MAARIT MYLLYPERKIÖ

Irradiation-induced DNA Strand Breaks in Normal Hematopoietic Cells and in Chronic Lymphocytic Leukemia

A Comet Assay Study of DNA Repair

University of Tampere
Tampere 2000

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
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Lenkkeilijänkatu 6, Tampere on June 2nd, 2000, at 12 o'clock.

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Tampere 2000

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LIST OF ORIGINAL COMMUNICATIONS

The dissertation is based on the following original communications, referred to in the text by roman numerals:

I Lankinen M, Vilpo L, Vilpo J (1996): UV- and γ-irradiation-induced DNA single-strand breaks and their repair in human blood granulocytes and lymphocytes. Mutation Research 352: 31-38.

II Lankinen M, Vilpo J (1997): Repair of γ-irradiation-induced DNA single-strand breaks in human bone marrow cells: Effects of a second irradiation. Mutation Research 373: 31-37.

III Lankinen M, Vilpo J (1997): Repair of γ-irradiation-induced DNA single-strand breaks in human bone marrow cells: analysis of unfractionated and CD34+ cells using single-cell gel electrophoresis. Mutation Research 377: 177-185.

IV Myllyperkiö M, Vilpo J (1999): Increased DNA single-strand break joining activity in UV-irradiated CD34+ versus CD34− bone marrow cells. Mutation Research 425: 169-176.

V Myllyperkiö M, Koski T, Vilpo L, Vilpo J (1999): γ-Irradiation-induced DNA single- and double-strand breaks and their repair in chronic lymphocytic leukemia cells of variable radiosensitivity. Hematology and Cell Therapy 41: 95-103.

VI Myllyperkiö M, Koski T, Vilpo L, Vilpo J (2000): Kinetics of excision repair of UV-induced DNA damage, measured using the comet assay. Mutation Research 448: 1-9.

In addition, some unpublished results are presented.

ABBREVIATIONS

AP	apurinic/apyrimidinic
BER	base excision repair
BMMNC	bone marrow mononuclear cell
C	cytosine
CFU	colony-forming unit
CD	cluster of differentiation
CLL	chronic lymphocytic leukemia
DNA	deoxyribonucleic acid
DSB	double-strand break
LGT	low gelling temperature agarose
NER	nucleotide excision repair
PBS	phosphate buffered saline
SSB	single-strand break
T	thymine
UV	ultraviolet
XP	Xeroderma pigmentosum

INTRODUCTION

The primary structure of DNA is dynamic and subject to constant change. Errors are induced spontaneously, especially during DNA replication and recombination (Lindahl 1993). These changes include mismatches (Modrich 1991), deamination and loss of bases (Lindahl 1979; Loeb and Preston 1986; Lindahl 1993), incorporation of uracil into DNA and oxidative damage to DNA (Lindahl and Nyberg 1974; Duncan et al. 1978; Ames 1989). Various kinds of DNA damage are also induced by environmental agents. These can be classified as physical, such as radiation, and chemical agents (Singer and Kusmierek 1982). Regardless of the cause of DNA damage, its repair is vital for the survival of an organism (Lindahl 1982). Unrepaired or incorrectly repaired lesions may lead to mutagenesis and apparently carcinogenesis (Lindahl 1993). Accurate DNA repair is important in the hematopoietic system, where a pluripotent stem cell gives rise after proliferation and differentiation to various lineage-committed progenitor cells and eventually mature circulating blood cells. Failure of DNA repair may lead to disruption of normal hematopoiesis and malignant transformations of hematopoietic cells, which may ultimately lead to leukemia (Sawyers et al. 1991; Hoffbrand and Pettit 1995).

Resistance to radiation and chemotherapeutic drugs constitutes a major obstacle in human cancer therapy. Cellular resistance to irradiation or drugs may result from a variety of different mechanisms. One such mechanism may be enhanced repair of induced DNA lesions. Little is known about the formation and repair of DNA damage in hematopoietic cells, and especially about possible differences in individual cells. This is mainly because of a lack of sufficiently sensitive methods for quantifying DNA lesions and the rate of their repair in small cell samples. However, comet assay provides a valuable tool for this kind of investigation, allowing the detection of formation and repair of DNA single and double-strand breaks in specific cell populations at single-cell level (Singh et al. 1988, Olive et al. 1991).

The purpose of this study was to investigate the kinetics of repair of ionizing and UV irradiation-induced DNA damage in normal hematopoietic cells of different maturation stages and in malignant cells of chronic lymphocytic leukemia at single-cell level.

REVIEW OF THE LITERATURE

1. Radiation

1.1. Ionizing radiation

The main forms of ionizing radiation are γ and x-rays, neutrons and α and β-particles. Most of the ionizing radiation received by humans comes from natural background radiation (Toivonen et al. 1987; Nias 1990; Hendry and Lord 1995). Ionizing radiation has direct and indirect effects. The deposition of energy may ionize a DNA molecule by directly removing the orbital electrons from the atoms. The indirect effects are formed as series of reactions that follow from the ionization of water molecules leading to various forms of aqueous free radicals. These in turn may react with other molecules, such as DNA and oxygen. As the aqueous free radicals react with oxygen, more reactive molecules are formed, which are toxic to biological structures (Johns and Cunningham 1983; Prasad 1984; Holmberg and Perkkiö 1988; Ward 1988; Nias 1990; Paile et al. 1996). The electromagnetic spectrum is illustrated in Figure 1.

1.2. Ionizing irradiation-induced DNA damage

In addition to causing degradation of carbohydrates, lipid oxidation and breakage of secondary and tertiary bonds of proteins in the cytoplasm of the cells, ionizing irradiation induces a great variety of DNA lesions (Somosy 2000). Chemically altered bases and sugars as well as single-strand breaks (SSBs) and double-strand breaks (DSBs) are formed (Hutchinson 1985).

Irradiation may induce several kinds of base modifications into DNA (McBride et al. 1991). The major site for attack is the bond between C-5 and C-6 in both thymine and cytosine. Ring saturated derivatives, such as thymine glycol, may also be formed (Téoule 1987). Thymine and cytocine appear to be more sensitive to radiation than adenine or guanine (Breimer et al. 1985). Damage to sugar residues may be less

frequent than damage to bases (Ward 1988). These lesions constitute alkali-labile sites, which convert to strand breaks if exposed to alkaline conditions (Hutchinson 1985).

Series of reactions that lead to the formation of SSBs are initiated by radical formation at deoxyribose. This may result either from direct ionization by radiation or from radical attack (Siddiqi and Bothe 1987). SSBs have a damaged sugar or base or a missing nucleotide (Price 1993). Fairly frequently two or more nearby damage sites, such as SSBs in both strands of the DNA, are formed producing a DSB (Hutchinson 1993). Two separate SSBs may be as much as 25 base pairs apart to form a DSB. In this case the formed DSB is repaired fast and it is most likely that the lesion is recognized as two individual damaged sites by the repair machinery (Ward 1999). The formation of DSBs may occur by radical transfer to the opposite strand (Siddiqi and Bothe 1987) or by multiple independent SSBs (Ward 1990). DSBs have been identified as the most likely cause of the lethal effects of ionizing radiation due to a failure to repair the lesion (Ward 1990; Price 1993). DSBs usually have damaged termini containing phosphoryl and phosphoglycolate groups (Henner et al. 1982; Isabelle et al. 1995).

1.3. Clinical use of ionizing irradiation

Ionizing irradiation is clinically used as radiotherapy. Therapy is intended to eradicate tumour tissue without causing too much damage to normal tissue. This is best achieved by giving the total dose of radiation in a number of fraction doses over a period of time, which gives a chance for normal tissues to repair their sublethal DNA damage (Hendry and Lord 1995). Radiotherapy is also used in the treatment of chronic lymphocytic leukemia (CLL) in order to treat lymph nodes and also for reducing the spleen size in patients with hypersplenism (Paule et al. 1985; Bessler et al. 1994; Hoffbrand and Pettit 1995). Ionizing irradiation is routinely used in the prevention of graft-versus-host disease by treating cellular blood products with gamma irradiation (Pelszynski et al. 1994; Waller et al. 1999). Ionizing radiation is also commonly used in diagnostic radiology (Nias 1990).

1.4. Ultraviolet radiation

Ultraviolet (UV) radiation causes excitations, where the orbital electrons within the atoms are raised to higher energy levels (Nias 1990). Visible light, UV and infrared radiation as well as radio waves are the most typical non-ionizing irradiation (Toivonen et al. 1987). The UV radiation spectrum is subdivided into three groups according to their wavelengths: UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (200-280 nm). Solar UV radiation exposure consists mainly of UV-A and UV-B, since UV-C is absorbed in the earth´s atmosphere (Arlett et al. 1993). UV radiation is a part of the electromagnetic spectrum, which is illustrated in Figure 1.

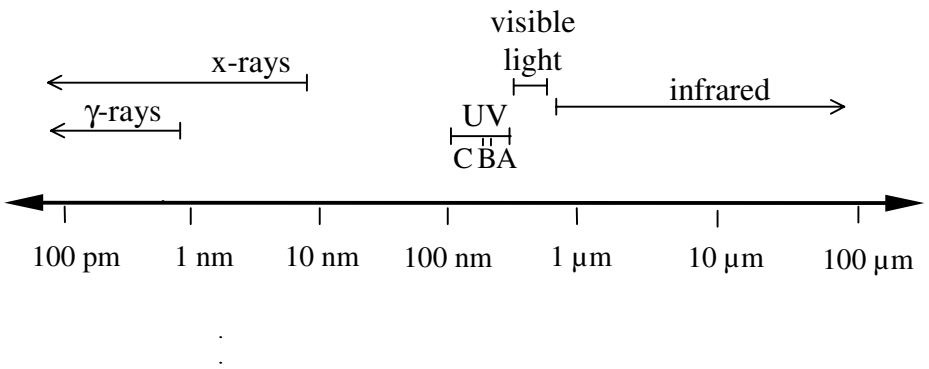

Figure 1. Electromagnetic spectrum according to wavelength (Modified from Nias 1990).

1.5. UV irradiation-induced DNA damage

UV irradiation induces several kinds of lesions into DNA. UV-A is absorbed by cellular organelles (Green et al. 1999) and thymine glycols (Demple and Linn 1982) and pyrimidine hydrates (Boorstein et al. 1990) can be formed. The main lesions, however, are cyclobutane pyrimidine dimers and pyrimidine-(6-4)-pyrimidone photoproducts, which are induced by UV-B and more efficiently by UV-C (Bianchi et al. 1990; Arlett et al. 1993; Clingen et al. 1995; Alapetite et al. 1996; Gentil et al. 1997). In double-

stranded DNA these lesions are formed in a ratio of 3:1 (Aboussekhra and Wood 1994). These lesions generate SSBs when they are repaired by the action of NER (Friedberg 1996).

Cyclobutane pyrimidine dimers occur by symmetric reaction of the 5,6 bonds of any two adjacent pyrimidines in the DNA strand (Franklin et al. 1982; Mitchell et al. 1990). The yield of these lesions in DNA is influenced by nucleotide composition. More than half of the cyclobutane pyrimidine dimers occur in thymine-thymine (T-T) doublets and in cytosine-thymine (C-T) doublets (Mitchell et al. 1992; Tornaletti et al. 1993). These lesions can produce extensive bending of 7 to 44 degrees into DNA helix (Husain et al. 1988; Wang and Taylor 1991; Lee et al. 1998).

Pyrimidine-(6-4)-pyrimidone photoproducts are formed by a stable bond between positions 6 and 4 preferentially of a T-C doublet followed by C-C and T-T doublets. C-T doublets do not occur (Franklin et al. 1982; Mitchell et al. 1990). This lesion introduces a major distortion in the double-helical structure of DNA (Taylor et al. 1988; Menichini et al. 1991). Pyrimidine-(6-4)-pyrimidone photoproduct can convert to its secondary structure, Dewar isomer, when the lesion is irradiated with UV-B light (Mitchell et al. 1990; Clingen et al. 1995).

1.6. Clinical applications of UV irradiation

Both UV-A and UV-B, but not UV-C irradiation are used in the treatment of many kinds of skin diseases (Krutmann and Morita 1999). Phototherapy is used as a standard therapy for severe psoriasis, mycosis fungoides, vitiligo and other dermatoses (Honigsmann 1990).

1.7. Oxidative DNA damage

Reactive oxygen radicals that induce oxidative DNA damage include singlet oxygen ($\cdot O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$) (Riley 1994). These can be induced by indirect effects of ionizing irradiation and UV-A irradiation. Most of the DNA damage caused by ionizing irradiation is in fact due to attacks of reactive oxygen

species. Oxidative DNA damage includes various kinds of DNA base modifications (McBride et al. 1991; Breen and Murphy 1995), damage to sugar residues (Ward 1988) and formation of ring saturated derivatives, such as thymine glycol (Téoule 1987). In addition to direct DNA damage, oxygen radicals may induce reactive intermediates from other cellular components such as lipids (Marnett 2000).

2. Basics of DNA repair

Most sites of DNA damage are repaired efficiently, with half-times varying from 4 min. for base damage to 90 min. for the slow component of double-strand break repair (Price 1993). Half-times of DNA damage measured by comet assay for lymphocytes after ionizing irradiation dose of 2-2.5 Gy vary from <10 min (Singh et al. 1994) to 30 min (Bauch et al. 1999), whereas in tumour cells (MeWo and PECA 4197) the half-time of DNA damage after irradiation dose of 5 Gy was approximately 30 min (Müller et al. 1994). Human skin fibroblasts also show very similar half-times (Oppitz et al. 1999). DNA repair is of clinical importance when fractionation of radiotherapy is performed.

2.1. Base excision repair

DNA damage induced by ionizing irradiation, such as damaged, mispaired or inappropriate bases, such as uracil, in the genome, are repaired by base excision repair (BER). The repair is initiated by the action of a specific class of DNA repair enzymes: DNA glycosylases. These enzymes catalyze the hydrolysis of the glycosyl bonds linking the base to the deoxyribose phosphate backbone of DNA and induce a site of base loss called apurinic or apyrimidinic (AP) site (Lindahl 1979). The abasic site is repaired by AP endonucleases which generate 5´terminal deoxyribose-phosphate residue. However, some DNA glycosylases are associated with AP lyase activity (Doetsch and Cunningham 1990; Tsai-Wu et al. 1992). To remove the residues generated by the AP endonucleases, yet another class of enzymes is needed: exonucleases, which remove these residues from single-stranded DNA. The action of these enzymes induces a single-strand break. Thereafter the repair is continued with repair synthesis by the action of DNA polymerase and through the action of DNA ligase

(Teebor and Frenkel 1983; Price 1993; Dianov and Lindahl 1994). The schematic representation of BER is illustrated in Figure 2.

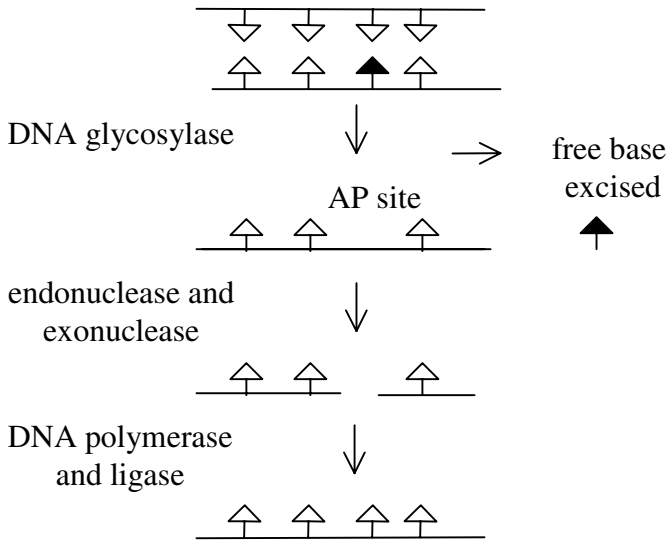

Figure 2. Schematic representation of BER.

2.2. Nucleotide excision repair

Nucletide excision repair (NER) is the most efficient mechanism for the repair of UV-C irradiation-induced DNA helix-distorting cyclobutane pyrimidine dimers and pyrimidine-(6-4)-pyrimidone photoproducts (Friedberg 1996). The complex pathway of NER is initiated by an action of DNA binding proteins and thereafter by damage-specific endonucleases, each of which operates on one side of the lesion. Two major phases have been revealed after damage recognition. First, damage specific endonucleases incise on both sides of the damaged DNA strand and generate a potential oligonucleotide fragment and a SSB. The fragment includes the lesion and is excised from the genome. Second, repair synthesis and ligation complete the repair process (Friedberg 1996; Wood 1996). For the proper function of NER about 30 different polypeptides are required (Aboussekhra et al. 1995; Mu et al. 1996). The schematic representation of NER is illustrated in Figure 3.

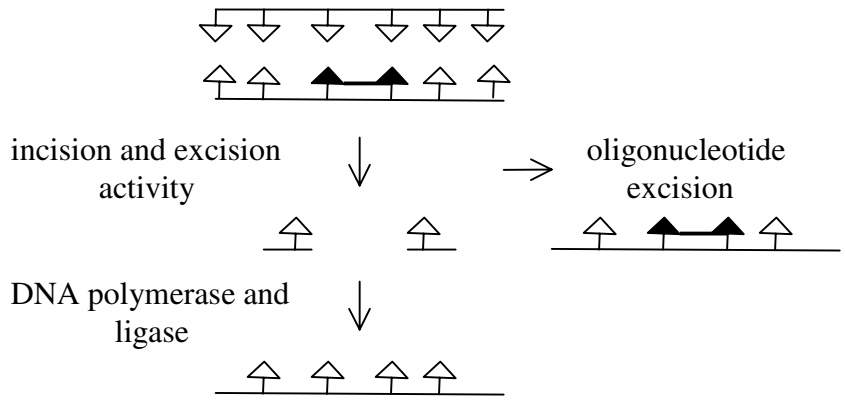

Figure 3. Schematic representation of NER.

2.3. Other repair mechanisms

The main function of mismatch repair is the repair of a particular type of DNA damage: mispared bases in DNA. The repair may occur through several biochemical pathways and also by BER (Folger et al. 1985; Glazer et al. 1987). In direct repair, the damage is repaired without a multistep pathway. An example of this kind of repair is the action of O^6-methylguanine DNA methyl transferase. It is a suicide enzyme that directly removes the methyl or other alkyl group from O^6-position of guanine and is present in all species tested (Lindahl 1982; Sancar 1996; Abril et al. 1999).

2.4. DNA repair in hematopoietic cells

Even though lymphocytes have been the subject of many studies of toxicology (Fairbairn et al. 1995), little is known about the DNA repair in normal human hematopoietic progenitor cells. It has even been stated that bone marrow stem cells are unable to repair radiation damage (Cosset et al. 1995). The activity of BER enzyme uracil-DNA glycosylase has been carefully studied in the hematopoietic system. The

activity of the enzyme in the granulocytic and erythroid series decreased towards maturity being almost absent in granulocytes and erythrocytes (Koistinen and Vilpo 1986a). However, in bone marrow cells the activity was lower than in mature lymphocytes (Koistinen and Vilpo 1986b). In other studies, human bone marrow cells and CD34+ progenitor cells have been reported to contain low levels of a direct DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase (Gerson et al. 1996).

2.5. Diseases with defective DNA repair

The survival of an organism requires that the genetic inheritance is accurately maintained (Lindahl 1982; Lindahl and Wood 1999). Failure in the DNA repair mechanisms leads to mutagenesis and apparently carcinogenesis (Lindahl 1993). Several diseases characterized by defective DNA repair have been identified. The most famous of these diseases is Xeroderma pigmentosum (XP), in which NER function is incomplete (de Boer and Hoeijmakers 2000). XP is clinically characterizrd by the early onset of severe photosensitivity of the exposed skin areas, a high incidence of skin cancers, and to a lesser extent, to leukemia. Frequent neurological abnormalities occur (Fujiwara et al. 1987; Cleaver 1990; Kraemer et al. 1994; van Steeg and Kraemer 1999). Another disease is Cockayne´s syndrome. Patients with this disease have arrested growth and development, which results in a dwarfed appearance (Fujimoto et al. 1969). These patients are sensitive to sunlight and they may have defective repair of UV irradiation-induced damage to DNA (Marshall et al. 1980; Deschavanne et al. 1984). In about half of the patients with trichothiodystrophy a defective NER of UV-induced DNA damage has been reported and so these patients suffer photosensitivity (Stefanini et al. 1986). Some cells from patients with Fanconi´s anemia have also been reported to be defective in the repair of DNA inter-strand cross-links (Moustacchi et al. 1987).

3. Hematopoiesis and chronic lymphocytic leukemia

3.1. Sites of hematopoiesis

In adults, the pelvis and vertebrae are the major functional sites that make up about 60% of the total bone marrow. The ribs, sternum, skull and proximal portions of the femur and humerus also contain functioning marrow (Mauch et al. 1995). In these hematopoietic areas, approximately half of the marrow consists of fat (Hoffbrand and Pettit 1995). Bone marrow sinuses are the finer branches of a complex vascular network through which blood flows in the bone marrow. The hematopoietic cells are formed in the extravascular spaces between these sinuses. (Lichtman 1981; Mauch et al. 1995). As the hematopoietic cells differentiate they lose cell adhesion molecules. This contributes to the capacity of hematopoietic cells to leave the marrow and enter the circulation (Hoffbrand and Pettit 1995).

3.2. Hematopoietic stem and progenitor cells

Hematopoiesis involves complex patterns of proliferation and differentiation of pluripotent stem cells into various lineage-committed progenitor cells and eventually mature circulating blood cells (Reems and Torok-Storb 1995; Scott 1995). The main progenitor cell lines are erythroid, granulocytic and monocytic, and megakaryocytic, and also T and B cell lines. The earliest myeloid precursor cell, CFU-GEMM, gives rise to granulocytes, erythrocytes, monocytes and megakaryocytes. CFU refers to a colony-forming unit in agar culture. CFU-E gives rise to erythrocytes, CFU-M to megakaryocytes. CFU-GM gives rise to both granulocytes and monocytes/macrophages but CFU-G only to granulocytes. The lymphoid stem cell gives rise to lymphoid cells (Bot et al. 1989; Hoffbrand and Pettit 1995). Progenitor cell lineages are illustrated in Figure 4.

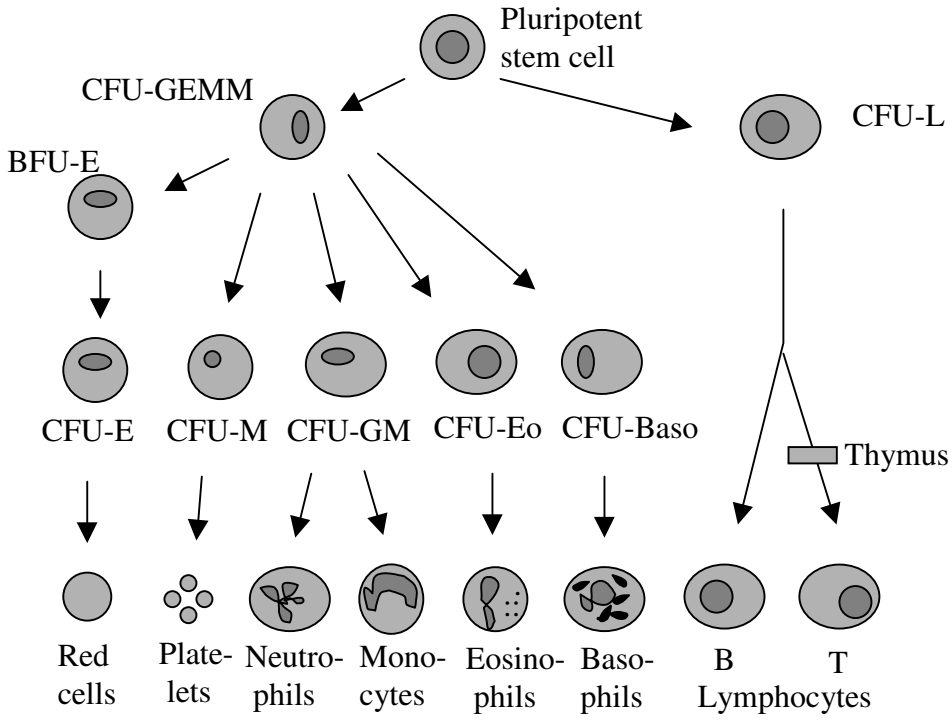

Figure 4. Hematopoietic cell lineages and mature blood cells (Modified from Hoffbrand and Pettit 1995).

Stem cells owning a capability of self-renewal constitute only about 0.1% of the bone marrow cells (Hoffbrand and Pettit 1995; Scott 1995). These cells as well as other early lymphohematopoietic cells express CD34 antigen on their surface (Kreja et al. 1993; Krause et al. 1996). CD34 is also expressed on embryonic fibroblasts and small-vessel endothelial cells (Fina et al. 1990; Krause et al. 1996).

CD34+ cells constitute 1-5% of adult (Horny et al. 1995; Scott 1995) and 5-12% of fetal bone marrow cells (Olweus et al. 1995). These cells replicate and differentiate along lymphoid or myeloid lineages that are regulated by hematopoietic growth factors and cellular interactions (Clark and Kamen 1987; Mauch et al. 1995). Expression of CD34 declines progressively as the progenitor cells differentiate and become committed to

particular lineages (Horny et al. 1995). Progenitor cells may have an age-related limited proliferation potential. Stem cells with a CD34+CD38low phenotype have shorter telomeres than cells from fetal liver or umbilical cord blood. A proliferation-associated loss of telomeric DNA has also been shown (Vaziri et al. 1994; Simmons and Haylock 1995).

3.3. Mature leukocytes

Leukocytes or white blood cells are generally involved in the cellular and humoral defences of the organism. Granulocytes and monocytes have a capacity for ameboid movement. There is a range average of 4-10$\times 10^9$/l leukocytes in normal human blood. Leukocytes are classified into granulocytes, monocytes and lymphocytes. Granulocytes constitute 65-75% of leukocytes in blood. Monocytes make up from 3-8% and lymphocytes 20 to 35% (Leeson et al. 1988).

3.3.1. Granulocytes

Granulocytes are of three types: neutrophils, eosinophils and basophils. The function of granulocytes is to protect the body against infection. The majority of granulocytes in blood are neutrophils (Leeson et al. 1988). They have a characteristic nucleus that is made up of three to five segments connected by filaments (Heckner et al. 1994). Neutrophils are phagocytes. They destroy foreign particles by microbicidal mechanisms (Roitt 1991). In eosinophils the cytoplasmic granules are coarser than in neutrophils and there are rarely more than three nuclear lobes. Eosinophils have a special role in allergenic responses and in defence against parasites, which cannot physically be phagocytosed (Leeson et al. 1988; Roitt 1991; Hoffbrand and Pettit 1995). The last group of granulocytes is basophils. They constitute only <1% of the circulating leukocytes (Dacie and Lewis 1995).

3.3.2. Monocytes

Monocytes, together with granulocytes, are phagocytes. Monocytes are larger than other peripheral blood leukocytes. They possess a large bean shaped nucleus. Granules are present in cytoplasm. From blood, monocytes settle in the tissues as macrophages. They

constitute the mononuclear phagocyte system as they filter off foreign material (Roitt 1991; Heckner et al. 1994; Hoffbrand and Pettit 1995).

3.3.3. Lymphocytes

Lymphocytes are immunologically competent cells which assist the phagocytes in the defence of the body against infection and other foreign invasion and add specificity to the attack. The lifespan of lymphocytes is from days to years. Lymphocytes are divided into T and B cells. 60-80% of lymphocytes are T cells. These cells originate in the bone marrow but undergo processing in the thymus. T cells are responsible for cell-mediated immune reactions and have surface receptors that are specific for foreign antigen recognition. B cells derive from the bone marrow stem cells but it is uncertain whether any of the cells are processed outside of the bone marrow to become mature B lyphocytes. B cells are responsible for the humoral immune reaction. They produce antibodies, which bind specifically to foreign antigens. Less than 10% of lymphocytes are so-called natural killer cells. These cells recognize virally infected cells and kill these cells before the virus has had a chance to reproduce (Leeson et al. 1988; Roitt 1991; Heckner et al. 1994; Hoffbrand and Pettit 1995).

3.4. Chronic lymphocytic leukemia

3.4.1. Clinical aspects

CLL is the most common form of leukemia in Western countries. The median age at diagnosis is approximately 60 years (Douglas et al. 1997) and the male to female ratio is 2:1 (Hoffbrand and Pettit 1995). CLL is characterized by an increased number of mature monoclonal B-lymphocytes in the blood, bone marrow, lymph nodes and spleen (Begleiter et al. 1991; Begleiter et al. 1996; Ribrag et al. 1996). The clinical course of CLL is extremely variable (Ribrag et al. 1996) and the etiology is unknown. Some patients remain asymptomatic while others progress rapidly (Melo et al. 1986). Anemia is a common feature as the disease progresses. Thrombocytopenia also occurs frequently (Hoffbrand and Pettit 1995). Patients with CLL have a greater susceptibility to infection due to reduced humoral and cellular immune processes including impaired granulocyte function (Itälä et al. 1996; Rossi et al. 1996; Schlesinger et al. 1996; Veenstra et al. 1996; Kipps 1997). CLL patients also frequently develop autoimmune

disease (Caligaris-Cappio 1996). The median survival of patients with CLL is 3-10 years (Molica 1991; Hoffbrand and Pettit 1995).

3.4.2. Leukemic B cells

CLL is thought to arise by expansion of non-cycling CD5+ B cells (Douglas et al. 1997), which are normally seen in the mantle zone of peripheral lymph nodes (Hoffbrand and Pettit 1995). As well as pan-B cell markers CD19 or CD20 and CD 22, leukemic B cells also express CD5. Typically expression of surface Ig and CD20 is low (Zomas et al. 1996; Kipps 1997). A small subset of cells expresses CD38, which is generally low or absent (Zupo et al. 1996). Circulating cells express apoptosis-regulation proteins including Bcl-2, Mcl-1, BAG-1, Bax, Bak and Caspase-3 (Kipps 1997; Kitada et al. 1998; Reed 1998). Defective apoptosis leads to continued cell expansion (Hanada et al. 1993; Miyashita and Reed 1993; Reed 1998) and is associated with resistance to chemotherapeutics (Gottardi et al. 1996; Pepper et al. 1996; Petersen et al. 1996). In approximately half of CLL patients clonal karyotypic changes have been detected in leukemic cells, but a recently developed method using optimized mitogen has revealed as much as 77% of karyotypic abnormalities (Larramendy et al. 1998). The most common aberrations are structural abnormalities of chromosome bands 13q14 and 14q32, trisomy 12 (Knuutila et al. 1986; Anastasi et al. 1992; Bullrich et al. 1996; Döhner et al. 1997; Kipps 1997) and a partial loss of the 11q region (Karhu et al. 1997; Zhu et al. 1999).

3.4.3. Treatment of CLL

Treatment of CLL becomes necessary as the disease progress (Molica 1991; Kipps 1997). Alkylating agents, such as chlorambucil and cyclophosphamide, are commonly used to treat patients with progressive CLL. These compounds reduce the lymphocyte mass by alkylating and cross-linking DNA and inducing apoptosis. Most patients, however, eventually become resistant to these drugs. (DeNeve et al. 1989; Geleziunas et al. 1991; Begleiter et al. 1994; Hoffbrand and Pettit 1995; O'Brien et al. 1995; Begleiter et al. 1996). Anti-purine drugs, fludarabine and chlorodeoxyadenosine, are also used in the treatment of CLL (Begleiter et al. 1996; Kipps 1997). Alkylating agents and purine nucleosides can probably inhibit NER and continual DNA "housekeeping" (Sandoval et al. 1991). Combination of cyclophosphamide, doxorubicin, vincristine and prednisolone

is often effective if the patient is resistant to alkylating agents (Hoffbrand and Pettit 1995; Montserrat and Rozman 1995; Ribrag et al. 1996). However, at present there is no curative treatment by drugs (Molica 1991). With advanced CLL splenectomy may ameliorate the hemolytic anemia (Seymour et al. 1997). Radiotherapy may be used as supportive treatment (Hoffbrand and Pettit 1995). Bone marrow transplant may be beneficial for younger patients (Provan et al. 1996).

4. Comet assay - single-cell gel electrophoresis

4.1. Development and principle of the method

In 1978 Rydberg and Johanson were the first to directly quantitate DNA damage in cells by lysing cells embedded in agarose on microscope slides under mild alkali conditions to allow the partial unwinding of DNA. The cells were stained with acridine orange and the extent of DNA damage was quantitated by measuring the ratio of green (indicating double-stranded DNA) to red (indicating single-stranded DNA) fluorescence using a photometer (Rydberg and Johanson 1978).

In 1984 Östling and Johanson improved the sensitivity of detecting DNA damage in individual cells by developing an electrophoretic microgel technique. The cells embedded in agarose on a microscope slide were lysed by detergents and salts at high concentrations. Then the electrophoresis was performed, which caused some of the DNA to stretch out as a tail in the direction of the anode (Östling and Johanson 1984).

The generally adopted comet assay technique is the modification by Singh and colleagues of the procedure of Östling and Johanson. In 1988 Singh and colleagues performed the electrophoresis in alkali conditions and at high salt concentration. Thus single-strand breaks and alkali-labile sites were revealed. The principle is that the discontinuities in single strands can be detected only if the base-pairing is disrupted by alkali. Following staining with ethidium bromide, increased extension of the DNA from the nucleus towards the anode was observed. The damaged cells had the appearance of a comet with a brightly fluorescent head and tail, while the undamaged cells appeared as

intact nuclei without tails (Singh et al. 1988). The comets were formed as broken ends of the DNA became free to migrate in the electrophoresis. In alkaline comet assay SSBs can be detected since the alkaline environment separates the DNA strands, which is not the case in neutral method, where the DNA is double-stranded during the electrophoresis. The continuity of the double-stranded DNA is not affected by occasional SSBs at neutral pH (McKelvey-Martin et al. 1993; Fairbairn et al. 1995).

4.2. Technical aspects

There has been increasing interest world-wide in the comet asssay in the past few years and several laboratories have performed the assay in either its original or modified form (McKelvey-Martin et al. 1993). The ability to measure DNA damage is dependent upon several biological and technical factors. As there are only few differences in the preparation of slides, much of the variation in the assay protocols is found during the lysis (Müller et al. 1994; Singh et al. 1994; Singh et al. 1995; Speit et al. 1999) and electrophoresis (Olive et al. 1990; Vijayalaxmi et al. 1992; Müller et al. 1994; Singh et al. 1994; Singh et al. 1995; Alapetite et al. 1996; Lai and Singh 1996). Lysis conditions, salt concentrations, pH and in particular lysis time, affect the ability to detect damage (Olive et al. 1992; Singh et al. 1994; Klaude et al. 1996). The voltage and time of electrophoresis is related to the levels of damage to be detected (Olive et al. 1992).

Following electrophoresis the slides are stained with a fluorescent DNA binding stain. A variety of stains have been used effectively (Olive et al. 1992; Singh et al. 1994; Singh et al. 1995; Collins et al. 1997). However, the most commonly used stains are ethidium bromide (Anderson et al. 1994; Betti et al. 1994) and propidium jodide (Olive et al. 1990; Bauer et al. 1998). There is also great variation in comet image analysis. The simplest technique is to score the comets empirically on the basis of damage extent (Visvardis et al. 1997). The distance of DNA migration from the nuclear core is also used (Lai and Singh 1996, Visvardis et al. 1997). Comet image analysis systems are used increasingly and the tail moment, the product of tail length and the fraction of DNA in the comet tail, has become a popular evaluation endpoint (Ashby et al. 1995; Hellman et al. 1995; Olive and Banáth 1995a; Bauer et al. 1998).

Studies using preserved comet assay slides (Woods et al. 1999) and even a CometAssay kit (Lemay and Wood 1999) have also been made.

For the present study the comet assay technique presented by Singh et al. 1988 was chosen with minor modifications as it is the most generally adopted method.

4.3. Applications

Comet assay was first developed to measure DNA damage induced by ionizing irradiation. At present there are also many agents that are used to induce DNA damage. A wide variety of chemicals and radiation have been used (Fairnbairn et al. 1995; Lai and Singh 1995; Malcolmson et al. 1995; Vaghef and Hellman 1995; Hartman and Speit 1996; Kasamatsu et al. 1996; Mattii et al. 1998). Comet assay also has applications in genetic toxicology (Pandrangi et al. 1995; Singh and Khan 1995; Rojas et al. 1996; Tuo et al. 1996) and in visualizing apoptotic cells (Olive et al. 1993; Gopalakrishna and Khar 1995; Olive and Banáth 1995b; Godard et al. 1999). Most studies have investigated DNA damage in human peripheral blood lymphocytes (Singh et al. 1994; Rojas et al. 1996; Visvardis et al. 1997) but tumor cells (Gedik et al. 1992; Olive and Banáth 1995a; Siim et al. 1996), animal tissues (Khan et al. 1995; Klaude et al. 1995; Pandrangi et al. 1995; Kreja et al. 1996; Vaghef et al. 1996) and even plant cells (Koppen and Verschaeve 1996) and bacterial cells (Singh et al. 1999) have been used. One of the applications of the comet assay is its use as a biomonitoring tool in human population studies investigating the effects of diet and infection (Betancourt et al. 1995), age (Singh et al. 1990), gender (Betti et al. 1994) and smoking habits (Betti et al. 1994; Sardas et al. 1995) on DNA damage.

AIMS OF THE STUDY

The present work was undertaken to study the kinetics of DNA excision repair in normal human hematopoietic cells and in chronic lymphocytic leukemia cells. UV and γ-irradiation were used to induce DNA damage, which is repaired by two repair pathways: NER or BER. The specific aims were as follows:

1. to study DNA single-strand break formation and repair in mature blood cells (Study I);

2. to analyze the effects of a repeated γ-irradiation in the repair of DNA single-strand breaks in bone marrow mononuclear cells (BMMNCs) (Study II);

3. to investigate DNA single-strand break formation and repair in bone marrow mononuclear cells and in immature CD34+ bone marrow cells (Studies III and IV);

4. to study DNA single- and double-strand break formation and repair in chronic lymphocytic leukemia cells (Studies V and VI);

5. to investigate the relation of radiation-sensitivity or –resistance on DNA strand break formation and repair in chronic lymphocytic leukemia cells (Studies V and VI).

MATERIALS AND METHODS

1. Cell isolation

1.1. Separation of lymphocytes and granulocytes

Human peripheral blood was obtained from healthy volunteers and anticoagulated with preservative free heparin (Noparin, Novo Nordisk AS, Bagsværd, Denmark). The mononuclear cell fraction was isolated by centrifugation over a Lymphoprep layer (Nycomed Pharma AS, Oslo, Norway) of density 1.077 g/ml. The cells were washed once with phosphate-buffered saline (PBS, pH 7.4) and once with culture medium consisting of RPMI 1640 (20 mM Hepes, ICN Biomedicals, Costa Mesa, CA, USA), 10% heat-inactivated fetal calf serum (Gibco BRL, Paisley, UK) and 2 mM L-glutamine (Gibco). Adherent cells were removed by a 4-hour incubation on the culture dishes at + 37 °C in culture medium. The presence of monocytes was less than 5% as assessed with esterase staining of cytocentrifuge preparations employing α-naphtyl butyrate as substrate (Yam et al. 1971). Granulocytes were separated from similar venous samples. Unfractionated leukocytes were obtained by an enhanced sedimentation of red cells using methylcellulose, whereafter the granulocytes were separated on Lymphoprep. This procedure resulted in the granulocyte preparation with less than 5% of contaminating mononuclear cells and 10-40% of red cells, as determined from the May-Grünwald-Giemsa-stained cytocentrifuge preparations. >95% of the granulocytes were mature (polymorphonuclear) neutrophils.

1.2. Separation of bone marrow mononuclear cells, CD34+ and CD34– cells

Bone marrow samples were obtained by routine diagnostic aspiration from patients with no obvious malignant hematological involvement in the bone marrow. The mononuclear cell fraction was isolated on Lymphoprep as described. Separation of CD34+ cells from the BMMNC fraction was performed using a VarioMACS magnetic cell sorting system in combination with a MACS RS+ column (Miltenyi Biotec, Auburn, CA, USA). A CD34 Progenitor Cell Isolation Kit (QBEND/10) was used according to the

manufacturer´s instructions (Miltenyi Biotec, Sunnyvale, CA, USA). Two purification rounds were performed routinely. CD34- cells represented the first flow-through fraction from the magnetic separation column. The cells were then suspended in culture medium. The purity of CD34+ cells in the selected population always exceeded 80% as assessed by immunofluorescence microscopy using phycoerythrin-conjucated mouse monoclonal anti-HPCA-2 antibody (Becton Dickinson, San Jose, CA, USA). Homogeneous morphology of CD34+ cells was determined in the May-Grünwald-Giemsa-stained cytocentrifuge preparations.

1.3. Separation of CLL cells

Clinical specimens were obtained after informed consent from 36 consecutive patients referred to the CLL outpatient clinic of Tampere University Hospital. The diagnosis and staging of CLL were based on standard clinical, morphological and immunophenotyping criteria. CLL cells were isolated from peripheral blood samples by cenrifugation over a Lymphoprep layer. The CLL cells were washed twice with PBS (pH 7.4) and once with complete CLL medium consisting of RPMI 1640 (20 mM Hepes, ICN Biomedicals, Costa Mesa, CA, USA), 10% heat-inactivated fetal calf serum (Gibco BRL, Paisley, UK), 2 mM L-glutamine and antibiotics (Gibco; 50 U penicillin/ml and 50 μg streptomycin/ml). Thereafter the cells were washed once with CLL medium without L-glutamine and antibiotics and resuspended in RPMI 1640, 50% heat-inactivated fetal calf serum and 10% dimethyl sulfoxide (DMSO). The cells were placed at −70 °C overnight and moved into liquid nitrogen the following day. For the comet assay experiments, cells were thawed rapidly at +37 °C, washed twice and resuspended in complete CLL medium, where the cell viability was >98% as determined by trypan blue dye exclusion. As the problems in separating CLL cells have been recognized (Vilpo et al. 1998), the numbers of polyclonal monocytes, B cells and T cells were always determined by flow cytometry. Their number was always less than 10%, indicating that the clonal B-CLL cells represented >90% of the isolated cells.

2. Irradiation and DNA repair

2.1. γ-Irradiation

γ-Irradiation was carried out using a ^{137}Cs source (Mølsgaard Medical, Denmark). The cells were kept in an ice-bath during irradiation and at all times before repair incubation. Non-irradiated control cells were treated in a similar way. The dose rate was 0.07 Gy/s, which was confirmed by using thermoluminesence dosimeters (kindly performed by Dr. Maunu Pitkänen). Cell viability after irradiation and repair incubations was >98% as assessed by trypan blue dye exclusion. Doses which produced accurately measurable amount of DNA damage for comet assays and were clinically relevant were used. The doses were 0.93, 1.43, 2.43, 5.43 and 10.43 Gy for comet assays and 0.7, 2.4, 5.4, 15 and 30 Gy for cytotoxicity tests.

2.2. UV-C irradiation

UV-C irradiation (peak wavelength 253.7 nm) was carried out at room temperature using a Universal Unit (Gelman Sciences, Ann Arbor, MI, USA) at a dose rate of 1 J/m^2 per s ± 0.3 J/m^2, when total dose was 2 J/m^2 and 1 J/m^2 per s ± 0.05 J/m^2, when total dose was 50 J/m^2. The dose rate was confirmed before every irradiation by using a Black-Ray (J-225) short wave UV meter (UVP, Inc, CA, USA) calibrated in the Finnish Centre for Radiation and Nuclear Safety (STUK). Cell viability after irradiation and repair incubations was > 80% as assessed by trypan blue dye exclusion. Doses of 2, 10 and 50 J/m^2 were used to induce an amount of DNA lesions, which were properly measurable by comet assays. Doses of 5, 10, 20, 50 and 100 J/m^2 were used for cytotoxicity tests.

2.3. DNA repair

The formation of DNA strand breaks and rejoining were allowed to proceed by incubating the cells for the indicated time periods in the culture medium at +37 °C. Thereafter, the cells were transferred to an ice-bath.

3. Comet assays

3.1. Alkaline comet assay

Alkaline comet assays were performed in order to study DNA single-strand breaks.
Cells (5µl, ~10 000 cells) were added to 25 µl of 0.5% low gelling temperature agarose (LGT) (Sigma: type vii, St.Louis, MO, USA) (kept at +37 °C) on microscope slides prelayered with 0.5% regular agarose. After solidification of the cell containing LGT, a top layer of 25µl LGT was added. After the top layer had solidified, the slides were immersed for 1 hour in a lysing solution consisting of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl (pH 10.0), 1% sodium sarcosinate and 1% Triton X-100. Thereafter the slides were placed in a horizontal electrophoresis unit, and the unit was filled with freshly prepared alkaline solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13.0) to a level of ~0.2 cm above the slides. The cells were exposed to the alkali for 20 minutes at room temperature. Next, electrophoresis was conducted for 15 minutes by applying an electric current of 300 mA at a voltage of 1 V/cm. After electrophoresis, the slides were washed with 0.4 M Tris-HCl (pH 7.5) to remove the alkali, stained with ethidium bromide (20 µg/ml distilled water) and coverslips applied. The edges were sealed with nail varnish. The slides were stored at +4 °C. They were brought back to room temperature and analyzed the following day.

The reproducibility of the method was tested by performing duplicate or triplicate experiments with the same donor.

3.2. Neutral comet assay

Neutral comet assays were performed in order to study DNA double-strand breaks.
Cells (15 µl, ~30 000 cells) were added to 75 µl of 0.5% LGT (kept at +37 °C) on microscope slides prelayered with 0.5% regular agarose. After solidification of the cell containing LGT, a top layer of 75 µl LGT was added. After the top layer had solidified, the slides were immersed for 1 hour in a lysing solution consisting of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM tris-HCl (pH 10.0), 1% sodium sarcosinate and 1% Triton X-100 at +4 °C.

After lysis the slides were immersed for 2 hours in Ribonuclease A (10 μg/ml; Sigma) in lysing solution without Triton X-100, pH 7.0 at + 37 °C. Thereafter the slides were immersed overnight in Proteinase K (1 mg/ml; Sigma) in lysing solution without Triton X-100, pH 7.4 at +37 °C. The following day the slides were placed in a horizontal electrophoresis unit, and the unit was filled with freshly prepared neutral solution (300 mM sodium acetate, 100 mM Tris-HCl, pH 8.5) to a level of ~0.2 cm above the slides. The cells were exposed to the electrophoresis buffer for 20 minutes at room temperature. Next, electrophoresis was conducted for 1 hour by applying a voltage of 0.4 V/cm. After electrophoresis, the slides were exposed to alkali for 10 minutes (300 mM NaOH) at room temperature and washed with 0.4 M Tris-HCl (pH 7.5) for 1 hour to remove the alkali. DNA was stained with ethidium bromide (20 μg/ml distilled water) and coverslips applied. The edges were sealed with nail varnish. The slides were stored at + 4 °C. They were brought back to room temperature and analyzed the following day.

3.3. Image analysis

Observations were made at 500 × magnification using fluorescence microscope (Leitz Labolux D, equipped with a 515-560 nm excitation filter, a 590 barrier filter, and a Leitz NPL Fluotar 50/1.00 oil immersion objective) connected through a Sony 3CCD Video Camera and a DXC-750P Camera Control Unit to a Macintosh IIci computer. The image of each individual cell was acquired immediately after opening the microscope shutter to the computer monitor (Applecolor High-Resolution RGB), employing the ColorCapture program by Data Translation (Marlboro, MA, USA). Images of 46 (Study I), 50 (Studies II-VI) randomly selected cells were analyzed in each sample. The length of DNA migration (tail) was read directly from the computer monitor with the help of a calibrated scale on the screen.

4. Cytotoxicity tests

The cytotoxic effects of γ and UV-C irradiation on human peripheral blood mononuclear cells from CLL patients and healthy donors were assessed using 4-day cultures on 96-well microplates. 200 000 irradiated cells per well were added in a volume of 100 µl, i.e. 2×10^6 per ml. The effects of irradiation were monitored by assessing protein synthesis in duplicate cultures using [^{14}C]- or [^{3}H]-leucine incorporation. The cells were cultured all together for 4 days. Labelled leucine was added for the final 24 hours of culture. After incubation, the proteins were precipitated with 0.2 M perchloric acid and collected on glass fiber filters using a multiple cell harvester (LKB Wallac 1295-001, Turku, Finland). The radioactivity incorporated was measured in a liquid scintillation counter (Wallac 1410). The radiosensitivity of the cells was calculated from the dose-response curves: an ID50 and ID80 values mean a calculated dose causing a 50 and 80% decrease of the viability of cells as determined on the basis of macromolecular protein synthesis.

5. Apoptosis detection

The proportion of apoptotic cells was assessed in non-irradiated control cells and in UV-C irradiated cells after an irradiation dose of 10 J/m^2, using cells from index CLL patients 15 and 35 (Study VI). Genzyme TACS Annexin V-FITC Apoptosis Detection Kit was used as described by the manufacturer (Genzyme Diagnostics, Cambridge, MA, USA). The proportion of apoptotic cells was analyzed by fluorescence microscopy immediately after staining. Over 100 cells were counted to obtain the percentage of apoptotic cells.

6. Statistical analyses

Statistical analyses of the groups were done using non-parametric two-tailed ANOVA according to Kruskall-Wallis (Instat, GraphPad Software), (BMDP3S by BMDP Statistical Software, Inc., CA, USA), (Statistica 5.1., Statsoft, OK, USA) and the non-parametric Mann-Whitney *U*-test (Statistica 5.1., Statsoft, OK, USA).

7. Ethics

The studies were approved by the ethics committee of Tampere University Hospital, Tampere, Finland.

RESULTS

1. DNA single-strand breaks and their repair in lymphocytes and granulocytes

1.1. Effects of γ-irradiation (Study I)

Lymphocytes and granulocytes showed quite similar dose-dependent increase in the length of DNA migration following γ-irradiation exposure to 0, 0.93, 2.43 and 5.43 Gy, although slightly longer comets were observed with granulocytes. The repair of DNA single-strand breaks was demonstrable as a 50% decrease in comet lengths among ¾ of donors already after a 15-minute repair period in both lymphocytes and granulocytes after an irradiation dose of 2.43 Gy. There was a tendency for lymphocytes to repair their DNA faster and to a greater extent than granulocytes.

1.2. Effects of UV-C irradiation (Study I)

The kinetics of the formation of DNA single-strand breaks after UV-C irradiation was different from that of the γ-irradiation-induced breaks. With lymphocytes the maximum DNA migration was observed after 0.5 hours and with granulocytes after a 1 hour incubation period after irradiation dose of 10 J/m^2. Thereafter, a decrease in comet lengths was recorded between 0.5 hours and 2.5 hours with lymphocytes and between 1.0 h and 2.5 h with granulocytes. The repair process was not complete at the end of the 2.5 hour observation period in either cell type.

2. DNA single-strand breaks and their repair in bone marrow cells

2.1. Effects of γ-irradiation (Studies II and III)

In Study III human bone marrow mononuclear cells (BMMNCs), CD34+ and CD34− cells showed similar dose-response curves when γ-irradiation doses up to 5.43 Gy were tested. There was a certain amount of intra- and interindividual variation in all cell populations. Similar repair kinetics were observed in the BMMNCs as in the CD34+ and CD34− populations. The kinetics of DNA repair was dose-dependent. Cells irradiated with 0.93 Gy repaired their DNA almost completely in 1-3 hours incubation period, which was faster than with higher irradiation doses. Cells having received 5.43 Gy repaired their damage almost completely within 24 hours after irradiation. 50% of the damage was repaired in 15-25 minutes after an irradiation dose of 2.43 Gy.

In Study II the effect of a second irradiation was investigated. The purpose was to see if 2.43 Gy pre-irradiation, 24 hours previously would change the dose-response or DNA repair kinetics in BMMNCs. The dose-responses were remarkably similar and no difference in the extent of single-strand breaks was seen when compared with previoulsy unirradiated controls. This was also the case with the repair process.

2.2. Effects of UV-C irradiation (Study IV)

Three doses, 2, 10 and 50 J/m^2, of UV-C irradiation were tested to analyze SSB formation and repair in CD34+ cells, BMMNCs and CD34− cells. After an irradiation dose of 2 J/m^2 the maximum DNA migration was seen at 0.5 hours after exposure. Thereafter, during the next 0.5-1 h, the comet lengths diminished significantly in the BMMNCs and in 50% of the cases of CD34+ cells.

After irradiation with a dose of 10 J/m^2 the strand break formation was faster than after irradiation with a dose of 2 J/m^2. The maximum DNA migration was seen at 0.5 hours after exposure except in one case, where it was at the 1 hour time point. The extent of DNA migration was greater after a dose of 10 J/m^2 than after 2 J/m^2. The repair of SSBs

was slower than after a dose of 2 J/m^2. CD34+ cells repaired their damage faster than BMMNCs within 1.0-1.5 hours after irradiation.

There were more SSBs immediately after irradiation with 50 J/m^2 than after a dose of 2 J/m^2. The extent of DNA migration was almost the same with both 10 and 50 J/m^2. The maximum migration was seen at 0.5 hours after irradiation with 50 J/m^2 except in one case, where it was at the 1 hour time point. The repair of SSBs was slow. Neither CD34+ cells nor BMMNCs had repaired all their strand breaks in 24 h. CD34+ cells repaired SSBs to a greater extent than did BMMNCs during 1.5-23 hours after irradiation with a dose of 50 J/m^2. The superior repair capability of CD34+ cells was further substantiated by comparison with CD34– cells from the same donors. In every case the comet lengths of CD34+ cells were shorter than those of CD34– cells 24 hours after irradiation with a dose of 50 J/m^2.

3. Effects of irradiation on CLL cells

3.1. Effects of γ-irradiation (Study V)

In the cytotoxic effects of γ-irradiation there were remarkable interindividual differences among the 36 CLL patients. The ID50 values varied from 0.48 Gy to 48 Gy (median 2.2 Gy). A great variation was also observed among the 8 healthy donors (0.95 Gy – 25 Gy, median 7.8 Gy).

Formation and repair of DNA SSBs and DSBs in CLL cells were studied using alkaline and neutral comet assay respectively. A dose-dependent increase in the comet lengths was observed following exposure up to 2.43 Gy (SSBs) and 10.43 Gy (DSBs) of γ-irradiation. Cells were allowed to repair their SSBs and DSBs after irradiation doses of 2.43 and 5.43 Gy. During the 3-hour incubation period almost all DNA single and double-strand breaks were repaired. However, the half-time of the damage was <30 minutes. The formation and repair of DNA strand breaks were similar to those of normal cell types. Despite great differences in radiosensitivity of the cell populations the DNA SSB and DSB repair kinetics were essentially similar.

3.2. Effects of UV-C irradiation (Study VI)

As with γ-irradiation, there were great interindividual differences in the cytotoxic effects of UV-C irradiation among the 36 CLL patients. The ID80 values varied from 5 J/m^2 to 92 J/m^2 (median 20.5 J/m^2). The variation was not so remarkable among the 8 healthy donors (14.5 – 38 J/m^2, median 21 J/m^2). For the comet assay studies the patients were classified as resistant (ID80> 45 J/m^2), intermediate or sensitive (ID80< 14 J/m^2).

Formation and repair of SSBs were studied after an irradiation dose of 10 J/m^2. The DNA migration maximum was seen between 0.5 hours and 1.5 hours after irradiation. The interindividual variation between the mean maximum migrations was more remarkable in radiation sensitive patients than in resistant patients. 2/3 of radiation-resistant CLL patients repaired their SSBs to a greater extent than 2/3 of sensitive patients. In most cases two cell populations were observed at 24 hours after irradiation, which was not seen with normal blood mononuclear cells. The two cell populations were clearly distinct according to short and long comet lengths. A possible role of apoptosis was shown in the cell population with long comet lengths.

Reproducibility of the method was confirmed by performing duplicate or triplicate experiments of the same donor. Here, the UV-C induced DNA damage and its repair is illustrated by CLL index patient 35. As in all experiments, the intraindividual variation was remarkable. The results (mean migration of DNA: μm, standard deviation, 25% and 75% quartiles as well as median) of three sets of alkaline comet assay experiments after UV-C irradiation dose of 10 J/m^2 are illustrated in Tables 1-3. 50 cells were counted for each time point.

Table 1. Results of the first experiment of CLL index patient 35. A UV-C irradiation dose of 10 J/m^2 was used.

Time after irradiation	Mean (µm)	Standard deviation	25%	Median	75%
Ctrl 0h	8	15	0	0	10
Ctrl 24h	7	16	0	0	10
0h	12	18	0	0	15
0.25h	29	20	15	30	40
0.5h	36	25	15	30	60
1.0h	44	26	20	40	60
1.5h	27	34	0	10	45
24h	42	51	0	10	100

Table 2. Results of the second experiment of CLL index patient 35. A UV-C irradiation dose of 10 J/m^2 was used.

Time after irradiation	Mean (µm)	Standard deviation	25%	Median	75%
Ctrl 0h	5	14	0	0	0
Ctrl 24h	13	29	0	0	10
0h	6	14	0	0	0
0.25h	16	18	0	10	20
0.5h	35	23	5	30	50
1.0h	48	31	25	50	70
1.5h	34	30	10	30	60
24h	53	57	0	10	115

Table 3. Results of the third experiment of CLL index patient 35. A UV-C irradiation dose of 10 J/m^2 was used.

Time after irradiation	Mean (µm)	Standard deviation	25%	Median	75%
Ctrl 0h	8	18	0	0	10
Ctrl 24h	9	18	0	0	10
0h	10	21	0	0	10
0.25h	29	17	20	30	40
0.5h	39	25	10	30	60
1.0h	34	25	10	30	60
1.5h	27	34	0	10	45
24h	50	54	0	10	110

DISCUSSION

1. Formation and repair of DNA damage

1.1. Ionizing irradiation

All cell types investigated in this study, whether mature or progenitor cells, whether normal or malignant cells, showed a surprisingly similar response to γ-irradiation when SSBs were investigated. The susceptibility of human granulocytes to low doses of γ-irradiation has been reported to be similar to that of lymphocytes (Vijayalaxmi et al. 1993), which was confirmed here. However, the result was still somewhat surprising. DNA SSBs induced by γ-irradiation are repaired by the action of enzymes in the BER pathway (Price 1993). In a previous study, a very low activity of BER enzyme uracil-DNA glycosylase was present in mature granulocytes. The enzyme activity was only 2-3% of that of lymphocytes, suggesting that short-lived non-dividing mature cells do not necessarily need such an effective DNA repair capacity (Vilpo 1988). Hence, a reduced capacity of mature granulocytes to repair the γ-irradiation-induced DNA damage would have been expected. The observation that granulocytes are able to repair SSBs induced by γ-irradiation means that BER for these lesions is operative in these cells. In addition, it has become evident that granulocytes can regulate their steady-state levels of mRNA expression (Arnold et al. 1994) and hence, template activity is also required in these cells.

Human lymphocytes have been reported to have an age-dependent increase in DNA damage immediately after irradiation (Singh et al. 1990). With granulocytes this was not observed in the present study. However, with lymphocytes, although with very limited results, significantly longer DNA migration lengths were observed with the two older donors. Peripheral blood lymphocytes have been the subject of many investigations, where irradiation or H_2O_2-induced DNA damage and repair has been studied with comet assay (Singh et al. 1990; Singh et al. 1991; Green et al. 1992; Vijayalaxmi et al. 1992; McKelvey-Martin et al. 1993; Vijayalaxmi et al. 1993; Collins et al. 1995). The repair of ionizing irradiation-induced DNA SSBs is a rapid event.

More than 50% of the total repair was observed among ¾ of donors during the first 15 minutes after 2.43 Gy of γ-irradiation. This is in accordance with the previous study, where X-irradiation of the same dose was employed (Singh et al. 1990).

As in mature blood cells, a great variation between the cells was also observed in BMMNCs and in CD34+ progenitor cells. Not all the immature cells are in the G_0 cell cycle phase as are mature leukocytes. However, different cell cycle position of individual cells cannot totally explain this variation (Green et al. 1992). Even if human BMMNCs represent a very heterogeneous population, the formation and repair kinetics of SSBs were essentially similar to those of more immature CD34+ cells or more mature leukocytes. This result is in good concordance with previous studies (Plappert et al. 1995; Ziegler et al. 1998). In this view, the repair of irradiation-induced DNA SSBs in hematopoietic cells, even in immature cells, is normal. Even when BMMNCs were exposed to two irradiation doses of 2.43 Gy 24 hours apart, which simulates the clinical radiotherapy treatment, the formation and repair kinetics of SSBs were similar. However, the lethal effects of irradiation may still be different since it is widely accepted that after ionizing radiation, cell death mainly results for other reasons than DNA SSBs (Ward 1985; Ward 1986; Dikomey 1993).

Cytotoxic effects of γ-irradiation varied remarkably between the 36 CLL patients investigated. It has been demonstrated that resistance to irradiation may develop in parallel with resistance to chlorambucil (Bentley et al. 1995). Other studies on DNA repair and drug resistance in CLL cells indicate that there is no correlation between the clinical outcome and the DNA repair activity (Begleiter et al. 1991; Joncourt et al. 1993). However, increased expression of a DNA excision repair gene, ERCC1, in cell extracts and enhanced repair of DNA cross-links in cells from therapy-resistant CLL patients have been reported (Torres-Garcia et al. 1989; Geleziunas et al. 1991). Peculiar to CLL cells appear to be alterations in mismatch repair and a loss of stringent control of the DNA repair process (Gartenhaus et al. 1996; Buschfort et al. 1997). The great variation in the cytotoxic effects induced by γ-irradiation among the CLL patients provided a good opportunity to compare the repair of DNA SSBs and DSBs in radiation-resistant and radiation-sensitive leukemic cells. DSBs are considered to be of great biological consequence since they directly lead to chromosome aberrations and

loss of genetic material (Olive et al. 1991). Although the kinetics of formation and repair of the damage was similar in leukemic cells and in normal cells and the differences in CLL cell tolerance to irradiation did not correlete with the repair kinetics *in vitro*, it does not rule out its role *in vivo.* No correlation between reduced intrinsic radiosensitivity and the delay in death of lethally-irradiated CLL cells has been found (Thomson et al. 1991). Temporary, reversible effects occurring in individual patients are still possible. It is likely that the ultimate reason for cell death after γ-irradiation is not a single form of DNA damage but a combination of multiple lesions. However, the most frequent mechanism would appear to be failure to repair DSBs. This lead to chromosome aberrations preventing cell division at the next mitosis (Ward 1990; Price 1993; Olive 1998). Their role in quiescent cells, such as CLL cells, is not known.

1.2. UV-C irradiation

The main mechanism to repair UV-C induced DNA cyclobutane pyrimidine dimers and pyrimidine-(6-4)-pyrimidone photoproducts is NER (Friedberg 1996; Wood 1996). In this study, the first 0.5-1.5 h after UV-C irradiation represented a period during which the incision-excision phase of NER predominated, whereafter the strand synthesis and ligation predominated. In a previous study, little repair was noted after 1 hour when lymphocytes were irradiated with a UV dose of 7.5 J/m^2 (Green et al. 1992). According to the difference in time of occurrence of the longest comet tail lengths, the damage recognition or incision/excision tend be slower in granulocytes than in lymphocytes thereby delaying the formation of SSBs. After SSB formation, the lymphocytes had more time for synthesis and ligation than the granulocytes. In this regard, the second phase of NER was more effective in the granulocytes. A similar order of repair kinetics has been noted in UV-C irradiation-damaged immunoglobulin constant κ and μ genes, when human granulocytes and lymphocytes were allowed to repair the damage for 24 hours (Bianchi et al. 1990).

The long-lived stem cells are targets of leukemogenesis. NER is important in preventing leukemogenesis and skin carcinogenesis (Wood 1996). The present results demonstrate a stronger NER protection of UV-induced helix-distorting DNA damage in CD34+ progenitor cells than in more mature hematopoietic cells. Thereby it can be assumed

that CD34+ cells are well protected against helix-distorting drugs such as cisplatin. This information may be useful in the development of novel strategies to improve selectivity in cancer chemotherapy and yields in tumor cell purging for stem cell transplantation. An alkylating agent, maphosphamide, is selectively toxic to against leukemic progenitor cells (Carlo-Stella et al. 1992). The drug is used for negative purging of stem cell transplants. The significance of NER in the prevention of cisplatin toxicity and helix-distorting acetylaminofluorene adducts has been demonstrated (Vilpo et al. 1995). Perhaps irradiation or drugs, such as UV-irradiation or cisplatin, requiring NER for the removal of their adducts, could provide improved selectivity in the future.

There were remarkable interindividual differences in the cytotoxic effects of UV-irradiation among the CLL patients. The delayed formation of SSBs after UV-irradiation in radiation-sensitive patients observed in the present study indicates a slower damage recognition and incision/excision step of NER when compared to radiation-resistant patients. Hence, a slower NER function might partly explain the different responses to radiation. There was a trend of CLL patients to have a population of cells with extremely long comet tail lengths at 24 hours after irradiation, which was not seen in healthy donors. Apoptotic cells have a characterized appearance with most of the DNA in the comet tail and therefore can be detected by comet assay (Olive and Banáth 1995b, Fairbairn et al. 1996). Spontaneous apoptosis in CLL cell culture has been reported (Robertson et al. 1996; Tangye and Raison 1996; Bogdanov et al. 1997), but was not observed in this study. The increased frequency of cells with long comet tail lengths represents cells with incomplete excision repair sites, delayed repair or most probably an increased incidence of cells undergoing apoptosis. These cells may have a role in sensitivity to UV-C irradiation. This kind of intraindividual cell population formation was not seen in any of the normal cell types. Hence, it can be suggested as in a previous study (Tuck et al. 2000) that normal mature and immature hematopoietic cells have more NER protection against UV-C-induced DNA damage than CLL cells. This kind of information could be useful in the future when treatment of CLL patients is further improved.

2. Comet assay

Comet assay has been increasingly used in studies of DNA strand breaks in the past few years and several laboratories have performed the assay in either its original or modified form (McKelvey-Martin et al. 1993). The method performed by Singh et al. 1988 was chosen for this study as it is commonly used and suitable for the laboratory environment. The method is fast, visual, sensitive and can be performed with a small cell samples. A high reproducibility can now be added to the advantages of the technique. Almost identical results can be obtained in a different series of experiments, as was the case with lymphocytes and granulocytes. Despite all the advantages, questions have arisen concerning the method. UV-irradiation produces DNA-protein cross-links (Love et al. 1986). Although use of highly alkaline electrophoresis solution in the alkaline version of comet assay disrupts most of the DNA-protein cross-links, their existence during the experiment could be questioned. If there were any significance of these lesions in the experimental setting, they should prevent the DNA migration in electrophoresis. However, after UV-irradiation a significant and similar increase in comet tail lengths was observed when higher UV doses were used. In neutral comet assay, when the solution is not capable to break DNA-protein cross-links, appropriate enzymes are used.

Comet assay measures strand breaks and alkali-labile sites (Fairbairn et al. 1995). The reason for long comet tail lengths observed right after UV-irradiation with high dose is also questioned as UV-irradiation does not induce strand breaks directly. The explanation may simply be in the irradiation time. For a higher UV dose a longer irradiation time is needed, for 50 J/m^2 it was 50 seconds. The damage recognition and incision/excision of NER begins apparently so rapidly that some of the damage is processed and generated into strand breaks already during the irradiation, although the cells were at room temperature. Hence, it is obvious that both phases of NER operate simultaneously, although the kinetics demonstrate the predominance of one phase at a time.

Although the comet assay is highly reproducible, a great intraindividual heterogeneity has been observed. In addition to intracellular factors, the marked differences in DNA

migration in cell populations may partially be linked to methodological variables. These include variable susceptibility of individual cells to detergents and alkali, variable sizes of nuclei (DNA distribution) and other determinants which remain to be investigated. Further work is needed for a more complete characterization and understanding of biochemical mechanisms in comet formation and repair.

SUMMARY AND CONCLUSIONS

Ionizing and UV-irradiation induce a variety of DNA damage. The effective repair of DNA damage is vital for the survival of an organism. Defects in repair can lead to mutagenesis and carcinogenesis. The main mechanisms of cells to repair DNA damage are nucleotide excision repair (NER) and base excision repair (BER). DNA lesions induced by ionizing irradiation are mainly repaired by the action of enzymes of BER and damage induced by UV-irradiation are most efficiently repaired by NER. Unlike ionizing irradiation, UV-irradiation do not directly cause DNA strand breaks, but they are formed during the NER process.

Comet assay is a fast, visual and sensitive method for the detection of DNA single and double-strand breaks in individual specifically separated cells. It can be performed with small cell samples and is highly reproducible, which are major advantages. With comet assay it is possible to investigate the function of NER and BER when the DNA damage is induced specifically.

The purpose of this study was to investigate UV and γ-irradiation-induced DNA strand break formation and repair in normal human hematopoietic cells: mature lymphocytes and granulocytes, immature bone marrow cells and in CD34+ progenitor cells, and in chronic lymphocytic leukemia cells. Contrary to previous assumptions on the basis of expression of the BER enzyme uracil-DNA glycosylase, the kinetics of excision repair after γ-irradiation was surprisingly similar in every cell type investigated. However, a better NER function in immature CD34+ progenitor cells than in more mature bone marrow cells after a high dose of UV-irradiation was observed. Interesting features of CLL cells after a moderate dose of UV-irradiation were revealed. Two cell populations, one evidently apoptotic, were formed 24 hours after irradiation. The proportion of these cells might be one determinant in radiation-resistance or sensitivity. It is possible that such information can promote the development of novel CLL treatment strategies in the future.

YHTEENVETO

Ionisoiva ja ultravioletti (UV)-säteily aiheuttavat DNA-vaurioita. Ionisoiva säteily aiheuttaa monenlaisia DNA:n emäs- ja sokerivaurioita, mutta voi katkaista DNA-säikeen suoraakin. UV-säteily aiheuttaa DNA:n kaksoiskierteistä rakennetta vääntäviä vaurioita. Korjaamattomina nämä vauriot voivat aiheuttaa mutaatioita sekä vaikuttaa syövän syntyyn. DNA-vaurioiden tehokas korjaus onkin ensiarvoisen tärkeää eliön elinkyvyn ylläpitämiseksi. Soluilla on eri keinoja vaurioiden korjaamiseen. Tärkeimmät ovat nukleotidiekskiisiokorjaus (NER) ja emäsekskiisiokorjaus (BER). Ionisoivan säteilyn aiheuttamat DNA-vauriot soluissa korjaa NER ja UV-säteilyn aiheuttamat vauriot korjaa BER. UV-säteily ei katkaise DNA-säiettä suoraan, vaan ketjun katkeaminen tapahtuu osana korjaustapahtumaa.

Komeettamenetelmä on metodi, jolla voidaan tutkia DNA:n yhden ja kahden ketjun katkoksia yksittäisissä spesifisesti erotelluissa soluissa. Menetelmän etuja ovat nopeus, visuaalisuus ja hyvä toistettavuus. Komeettamenetelmällä voidaan myös tutkia erittäin pieniä solupopulaatioita. Menetelmä on oivallinen työkalu tutkittaessa DNA:n eri korjausreittejä. Tämä on mahdollista silloin, kun DNA-vaurio aiheutetaan tunnetusti, esimerkiksi säteilyllä, jolloin tiedetään, mitä korjausreittiä solu käyttää muodostuneen vaurion korjaamiseen.

Tämän tutkimuksen tarkoituksena oli kartoittaa ionisoivan ja UV-säteilyn aiheuttamien DNA:n yhden ja kahden ketjun katkosten muodostumista ja korjaantumista ihmisen normaaleissa kypsissä verisoluissa: lymfosyyteissä ja granulosyyteissä, epäkypsissä luuytimen soluissa ja luuytimen CD34+ kantasoluissa sekä kroonisen lymfaattisen leukemian leukeemisissa soluissa. Ionisoivan säteilyn aiheuttamien DNA:n ketjukatkosten muodostuminen ja korjaantuminen oli yllättävän samankaltaista kaikissa tutkituissa solutyypeissä. Luuytimen CD34+ kantasolut osoittautuivat kypsempiä luuytimen soluja tehokkaammiksi suuren UV-säteilyannoksen aiheuttamien vaurioiden korjaajiksi. Leukeemisissa soluissa todettiin normaaleista soluista poikkeava DNA:n korjaantuminen UV-sädetyksen jälkeen. 24 tunnin kuluttua sädetyksestä solut olivat muodostaneet kaksi ryhmää, josta toisessa solut olivat apoptoottisia. Näiden kuolevien solujen osuus saattaa olla yksi määräävä tekijä potilaiden sädeherkkyydessä. On

mahdollista, että tämänkaltainen tieto auttaa tulevaisuudessa kehittämään uusia kroonisen lymfaattisen leukemian hoitomuotoja.

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