DNA REPAIR IN MAMMALIAN CELL LINES AND IN LYMPHOCYTES OF PATIENTS WITH ALZHEIMER'S DISEASE

Mónika Mórocz

Summary of PhD. thesis

Supervisor: Dr. István Raskó

University of Szeged, **Molecular and Cell Biology PhD.** Programme,

Biological Research Center of Hungarian Academy of Sciences,

Institute of Genetics, Human Molecular Genetics Research Group

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INTRODUCTION

The large genomes of mammalian cells are vulnerable to an array of DNA-damaging agents, of both endogenous and environmental origin. Unrepaired DNA damage in the mammalian genome results in the induction of mutations during replication. These mutations may interfere with cellular viability or, when present in genes involved in cellular growth control, predispose the cell to develop into malignant cancer. To counteract these threats cells have evolved various DNA repair pathways. Among these, nucleotide excision repair (NER) is the most versatile, capable of removing a wide range of helix distorting lesions. The removal of these DNA lesions by NER occurs via a reaction mechanism that includes DNA damage recognition, lesion demarcation, dual assymetrical incisions at the 5' and and 3' sites flanking the lesion, excision of 30 nucleotide from the single-stranded oligonucleotide, containing the lesion, and gap-filling by DNA synthesis and ligation (de Laat 1999). Two NER sub-pathways, the transcription-coupled repair (TCR) and the global genome repair (GGR) have been identified as acting on the transcribed strand of active genes and on trancriptionally inactive DNA, respectivelly. TCR is believed to underlie the recovery of RNA polymerase II dependent RNA synthesis that is inhibited following exposure to genotoxic agents.

In spite of the fact that the concerted action of NER proteins has been reproduced *in vitro* using UV irradiated plasmid DNA and purified protein components (Aboussekhara 1995) little is known about NER mechanisms in chromatin environment. Information concerning the repair of heterochromatic (especially pericentric, centromeric and telomeric) sequences is scarce, however there is probably a direct link between chromatin structure, DNA repair and chromosome fragility (Surralles 1998).

It is well known that multiple levels of heterogeneity exist in the DNA repair of photoproducts. Induction and repair of CPDs can vary significantly along human genome and different repair rates are often described even between neighbouring sequences. UV damage is repaired more rapidly in transcriptionally active DNA than in the whole genome, largely due to faster repair in the transcribed strand than in the non transcribed strand of genes (Bohr 1987; Gao 1994; Hanawalt 1991; Mellon 1987; Tommasi 2000; Tornaletti 1994). Nevertheless little is known about cell type specific differences in the repair capacity of different human cell lines.

Several endogenous products of normal metabolisms have been demonstrated to or suspected of damaging DNA. These include a variety of damaged bases, such as

spontaneously formed, oxidised, alkylated or mismatched bases that are normally repaired by base excision repair (BER) although many of these lesions are also substrates for NER. (Memisoglu 2000). BER is generally belived to constitute the primary defence against lesions formed by endogenous DNA damaging agents. Free radical mediated oxidation of macromolecules (lipids, proteins, RNA and DNA) often contributes to pathological alterations and diseases. The imbalance between oxidative free radicals and defence mechanisms (enzymatic and nonenzymatic antioxidants, repair systems) leads to oxidative stress, which can affect processes leading to clinical manifestation.

The fundamental difference between NER and BER is that NER uses a limited number of proteins to recognise many different types of damage, whereas BER uses a number of damage-recognition proteins, each specific for a limited number of lesions. In the first phase of BER, the damaged base is removed by a DNA glycosilase. The abasic site is then recognised by an apurinic/apyrimidic (AP) endonuclease, which cleaves the phosphodiester bond that is 5' to the lesion, and leaves behind a strand break with a normal 5'-abasic terminus. Replacement of the abasic residue with the correct nucleotide relies on both the endonuclease and polymerisation activities of DNA polymerase β (and in certain situations the flap endonuclesase FEN1), and is followed by ligation of the break.

These two DNA repair pathways (BER and NER) play an important role in protecting the somatic cells of higher eukaryotes against cytotoxic, mutagenic and carcinogenic effects of a wide variety of DNA damaging agents. In contrast to somatic cells, mutations occuring in germ cells are transferred to the progeny and may lead to malformations and genetic defects in the offspring.

AIMS

I. Methods used previously for examining different aspects of DNA repair are not sufficiently specific, do not give accurate information, often require a large amount of cell/DNA and therefore are expensive. PCR based DNA repair methods are fast and accurate, and produce reproducible results from small amounts of DNA. Using PCR-based methods, specific genomic regions (which are not accessible by other techniques) can be examined.

The aim of our research were as follows:

- to develop a highly sensitive and specific quantitative PCR (QPCR) method for the study of DNA repair in human pericentric heterochromatin

- to obtain further knowledge of repair kinetics of UV induced DNA damage in human cell lines using the QPCR method

II. There is increasing evidence that free radical damage is involved in neuron death in neurodegenerative disorders (Christen 2000; Markesbery 1997; Markesbery 1999; Smith 2000). Recent publications support this and suggest that oxidative stress is also present in peripheral tissues (Matsushima 1995; Mecocci 1998; Parker, Jr. 1995).

Therefore we decided to examine this link in peripheral lymphocytes of patiens with Alzheimer's disease.

The aim of our experiments were using the alkaline Comet assay:

- to measure the level of oxidative DNA damage in peripheral lymphocytes (from patients with Alzheimer's disease versus healthy, age matched controls) **without any treatment**
- to measure the level of oxidative DNA damage in peripheral lymphocytes (from patients with Alzheimer's disease versus healthy, age matched controls) **after a short repair period following *in vitro* oxidative stress**

MATERIALS AND METHODS

I. *Tissue cultures*: 12 human (diploid human fibroblast, RVH421, R13, A375, TPC1, HT1080, EJ30, 3 different Xeroderma pigmentosum (two XPA and an XPC), HeLa) and two rodent/human hybrid (15A (chinese hamster/human) and A9+15 (mouse/human)) cell lines were examined.
Quantitative PCR method (detailed description of method is part of section Results and Conclusions)
Denaturing polyacrylamide gelelectrophoresis
Autoradiography and quantitative analysis using PhosphorImager Analyser
Hydroxiapatite chromatography
UV survival assay
Inhibition of DNA repair

II. *Separation of human lymphocytes by Histopaque 1077*
Alkaline Comet assay with DNA oxidation specific endonucleases (Fpg és Endo III)
Fluorescent microscopy
Statistical analysis

RESULTS AND CONCLUSIONS

This Ph.D thesis focuses on the induction and removal of DNA damage repaired by two DNA repair pathways NER and BER.

- In order to examine the repair characteristics of UV photoproduct repair in human cell lines a quantitative PCR method employing a particularly A+T rich pericentromeric region as a probe, was developed. The method is based on the inhibitory effect of UV photoproducts on replication by Taq polymerase (Kalinowski 1992; Murray 1992; Ponti 1991).
- The target sequence was chAB4, a sequence element originally discovered in the polydisperse DNA of an angiofibroma cell lines. It is a low copy number repetitive element, occuring about 50 times in the haploid human genome, and in an evolutionary sense is from an unusually unstable sequence family (Assum 1994);(Assum 1998). An advantage of studying the chAB4 sequence is that it contains long arrays of potential pyrimidine dimers, and thus serves as a sensitive target for ultraviolet light.
- We developed precise conditions for the quantitative amplification of the chAB4 target sequence from nanogramme quantities of genomic DNA, and for the detection of UV-induced photoproducts at biologically relevant doses. The optimal PCR conditions (which ensure that amplification remains in exponential phase), were used to examine the repair kinetics of 11 human and 2 human/rodent hybrid cell lines.
- Using QPCR method we detected efficient repair (100% repair in 12 or in 24 hour) in diploid human fibroblast, in HT1080, in A375, A9+15 and in TPC1 cell lines, which is not usual in human heterochromatin and a quite unexpected phenomena in rodent/human hybrid as A9+15.
- 12 hours after UV irradiation an unexpected UV induced strand breakage accumulation in he human chromosome 15 was detected by QPCR after seemingly complete repair in 15A hamster/human hybrid cells. Elevation of the level of DNA strand breaks was confirmed by Comet assay, however, explanation of this process needs further investigation.
- Deficient repair was detected in some tumour cell lines (R13, RVH421, HeLa, EJ30) and in cell lines derived from NER deficient Xeroderma pigmentosum patients (XPA and XPC).

- This work shows that making chromatin accessible via sodium-butyrate treatment does not improve the repair of heterochromatin but improve overall repair in HeLa cells. This suggests that the lack of UV damage repair (accesibility of lesions) in pericentric heterochromatin maybe under the control of yet unidentified repair factors.
- High yields of pyrimidine dimers produced in the relatively short chAB4 sequence could cause considerable torsion of DNA, which could in turn lead to perturbation of chromatin organisation and structural instability. This may be relevant to pericentric heterochromatic regions, these being hot spots for duplication, microdeletion and microduplication syndromes (Amos-Landgraf 1999; Chen 1997; Horvath 2001; Padilla-Nash 2001; Reiter 1997; Shaikh 2001). The correct repair of pericentric lesions would, therefore be then important as a defence against gross, potentially oncogenic genomic rearrangements. Thus deficiency in repair in this unstable pericentric heterochromatin region may promote oncogenic changes.
- Given this cellular diversity of response to chAB4 damage, we can conclude that repair of this pericentromeric heterochromatin sequence is governed by factors which are too complex to be understood at present; in accordance with the current understanding that the damage recognition mechanism for global genomic nucleotide excision repair is significantly complex process.
- In order to examine DNA oxidation markers in Alzheimer's disease we used an alkaline Comet assay with lesion specific endonucleases (Fpg recognises the oxidised purines, EndoIII recognises oxidised pyrimidines). This method allows precise and reproducible conditions for detecting oxidative base modifications without causing formation of oxidative artifacts.
- The Comet assay showed significant elevations of oxidised purines in the basal state of peripheral lymphocytes in Alzheimer' patients (AD). This result follows the findings of (Mecocci 1994) who, using HPLC, detected significantly higher levels of 8OhdG in lymphocytes of AD patients than in controls.
- The level of single strand breaks and oxidised pyrimidines we found slightly, but non-significantly elevated in AD patients compared to healthy, age matched control patients.
- We compared the DNA repair capacity of oxidative damage in AD lymphocytes and controls. After a 1 hour repair period, the amount of remaining single stand breaks and alkali-labile sites of lymphocyte DNA did not differ significantly between AD patients and controls, reflecting similar effectiveness of repair. The increase of Comet tail DNA showed

significantly higher levels of oxidised purines remaining after repair period in AD patients, indicating a difference between the repair kinetics of Fpg sites in the two groups. The amount of oxidised purines removed during repair period was higher in controls than in AD patients. Removal of oxidised pyrimidines was more efficient than that of purines and interestingly was significantly more effective in AD. This significant decrease of EndoIII sites indicates effective repair of pyrimidins in AD patients.

- These findings showed a differerence between the repair kinetics of oxidised purines and pyrimidines in AD patients. While there is no significant difference between single strand break repair kinetics of AD lymphocytes and controls, and probably no defect in the essential components of this repair pathway, our results not only support the concept that AD lymphocytes are subject to increased intracellular oxidative stress, but also suggests that AD cells may have deficiencies in oxidation-specific DNA repair mechanisms leading to accumulation of oxidised purines.
- In accordance with our results a statistically significant decrease of the activity of 8-oxoguanine glycosylase (responsible for the excision of 8OdHG) and increased DNA helicase activity has been found in nuclear protein samples from AD brain tissue (Lovell 2000). Jaruga (1996) have shown that purines are the most susceptible bases for oxidative damage and the deficiency in cultured human lymphoblastoid cells of their repair system leads to an accumulation of 8OHdG. Guanine was also found to be the most vulnerable base in oxidative stress in brain tissue, and Gabbita (1998) suggested that damage to this base could cause functional changes in AD brains.
- In conclusion, our study demonstrates that Alzheimer's disease is associated with elevated levels of oxidative DNA damage in peripheral lymphocytes, as well as the previously-reported neuronal damage, and showed that removal of oxidised purines in AD lymphocytes is impaired. We also demonstrated that the Comet assay with oxidative lesion specific endonucleases offers a simple alternative methodology for detection of increased free radical damage to nuclear DNA in Alzheimer’s disease and can verify that oxidative stress in AD is present not only in neuronal tissues but also in peripheral lymphocytes.

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PUBLICATIONS

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3. Mórocz Mónika, Kálmán János, Juhász Anna, Sinkó Ildikó Stephen Downes, Janka Zoltán, Raskó István; Emelkedett szintű sejtmagi oxidatív DNS károsodás kimutatása Alzheimer kóros betegek limfocitáiban Comet assay alkalmazásával; ***Clinical Neuroscience* 2000. 53(1) 26-27.*(poszter absztrakt*)**

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3. **M. Morocz,** A. Csiszar, T. Kalmar, I. Cserpan, I. Rasko; A multiplex, low copy number repetitive sequence based quantitative PCR Method to detect UV photoproduct; **Victor Rotschild Memorial Symposia, Hebrew University, Jerusalem, Israel, (02-07. May, 1999).**

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