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AN EXPERIMENTAL MODEL FOR ASSESSMENT OF GLOBAL DNA REPAIR CAPACITY

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ABSTRACT

Cellular DNA is constantly subjected to a significant amount of damage resulting from extracellular as well as intracellular processes. There are various mechanisms to repair DNA damage among which nucleotide excision repair (NER) pathway has the highest degree of versatility for recognizing and repairing potentially hazardous DNA modifications. It is difficult, however, to measure the rate of DNA repair as the synthesis of DNA related to repair constitutes only a small fraction of the overall rate of DNA synthesis in the cell. The current paper presents an experimental setup that allows measuring the rate of repair DNA synthesis based on suppression of the replicative DNA synthesis in order to differentiate the repair-associated synthetic activity. The proposed methodology allows for assessment of repair rate of the DNA damage of any type and in any DNA region undergoing repair irrelevant of the structure of the region in question. It could serve as a basis for development of diagnostic methods for assessment of the capacity for global DNA repair.

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Introduction

Cellular DNA is subjected daily to a considerable amount of damage produced by a variety of factors of both external and internal origin. There are various mechanisms to repair the damage which may deal only with a particular lesion (e.g. designated glycosylase activities for excision of different modified bases) or with a variety of lesions, provided that they fall within a certain broadly defined type (e.g. large DNA adducts). Nucleotide excision repair (NER) repair pathway is the most versatile type of DNA repair, recognizing and repairing a wide spectrum of potentially harmful DNA modifications – usually bulky, helix-distorting lesions. Such are dimers in DNA (for example, UV-induced cyclobutan-pirimidine dimers), large adducts (such as bensopyrene adducts caused by smoking, cisplatine derivatives and other bulky DNA-binding complexes resulting from chemotherapy), etc. (5).

DNA repair has been extensively studied but the mechanisms that the cells use in the process of their "decision-making" about what damages to repair and when to initiate repair have not been completely clarified yet. It is known that some regions in DNA are more actively repaired than others – e.g. actively transcribed genes are repaired with priority over those that are transcriptionally inactive; the transcribed strand is repaired before the non-template strand, etc. (3, 7). In 2002, Hu et al. (4) discovered prioritization in the DNA repair for different exons of the same gene. That same year it was revealed that even some individual nucleotides (e.g. those found in the transcription initiation sites) could be repaired with priority (1). Nevertheless, the fine detail is still elusive. There is also increasing evidence that the chromatin structure plays a role in the prioritization of DNA repair (10, 11, 14).

DNA repair constitutes a specific area of research interest as defects in some of the repair genes are directly related to a number of human diseases and conditions (inherited diseases as well as cancers and immunodeficiencies). There has been increasing evidence that some polymorphisms in genes whose products are implicated in DNA repair may influence outcomes after allogeneic transplantation (12). Therefore, the repair capacity and the molecular mechanisms it is contingent on are under extensive study in order to identify and calibrate molecular markers for research and diagnostic purposes as well as for assessment of eligibility for various types of anticancer therapy based on individual tolerance (2, 6, 8).

In this study we present a model system for direct assessment of global repair activity measured by rate of DNA synthesis in cell cultures in which the replicative synthetic activity has been inhibited by treatment with hydroxyurea.

The relative proportion of the repair-associated DNA synthesis compared to the overall synthetic rate (formed predominantly by the level of replicative DNA synthesis) constitutes no more than 5%. Presence of DNA lesions, caused by low-dose DNA damage (e.g. UV irradiation) tend to stimulate both types of DNA synthesis which results in impossibility to determine the separate rates of the two synthetic activities. We propose that it is possible, however, to reliably assess the rate of repair-associated DNA synthesis by decreasing the replication-associated synthetic activity.

Hydroxyurea (HU) has been widely used in clinical medicine for many years as an antineoplastic agent (mainly for myelodysplastic syndromes and hematological malignancies) and in treatment of sickle cell anemia. Recently, its applicability as an antiretroviral agent was extensively studied for the purposes of management of HIV-associated conditions (9). HU is a potent inhibitor of the ribonucleoside diphosphate reductase and works by effectively starving the cell for all 4 deoxynucleotide triphosphates. In relatively low doses (which, however, fall within the therapeutic window) hydroxyurea effectively blocks DNA replication (which is the mainstay of its anticancer effect), while affecting the repair-associated DNA synthesis only minutely or not at all (13). In higher doses, however, hydroxyurea inhibits both repair and transcription-related DNA synthesis. Thus, under carefully dosed hydroxyurea blockage, the repair capacity could be assessed reliably without interference from the replicative synthetic activity. The model system for assessment of global repair capacity we describe here has been created and tested under conditions which render the possibility for interference from replicative DNA repair negligible.

Materials and Methods

We used human fibroblast WISH (CCL25) cells grown in monolayers. The cells were grown in DMEM supplemented with 7% calf fetal serum (CFK). Short-wavelength UV light (254 nm) was used as a DNA damaging agent in order to ensure random damage of DNA.

Cultured cells in exponential growth phase were incubated in medium containing hydroxyurea in different concentrations (2.5 – 15 mM) for different periods of time (2, 3 and 5 h). Subsequently, the hydroxyurea-containing medium was drained and the monolayers were rinsed briefly with fresh DMEM followed by irradiation with UV light at a fluence rate of 0.05 J/m^2/s for varied periods of time (0-10 min). WISH cells in exponential growth phase cultured under identical conditions but not treated with hydroxyurea were used as a positive control.

In order to assess the rate of DNA synthesis we carried out pulse labeling of the newly synthesized DNA with ^{3}HdT by incubating the cells in nutrient medium (with or without hydroxyurea, respectively) containing ^{3}HdT at a specific activity of 3 mCi/ml for 60 min, followed by a rinse with ice-cold 1x PBS to remove unincorporated label.

Total cell DNA was extracted by Proteinase K treatment, deproteinization with phenol and ethanol precipitation.

Assessment of the incorporation of the ^{3}HdT was carried out by liquid scintillation counting.

Results and Discussion

In the present study we aimed to determine the optimal concentration of hydroxyurea in order to achieve maximal (but discriminative) suppression of the replicative DNA synthesis. Five different concentrations (2.5 – 15 mM) of the inhibitors were used in three different treatment intervals (2, 3 and 5 h). The results are presented graphically in **Fig. 1.**

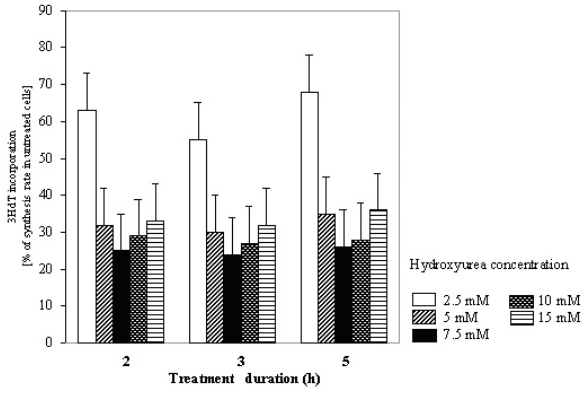

Fig. 1. Effect of the hydroxyurea concentration on the rate of DNA synthesis. The synthesis rate of untreated control is set at 100%. Values represent median results from 6 independent experiments.

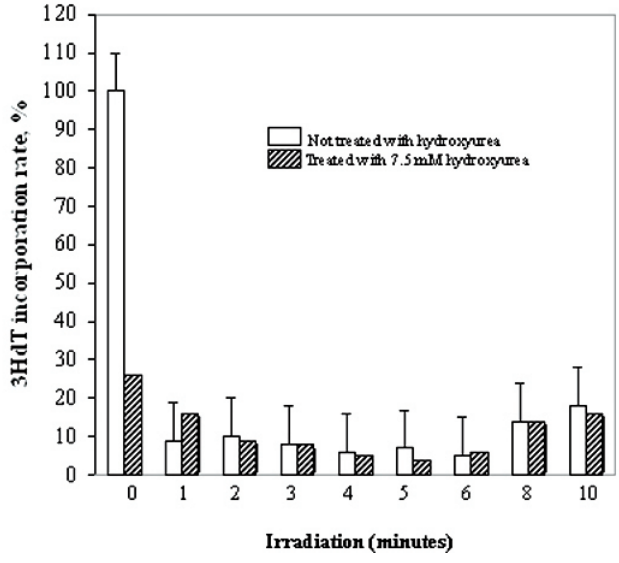

Fig. 2. Effect of the dosage of UV irradiation, delivered in longer time intervals, on the DNA synthesis in cells treated with 7.5 mM hydroxyurea. Values represent median results from 6 independent experiments.

The concentration of 7.5 mM hydroxyurea was considered to be optimal as, on the one hand, under these conditions the suppression of the DNA synthesis was at its highest (estimated by the rate of incorporation of the labeled nucleotide), and, on the other hand, the replication blockage proved to be relatively stable for at least 5 hours. The rate of residual DNA synthesis, however, remained relatively high (about 25% of the synthetic rate in the untreated controls). Nevertheless, with irradiation this rate drops (as cells tend to stop their cycling in order to repair the damage). The decrease thus gained affects, once again, predominantly the replicative synthesis, rendering the repair-associated DNA synthesis accountable for the significant part of the generated measurable output. Complete inhibition of the replicative DNA synthesis, however, is neither feasible nor desirable, as there would always be some synthetic activity at replication origins, albeit at basal level and doses

of hydroxyurea that ensure total suppression of the replicative synthetic activity would affect adversely the reparative DNA synthesis as well.

In order to identify the optimal dose of irradiation, cells treated with 7.5 mM hydroxyurea were irradiated with UV light at a fluence rate 0.05 J/m^2/s for varied time intervals (initially, 1-10 min) and labeled with ^{3}HdT (**Fig. 2**).

The decrease of the rate of incorporation of the labeled nucleotide in all irradiation intervals compared to the controls was a result of the suppression of the replicative DNA synthesis because of the UV-induced damage. Clear-cut and statistically significant differences in synthetic activity between hydroxyurea-treated and untreated cells were achieved at irradiation time of 1 min.

In order to fine-tune the dose of irradiation at which the signal from repair activity could be unequivocally differentiated from the background, a second series of irradiation experiments was performed in which the irradiation times were shorter than in the previous experiment and the individual doses were more narrowly spaced (**Fig. 3**).

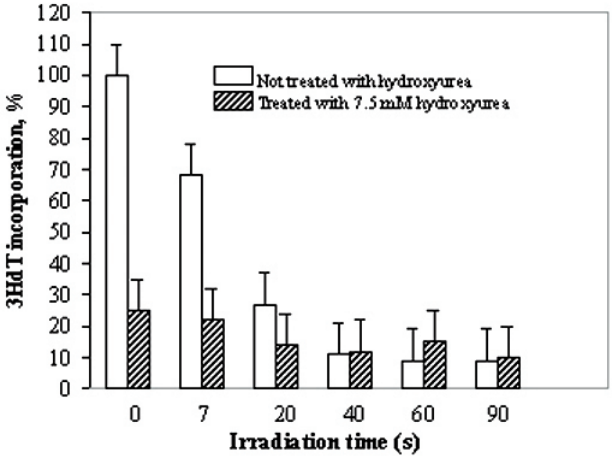

Fig. 3. Effect of the dosage of UV irradiation on the rate of DNA synthesis in 7.5 mM hydroxyurea-treated cells. Values represent median results from 6 independent experiments.

Again, 1 min seemed to be the optimal time interval to permit distinctive differentiation of the repair-associated DNA synthesis. After analysis of the experimental results we selected 3 J/m^2 as an optimal irradiation dose corresponding to irradiation time of 1 min.

Thus, the optimal conditions for eliciting specifically the repair-associated DNA synthetic activity in cultured human cells using hydroxyurea as a replicative inhibitor was determined as treatment with 7.5 mM hydroxyurea followed by irradiation with short-wavelength (254 nm) UV light at a dose of 3 J/m^2 (60 s at irradiation rate of 0.05 J.m^2/s).

Conclusions

The experimental setup described above is based on suppression of the replicative DNA synthesis in order to differentiate the repair-associated synthetic activity. Potentially, this could be used for assessment of individual capacity for global DNA repair. The method has the advantage of being independent from the type of the DNA damage and the type and structure of the DNA region under repair.

Apart from their importance as a source for research data, the results of studies of DNA repair have the potential to be used in clinical settings. Further development of the methods for assessment of repair capacity may lay the basis for specific diagnostic methodologies and identify markers for assessment of eligibility for various treatments (chemotherapy, radiotherapy, etc.). In addition, assessment of individual repair capacity similar to that proposed in the present study may, in the long run, play a role in the monitoring and management of conditions (naturally occurring as well as iatrogenic) in different patients and the assessment of risks for complications. The method proposed hereby may present a contribution to the field of individualized medicine, in its diagnostic as well as in its therapeutical branch.

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