涯リスクレベルは一般的に百万分の1(10^{-6})が採用されている．10^{-6}の生涯リスクとは日本の人口(10^{8})と，平均寿命(80)から計算すると($10^{8}\times1/80\times10^{-6}=1.25$)1年間に1.25人のがんによる死者が増えることを意味する．がんは今や先進諸国では死亡原因の1位であり，我が国においても年間約35万人が，がんで死亡していることを考慮すると1.25人の増加は社会的に許容できるといえよう．VSDは一般にげっ歯類を用いた発がん試験で得られた半数がん誘発用量(TD 50)からマルチステージモデル，もしくは直線外挿により得られる(図3)．このような発がん化学物質を生涯発がんリスクレベルで評価し，管理に用いる手法は，現在，水道水や大気中に含まれる汚染物質の新しい環境基準値の設定に用いられている．

Cheesemanらは約500種類の発がん化学物質に関する動物実験でのTD 50からVSDを算出し，その算定曝露分布の結果から，ほとんどの発がん化学物質については0.5 μg/kg(0.5 ppb)以下の食事中濃度で百万分の1のがん生涯リスクよりも低くなることを示した．[1] 1人の1日食事量を3 kg(固形食品1.5 kg，飲料1.5 kg)とし，その化学物質が全食事にムラなく入っていると仮定すると，1日曝露量は1.5 μg/人と計算できる．つまり，大部分の化学物質については1日の摂取量が1.5 μg/人以下であれば，たとえそれが発がん物質であっても実質的な健康危害はほとんどないだろうとすることができる．このような包括的な閾値を「毒性学的懸念の閾値(threshold of toxicological concern；TTC)」という．TTCは化学構造を考慮すればその毒性が分かっていないものも含め，多くの化学物質に適用できる．我が国では食品衛生法に基づき残留農薬のポジティブリスト制が導入されたが，ここでは残留基準値が設定されていない農薬に関しては一律基準値として0.01 ppmが設定された．この値もTTC(1.5 μg/人)に基づくものであり，個々の農畜産物の1日摂取量は米を除いて150 gを超えることがないという国民栄養調査から計算されている(1.5/150＝0.01)．TTCは既に米国FDAがプラスチック容器から溶出する化学物質(間接添加物)のリスク管理に用いており，またJECFA(FAO/WHO合同食品添加物専門家委員会)は食品に添加する香料物質に適用している．

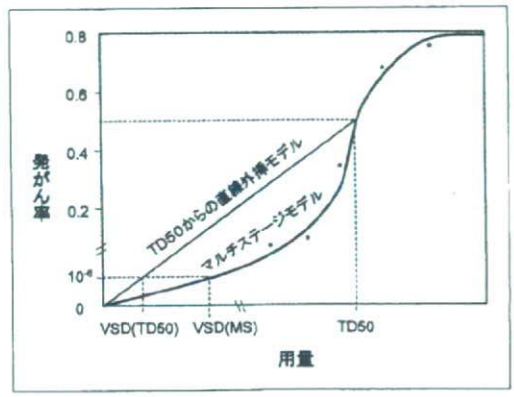

図3　Virtually Safety Doseの算出法

しかしながら，TTC レベルはその発がん物質に遺伝毒性があった場合にはより慎重な取り扱いが必要となる．Kroes らは 600 以上の発がん化学物質を比較して，TTC を 1.5 μg/人とした場合，遺伝毒性もしくは要注意構造を持つ遺伝毒性物質の幾つかについて高い発がんリスクを懸念している．[2] このため，多くの専門家は食事中に低レベルで存在する遺伝毒性／要注意構造を持つ発がん物質に関しては TTC を 1 桁低い 0.15 μg/人とすることを推奨している．さらに，TTC が適応できないような極めて強力な遺伝毒性発がん物質としてアフラトキシン類，アゾキシ化合物，ニトロソ化合物を挙げている．これら化合物に関しては個別の毒性データとリスク管理が必要であり，TTC を適用すべきではない．

一方，医薬品に関しては別の TTC の考え方がある．医薬品そのものに遺伝毒性があることは許されないが，そこに含まれる不純物に遺伝毒性がある場合，TTC の概念を取り入れた不純物のリスク管理が米国，EU でガイドライン化されつつある．これらガイドラインでは場合によっては，不純物に遺伝毒性があっても 1 日あたり 120 μg/人までの TTC が許容される．医薬品は食事と異なり，摂取(服用)期間が限られていること，また医薬品のベネフィットを考慮した 10^{-5} のリスクレベルなどが採用された段階的 TTC が提唱されている．[3]

6 おわりに

発がん率は人口あたりで発生するがん患者の数であり，動物実験による発がん性試験は担がん動物の数によって評価される．その単位は/人口，/動物数であり普遍である．一方，遺伝毒性の単位は試験系によって異なる．エームス試験は/plate，染色体異常試験は/cell，遺伝子突然変異試験は/gene によって評価される．単位が違えばその検出レベルも異なり，そこで仮に閾値が観察されたとしても，その値は試験系に依存する．また，発がん性は種差，個体差等によって変動することは当然考えられるが，遺伝毒性とは「DNA や染色体の構造的もしくは量的変化を引き起こす性質」であり，DNA や染色体がすべての生物で共通であることを考慮すると，それは普遍でなくてはならない．もし，遺伝毒性に閾値が存在するのであれば，試験法によってそれが変動すること自体が矛盾である．したがって，遺伝毒性とはそもそも閾値を論じるような性質のものではないといえるのかも知れない．

遺伝毒性発がん物質に無理に閾値を設定し，ゼロリスクを求めるよりも，低レベルのリスクを，無視しうる(negligible)，もしくは許容できる(acceptable)リスクとして評価し，社会が受け入れることの方が現実的と考える．文明社会で生活する限り，多くの化学物質の摂取は不可避であり，そのベネフィットとリスクのバランスを考えることが重要である．また，我々人間は自然の食物からも多くの化学物質を摂取しており，それらが遺伝毒性発がん物質であることも少なくない．これら化学物質の中には，一般化学物質よりも高い 10^{-4}～10^{-5} というリスクレベルでないと管理できないものもある．Doll と Peto が言うようにがんの最大の原因は我々の日常の食べ物にあり，残留農薬や食品添加物にあるのではない．[4] もちろん，これらリスクはできるだけ回避することは必要であるが，やはりここでもバランスが重要である．このバランス感覚を身につけることが，成熟した社会での安心した生活に繋がるものと考える．

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Short Communication

Effects of Tamoxifen on L-Glutamate Transporters of Astrocytes

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Abstract. Tamoxifen (Tam) decreased the clearance of L-glutamate (L-Glu) by cultured astrocytes at 1 pM, 1 nM, and 1 μM, but became toxic at 10 μM. When L-Glu transporters were mostly inhibited by *threo*-β-benzyloxyaspartate (TBOA) (1 mM) or D,L-*threo*-β-hydroxyaspartate (THA) (1 mM), Tam (1 nM) did not change extracellular L-Glu concentration, confirming that Tam attenuates L-Glu transport through L-Glu transporters. ICI182,780, LY294002, and U0126 inhibited the effect of Tam dose-dependently, suggesting the involvement of estrogen receptors (ERs), the phosphatidylinositol 3-kinase (PI3K) cascade, and the mitogen-activated protein kinase (MAPK) cascade in the effect of Tam.

Keywords: tamoxifen, L-glutamate transporter, astrocyte

L-Glutamate (L-Glu) is a major excitatory neurotransmitter in the central nervous system (CNS). L-Glu transporters are the only significant mechanism for removal of L-Glu from extracellular fluid and maintenance of low and non-toxic concentrations of L-Glu (1). In addition, a growing body of evidence has suggested the importance of L-Glu transporters in synaptic transmission: their function has correlation with the time course of neuronal activation and the number of neurons activated by L-Glu (2). Therefore, the elucidation of interactions between L-Glu transporters and chemical compounds would be important to clarify their influence on the CNS.

Tamoxifen (Tam) is a synthetic estrogen analog used clinically in breast cancer treatment to inhibit the proliferative action of estrogens (3). Tam is also known to have estrogen receptor (ER)-agonist properties depending on cell types and promoter context (4). In the follow-up study of women with primary breast cancer, it was suggested that current use of tamoxifen may adversely affect cognition (5). However, little information is available yet concerning the influence of Tam on brain function. We therefore investigated the effect of Tam on L-Glu transporters in this study.

According to the report by Kisanga et al. (6), the concentration of Tam in serum during conventional dosage for breast cancer is in the range of 20 – 225 nM. However, the exact concentration in the brain tissue has not been reported and may vary according to the circumstances such as body temperature, integrity of blood brain barrier (BBB), and so on. Therefore, we used a broad range of concentrations of Tam in this study. We here report that Tam inhibited L-Glu transporters at quite low concentrations (1 pM – 1 μM).

All procedures in this study were in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society and the guidelines of the National Institute of Health Sciences, Japan. Primary cultures of astrocytes were prepared from the cerebral cortices of 3-day-old neonates of Wistar rats, as described previously (7). Briefly, dissociated cells were suspended in modified Dulbecco's medium (DMEM) containing 10% fetal bovine serum (FBS) and plated on uncoated 75-cm^2 flasks at the density of 600,000 cells/cm^2. Non-astrocytes were detached from the flasks by shaking and were removed by changing the medium. Astrocytes in the flasks were dissociated by trypsiniza-

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tion and resceded on uncoated 96-well microtiter plates. When astrocytes became confluent, the effects of Tam on L-Glu clearance by cultured astrocytes were assayed in the following way: Tam was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and diluted to final concentrations with the culture medium. Cells were incubated with Tam for 24 h and then L-Glu clearance was measured. *threo*-β-benzyloxyaspartate (TBOA) and D,L-*threo*-β-hydroxyaspartate (THA) were dissolved at 1 M in phosphate-buffered saline (PBS) and diluted to final concentrations with the culture medium. ICI182,780, LY294002, and U0126 were dissolved at 1, 5, and 5 mM in DMSO, respectively, and were diluted with the culture medium to yield the final concentrations. These inhibitors were co-applied with 1 nM of Tam for 24 h. L-Glu clearance was measured as described previously (8, 9). Briefly, after the treatment with Tam, the culture medium was changed to that with 100 μM L-Glu and without Tam. After 1 h, 50 μl of culture supernatant was mixed with 50 μl of substrate mixture consisting of 20 U/ml glutamate dehydrogenase (GDH), 2.5 mg/ml β-nicotinamide adenine dinucleotide hydrate (β-NAD), 0.25 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), 100 μM 1-methoxy-5-methyl-phenazinium methyl sulfate (MPMS), and 0.1% (vol/vol) Triton X-100 in 0.2 M Tris-HCl buffer (pH 8.2). After 10-min incubation at 37°C, the reaction was stopped by adding 100 μl of stop solution containing 50% (vol/vol) dimethylformamide and 20% (wt/vol) sodium dodecyl sulfate (SDS) (pH 4.7). The amount of the reaction product (MTT formazan) was determined by measuring the absorbance with a microplate reader at 570 nm (test wavelength) and at 655 nm (reference wavelength). The extracellular L-Glu concentration was estimated from a standard curve, which was constructed in each assay using cell-free media containing known concentrations of L-Glu. L-Glu clearance was shown as the decrease in the amounts of L-Glu in the medium. MTT reduction and lactate dehydrogenase (LDH) activity in the medium was evaluated according to the method of Abe et al. (10).

For statistical analysis, the values were averaged for 6 wells in each treatment (including control) in one experiment. Using these averaged values obtained from 6 independent experiments (separate platings), the mean and S.E.M. were calculated for both control and treated groups. Statistical analysis was performed by one-way repeated-measure analysis of variance (ANOVA) and the post hoc Tukey's test for multiple pairwise comparisons. MTT and pharmacological data are shown as the data normalized to the control values.

Firstly, we examined the effects of Tam on L-Glu clearance by cultured astrocytes (Fig. 1A). Tam (24 h) significantly decreased the L-Glu clearance at 1 pM, 1 nM, and 1 μM. The extent of the inhibition at each concentration was almost the same (63.1% – 69.6% control). In contrast, Tam increased the L-Glu concentration in the medium at 10 μM. We then checked the cytotoxicity of Tam. Tam had no effect on MTT reduction and LDH leakage at the range of concentration from 1 pM – 1 μM. At 10 μM, LDH leakage was significantly increased whereas MTT reduction was attenuated. DMSO, the solvent for Tam, alone had no effect on the L-Glu clearance (data not shown). These results indicate that Tam has an inhibitory effect on the L-Glu clearance at 1 pM – 1 μM and that it becomes toxic at 10 μM, thereby causing the leakage of L-Glu from damaged cells. A typical image of astrocytes damaged by 10 μM of Tam is shown in Fig. 1C (right), in which cell shapes have changed to fibrillary ones with many granules on the cell surface. We checked whether a shorter period of the application of Tam is also inhibitory or not. The application of Tam for 10 min significantly inhibited the L-Glu clearance at 1 pM, 1 nM, and 1 μM, suggesting that Tam produced an immediate inhibitory effect, and this is thus a non-genomic event.

TBOA is an L-Glu–transporter blocker that is not a substrate of the transporter, and it potently suppresses the activity of glial L-Glu transporters (11). In our experiments, 95.9% inhibition was achieved by the application of 1 mM TBOA for 24 h (Fig. 2A). When Tam (1 nM) was co-applied with TBOA (1 mM) for 24 h, Tam did not change the extracellular L-Glu concentration (Fig. 2C), confirming that Tam-induced decrease in L-Glu clearance was caused by attenuating L-Glu transport through L-Glu transporters. The transporters may mainly be GLAST, which is the predominant L-Glu–transporter subtype in our culture condition (8). THA, another L-Glu–transporter blocker that is a substrate of the transporters and does not inhibit heteroexchange (12), also markedly inhibited the glial L-Glu transporters (94.7% inhibition at 1 mM, Fig. 2B). When Tam was co-applied with THA (1 mM), Tam did not change the extracellular L-Glu concentration, either, indicating further that Tam did not cause heteroexchange but attenuated L-Glu transport via GLAST.

We investigated the mechanisms underlying the effect of Tam pharmacologically (Fig. 3). Because Tam is a partial agonist to ERs (4), we first checked the involvement of ERs. ICI182,780, a potent antagonist for ERs (IC_{50}: 0.29 nM), inhibited the effect of Tam dose-dependently (Fig. 3A). At 100 nM and higher concentrations, ICI182,780 completely suppressed the effect of Tam. In our previous reports, we confirmed that the predominant ER subtype in cultured astrocytes was ERα

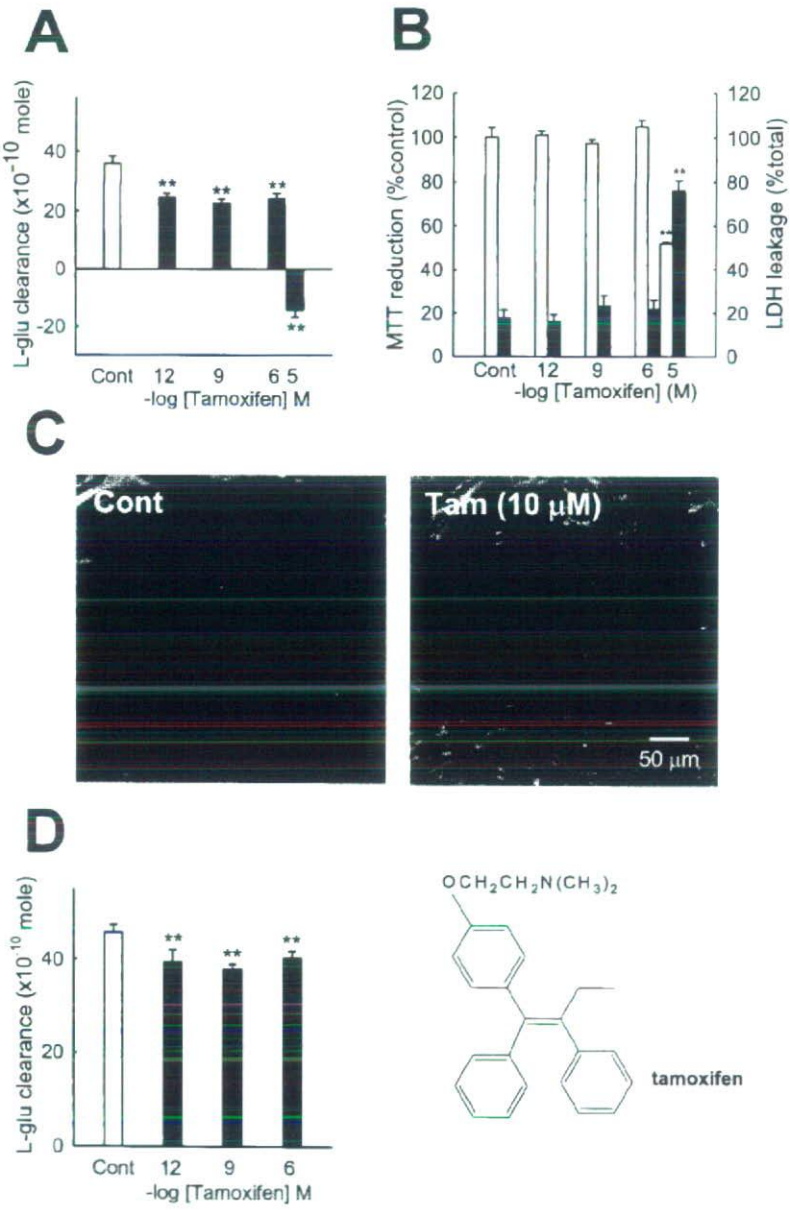

Fig. 1. Tamoxifen (Tam) inhibited L-glutamate (L-Glu) clearance by cultured astrocytes. A: Tam significantly decreased L-Glu clearance at 1 pM, 1 nM, and 1 μM. At 10 μM, Tam inversely increased the concentration of L-Glu in the medium. B: Tam had no effect on MTT reduction (white bar) and LDH leakage (black bar) at the concentration range of 1 pM – 1 μM. At 10 μM, Tam suppressed MTT reduction and increased LDH leakage from cultured astrocytes. C: Typical DIC images of cultured astrocytes in the control group (left) and in the group treated with 10 μM of Tam (right). D: Ten-minute application of Tam revealed the inhibitory effect on L-Glu clearance (left). The structure of Tam is also shown (right). $^{**}P<0.01$ *vs* the control group. N = 6, Tukey's test following ANOVA.

(8). Taken together, this result suggests that the effect of Tam was mediated by ERα. We then investigated the involvement of the phosphatidylinositol 3-kinase (PI3K) cascade and the mitogen-activated protein kinase (MAPK) cascade. LY294002, a selective PI3K blocker (IC_{50}: 10 μM), inhibited the effect of Tam dose-dependently (Fig. 3B). Complete suppression was achieved at 10 nM and higher concentrations. U0126, a selective and potent blocker of the MAPK cascade, which inhibits MEK1 (IC_{50}: 70 nM) and MEK2 (IC_{50}: 60 nM), activators immediate upstream of MAPK, also inhibited the effects of Tam at 10 and 100 nM (Fig. 3C). We confirmed that ICI182,780, LY294002, and U0126 alone had no effect on the L-Glu clearance (data not

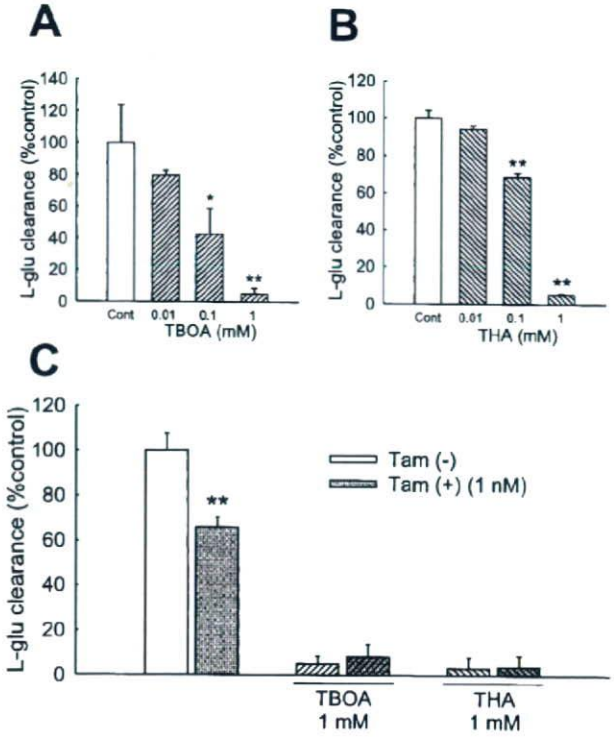

Fig. 2. Tamoxifen (Tam)-induced decrease in L-glutamate (L-Glu) clearance was caused by attenuating L-Glu transport through L-Glu transporters. A: Dose response curve for *threo-β*-benzyloxyaspartate (TBOA) on L-Glu clearance. L-Glu clearance was mostly inhibited by 1 mM TBOA. B: Dose-response curve for THA on L-Glu clearance. L-Glu clearance was mostly inhibited by 1 mM D,L-*threo-β*-hydroxyaspartate (THA). C: In the presence of 1 mM TBOA or 1 mM THA, Tam did not change the extracellular L-Glu concentration. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the control group. N = 6, Tukey's test following ANOVA.

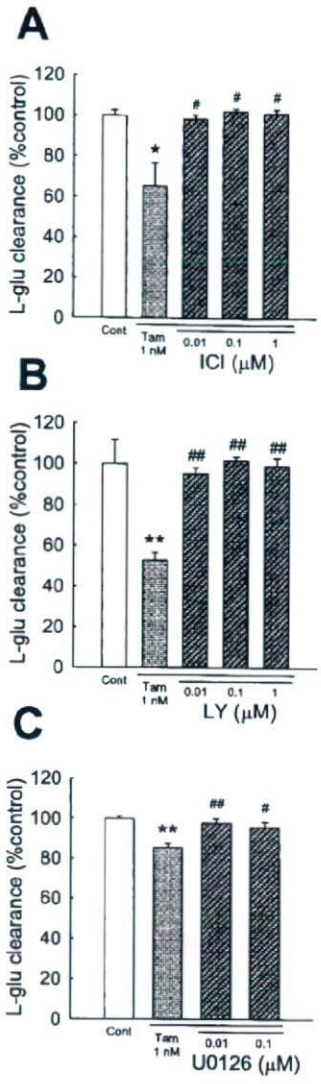

Fig. 3. Estrogen receptors (ERs), the phosphatidylinositol 3-kinase (PI3K) cascade, and the mitogen-activated protein kinase (MAPK) cascade are involved in the effect of Tam. A: ICI182,780 (ICI) dose-dependently inhibited the effect of Tam. B: LY294002 (LY) dose-dependently inhibited the effect of Tam. C: U0126 inhibited the effect of Tam. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* the group treated with Tam (1 nM). N = 6, Tukey's test following ANOVA.

shown). These results indicate that the activation of the PI3K and the MAPK cascades is involved in the effect of Tam. Our data demonstrated that Tam inhibited GLAST-mediated L-Glu transport through activation of ERα, the PI3K cascade, and the MAPK cascade.

We previously clarified that 17β-estradiol inhibited GLAST via a non-genomic pathway starting from membrane-associated ER (mERα) (8). Most recently, we elucidated that this pathway involves the PI3K cascade and causes the increase in K_m without affecting V_{max} and subcellular localization of GLAST (unpublished observation). Our pharmacological data strongly suggest that the same mechanisms are also activated by Tam. Such attenuation of L-Glu transport by Tam might be related to the effects of Tam on cognition. In addition, our data raise the possibility that Tam could also act as a partial agonist for mERα.

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