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A transformational alternative for genotoxicity hazard and risk assessment is proposed to the current standard regulatory test battery. In principle, the proposed approach consists of a single in vitro test system with high genomic sequence homology to humans that addresses the relevant principal genetic lesions assessed in the current test battery. The single test system also possesses higher throughput attributes to permit the screening of large numbers of compounds and allow for an initial differentiation of genotoxic mechanisms (i.e., direct vs. indirect mechanisms) by how the hazard end point is measured. To differentiate compounds showing positive results, toxicogenomic analysis can be conducted to evaluate genotoxic mechanisms and further support risk assessment. Lastly, the results from the single test system can be followed up with a complementary in vivo assessment to establish mechanistic relevance at potential target tissues. Here, we propose the in vitro (yeast) DNA deletion (DEL) recombination assay as a single test alternative to the current genotoxicity test battery with a mechanistic follow up toxicogenomic analysis of genotoxic stress response as one approach that requires broader evaluation and validation. In this assay, intrachromosomal recombination events between a repeated DNA sequence lead to DNA deletions, which have been shown to be inducible by a variety of carcinogens including those both negative and positive in the standard Salmonella Ames assay. It is hoped that the general framework outlined along with this specific example will provoke broader interest to propose other potential test systems.

Key Words: genotoxicity; carcinogenesis; mechanisms; risk assessment; toxicogenomics.
information regarding mechanisms or relevance and often lead to the early conduct of costly and time-consuming carcinogenicity assessments in rodents.

As the ICH testing guidelines undergo discussions for revision, a transformational alternative to the current test battery is proposed. This transformational paradigm proposes the use of a single in vitro test system, the yeast DNA deletion (DEL) recombination assay. This test system possesses high genomic sequence homology to humans that addresses the relevant principal genetic lesions assessed in the current test battery and appears to possess higher predictivity for carcinogenic outcomes. This test system is also amenable to larger scale compound screening, applying toxicogenomics to evaluate mechanism of action and a complementary follow up assessment in vivo to establish mechanistic relevance at potential target tissues of highest exposures.

CHARACTERISTICS OF A SINGLE TEST SYSTEM

An ideal single test system should detect the principal forms of DNA damage with high concordance to carcinogenic outcome, namely mutational events at the DNA sequence level as well as larger chromosome effects. In the current in vitro test battery, the detection of mutations is specifically addressed using the bacterial reverse mutation assay. A second mammalian cell assay is also required to determine if a test article causes structural or numerical chromosome effects. The two regulatory assays used for this purpose are the in vitro chromosome aberration assay and the mouse lymphoma thymidine kinase forward mutation assay, with the in vitro micronucleus assay also now being considered as a viable alternative. Although these assays are effective in the identification of chromosome damaging compounds, they also suffer from a high rate of false positive results in terms of predicting carcinogenic outcomes (Kirkland et al., 2005; Muller et al., 1999; Snyder and Green, 2001).

The ideal single test system should sensitively detect currently recognized genotoxic compounds, particularly those with known carcinogenic or germ-line effects. It will be critical to evaluate and validate the new assay system against a wide variety of compounds, preferably with well-characterized genotoxicity, carcinogenicity, or germ-cell data as well as with mechanism of action information to permit an unbiased assessment of predictivity and overall utility of the test system. The test system also needs to be amenable to the testing of the wide variety of chemical entities from environmental contaminants, industrial chemicals, and biologically active compounds and also have the potential to evaluate biologic test substances, particularly those containing modified RNA and DNA bases. Ideally, the system would also provide metabolism of the test article in a manner similar to human exposure with both primary and secondary metabolic reactions that would adequately mimic relevant in vivo physiological conditions.

Due to the genetic nature of many human cancers and important genetic differences between humans and the rodent carcinogenicity models as well as the in vitro systems currently used to assess genotoxicity, an ideal system should have a genome with high-sequence homology to humans. Provided a similar genome, functional genomic and systems biology tools could be applied to increase the power and predictivity of the new model. For example, analyses of effects on specific oncogenes, tumor suppressor genes, or other cellular processes critical to homeostatic functions could provide clues to the mechanism of action and could even provide information on genetic versus epigenetic causes for cancer induction.

An understanding of mechanism of action is crucial for evaluating the biological relevance of genotoxicity and carcinogenicity findings and ultimately assessing cancer risk associated with chemical exposure. Therefore, a single test system should incorporate mechanism-based risk assessment in addition to hazard identification. Conceptually, a single test system should (1) detect the principal genetic lesions as outlined previously and have a higher predictivity of carcinogenic outcomes; (2) provide insight into genotoxic and carcinogenic mechanisms; (3) enable human risk assessment by evaluating the biological relevance of findings by translating the test system’s outcomes to in vivo, and (4) for purposes of efficiency possess higher throughput attributes to enable the screening of large numbers of chemicals. Here, we propose one alternative system which involves initially assessing the effects of chemicals on genome integrity followed by investigating genotoxic mechanisms via functional genomic and system biology approaches.

A PROPOSED ALTERNATIVE MODEL

We propose the in vitro (yeast) DNA deletion (DEL) recombination assay as a single test alternative to the current genotoxicity test battery. It is well accepted that genetic instability such as inversions, translocations, deletions, and microsatellite instability are mechanistically involved in the development of cancer, and several human cancer–prone diseases feature an elevated frequency of recombination and genome rearrangements (consult citations in Bishop and Schiestl, 2003). Because of the association among recombination, deletion, and carcinogenesis, assay systems detecting the frequency of intrachromosomal homologous recombination, resulting in genomic deletions (DEL), have been developed in several organisms. These include yeast (Schiestl, 1989), human cells (Aubrecht et al., 1995), and mice (Schiestl et al., 1997a). In all of these systems, genotoxic stress induces DNA deletions.

The association between carcinogenesis and DEL recombination has been established twofold. First, transgenic mouse models that are predisposed to cancer such as through mutations in Atm (Bishop et al., 2003; Reliene et al., 2004), p53 (Bishop et al., 2003), and Wrn (Lebel, 2002) show elevated frequencies of DEL events. Furthermore, an
antioxidant that causes a significant reduction in tumor frequency in Atm-deficient mice (Reliene and Schiestl, 2006) also significantly reduced the frequency of DEL events (Reliene et al., 2004). Secondly, DEL recombination in yeast (Brennan and Schiestl, 1997a,b, 1998a,b, 1999, 2004; Brennan et al., 1996; Carls and Schiestl, 1994; Schiestl, 1989; Schiestl et al., 1989a,b; Schiestl et al., 1997b) as well as in vivo in mice (Kirpnick-Sobol et al., 2006; Reliene et al., 2004; Schiestl et al., 1994; Schiestl et al., 1997a,b) is induced by a broad range of compounds, which, although tumourigenic in long-term rodent bioassays, are poorly detected by most other genotoxicity assays (Supplemental Table S1). Mechanistically, the DEL recombination event is initiated mostly by DNA breaks and results in a deletion (Galli and Schiestl, 1995) (Fig. 1). Consequently, it appears that there may be a high correlation between clastogenicity and DEL recombination activity (Kirpnick et al., 2005). In addition, by changing the selection scheme, the assay can be used to detect aneugenic activity (Howlett and Schiestl, 2000, 2004). Thus, we propose to use this selection scheme in addition to detect aneugenic activity.

The DEL assay is sensitive to a variety of DNA lesions occurring as a consequence of compound-DNA interaction or in response to indirect chemically induced stresses such as reactive oxygen species generation (Brennan and Schiestl, 1997b, 1998c, 1999; Brennan et al., 1994, 1996). Regarding underlying genotoxic mechanisms, DEL recombination can differentiate direct (DNA reactive) from indirect mechanisms based on the shape of the dose response. Direct-acting compounds display a linear dose response, whereas indirect-acting compounds display a threshold response, only at cytotoxic concentrations (Galli and Schiestl, 1995). In addition, the assay has been shown to differentiate between structurally similar carcinogenic and noncarcinogenic isomers, ethionine/methionine, o-toluidine/2,4-dimethoxy aniline as well as 2,4-diamino toluene/2,6-diamino toluene (Brennan and Schiestl, 1997b; Carls and Schiestl, 1994).

To address the needs for higher screening efficiency, the yeast DEL assay has recently been developed into a high throughput version in 384-well plates (Hontzeas et al., in press). Since the yeast cell wall limits uptake of particularly large lipophilic compounds, a strain with a permeable cell wall has been recently developed (Hontzeas et al., in preparation) and could enhance the utility of the yeast DEL assay for evaluating a broader spectrum of chemicals. Since the principle of the yeast DEL recombination assay is easily transferable to the in vitro mammalian cell (Aubrecht et al., 1995) and in vivo models (Schiestl et al., 1997a), it provides the opportunity to establish the biological relevance of the in vitro findings and relate it to the whole animal.

The cellular response to stress triggers a complex web of molecular pathways involved in repair, survival, and/or cell death. Any component of the response can be regulated at the transcriptional or translational levels, during posttranslational modifications, and/or by specific molecular interactions. Thus, the development of testing approaches with potential to provide a basis for generating mechanistic information in the framework of the proposed ‘‘single test-based’’ paradigm relies on a subsequent application of functional genomic and systems biology tools to evaluate genotoxic mechanisms. Although these molecular approaches have been established only recently, there are several lines of evidence suggesting their utility. For instance, functional genomic approaches utilizing panels of yeast mutants have been used to assign protein networks involved in damage by a simple alkylating agent, an agent producing bulky DNA-base adducts, UV radiation, and an oxidizing agent (Said et al., 2004). Furthermore, 60 human tumor lines–based approach provided patterns or ‘‘fingerprints’’ for six general mechanisms of action, and a neural network analysis was capable to assign these 129 agents to the correct category (Weinstein et al., 1992). A gene expression profile analysis, toxicogenomic approach, has been proposed to study toxic mechanisms (Nuwaysir et al., 1999), and up to date there are more than 1100 publications involving stress-gene expression profiling. In regard to DNA-damaging agents, the initial studies evaluating responses to treatment with prototypic genotoxic agents in the yeast (Jelinsky and Samson, 1999) and mammalian cells (Fornace et al., 1999) showed triggering of a complex web of genes and pathways and provided foundation for further studies.

![FIG. 1. The yeast DEL recombination system measures the recombination frequency between two tandem his3Δ alleles (his3Δ3′, his3Δ5′), each containing ~400 bp of homology to the other allele. Homologous recombination between the two his3Δ alleles results in the deletion (DEL) of intervening sequences to give a functional HIS3 gene and a reversion to histidine prototrophy. Possible mechanisms that can lead to recombination between the two his3Δ alleles are single-strand annealing, one stranded invasion, intrachromosomal crossing over, sister chromatid exchange, and sister chromatid conversion (Galli and Schiestl, 1995; Galli and Schiestl, 1998). Cells are exposed to test agents in liquid culture and subsequently washed, counted, diluted, plated, and scored for survival and HIS reversion on selective media plates.](image-url)
The potential for gene expression profiling to facilitate genotoxicity risk assessment has been extensively reviewed (Aubrecht and Caba, 2005). Briefly, the cellular stress response in mammalian cells is quite complex involving hundreds and possibly thousands of genes (Amundson et al., 2005). It has been shown that toxicogenomic analysis can differentiate genotoxic from nongenotoxic carcinogens in HepG2 cells and direct from indirect genotoxic mechanisms including cytotoxicity in TK6, L5178Y, HepG2 cells, and yeast (consult citations in Aubrecht and Caba, 2005). Despite the variability in reported gene expression profiles, common pathway features are apparent. Interestingly, only a relatively small number of DNA repair genes have been shown to be DNA damage inducible, and the majority of inducible genes mediate regulatory processes involved in cell cycle control, apoptosis, senescence, and inflammation (Amundson and Fornace, 2003). The genotoxic stress response involves activation of several pathways including p53, mitogen-activated protein kinase cascades, nuclear factor-kappa B, and the API transcription factors (consult citations in Amundson and Fornace, 2003; Aubrecht and Caba, 2005). There is increasing evidence that the same molecular pathways observed in in vitro test systems are also found in animal studies (Ellinger-Ziegelbauer et al., 2004, 2005) and might be useful as potential biomarkers of exposure (Amundson et al., 2000). Recently published studies provide further evidence that genomic- and system biology–based approaches might provide tools applicable for investigating mechanisms of toxicity (Amundson et al., 2005; Lamb et al., 2006). The most comprehensive study published to date interrogated gene expression profiles of 13 agents with a broad variety of mechanisms of action (Amundson et al., 2005). The results clearly indicate the potential of the toxicogenomic analysis of the stress response to differentiate genotoxic from nongenotoxic stresses. It also shows that key regulatory factors such as p53 pathway display important quantitative and qualitative differences in their activation by different types of genotoxic stress.

Regarding the dose-response relationship for observed changes in toxicogenicomic analysis of the genotoxic stress response, studies with ionizing radiation have conclusively shown that gene induction of typical p53-regulated genes such as Cdkn1a (p21) and Gadd45a shows a linear dose response and occurs at low doses in both human cell lines (Amundson et al., 2000) and in primary cells (Amundson et al., 1999). On the other hand, the detection of benzo(a)pyrene diol epoxide-DNA adducts and DNA-cisplatin crosslinks occurred at lower concentrations than the gene induction (Akerman et al., 2004; Dickinson et al., 2004), suggesting low sensitivity of the toxicogenicomic approach. Although one cannot exclude that stress responses at low doses might provide unique responses in some cases (Coleman et al., 2005), it has been suggested that persistent DNA damage capable of invoking a global DNA damage response is necessary for robust activation of signaling pathways that can affect gene expression detectable on the mRNA level (Rouse and Jackson, 2002). There are several implications to such observations. The first is that the signal to noise ratio will be greater at higher doses for many stress response genes. The second is that since many of these responses are immediate early genes, robust changes in gene expression can be detected within several hours and prior to the onset of appreciable cytotoxicity (Amundson et al., 2005). Taken together, it might be preferable to choose a dose that induces a robust response, particularly if the toxicity of the drug exhibits a threshold pattern, when developing gene signatures for classifying chemical agents. Therefore, an appropriate experimental design to apply toxicogenomics to study mechanisms in this proposed test system is essential.

CONCLUSION

A proposed alternative model to genotoxicity testing exploits the mechanistic link between genome instability and carcinogenesis for the initial hazard assessment followed by molecular mechanistic analysis of the stress response to support risk assessment of positive findings. The proposed single test system consists of the in vitro (yeast) DEL recombination assay supplemented by mechanistic characterization of stress responses via toxicogenomic analysis for agents inducing DEL recombination. The potential acceptance of this proposed alternative by the scientific community and regulatory agencies will require broader evaluation and a more thorough validation using chemicals with known genotoxic and carcinogenic properties (both those tested through the National Toxicology Program and pharmaceutically relevant agents) via the Interagency Coordinating Committee on the Validation of Alternative Methods/European Centre for the Validation of Alternative Methods processes. The scientific and technological characteristics of the proposed testing approach, if validated and implemented, would facilitate genotoxic risk assessment for both drug candidates and also industrial chemicals as required by the EU Registration, Evaluation, Authorisation and Restriction of Chemicals initiative (Combes et al., 2006). The advantage of the DEL assay resides in its ability to detect cancer-relevant changes in a single test system amenable to higher throughput and automation. Furthermore, the shape of the dose response for DEL recombination induction in relation to cytotoxicity could be used as a first criterion to differentiate direct (DNA reactive) from indirect genotoxic mechanisms, and thereby serve to prioritize the need for further toxicogenomic evaluation. With the appropriate experimental design, toxicogenomic analysis would be directed to investigate the nature and biological relevance of DEL recombination induction in mammalian cells or in animals in vivo, thus enabling a more mechanism-based risk assessment as a follow up in place of conducting the conventional secondary genotoxicity tests that may have questionable relevance to evaluating carcinogenic risks.
SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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