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RELATIONSHIP BETWEEN DNA-ADDUCT FORMATION, DNA REPAIR, MUTATION FREQUENCY AND MUTATION SPECTRA

A.A. VAN ZEELAND, G.R. MOHN, L.H.F. MULLENDERS, A.T. NATARAJAN, M. NIVARD, J.W.I.M. SIMONS, J. VENEMA, E.W. VOGEL, H. VRIELING, M.Z. ZDZIENICKA and P.H.M. LOHMAN

Department of Radiation Genetics and Chemical Mutagenesis, Sylvius Laboratories, State University of Leiden, Leiden, The Netherlands

Summary. - *DNA-adduct formation by a series of ethylating agents was determined and correlated with induction of gene mutations. This approach gave information concerning the DNA-adduct(s) likely to be responsible for the observed mutations. A methodology has been developed which is used for the DNA sequence analysis of point mutations in the HPRT gene of mammalian cells. This method can be used to obtain mutation spectra and to determine whether the base-pair changes do occur at those sites where DNA-adducts are likely to occur. Measurements of DNA repair in specific DNA sequences show that actively transcribed genes are repaired faster than the genome overall. This stresses the importance of studying removal DNA-adducts in the gene that is used for the analysis of mutation induction.*

Riassunto (Relazione tra la formazione di addotti sul DNA, la riparazione del DNA, la frequenza di mutazione e il tipo di mutazione indotta). - *In questo lavoro la formazione di addotti sul DNA dopo la esposizione ad agenti etilanti è stata determinata e correlata con l'induzione di mutazioni geniche. Questo tipo di approccio permette di ottenere informazioni sugli addotti al DNA che sono probabilmente responsabili delle mutazioni osservate. Inoltre abbiamo sviluppato una metodologia per determinare la sequenza di mutazioni puntiformi nel gene HPRT di cellule di mammifero. Questo metodo può essere usato per ottenere gli spettri di mutazione e stabilire se le alterazioni nella sequenza corrispondono ai probabili siti di formazione di addotti al DNA. La misura della riparazione del DNA in sequenze specifiche mostra che i geni attivamente trascritti sono riparati più velocemente rispetto al genoma totale. Queste osservazioni sottolineano l'importanza dello studio della rimozione degli addotti al DNA nello stesso gene usato per l'analisi dell'induzione di mutazione.*

Introduction

The mutagenic potential of chemical and physical mutagens is primarily determined by the nature, quantity and site of the DNA lesions introduced in the genome. Since mutagens often cause more than one type of DNA lesion, each of which can have different mutagenic properties, many types of mutagenic changes can occur. In order to determine which DNA lesions are responsible for the mutagenic effects observed for a particular class of mutagens, we are carrying out studies in which we are measuring the frequency of mutation induction and the frequency of DNA-adduct formation in a particular assay system under identical conditions. This will allow us to quantitatively correlate mutation induction with the presence of a specific DNA-adduct. This approach was applied to a series of ethylating agents which all introduce similar types of DNA-adducts, but in different proportions.

A second approach which we follow to relate DNA damage to mutation induction and which gives fundamental information about the molecular mechanisms underlying mutation induction is the determination of mutation spectra at the DNA sequence level. This approach will give detailed information concerning the position of base pair changes in a gene which has been mutated and makes it possible to directly relate possible positions of DNA-adducts with specific changes in the DNA sequence. We have developed for this purpose a methodology for sequence analysis of point mutations in an endogenous gene of mammalian cells (HPRT gene) and applied this technique to the analysis of mutation spectra in normal and repair deficient mammalian cells.

DNA repair processes can strongly influence the type and amount of mutagenic events induced by chemical or physical mutagenic agents. Most studies on DNA repair

focussed on the persistence of particular DNA-adducts measured in the genome overall. However, since it is now known that DNA repair processes in mammalian cells are not operating equally efficient for all parts of the genome [1] we also determine the kinetics of removal of DNA damage from specific DNA sequences. This approach will make it possible to relate mutation induction in a particular gene directly to the action of DNA repair processes in the same gene.

Materials and methods

Molecular dosimetry

DNA adduct formation, in various organisms, was measured after treatment with the following four ethylating agents: N-ethyl-N-nitrosourea (ENU), ethyl methanesulphonate (EMS), N-ethyl-N'-nitro-N-nitrosourea (ENNG) and diethyl sulphate (DES). *Escherichia coli (E. coli) K12* strain 343/113 [2] and V79 Chinese hamster cells were treated in suspension in phosphate buffered saline (PBS) for 1 hour (EMS for 2 hours) at 37 °C as described [2]. DNA adduct formation in (101xC3H) F1 male mice, (obtained from Dr. U.H. Ehling, Neuherberg, FRG), was monitored two hour after i.p. injection with 0.5 ml stock solution of EMS, ENU or DES. Radioactively labelled mutagens were ^{3}H-labelled in the ethyl group. Ethylation products in DNA were determined as described previously by high pressure liquid chromatography of hydrolyzed DNA and subsequent quantitation of the amount of ^{3}H-label in the various fractions of the chromatogram [2].

Determination of mutation induction

The induction of gene mutations in *E. coli* was determined as resistance to nalidixic acid (NAL^r). In V79 Chinese hamster cells mutation induction was determined at the HPRT gene [3]. Treatment conditions were exactly as described for the determination of DNA adducts, except that unlabelled mutagens were used.

Sequence analysis of mutations at the HPRT gene

The methodology used for the analysis of HPRT mutants has been described elsewhere [4]. Briefly, total cytoplasmic RNA was isolated and used as starting material for the synthesis of HPRT cDNA using an HPRT specific primer. The polymerase chain reaction (PCR) was used for the amplification of a 740 bp fragment which covered the complete coding region of the HPRT gene. The amplified material was restricted with appropriate restriction enzymes, cloned in a M13 based vector and sequenced using the dideoxy method.

DNA repair in specific DNA sequence

The methodology for the determination of the frequency of pyrimidine dimers in specific DNA sequences was carried out as described [5].

Results and discussion

Quantitative comparison between the frequency of ethylation products in DNA and the frequency of mutation induction

Mutation induction and DNA adduct formation by ethylating agents were studied in *E. coli* as well as in cultured V79 Chinese hamster cells [2]. Because both endpoint were determined in each system using identical treatment conditions, the frequency of mutation induction could be directly compared with the frequency of the various ethylation products formed in DNA. When compared on the basis of the exposure concentration of the chemical, ENNG was by far the most mutagenic followed by ENU, DES and EMS. In the case of V79 Chinese hamster cells the data are shown in Fig. 1 and 2. For most DNA-adducts there was no direct relation between the frequency of the DNA-adduct and the frequency of mutation induction. However, the frequency of mutations induced by all four chemicals used was the same when compared on the basis of the amount of O^6-ethylguanine in DNA (Fig. 2). The same results were

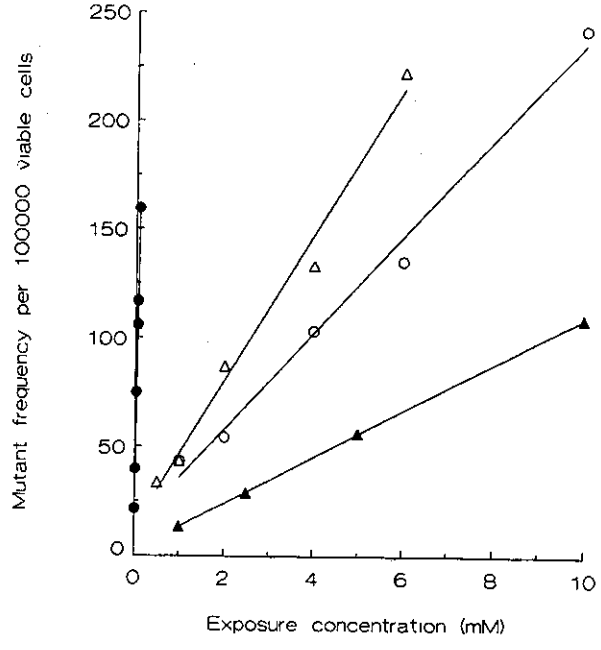

Fig. 1. - Induction of HPRT deficient mutations in V79 Chinese hamster cells as a function of the exposure concentration of ENNG (●), ENU (Δ), DES (○) and EMS (▲).

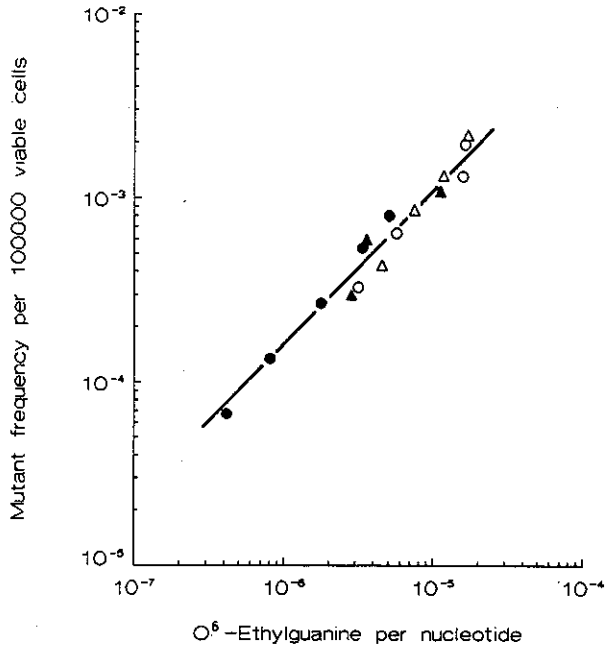

Fig. 2. - Induction of HPRT deficient mutations in V79 Chinese hamster cells as a function of the frequency of O^6-ethylguanine in DNA immediate after treatment with ENNG (●), ENU (Δ), DES (O) and EMS (▲).

obtained in *E. coli* [2]. These data indicate that this DNA adduct can be used as a dosimeter for the frequency of gene mutations induced by ethylating agents in various *in vitro* mutagenicity assay systems. The experiments described above were all performed with bacteria or mammalian cell lines which were proficient in DNA excision repair processes. When the frequency of mutation induction by alkylating agents was compared in cell lines or organisms which are deficient in DNA excision repair, differences are observed in the relation between O^6-alkylguanine and mutations. These differences are related to the Swain-Scott value of the alkylating agent used. For instance, alkylating agents such as ENU do have a relatively low Swain-Scott value and therefore do not have a strong preference for alkylating N atoms in DNA. This means that these type of alkylating agents cause a relatively high frequency of O^6-alkylguanine compared with the frequency of N-alkylations. Alternatively, alkylating agents with a rather high Swain-Scott value such as MMS cause very little O^6-alkylguanine in comparison with the amount of N-alkylations. The observation was made that chemicals which induce relatively high levels of O^6-alkylguanine (e.g. ENU) are equally mutagenic in normal cells compared with excision repair deficient cells, although cell killing is very much enhanced in the excision repair deficient cells. On the contrary mutation induction induced by chemicals which cause preferentially N-alkylations (e.g. EMS and MMS) is higher in DNA excision repair deficient cells compared to normal cells.

These observations are made in *Drosophila* as well as in cultured Chinese hamster ovary cells [6, 7]. O^6-alkylguanine is not removed by excision repair in normal hamster cells and is therefore a very persistent lesion in normal as well as in the repair deficient cells. Our interpretation is that in DNA repair deficient cells, besides O^6-alkylguanine, other DNA lesions are also mutagenic. The mutagenic effect of these other DNA lesions is not evident under conditions where high levels of O^6-alkylguanine are present (e.g. ENU), or under conditions of efficient excision repair, but is very clear in those cases where very little O^6-alkylguanine is induced (e.g. MMS) and where repair of other DNA-adducts is affected.

We have also determined the frequency of alkylation products in DNA from mice treated with ENU, EMS or DES. The data show that in testicular DNA, isolated 2 hours after i.p. injection with the ethylating agent, the frequency of O^6-ethylguanine is highest after treatment with ENU, whereas DES is the least potent ethylating agent to induce O^6-ethylguanine in testicular DNA (Fig. 3). However, when the three ethylating agents ENU, EMS and DES are compared on the basis of their capacity to induce 7-ethylguanine in testicular DNA, the ranking is different (Fig. 4). EMS is the most powerful in introducing 7-ethylguanine followed by DES and ENU. Parallel to these DNA adduct measurements, the induction of gene mutations in the same strain of mice was determined in the laboratory of U.H. Ehling (Neuherberg, FRG) using the specific locus test [2, 8, 9]. These comparisons show that in spermatogonia

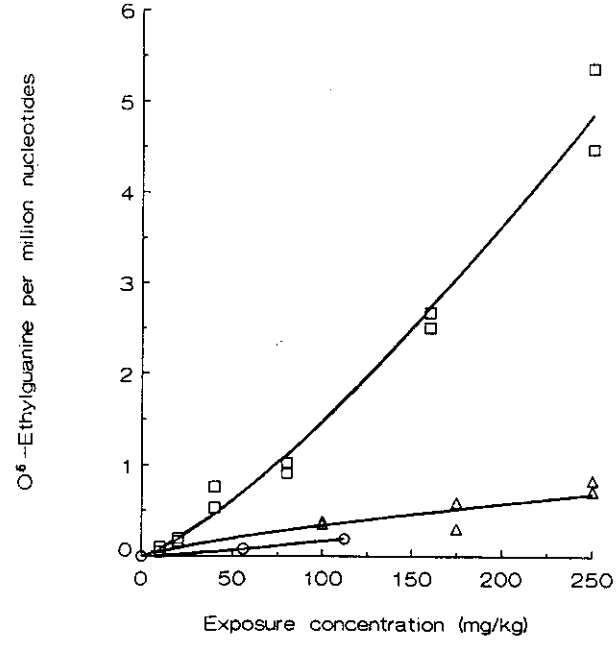

Fig. 3. - Frequency of O^6-ethylguanine in testicular DNA of mice as a function of the exposure concentration of ENU (□), EMS (Δ) and DES (O).

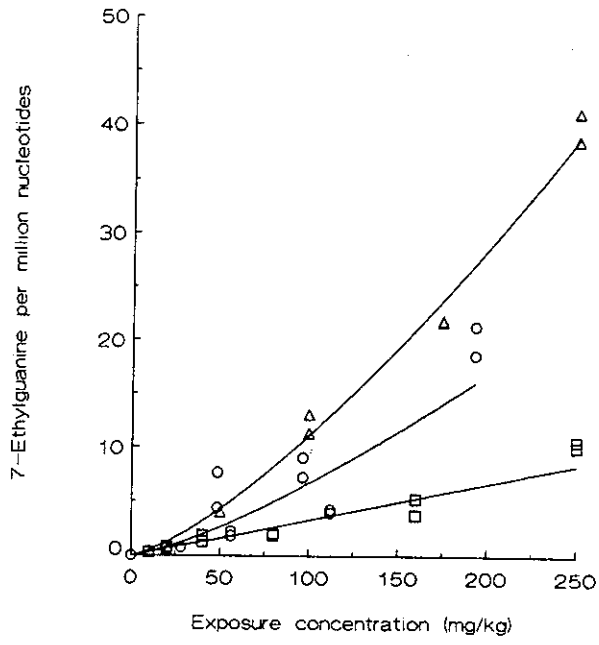

Fig. 4. - Frequency of 7-ethylguanine in testicular DNA of mice as a function of the exposure concentration of ENU (□), EMS (Δ) and DES (○).

ENU is by far the most powerful mutagen whereas EMS is weakly mutagenic and DES is negative. However, in post meiotic cells EMS is the most powerful mutagen followed by DES. ENU is hardly mutagenic in post-meiotic cells. The ranking of the capacity of the three chemicals to induce mutations in spermatogonia seems to be correlated with their capacity to induce O^6-ethylguanine in testicular DNA. However, the mutagenic potential in post-meiotic cells correlates with their capacity to induce 7-ethylguanine in testicular DNA. Since spermatogonia are mitotically dividing cells, O^6-ethylguanine can cause mutations by mispairing in the first round of DNA replication following treatment. In post-meiotic cells there is no DNA replication for a relatively long period of time. The first round of DNA replication occurs only after fertilization. The observation that mutation induction in post-meiotic cells seems to correlate better with the capacity of the compounds to induce 7-ethylguanine leads us to suggest that most mutations in post-meiotic cells are not caused by O^6-ethylguanine but by N-alkylations. In analogy with the observations made in repair deficient mutants of cultured mammalian cells and *Drosophila*, we propose that most mutations in post-meiotic cells are caused by apurinic sites which arise because N-alkylations are chemically unstable and are converted into apurinic sites with time. These apurinic sites are probably not immediately repaired by excision repair because the more mature germ cell stages are known to be repair deficient. Since EMS and DES induce considerably more N-alkylations than ENU this hypothesis would explain why EMS and DES are more mutagenic than ENU in post-meiotic stages of the male germ cells in mice.

Sequence analysis of mutations at the HPRT gene

Studies on mutation induction in mammalian cells are usually carried out with genes which are X-linked (e.g. HPRT gene) or with autosomal genes in cell lines which are heterozygous for the genetic marker under study (e.g. TK and APRT gene). Until recently the molecular analysis of mutants at these loci was very difficult and time consuming, because no methods were available which allowed the rapid investigation of relatively large numbers of mutants in order to identify mutation spectra at these endogenous genes. For this reason many investigators started to use systems in which genes on shuttle vectors are being mutagenized in a mammalian host and then subsequently transferred to *E. coli* followed by cloning of the mutated gene and determination of the DNA sequence. We have developed a new method which allows the rapid sequencing of point mutations in an endogenous gene, *i.e.* the HPRT locus in mammalian cells [4]. This methodology can be used to answer many important questions such as what is the nature of mutation spectra induced by various chemical or physical mutagens, or what are the differences between these spectra in normal and in repair deficient cells. Moreover, possible differences between spectra obtained from genes on shuttle vectors and from endogenous genes can be investigated. Since most point mutations which lead to loss of HPRTase activity are expected to occur in exon sequences of the gene, mRNA was used as starting material. First a cDNA copy was made, which was then used in an *in vitro* amplification procedure. The polymerase chain reaction (PCR) was employed to specifically amplify the entire coding region of HPRT cDNA. The amplified fragment was subsequently cloned in M13 based cloning vectors and sequenced. We have applied this methodology to 3 HPRT mutants obtained from a mouse lymphoma cell line (GRSL 13-2) which was treated with ENU. In these experiments Klenow fragment of *E. coli* DNA polymerase I was used for the amplification. The first mutant contained a point mutation at position 392 of the HPRT coding region as a result of a AT to GC transition. This transition leads to a replacement of leucine by serine. The second mutant contained a point mutation at position 585 of the coding region as a result of a TA to AT transversion. This mutation causes a change of a codon for tyrosine (UAU) into a stop codon (UAA). Sequence analysis of the third mutant showed in two independent amplified derivatives that precisely exon 2 was missing. In a third amplified derivative exon 2 plus 3 were missing. These results can be explained

by a mutation in the 3' splice site of intron 1. Whether this would be a base pair change or a small deletion is not clear. However the restriction pattern of AccI, which cuts within the second exon, was unchanged compared with wild type, ruling out the possibility that the entire second exon has been deleted from the genome. It is clear that in this mutant at least two alternative 3' splice sites are used. The 3 mutants from which the DNA sequence change was determined were part of a group of 29 ENU induced HPRT mutants. All 29 mutants showed normal restriction pattern on Southern blots, but 5 of them showed a reduced level of HPRT mRNA and in another 5 no HPRT mRNA was detectable. This last category might be due to instability of HPRT mRNA caused by a mutation in the coding sequence or possibly to a mutation in the promotor region of the HPRT gene [10].

In addition to these mouse lymphoma mutants we have also investigated a number of UV-induced HPRT mutants obtained from normal V79 Chinese hamster cells as well as from V-H1, a UV-sensitive derivative of V79. V-H1 is a repair deficient mutant with the interesting property of extreme sensitivity to UV-killing combined with a high level of DNA repair replication. The mutant is 10 times more sensitive to UV (D_{10} value) and has a 7 fold higher UV-induced mutation frequency at the HPRT locus then V79 cells. UV-induced DNA repair synthesis in V-H1 cells is 30-50% lower compared with V79 cells [11]. Southern analysis of 20 HPRT mutants from V79 and 22 mutants from V-H1 showed identical restriction patterns compared to HPRT wild type cells. Northern analysis showed reduced *hprt* mRNA levels in 2 mutants derived from V79 and in 4 mutants derived from V-H1. Sequence analysis of 19 mutants obtained from V79 cells and of 17 derived from V-H1 cells shows that the spectrum of mutation induction in V-H1 cells differs from the spectrum obtained from normal V79 cells (Table 1). These differences can be summarized as follows: i) all observed mutations except one were found at potential sites for UV photoproduct formation (dipyrimidines); ii) the single base pair mutants in V79 cells represent all classes of base pair substitutions except GC to CG with no preference for any specific type of change; iii) in contrast all base pair mutations in V-H1 are GC to AT transitions.

Table 1. - *Types of single and tandem base pair substitutions in UV-induced HPRT mutants from DNA repair proficient V79 and deficient V-H1 Chinese hamster cells*

	V79	V-H1
Transitions	6 (32%)	15 (100%)
GC > AT	3	15
AT > GC	3	0
Transversions	13 (68%)	0 (0%)
GC > TA	5	0
GC > CG	0	0
AT > TA	4	0
AT > CG	4	0
Total	19	15

DNA repair in specific DNA sequence

Since it is clear that the frequency of UV-induced mutations and the nature of the DNA sequence changes involved in these mutants is strongly influenced by DNA repair mechanisms, it is important to analyze the relationship between DNA repair and mutagenesis. Until recently most investigations on removal of damage from DNA in mammalian cells were focussed on repair of the genome overall. Classical methods for the detection of repair of pyrimidine dimers such as chromatography of hydrolyzed DNA [12] or alkaline sucrose centrifugation of DNA treated with UV-specific endonucleases [13] all result in average repair rates for the genome. However, it has been shown recently that certain regions of the genome are repaired at a different rate as observed for the genome overall [14]. Moreover, it has been shown that actively transcribed genes in cultured Chinese hamster cells are repaired to a greater extent than the genome overall [15, 16]. In human cells, the repair of pyrimidine dimers in transcribed genes is faster than the genome overall. Since we want to investigate the influence of DNA repair processes on mutation induction it is essential to measure both processes *i.e.* removal of DNA damage and mutation induction, in the same gene. We investigated already the rate of dimer removal from the adenosine deaminase locus (ADA gene) in cultured human fibroblasts. The ADA gene is transcribed in human cells in culture. Our data show that the removal of pyrimidine dimers from the ADA gene in normal human cells is faster than from the genome overall [17]. In contrast, the removal of dimers from the non-transcribed 754 locus which is located on the X-chromosome showed the same kinetics as found for the genome overall. These data indicate that the rate of repair is indeed related to the potential transcriptional activity of the DNA sequence investigated. At present we are developing the methodology for measuring removal of pyrimidine dimers from the HPRT gene in the same set of Chinese hamster cell lines used for the mutation experiments. Preliminary experiments show that in normal V79 Chinese cells pyrimidine dimers are very efficiently removed from the HPRT gene, whereas in V-H1 dimer removal is completely deficient.

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