Proof of Principle: Detection of Genotoxicity by a Fluorescence-Based Recombination Test in Mammalian Cells

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Summary
Genotoxicity tests available today have several shortcomings. The widely applied Ames assay measures mutations in bacteria, thereby disregarding the physiological particularities of the human cell and organism.
We provide first evidence for a new concept of genotoxicity detection in living human cell cultures. The data were obtained by use of a newly developed assay, which is based on the quantification of fluorescent signals, i.e. counting of the relative number of fluorescent cells in the sample. It is characterised by a short reaction time and fulfills the requirements for automated performance. The new system monitors chromosomal rearrangements and, therefore, is predicted to detect a broad spectrum of genotoxic substances. Indeed, we demonstrate the genotoxic effect of ionising radiation, of an Ames assay positive compound, and of two compounds which are poorly mutagenic in the Ames assay. The new assay will be optimised further and adapted to the requirements for routine analysis in order to help to further reduce animal experimentation in genotoxicity testing.

Zusammenfassung: Beweis des Wirkungsprinzips: Nachweis genotoxischer Aktivitäten mit einem Fluoreszenz-basierten Rekombinationstest in Säugerzellen
Die Aussagekraft der heute verfügbaren Genotoxizitätstests leidet unter gewissen Limitationen. So mißt beispielsweise der am häufigsten angewandte Test, der Ames Assay, Mutationen in Bakterien, wobei die physiologischen Besonderheiten der menschlichen Zelle und des menschlichen Organismus unberücksichtigt bleiben.
In dieser Arbeit erbringen wir erste Beweise für ein neues Prinzip zur Detektion von genotoxischer Wirkung direkt an kultivierten menschlichen Zellen. Die Daten wurden mit Hilfe eines neuentwickelten Tests erbracht, welcher auf der Quantifizierung von Fluoreszenzsignalen basiert, indem er einfach leuchtende unter nichtleuchtenden Zellen in der Probe zählt. Darüber hinaus zeichnet er sich durch eine kurze Reaktionszeit aus und erfüllt die Voraussetzungen für die Automatisierung des Verfahrens. Der neue Test weist chromosomale Rearrangements nach, weshalb die Detektion eines breiten Spektrums von genotoxischen Agentien vorhergesagt wird. In dieser Arbeit demonstrieren wir, dass der Test multiple genotoxische Effekte erfaßt, nämlich von ionisierender Strahlung, einer im Ames Assay positiven Substanz und zwei Substanzen, welche im Ames Assay nur schwer nachzuweisen sind. Im nächsten Schritt wird das Verfahren weiter optimiert und standardisiert werden, um eine Reduktion der Tierversuche bei der Genotoxizitätsprüfung zu unterstützen.

Keywords: genotoxicity, recombination repair, EGFP fluorescence

1 Introduction

Human beings are exposed to mutagens occupationally, accidentally, during medical treatments, or by their lifestyle. To minimise the risk of cancer, genotoxicity must be excluded before marketing a new physiological substance in pharmaceutic, cosmetic and food products. Cancer is a disease affecting mammals, and that is why it is still obligatory to directly assay potential carcinogens by testing whether they induce tumor growth. However, in addition to causing tumors in animal cells, most carcinogens are genotoxic, i.e. cause genetic alterations, and, thereby, accelerate the multistep process of cancer formation. Based on this insight, several genotoxicity tests have been developed. Today, the most frequently used test is the Ames assay or Reverse mutation assay (Ames et al., 1973). The assay is based on the reversion of mutations in the histidine (his) operon of the bacterium *Salmonella typhimurium*. Growth on histidine-free

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medium plates provides a selection method for revertant strains, which appear at an elevated frequency upon exposure to genotoxic agents. Depending on the strain and the mutagen used, base substitutions or frame shift mutations can be detected. However, the Ames assay has several limitations and can therefore not detect 10% of known human carcinogens (Ashby and Tennant, 1991). First, the detection of genotoxic effects is limited to point mutations, but carcinogens frequently cause large chromosomal genome alterations, such as translocations, deletions, expansions, and gene amplifications, which initiate leukemia and solid tumor formation (Tlsty et al., 1995; Loeb and Loeb, 2000). Therefore, in order to detect all human mutagens, the *Salmonella* assay should be combined with a cytogenetic assay that monitors chromosomal aberrations. Second, *Salmonella* does not possess the enzymatic machinery of mammals, which can convert pro-mutagens into reactive metabolites. In an attempt to overcome this problem, rat liver extracts are frequently added to the tests.

There are several assays utilising mammalian cells to detect the presence of potential mutagens. Thus, in the cultivated CHO hamster cell line, the appearance of cell clones which are resistant to 6-thioguanine in response to treatment with a harmful substance indicates point mutations within the HPRT gene (O'Donovan, 1990). Similarly, by selecting mutants in culture medium supplemented with the thymidine analogue trifluoro-thymidine, mouse lymphoma cells are used to determine whether a test material has the capacity to mutate the thymidine kinase gene (Moore et al., 2002). Cytogenetically, the effect of harmful substances is detected by the increase of sister chromatid exchanges (SCEs) in bone marrow cells (Hadnagy et al., 1989). Alternatively, the comet assay offers a visual method for quantitative assessment of DNA breakage (Speit and Hartmann, 1999). Another cytogenetic method, the micronucleus assay, has been used to monitor exposure to genotoxic agents by quantifying the formation of acentric chromosome fragments or complete chromosomes which are excluded from the nucleus (Tucker and Preston, 1996). These mammalian *in vitro* test systems have several shortcomings. Thus, the detection of 6-thioguanine- or trifluoro-thymidine-resistant cells requires the time consuming culture and quantification of colonies or the microscopic cell analysis after replicative incorporation of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU). On the other hand, it is still difficult and time consuming to quantitatively evaluate genetic alterations by conventional cytogenetics, as the cells have to be prepared, fixed, stained, and analysed microscopically. Moreover, a treatment with colchicine or BrdU is required before preparing cells for the detection of SCEs, and with cytochalasin B for the micronucleus assay. Problems with the Comet assay are related to the reproducibility of the results, as they are influenced by the fragility of the cells, the nuclear membrane, and other cellular components. Importantly, deletions or smaller rearrangements escape detection by all cytogenetic methods.

Since it is unclear whether mammalian liver homogenates added to the Ames assay accurately reflect the situation in the living mammal, metabolism dependent effects are still tested by animal experiments in genotoxicology. The most widely used transgenic mouse mutagenesis test is the Stratagene "Big Blue™ Transgenic Mouse Mutagenesis Assay System", which is marketed by the Stratagene Company. This system relies on multiple copies of a bacteriophage lambda shuttle vector, which were integrated into the genome of C57BL/6 mice. Upon genotoxic treatment of the animal, the phage genomes are transduced into bacteria, where point mutations within the lacI gene can be screened phenotypically with a chromogenic ß-galactosidase substrate. However, the Stratagene system does not detect several potent mutagens, like methylmethane sulfonate (Mirsalis et al., 1993), possibly due to the fact that unphysiologically high basal rates are generated in the bacteria by spontaneous mutations within the phage genomes (Friedberg, 1984). Other types of animal experiments are performed in cancerogenicity testing to watch tumor formation directly and to monitor physiological parameters like changes in body weight, water consumption, blood chemistry, and histopathological changes. At least three doses of the substance have to be tested and at least 50 animals have to be sacrificed per group from at least one mammalian species, so that ethic and economic reasons strongly support the development of alternative methods.

DNA recombination processes are the biochemical events underlying cancerogenic chromosome rearrangements like gene amplifications, deletions, and translocations (Pierce et al., 2001). To take into account that chromosomal rearrangements larger than the point mutations detected by the classical Ames assay are carcinogenic and to avoid shortcomings caused by the use of microorganisms, we developed a novel assay that monitors recombinative rearrangements in cultivated human and other mammalian cells. So far, systems for probing recombination in mammalian cells were either based on time consuming procedures selecting for survival phenotypes or on the rescue of Simian virus 40 in a plaque assay (Wiesmüller et al., 1996; Lambert et al., 1999). In contrast, we describe a fast read-out assay system which utilises an enhanced green fluorescent protein (EGFP) gene reporter.

2 3R-relevance

Clearly, there are differences in the metabolic activation of potentially harmful chemicals in bacteria and mammals, but, more importantly, also between rats and human beings (Doehmer and Jacob, 1994). Therefore, results from animal experimentation do not accurately describe the genotoxic and tumor promoting activities of chemical agents. This is why a genotoxicity assay, like the fluorescence-based assay described here, which is applicable to human cells or to cells which are human-like in terms of the responses to genotoxic treatment (DNA fragility and repair, growth regulatory and apoptotic response, metabolism), carries the potential to improve genotoxicity testing and to reduce animal experimentation.

3 Materials and methods

3.1 Cell culture and establishment of transgenic test cell lines

KMV cells, which represent the human myeloid leukemia cell line K562, stably expressing the Gal4-chimera, GalER-VP16 (Braselmann et al., 1993), were cultured at 37°C, 5% CO_2, in phenol red free RPMI 1640 (Gibco BRL Life Technologies). The medium was supplemented with 12% fetal calf serum, stripped by stirring 1 l with 10 g charcoal (Norit A, Merck) and 1 g Dextran 40 (Merck) for 30 min and subsequent centrifugation at 13,000 x g. Stable test cell lines, carrying the EGFP-based recombination substrates, were established after electroporation of KMV cells with vector EJ-EGFP/3'EGFP (KMV-EJ), Δ-EGFP/3'EGFP (KMV-Δ), and HR-EGFP/3'EGFP plus pSV53her (Roemer and Friedman, 1993) or HR-EGFP/3'EGFP only (KMV-HR), followed by soft agar cloning in phenol red-free RPMI with 12% fetal calf serum. Antibiotic selection of clones with genomically integrated constructs was achieved by the addition of 0.25 mg/ml puromycin. For genotoxicity testing we used clones which had been screened by genomic PCR for the integrity of the chromosomally integrated constructs. The generation of the recombination constructs and the PCR method were described previously in Akyüz et al. (2002).

3.2 Genotoxic treatment

For the targeted introduction of DNA double-strand breaks into each tester strain, 10^7 cells were electroporated (240 V, 1050 mF) with 10 µg of pCMV-I-SceI plasmid DNA each (Rouet et al., 1994). In parallel, 10^7 cells were electroporated with the same amount of pBS control vector. Subsequently, cultivation was continued in fresh medium until harvest.

For the analysis of the carcinogenic compound etoposide, KMV-EJ cells were seeded at a density of 10^5 cells per ml. 16 h afterwards, etoposide (Sigma) was added at increasing concentrations to the culture medium of split cultures, to result in final etoposide concentrations of 0 µM, 1 µM, 10 µM, or 100 µM. 2 h later the medium was replaced with fresh medium and the cells were incubated for a further 72 h. To analyse the effect of estradiol, KMV-EJ cells were cultivated in medium without or with 200 nM 17ß-estradiol (Sigma) during the whole incubation period of 72 h. NH_4OH, at a final concentration of 4 mM, was included in the culture medium of KMV-HR cells for 24 h.

To test the effect of ionising radiation on EGFP reactivation, we subjected parallel dishes of overnight cultures of different tester strains to γ-ray treatment (^{137}Cs source) at a dose of 5 Gy, followed by a change of medium. Care was taken to make sure that the cells were sedimented on the culture dish surface as a cell monolayer.

3.3 Detection of recombinative repair as the basis of the assay design

There is accumulating evidence that deregulated recombination processes are associated with cancer (Pierce et al., 2001) and with a broad spectrum of carcinogenic treatments (Schiestl, 1989; Brennan et al., 1994; Zhang et al., 1996; Schiestl et al., 1997; Aubrecht et al., 1999). To exploit DNA recombination as the endpoint for genotoxicity testing, we designed a short-term assay on the basis of fluorescence analysis in living, mammalian cells (Fig. 1).

Homologous recombination repairs DNA strand breaks, crosslinks, and other DNA lesions that remain unrepaired until encountered by a replication fork (Flores-Rozas and Kolodner, 2000; Khanna and Jackson, 2001). During conservative homologous recombination, identical or similar sequences that are located either on the same (intramolecular) or on different (intermolecular) DNA molecules are copied in such a way that the damaged acceptor copy is fully reconstituted by the donor template (Fig. 2).

Nonconservative events are accompanied by the loss of DNA sequences between the homologous sequences, and this in turn causes serious chromosomal alterations, such as deletions.

EGFP is a mutated version of the green fluorescent protein from marine vertebrates which emits bright autofluorescence upon excitation with light at a wavelength of 488 nm (Cubitt et al., 1995). EGFP owes its fluorescence characteristics to a chromophore generated by three central amino acids. The process of chromophore formation is extremely rapid, which is why EGFP is an attractive reporter molecule to study biological processes in real time. Therefore, our test system was designed in such a way that recombinative DNA exchange processes involving differentially mutated EGFP genes reactivate the intense autofluorescence of EGFP (Fig. 2). Thus, upon successful recombination, green fluorescent cells appear among the test cells, and the fraction of fluorescent cells within a population of nonfluorescent cells is easily quantified by well-established methods, such as fluorescence-activated cell analysis (FACS analysis) by use of a FACScan (Fig. 1). One of three

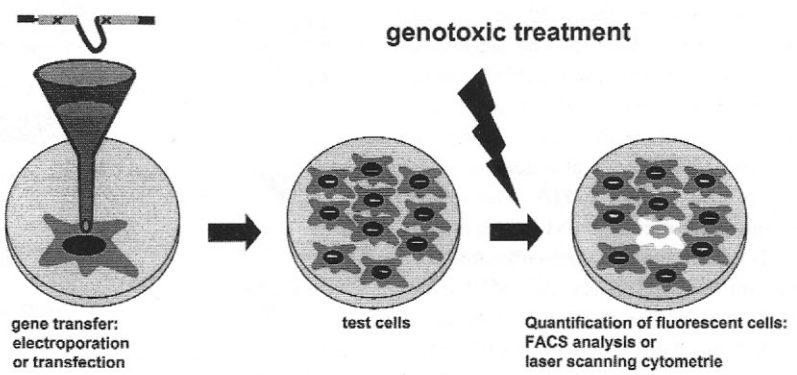

Fig. 1: Fluorescence based assay for the identification of genotoxicities.

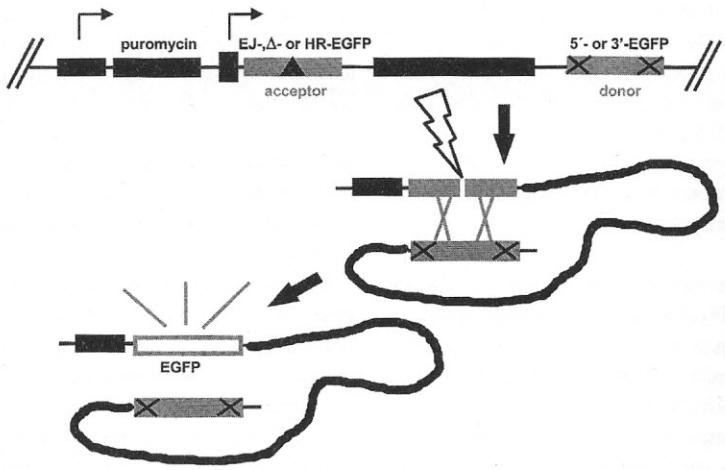

Fig. 2: Principle of the test system: recombination between EGFP genes.

EGFP gene variants (EJ-/Δ-/HR-EGFP), which were differentially mutated within the chromophore coding region, was inserted at the acceptor position following a promoter for EGFP protein production (Fig. 2). At the donor position we inserted either the 3'truncated 5'EGFP or the 5'truncated 3'EGFP. The three constructs, resulting from all possible acceptor-donor gene combinations, supported all classes of homologous recombination, i.e. conservative (5'EGFP and 3´EGFP) and nonconservative (3'EGFP) events involving short (Δ-EGFP) and long (HR-EGFP, EJ-EGFP) stretches of homologies (Akyüz et al., 2002).

3.4 Quantification of EGFP reactivation

At the indicated times, after the introduction of the EGFP-based recombination substrate, of the I-SceI expression vector, after radiation, or the addition of a test compound, 1-2 ml of the cell suspension were collected by centrifugation, washed in 1-5 ml phosphate buffered saline supplemented with 0.2% EDTA (PBS-EDTA), and resuspended at a density of 10^6 cells per ml in PBS-EDTA. The cells in the sample were analysed flow cytometrically in a fluorescence-activated cell analyser (Coulter Epics® XL-MCL). The EGFP reactivation frequency was determined from the fraction of green fluorescent cells. Mean values were calculated for one representative test cell line per tester strain (KMV-EJ, KMV-Δ, KMV-HR) and 3-6 independent measurements each. After ß-estradiol and etoposide treatment one representative KMV-EJ clone was tested accordingly. The KMV-HR tester strain served to analyse the genotoxic effect of NH_4OH.

4 Results

4.1 EGFP reactivation in test cells

In this work, we selected KMV cells, originating from the human erythroleukemia cell line K562, to generate test cells. For gene transfer, we applied the physical method of electroporation. In our first approach, we utilised the cells 72 h after electroporation, to demonstrate EGFP reconstitution as a consequence of homology-directed DNA exchange processes. We counted the number of green fluorescent cells flow cytometrically and separated the intensely green fluorescent cells from weakly orange autofluorescing cells by two-colour fluorescence dot plot analysis of the resulting FACS data (Fig. 3).

With the Δ-EGFP/5'EGFP recombination substrate, a fraction of 2.6% of green fluorescent cells appeared above the diagonal within the dot plot. For comparison, the negative control vector, devoid of a donor gene, did not cause the appearance of green fluorescent cells. The positive control vector with wild-type EGFP gave rise to 36.7% of EGFP positive cells. After electroporation with the Δ-EGFP/3'EGFP recombination substrate, 7.0% of the cells showed green fluorescence, indicating a recombination frequency increase from 2.6% to 7.0% due to the contribution of nonconservative homologous recombination events. Differently, the replacement of Δ-EGFP by the acceptor genes HR-EGFP or EJ-EGFP did not significantly alter the recombination frequencies. Thus, EGFP reconstitution was readily detectable, especially with constructs carrying the 3'EGFP donor gene. However, in these assays the basal recombination frequencies were extremely high even in the absence of any genotoxic stimulus. This observation can be explained by the fact that transiently electroporated cells carry multiple, naked DNA copies. The pres-

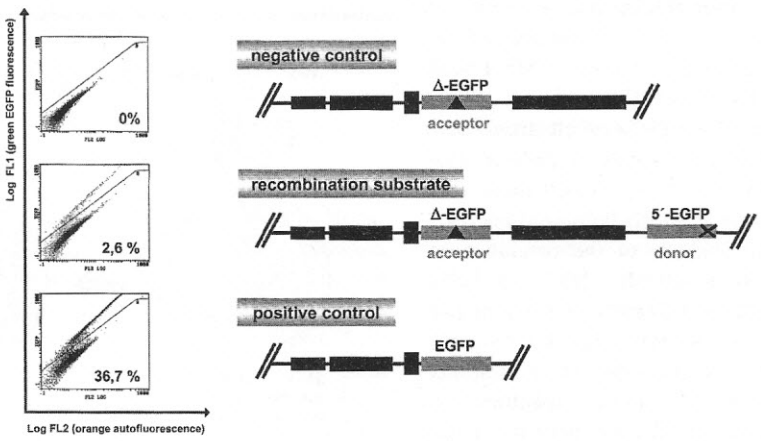

Fig. 3: Reconstitution of EGFP after electroporation with recombination plasmid.

Tab. 1: Recombination initiation by targeted double-strand break formation.

		EGFP reactivation frequency [10^{-4}]		
incubation time		48 h	72 h	96 h
I-SceI	cell lines			
- I-SceI	KMV-EJ	0	0	0
	KMV-Δ	0	0	0
	KMV-HR	0	0	0
+ I-SceI	KMV-EJ	4	12	15
	KMV-Δ	3	11	14
	KMV-HR	2	9	19

ence of naked DNA itself triggers repair mechanisms (Siegel et al., 1995). Consequently, we chose to stably introduce the recombination substrate into the genome of the mammalian cell, in order to achieve low basal levels and a satisfactory signal-to-noise-ratio for subsequent genotoxicity studies. We selected clones with stably integrated constructs on the basis of the co-transferred puromycin-resistance gene and screened for the integrity of the recombination substrates by genomic PCR. Green fluorescent cells did not appear spontaneously in the culture, indicating the desirable, low basal frequency of recombination in these new tester strains.

To demonstrate that the integrated DNA copies were actively participating in recombination, double-strand breaks were introduced. For that purpose we transiently expressed the I-SceI meganuclease which specifically recognises a sequence of 18 base pairs (Rouet et al., 1994). This recognition sequence formed part of the acceptor genes Δ-, EJ-, and HR-EGFP, so that double-strand breaks could be generated within the recombination substrate. Consequently, we electroporated our test cells with the I-SceI expression vector pCMV-I-SceI (Rouet et al., 1994) and performed flow cytometric analysis after 48 h, 72 h, and 96 h of further incubation (Tab. 1).

I-SceI expression (+ I-SceI) caused EGFP reconstitution with fluorescent signals increasing until 96 h after electroporation (14-19 x 10^{-4}). For cells carrying the EJ-EGFP/3'EGFP (KMV-EJ), the Δ-EGFP/3'EGFP (KMV-Δ), or the HR-EGFP/3'EGFP (KMV-HR) substrate, similar frequencies were determined. On the contrary, after electroporation with the control vector pBS (- I-SceI), EGFP-positive cells were below 10^{-4}, indicating a more than 10 fold recombination induction by specific I-SceI cleavage. Conversely, in transiently electroporated cells with high basal rates of up to 7%, I-SceI-mediated cleavage caused a less than twofold further increase (data not shown). Thus, with all three types of chromosomally integrated substrates we detected EGFP reactivation after targeted double-strand break formation exclusively, i.e. in response to the most severe genotoxic treatment, namely double-strand break induction.

4.2 Analysis of genotoxicities in mammalian cells

Next, we investigated the capacity of the fluorescence-based assay to detect genotoxic activities, which do not target the chromosomally integrated DNA construct specifically. Ionising radiation causes double-strand breaks, single-strand breaks and base modifications, and exerts lethal effects in Ames tester strains of *Salmonella* (Isildar and Bakale, 1985; Fernandez et al., 2001). To test the applicability of our newly developed assay for the identification of radiation-dependent DNA damage, we treated our KMV tester strains with γ-rays by use of a [^{137}Cs] source. The cells received a dose of 5 Gy and cultivation was continued for 24 h, 48 h, and 96 h before harvest. Subsequently, the cells were analysed flow cytometrically in comparison to untreated aliquots of the same cultures. The results showed that green fluorescing cells appeared in all three tester strains in response to radiation, whereas fluorescence signals were not detected in the parental cell line or in untreated controls (Tab. 2).

Etoposide is an antineoplastic drug that stabilises the complex formed between the topoisomerase and the cleaved end of the DNA, thus, causing the generation of double-strand and single-strand breaks, respectively (Burden and

Tab. 2: Appearance of fluorescent cells after irradiation.

		EGFP reactivation frequency [10^{-5}]		
incubation time		24 h	48 h	96 h
dose	cell lines			
0 Gy	KMV	0	0	0
	KMV-EJ	0	0	0
	KMV-Δ	0	0	0
	KMV-HR	0	0	0
5 Gy	KMV	0	0	0
	KMV-EJ	3	3	0
	KMV-Δ	0	0	3
	KMV-HR	0	3	10

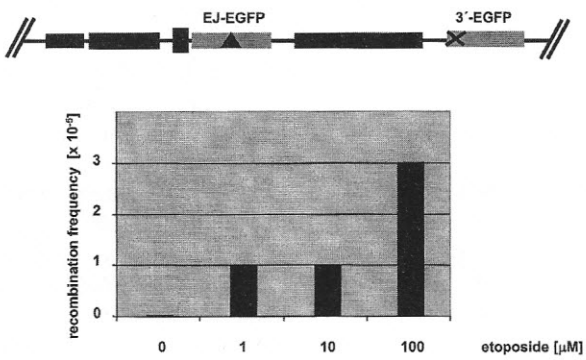

Fig. 4: Detection of etoposide treatment for 2 h.

Osheroff, 1998). Etoposide is carcinogenic and appears to produce primarily chromosome aberrations. Therefore, it is not detected by the reverse mutation *Salmonella* strains (Nakanomyo et al., 1986). When we added this chemotherapeutic agent at increasing concentrations (1-100 µM) to the culture medium of KMV-EJ cells for 2 h, we recorded the increasing appearance of green fluorescent cells 72 h later (Fig. 4).

For both radiation and etoposide treatment, rates in the order of 10^{-5} to 10^{-4} were determined. Similarly, NH_4OH at a concentration of 4 mM caused EGFP reactivation at a frequency of 1 x 10^{-4}. Consistently, ammonium ions display a strong mutagenic effect in the *Salmonella* strains 1538 and TA98 (Iwaoka et al., 1981). The addition of 200 nM 17ß-estradiol, corresponding to an intermediate therapeutic dose, raised the recombination frequency to 3 x 10^{-5}. This result is in agreement with epidemiologic data and animal experiments, which strongly suggest that steroid hormones, despite being ineffective in the Ames assay, increase the cancer risk by acting genotoxically (Djelic and Djelic, 2002). Taken together, our data indicate that the test system has the capacity to assess the effect of radiation in human cells, and of treatment with chemotherapeutic or other potentially harmful drugs by measurements of DNA-exchange activities between EGFP marker genes.

5 Discussion

In this work, we show that our novel, short-term, fluorescence-based test system identifies genotoxic activities in living mammalian cells. The system monitors recombinative repair processes in response to a genotoxic stimulus, and therefore reflects the association of genome rearrangements and tumorigenesis. Inducibility was demonstrated here for treatment with γ-rays, etoposide, ammonium hydroxide, and ß-estradiol.

Since certain genotoxic compounds are missed by the tests currently in use (Schiestl, 1989; Schiestl et al., 1997), there is a need for inexpensive and reliable short-term *in vitro* tests, to detect genotoxic activities without the need for animal experimentation. First, the well-known Ames assay cannot be used to detect all human mutagens. Thus, certain pharmaceutical compounds exist that cannot be tested in bacteria because of their therapeutic principle. For example, quinolones act specifically on the procaryotic gyrase which is structurally and functionally related to, but significantly different from, the eukaryotic topoisomerase II (Herbold et al., 2001). Second, many classes of known genotoxic compounds show poor responses in the Ames assay, such as certain metals, steroid hormones, and chlorinated hydrocarbons (Schiestl, 1989; Schiestl et al., 1997). Third, the Ames assay disregards DNA damage responses which are specific to the human cell (DNA repair and surveillance, growth control, apoptosis) or to the human organism (metabolic activation of harmful compounds).

In contrast, our assay can utilise mammalian cells from different species and different tissues and therefore fulfills the requirements to integrate the genotoxic effects related to the mammalian DNA damage response. Additionally, the system offers the possibility to assess the impact of human metabolites, not only by adding liver extracts but also by use of V79 Chinese hamster cells, which have been genetically engineered to mimic the metabolising profile of the human liver (Doehmer et al., 1991; Doehmer and Jacob, 1994). This combined approach of fluorescence-based genotoxicity testing and metabolic activation of harmful substances is extremely promising with respect to its potential to reduce animal experimentation in the future. Remarkably, rats, which are generally used for cancerogenicity testing in animals, show striking differences in the metabolite profile as compared to human beings (Doehmer and Jacob, 1994). Therefore, beyond economic and ethic reasons, this application has advantages in terms of the detectability of compounds which are harmful to human beings.

Referring to the spectrum of genotoxic compounds which are detectable by the classical Ames assay, it is important to note that this assay and other mutagenicity tests, like the HPRT gene assay in hamster cells, miss larger recombinative genome rearrangements. However, among the biochemical events underlying chromosomal instabilities, dysfunction of recombinative repair has been linked closely to tumorigenesis (Pierce et al., 2001). Consequently, Schiestl (1989) established the DEL assay, which measures the inducibility of nonconservative recombination events at the HIS3 locus in the yeast *Saccharomyces cerevisiae*. In their study, a large spectrum of mutagens that are detectable by a recombination-based assay was described. In agreement with these and other data (Djelic and Djelic, 2002), the Ames assay negative hormone estradiol was active in our assay using human cells. Likewise, we

detected the genotoxic activities of ionising radiation, etoposide, and ammonium hydroxide. Importantly, ionising radiation at a dose of 5 Gy causes the generation of only 75 double-strand breaks per genome, but base damage and single-strand breaks are generated far more frequently (El-Awady et al., 2001; Fernandez et al., 2001). Until several years ago, recombination was understood as a DNA repair process which is initiated by DNA double-strand breaks and crosslinks exclusively. According to this concept, EGFP gene reactivation would not have been detectable in our assay. Only recently it became clear that any kind of unrepaired lesion, like base damage and single-strand breaks, causes replication fork stalling (Flores-Rozas and Kolodner, 2000). Recombination serves to bypass lesions that inhibit chain elongation and, therefore, represents a unique repair process that senses any kind of lesion in mitotically growing, mammalian cells. This explains why clastogens and point mutagens like benzo[a]pyrene (Aubrecht et al., 1999), N-methyl-N'-nitro-N-nitrosoguanidune/ MNNG (Zhang et al., 1996) and ammonium hydroxide (this study) induce recombination. This is why recombinative rearrangements are excellent biological endpoints to monitor genotoxic activities from different sources, and why certain compounds are missed in the reverse mutation assay but not in a recombination-based test.

It is important to note that the EGFP-based assay was not designed to efficiently identify non-genotoxic carcinogens like 12-O-tetradcanoylphorbol-13- acetate (TPA). Yet, at least with respect to tumor promoting agents that lead to the loss of a G1-arrest, it may be speculated that these treatments could be picked up simply due to the fact that replication fork stalling occurs when cells progress into the S-phase without completing the repair of lesions existing previously in G1. However, the possibility of identifying non-genotoxic carcinogens by cellular fluorescence will have to await a systematic examination in future experiments.

A major advantage of the newly developed system, as compared to cytogenetic tests, lies in the possibility of automated performance. Cells growing in suspension culture like K562 were used in our study, as they are easy to handle. A new module for flow cytometric analysis has been developed by Becton Dickinson, which permits high throughput analysis. This autosampler allows quantification of the fraction of fluorescent cells in multiple samples within 96 and 384 well microtiter plates. In this report, we measured EGFP reactivation frequencies with a detection limit of 1×10^{-5}. At least with respect to etoposide treatment, the signals can be intensified by a prolongation of the incubation period (e.g. 24 h versus 2 h). Moreover, the detection limit can be lowered simply by analysing larger cell numbers. However, the latter approach would decrease the automatic scoring rate and increase the cost of the test. Therefore, we will concentrate our efforts on raising the sensitivity of the system further, in order to prepare systematic studies of dose-dependent relationships for a variety of genotoxic compounds. First, we will examine and select cell lines with distinct DNA repair defects for the development of new tester strains, which, after genotoxic treatments, maximally accumulate DNA lesions at replication forks and, therefore, perform recombinative repair at highly increased frequencies. Second, we will elevate recombination frequencies by chromosomal introduction of larger copy numbers of the recombination substrate into these cells.

In summary, the assay provides a procedure to identify genotoxic compounds as a consequence of their activity in increasing the frequency of genome rearrangements in mammalian cells. This method is expected to identify a broad spectrum of human mutagens and fulfills the requirements for screening agents on a large scale. Most importantly, it is also applicable to transgenic cell lines with human metabolic enzymes, and so carries the potential to further reduce animal experiments in genotoxicity testing. Recently, the EU authorities decided to prohibit the use of animals in the testing of cosmetic products and the import and marketing of these goods after January 2009. This decision marks a new direction in safety studies and further stresses the need for developing alternative ways of screening to reduce or replace animal testing.

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