Session 7.6
Non-genotoxic carcinogenicity: Mechanistic perspectives for alternatives

Poster

Use of Tg.AC mouse model as an alternative to the two-year bioassay: The US National Toxicology Program experience

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The Tg.AC transgenic mouse is a genetically initiated, tumour promoter–sensitive epidermal tumourigenesis model that is being evaluated as an alternative to the traditional two-year bioassay. The model requires fewer animals and shorter duration of exposure. Tg.AC mice develop epidermal papillomas in response to treatment with dermally applied carcinogens. The US National Toxicology Program (NTP) selected four chemicals to study in the Tg.AC mouse model by dermal route of exposure as a part of its effort to validate the model as a replacement for two-year dermal carcinogenicity studies. The chemicals studied were dicyclohexylcarbodiimide (DCC), diisocarbodiimide (DIC), trimethylolpropane triacrylate (TMPTA) and pentaerythritol triacrylate (PETA). The studies on TMPTA and PETA have been completed. Topical application of TMPTA and PETA at dose levels ranging from 0.75 to 12 mg/kg to Tg.AC mice for six months showed dose-dependent increases in squamous cell papillomas at the site of application, with decreases in the latency of their appearance in mice receiving 3 mg/kg or greater. papillomas were accompanied by a few squamous cell carcinomas, along with hyperplastic and inflammatory lesions. The results of these studies were presented to the NTP Peer Review Panel as definitive studies of carcinogenicity in lieu of two-year bioassay but were not accepted by the Panel. Currently, the NTP is conducting 2-year studies on TMPTA in rats and mice to identify its carcinogenic potential. Our experience suggests that additional mechanistic data is needed to gain acceptance of the Tg.AC mouse model as an alternate to the traditional animal bioassay.

Lecture

Non-genotoxic carcinogens: Mechanistic perspectives for alternatives

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Non-genotoxic carcinogens are thought to act via disturbance of the balance between cell death and cell proliferation, leading to clonal expansion of pre-neoplastic cells. Precise mechanisms that lead to this disruption are varied and this hinders the development of short term and *in vitro* tests to predict non-genotoxic carcinogenic potential. Biomarkers indicative of a chemical's ability to influence cell survival and proliferative advantage may be helpful in the interpretation of rodent carcinogenicity that may occur in the absence of genotoxicity, and may aid human risk assessment. The dose response relationships and species differences in response are critical parameters in this process. Biomarkers can be relatively non-specific relating to apoptosis and proliferation in target organs but may be optimised and more refined by interrogation of the modulation of specific molecules involved in key signalling processes. Both the modulation of connexin-mediated gap junction intercellular communication and the status of DNA methylation have shown particular promise as relevant biomarkers in conjunction with functional studies as above. For example, the rodent non-genotoxic hepatocarcinogens Wy-14,643, 2,3,7,8-tetrachlorodibenzo -p-dioxin, methapyrilene and hexachlorobenzene and the rat kidney carcinogens chloroform and p-dichlorobenzene all disrupt gap junction plaques containing connexin 32 in the target organs in the absence of toxicity and they all cause proliferation at the carcinogenic dose. Furthermore, compounds that deplete glutathione such as chloroform and carbon tetrachloride have the ability to induce a secondary genotoxicity through oxidative stress but only at high concentrations. Transcriptomic analyses have identified further novel potential biomarkers associated with species- and tissue- specific non-genotoxic carcinogenesis such as the activation of pathways involved in the control of cell cycle.

Poster

ECVAM Key Area Carcinogenicity and Genotoxicity: Summary of ongoing activities

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The ECVAM key area on carcinogenicity and genotoxicity has been reorganised with the initiation of several activities related both to validation and its applied research.

The carcinogenicity/genotoxicity taskforce was set up in January 2004. During the first meeting the experts agreed on the need to validate the cell transformation assay (CTA), as this is the only developed *in vitro* test which has the potential to detect both genotoxic and non-genotoxic carcinogens. In the area of genotoxicity, it was decided to validate the micronucleus test (MNT) *in vitro* based on existing data.

During the second taskforce meeting (November 2004) there was a consensus that, in addition to ongoing activities, it would be necessary to focus on the reduction and refinement of genotoxicity tests, since considerable *in vivo* testing is still required for confirmation of the genotoxic prediction.

Two workshops on CTA and MNT *in vitro*, respectively, have been organised in April 2004. In these workshops, it was agreed to prevalidate the CTA on both Balb/c 3T3 and SHE cell systems and to initiate a retrospective validation on the MNT *in vitro*.

The prevalidation studies on CTA are ongoing.

The retrospective validation of MNT *in vitro* is foreseen to be submitted to the ECVAM Scientific Advisory Committee (ESAC) for peer review in summer 2005.

In support to the validation activities, extensive research work is carried out in the ECVAM laboratories with the aim of developing a strategy which integrates the CTA, the MNT and the Comet assay *in vitro*.

The independent validation of the different tests will lead ultimately to the development of a refined strategy for genotoxicity and carcinogenicity, which has to be as safe as the current one but would consume less animals.

Lecture

Validation of toxicogenomics-based tests, a new generation of alternatives

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Toxicogenomics-based methods are being widely applied in toxicology and biomedical research. Since data are already being generated using these technologies, it is both timely and important to address the critical validation issues now with the aim of establishing a foundation that will facilitate future regulatory acceptance of scientifically valid toxicogenomics-based test methods. Addressing such issues early on, will also facilitate early buy-in and confidence in the technologies by the regulatory arena in its quest for new and improved methods by which to help ensure human health, protect the environment, and demonstrate responsiveness to animal welfare issues.

For that reason, the European Centre for the Validation of Alternative Methods (ECVAM), the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the National Toxicology Program (NTP) Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) have started to investigate the specific considerations necessary for adequate validation of toxicogenomics-based test methods. Experience in validation of conventional alternative test methods has led to an understanding that the validation approach will have to be adapted to the evaluation of methods based on toxicogenomics. The toxicogenomics field is rapidly evolving; therefore the validation process should accommodate the anticipated changes in the technology and must not be at the expenses of innovation. Moreover, other international Organizations as the OECD and the WHO/IPCS are currently drafting activity programs related to the possible use of toxicogenomics-based test methods for hazard and risk assessment purposes.

Poster

Modelling squamous metaplasia in an *in vitro* bronchial cell culture model: A flow cytometric analysis

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The development of squamous metaplasia in an *in vitro* bronchial cell culture model is being investigated as a tool to measure the effects of repeat exposure to airborne pollutants. Squamous metaplasia describes a potentially reversible event that may serve to protect the tracheobronchial lining from the effects of inhaling airborne pollutants but has also been linked to squamous cell carcinoma. The analytical methods used to assess pathological changes that occur in animals following repeated inhalation of toxic compounds are subjective and can not be quantified. Therefore it is difficult to obtain the reliable and reproducible data that is essential for the validation of a toxicity test. However, techniques and methods of analysis not practical in animal models can be combined in *in vitro* models. Squamous metaplasia is characterised by morphological and molecular changes including the replacement of the pseudostratified mucociliary epithelium with a stratified squamous epithelium and the expression of involucrin, filaggrin, a change in the cytokeratin profile, transglutaminase I and the formation of a cross linked envelope. Flow cytometry has been used to illustrate: Changes in the expression of these proteins to assess their contribution in response to different inducers; transglutaminase catalysed fluorescein cadaverine incorporation as a measure of activity; and annexin V binding as a measure of apoptosis, a form of programmed cell death that shares many characteristics in common with the squamous pathway. These investigations will lead to the establishment of a battery of end points signifying the initiation and development of squamous metaplasia.

Lecture

Animal carcinogenicity studies: Alternatives to the bioassay

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Traditional animal carcinogenicity tests take around three years to design, conduct and interpret. Consequently, only a tiny fraction of the thousands of industrial chemicals in use have yet been tested for carcinogenicity. Despite the cost of hundreds of millions of dollars, millions of skilled personnel hours, and millions of animal lives, several investigations have revealed animal carcinogenicity data to be lacking in human specificity (ability to distinguish human from animal carcinogens, where different), which severely limits its human utility. Causes include documented scientific inadequacies of the majority of carcinogenicity bioassays, and numerous serious biological and mathematical obstacles, which render attempts to accurately extrapolate human carcinogenicity assessments from animal data profoundly difficult, if not impossible. Proposed modifications have included the elimination of mice, the use of genetically-altered or neonatal mice, decreased timeframes, initiation-promotion models, greater incorporation of toxicokinetic and toxicodynamic assessments, quantitative structure-activity relationship (computerised) expert systems, *in vitro* assays, cDNA microarrays for detecting genetic expression changes, limited human clinical trials, and epidemiological research. Advantages of non-animal assays when compared to bioassays include superior human specificity results, greatly reduced timeframes, and greatly reduced demands on financial, personnel and animal resources. Inexplicably, however, regulatory agencies have been frustratingly slow to adopt alternative protocols. In order to minimise cancer losses to society, a substantial redirection of resources away from excessively slow and resource-intensive rodent bioassays, into the further development and implementation of non-animal assays, is strongly justified and urgently required.

Poster

Animal carcinogenicity studies: Poor human predictivity

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The regulation of human exposures to potentially carcinogenic chemicals constitutes society's most important use of animal carcinogenicity data. Environmental contaminants of greatest U.S. concern are listed in the Environmental Protection Agency's (EPA) Integrated Risk Information System (IRIS) chemicals database. However, of the 160 IRIS chemicals lacking even limited human exposure data but possessing animal data as of January 1, 2004, we found that in most cases (58.1%; 93/160) the EPA considered the animal data inadequate to support a classification of probable human carcinogen or non-carcinogen. For the 128 chemicals with human or animal data also assessed by the World Health Organization's International Agency for Research on Cancer (IARC), human carcinogenicity classifications were compatible with EPA classifications only for those 17 having at least limited human data ($p=0.5896$). For those 111 primarily reliant on animal data, the EPA was much likelier than the IARC to assign carcinogenicity classifications indicative of greater human risk ($p<0.0001$). The IARC is a leading international authority on carcinogenicity assessments, and the significant differences in human carcinogenicity classifications of identical chemicals between the IARC and the EPA indicate that: (i) in the absence of significant human data the EPA is over-reliant on animal carcinogenicity data, (ii) as a result, the EPA tends to over-predict carcinogenic risk, and (iii) the true human specificity, and hence utility, of animal carcinogenicity data is even poorer than indicated by EPA figures alone.

Poster

Animal carcinogenicity studies: Obstacles to human extrapolation

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Due to a paucity of human exposure data, risk classification and the consequent regulation of exposures to potential carcinogens has traditionally relied heavily upon animal tests. However, several investigations have revealed animal carcinogenicity data to be lacking in human specificity (the ability to distinguish human from animal carcinogens, where different). In order to investigate the reasons, we surveyed the 160 chemicals possessing animal but not human exposure data within the U.S. Environmental Protection Agency chemicals database that had received human carcinogenicity assessments as of January 1, 2004. We found a wide variety of species used, with rodents being predominant; a wide variety of routes of administration used, and a particularly wide variety of organ systems affected. The likely causes of the poor human specificity, and hence utility, of rodent carcinogenicity bioassays include (i) the profound discordance of bioassay results between rodent species, strains and genders, and further, between rodents and human beings; (ii) the variable and substantial stresses caused by handling and restraint and the stressful routes of administration endemic to carcinogenicity bioassays, with consequent effects on hormonal regulation, immune status and carcinogenesis predisposition; (iii) the differences in transport mechanisms and rates of absorption between test routes of administration and other important human routes of exposure; (iv) the considerable variability of organ systems in response to carcinogenic insults, between and within species, combined with the inability of commonly-used predictors of human carcinogenicity, such as the number of organ systems or sex-species groups effected, or fatalities, to withstand careful scrutiny; and (v) the inherent predisposition of chronic high dose bioassays towards false positive results, due to the overwhelming of physiological defences, and the unnatural elevation of cell division rates during ad libitum feeding studies. Such factors render attempts to extrapolate accurate human carcinogenicity assessments from animal data profoundly difficult, if not impossible.

Lecture

Humane endpoints and *in vitro* alternatives for sensitised genotoxicity screening

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Human risk-assessment for chemical compounds is often based on non-human model systems with arbitrary safety factors for extrapolation. In addition, large amounts of animals are used for this safety testing. Therefore, we propose to devise a human-based *in vitro* model system with a genome stability marker (lacZ-transgene) for mechanistic genome stability studies, and evaluate that model as a tool for realistic human risk assessment to genotoxic stressors. To increase the sensitivity of the human *in vitro* cell culture system, we will mimick the DNA-repair deficient phenotypes of specific knockout mice by knocking down DNA-repair proteins using an emerging innovative powerful tool called "RNA interference".

The DNA repair deficient phenotypes of two mouse models will be mimicked in our human system. The first knockout mouse model having a complete defect in nucleotide excision repair (NER), Xpa, was created in our own lab. The second mouse model, sensitive for genotoxic agents termed "clastogens" (genotoxic agents creating double-strand breaks), is deficient for Rad54 and its homologue Rad54B, two genes involved in DNA double-strand break repair (DSBR). Both mouse models are highly sensitive to genotoxic agents. Moreover, crosses of these mice with reporter mice carrying lacZ genes revealed mice able to detect genotoxic properties of chemical compounds in a wide variety of tissues. It is our expectation that comparing mutation frequency induction *in vivo* with *in vitro* and between species (human / mouse), will reveal whether *in vitro* assays are suitable for reliable safety measurements, and whether human cell systems will form a basis for a more accurate human risk-assessment.

This work was supported by ZonMw.

Poster

Inter-laboratory collaborative study of cell transformation assay for tumour promoters using Bhas 42 cells by non-genotoxic carcinogen study group in Japan

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The Bhas promotion assay is a cell culture transformation assay designed as sensitive and economical method for the detecting tumour-promoting activities of chemicals. In order to validate transferability and applicability of this assay, an inter-laboratory collaborative study was conducted with the participation of fourteen laboratories. After confirmation that these laboratories could obtain positive results with tumour promoters 12-O-tetradecanoylphorbol-13-acetate (TPA) and lithocholic acid (LCA), 12 chemicals were assayed under masked conditions. Each chemical was tested in four laboratories. For eight chemicals, all four laboratories obtained consistent results, and for two of the other four chemicals only one among the four laboratories showed inconsistent results. Thus, the rate of consistency was high. During the study, several issues were raised. Each issue was analysed step-by-step and resulted in protocol revision of the original assay. Among these issues were the importance of careful maintenance of mother cultures and the adoption of test concentrations for toxic chemicals. In addition, it is suggested that there are three different types of chemicals showing positive promoting activity in the assay. Those designated as T-type induced extreme growth enhancement, and included TPA, mezerein, PDD and insulin. LCA and okadaic acid belonged to the L-type category in which transformed foci were induced at concentrations showing growth-inhibition. In contrast, progesterone, catechol and sodium saccharin (M-type) induced foci at concentrations with little to slight growth inhibition. The fact that different types of chemicals similarly induce transformed foci in the Bhas promotion assay may provide clues for elucidating mechanisms of tumour promotion.

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Poster

The Syrian hamster embryo assay: An *in vitro* alternative to the rodent bioassay

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The Syrian hamster embryo (SHE) assay is actually the most predictive *in vitro* alternative to the 2 year rodent carcinogenesis (LeBoeuf et al., 1996). The assay is based on the ability of chemical/physical/biological agent to induce morphological transformation of SHE cells, which correlates with the potential to induce carcinogenesis. It is worthwhile noting that this assay possesses the ability to identify genotoxic as well as non-genotoxic carcinogens.

Hundreds (circa 400) of chemical/physical agents have been tested in the so called "low pH" version of the SHE assay with a sensitivity of 86%, a specificity of 83% and an 83% overall concordance with the rodent bioassay. Despite its good performances, the SHE assay has suffered a lot of criticism. This can be partly justified by the fact that the molecular events underlying the morphological transformation have not been fully elucidated. Furthermore, the visual scoring of the colonies (which is tedious and subjective) is far more the greatest weakness of this assay.

We have successfully set up and validated this assay in our laboratory. We are now committed in seeking molecular markers of morphological transformation. In doing so, we are confident that this will ease the scoring of the transformed phenotype and make it more objective. Preliminary steps of this research project addressed proteins involved in cell cytoskeleton (actin) and in cellular gap communication (connexin 43).

Poster

Differentially expressed genes in BALB/3T3 cells with exposure to non-genotoxic chemicals which promote cell transformation

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The cell transformation assay using BALB/3T3 cells is able to detect tumour promoters as transformation promoters. TPA, okadaic acid (OA), orthovanadate (VA) and p-nonylphenol (NP) significantly enhanced the cell transformation. To develop a short term assay identifying tumour promoters by detection of altered gene expressions, alterations in the gene expressions in BALB/3T3 cells exposed to these non-genotoxic transformation promoters were revealed using fluorescent mRNA differential display analysis and confirmed by RT-PCR. Elevated expressions of the following genes were induced: Ass1, Ly6e and Nudt9 by TPA and OA, Plat and Lgals3bp by OA, Ssb and Sned1 by NP. Decreased expressions of the following genes were induced: Thbs1 and an EST (BY594155) by TPA and OA, Vim by OA and NP, AI458795 and Sparc by TPA, Rbl3 by OA, ND1 by NP. TPA and OA caused common changes in the expression of several genes suggesting the existence of common actions on the cells between TPA and OA. However, the time courses of these changes were different between TPA and OA. No common gene was regulated by four non-genotoxic transformation promoters. It would be difficult to develop a simple assay method for tumour promoters utilizing detection of increased and decreased gene expressions. These data will contribute to clarifying the mechanisms of promotion by non-genotoxic chemicals during cell transformation and carcinogenesis.

Lecture

Detection of non-genotoxic carcinogens using ras-transfected Bhas 42 cells

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In vitro cell transformation tests can simulate the process of two-stage animal carcinogenesis, and propose a useful screening method for the detection of chemicals with carcinogenic activities. As formation of transformed foci is the consequence of the complex process of cell malignisation, the tests can be anticipated to be useful for the detection of not only tumour initiators but also tumour promoters and non-genotoxic carcinogens. In spite of this expectation, none of *in vitro* cell transformation tests have been accepted as a routine screening method, because the tests are thought to be laborious and time-consuming compared with the routine genotoxicity tests.

Ohmori et al. have developed an *in vitro* cell transformation assay for tumour promoters using Bhas 42 cells. The cells are v-Ha-ras-transfected BALB/c 3T3 cells. This transformation assay is a very sensitive method and has many advantages, such as shortened experimental period, use of less materials and simplicity of the procedure. Recently, in addition to the promotion assay we developed an assay method for the evaluation of initiating activity using these cells. When typical tumour initiators, were examined, transformed foci were induced in initiation assay but not in promotion assay. On the contrary, typical tumour promoters, were negative in initiation assay but positive in promotion assay.

Thus, Bhas initiation and promotion assays were suggested to be a highly sensitive screening method for the detection of chemicals with different mechanisms of transforming potential.

Lecture

The effect of the histone deacetylase inhibitor Trichostatin A on gap junctional intercellular communication in primary cultures of adult rat hepatocytes

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Background/aims: Trichostatin A (TSA), a histone deacetylase inhibitor, is known to induce cytodifferentation. The latter is partly controlled by gap junctional intercellular communication (GJIC). Hepatocellular gap junctions are composed of connexins Cx32 and Cx26, whereas Cx43 only becomes detectable upon dedifferentiation. Considering (i) the involvement of GJIC in cytodifferentiation, and (ii) the positive effects of TSA on this process, we raised the question whether TSA might affect hepatocellular GJIC.

Methods: Freshly isolated rat hepatocytes were cultivated and exposed to TSA. In another set of experiments, hepatocytes were isolated while TSA was present in the perfusion medium. Drug exposure was continued during consequent cultivation. RT-PCR was used to study Cx gene expression. Cx protein expression and localisation were investigated by means of immunoblotting and immunocytochemistry, while GJIC and albumin secretion were measured by using the scrape loading/dye transfer assay and ELISA, respectively.

Results: TSA induced the expression of Cx32, and downregulated the Cx26 production, the latter becoming located within the perinuclear compartment. TSA also promoted the appearance of Cx43, an effect that was only seen at the translational level. Overall, this resulted in enhanced GJIC and albumin secretion. Finally, the starting time of drug exposure was found to be a key parameter for the extent of the biological TSA outcome.

Conclusion: (i) TSA favours GJIC in primary cultures of rat hepatocytes, thereby suggesting a potential role for this compound in the optimisation of hepatocyte-based *in vitro* models. (ii) Cx proteins are likely to fulfil differential roles in the control of hepatocellular homeostasis.