Inter-individual differences in repair of DNA base oxidation, measured in vitro with the comet assay

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There is a need for a reliable, robust and sensitive assay for DNA repair, suitable for use with human lymphocyte samples in molecular epidemiological investigations. The comet assay (single cell alkaline gel electrophoresis) has been modified to measure the ability of a simple subcellular extract of lymphocytes to carry out the initial step of repair, i.e. incision, on a DNA substrate carrying specific lesions—namely, oxidized bases introduced by visible light in the presence of photosensitizer. The cell extract is free of non-specific nuclease activity, incising DNA only if the DNA has been treated with photosensitizer and light. The activity varies between individuals, but consistency is seen between samples from each individual taken on occasions several months apart. The lack of activity of extract from Ogg1– mouse cells (deficient in the glycosylase that excises 8-oxoguanine) in this assay confirms that the activity measured is predominantly excision repair of oxidized bases. This new DNA repair assay is simple, rapid and requires only small quantities of lymphocyte extract (obtainable from 10 ml blood).

Introduction

DNA damage, the initiating event of carcinogenesis, is subject to efficient repair by a sequence of cellular enzymes. An inherited defect in one of the various proteins involved in nucleotide excision repair causes the disease xeroderma pigmentosum, and enormously increases the risk of skin cancer following sun exposure (Araújo and Wood, 1999). Defective mismatch repair characterizes the disease hereditary non-polyposis colon cancer (HNPCC) (de la Chapelle and Peltomaki, 1995). However, it has yet to be established whether there are significant quantitative variations in intrinsic repair capacity among clinically normal individuals. Such variations might arise from minor genetic differences or by epigenetic influences causing induction, enhancement or inhibition of repair enzyme synthesis or activity. If such variations exist, they will surely contribute to an individual’s risk of contracting cancer.

Conventional assays for DNA repair depend on the measurement of repair DNA synthesis (‘unscheduled’ DNA synthesis, or UDS), or of DNA breaks occurring as intermediates in repair (the incision assay), or measure directly the removal of damaged bases. These methods are generally too insensitive or otherwise unsuitable for monitoring the responses of human lymphocytes to low doses of DNA-damaging agents. An alternative approach is to measure the in vitro repair activity of a cell extract, incubated with a DNA substrate containing specific DNA damage. We have modified the comet assay (single cell alkaline gel electrophoresis) to measure the capacity of human lymphocyte extract for repair of oxidized bases. The comet assay is a sensitive method normally employed for measuring DNA breaks. Cells embedded in agarose on a microscope slide are lysed with non-ionic detergent and high salt, leaving supercoiled matrix-attached DNA in a nucleoid. Under alkaline electrophoresis, DNA with breaks extends towards the anode, forming a ‘comet tail’ when viewed by fluorescence microscopy; the percentage of total fluorescence in the tail is linearly related to DNA break frequency up to about 2 per 109 daltons (Collins et al., 1996). Purified repair enzymes can be included in the comet assay to detect specific lesions. Formamidopyrimidine DNA glycosylase (FPG), for instance, added to the DNA in the gel after the lysis step, converts altered purines, including 8-oxoguanine, into DNA breaks (Dušinská and Collins, 1996).

The present assay works in the converse way. The nucleoids are derived from cells with a certain amount of specific DNA damage; they act as a substrate, and are incubated with lymphocyte extract of unknown activity, in place of the purified repair enzymes normally employed. The extraction procedure (based on that of Redaelli et al., 1998) is very simple, and sufficient material for several assays is obtained from the lymphocytes in 10 ml human blood. The extract is remarkably free of interfering nuclease activities. The method has been applied to human lymphocyte samples, and reveals consistent inter-individual differences in repair activity on a DNA substrate containing 8-oxoguanine. Analogous experiments performed on extract from cultured cells from the Ogg1– knockout mouse (Klungland et al., 1999), compared with cells from a wild-type mouse line, indicate that the activity we measure is predominantly that of the OGG1 protein.

Materials and methods

Preparation of extract from lymphocytes

Lymphocytes isolated from venous blood by standard centrifugation on a density gradient were washed in 3× diluted extraction buffer A (see below) and centrifuged (700 ×g, 5 min, 4°C). As much as possible of the supernatant was removed, the pellet resuspended by vigorously tapping the tube and 100 µl buffer A (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, adjusted with KOH to pH 7.8) was added for each 107 cells. The suspended cells, divided into 50 µl aliquots, were frozen in liquid nitrogen and stored at −80°C.

Just prior to an assay, a frozen aliquot was thawed, 12 µl 1% Triton X-100 in buffer A was added, and the lysate was centrifuged at 14 000 ×g for 5 min at 4°C to remove nuclei and cell debris. The supernatant was mixed with 4 vol reaction buffer B (45 mM HEPES, 0.25 mM EDTA, 2% glycerol, 0.3 mg/ml bovine serum albumin, adjusted to pH 7.8 with KOH, plus...
2.5 mM ATP and, in some experiments, 10 μg/ml creatine phosphokinase and 50 nM phosphocreatine; it was kept on ice until use.

**Preparation of substrate cells**

HeLa cells at 2x10^6 per 60 mm dish were incubated overnight in Glasgow-modified MEM (ICN Pharmaceuticals, Basingstoke, UK) with 5% calf serum and 5% foetal calf serum. They were washed twice with ice-cold PBSG (PBS with 0.1% glucose) and 2 ml 0.1 μM photosensitizer Ro 19-8022 (Hoffmann-La Roche, Basel) in PBSG, or 2 ml PBSG alone, was added to each dish. Cells were irradiated for 2 min on ice, at 330 mm from a 1000 W tungsten halogen lamp. After washing twice with PBSG, the cells were detached by gentle trypsinization, dispersed by gentle pipetting, split into four aliquots from each dish and centrifuged for 3 min at 200 g at 4°C. The pellet of cells was suspended in 170 μl low melting point agarose (Gibco-BRL) at 37°C for use in the comet assay.

**In vitro repair incubation; modified comet assay**

Two 85 μl aliquots of the substrate cells in agarose were placed on a microscope slide that had been pre-coated with agarose by dipping in a solution of 1% normal electrophoresis grade agarose (Gibco-BRL, Paisley, NI) and drying. This pre-coating ensures adhesion of agarose gels applied subsequently. Glass cover-slips were placed on the gels, which were left to set at 4°C and then placed in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris made to pH 10 with NaOH, and 1% Triton X-100) for 1 h at 4°C. The slides were immersed in three changes of buffer B (5 min each). Cell extract (45 μl) with ATP was added to each gel, covered with a cover-slip, and incubated for 45 min (or other times specified in Results) at 37°C in a humid chamber. Slides were then placed in an electrophoresis tank, immersed in 0.3 M NaOH, 1 mM Na_2EDTA (approximately pH 13), for 40 min, before electrophoresis at 25 V (0.8 V/cm), approximately 300 mA, for 30 min. After neutralization with 0.4 M Tris–HCl, pH 7.5, comets were stained with 4,6-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy. 100 comets per gel were classified visually into five categories, according to the intensity of DNA fluorescence in the tail relative to the head, and an overall score for each gel of between 0 and 400 was calculated; this score is linearly related to DNA break frequency over a wide range of damage (see Collins et al., 1997 for further details).

**Cellular and in vitro repair in mouse cell lines**

Wild-type and Ogg1– mouse fibroblast cell lines (6) were obtained from Dr Tomas Lindahl (Imperial Cancer Research Fund, London). They were cultured in D-MEM/F12 medium (Gibco-BRL) with 10% foetal calf serum. To measure the ability of these cells to remove 8-oxoguanine from their own DNA, replicate cultures in 35 mm dishes were washed with PBSG, and 2 ml 0.2 μM Ro 19-8022 was added. The cells were irradiated on ice for 2 min at 330 mm from the 1000 W tungsten halogen lamp. The cells were washed again with PBSG, and incubated in culture medium for up to 4 h at 37°C. At intervals, cells were lightly trypsinized and prepared for the standard comet assay, with FPG to detect the remaining 8-oxoguanine in the DNA, as previously described (Dušinská and Collins, 1996).

For the in vitro repair assay, cells of the two mouse cell lines were collected by light trypsinization of sub-confluent cultures grown in 90 mm dishes. Extracts were then prepared exactly as with lymphocytes, and incubated with nucleoid DNA substrate from HeLa cells treated with Ro 19-8022 and light.

**Protein concentration**

Aliquots (50 μl) of cell suspension (after thawing, without addition of Triton) were incubated overnight with 0.95 ml 0.1 M NaOH. Aliquots (0.1 ml) diluted with 0.9 ml water were analysed for protein concentration by the method of Lowry et al. (1951) calibrated against standard concentrations of bovine serum albumin.

**Results**

Initial experiments were carried out with ATP and the ATP-regenerating system (phosphocreatine and creatine phosphokinase) present in the reaction buffer, as we intended to devise a method applicable to both nucleotide excision repair, which requires ATP, and base excision repair. Later experiments, concentrating on base excision repair, retained the ATP for consistency, although in control experiments its omission did not affect the results. The ATP-regenerating system was omitted in the experiments comparing lymphocytes from different individuals and in the experiments with mouse cell lines.

**DNA incising activity of lymphocyte extract on various substrate DNAs**

(A) No extract, undamaged nucleoids. (B) No extract, nucleoids from cells treated with Ro 19-8022 and light. (C) Undamaged nucleoids incubated with extract. (D) Nucleoids from cells treated with light only, incubated with extract. (E) Nucleoids from cells treated with Ro 19-8022 and light, incubated with extract. Mean DNA breakage levels are shown, from incubations with lymphocyte extracts prepared from three individuals in several experiments. Bars indicate standard error of mean.

**Nicking activity of extract is dependent on oxidative DNA damage**

Damage was introduced to the nucleoid DNA by prior treatment of the HeLa cells with the photosensitizer Ro 19-8022 and irradiation with visible light. The predominant form of damage is 8-oxoguanine (Pflaum et al., 1998). Figure 1 shows the results of incubating these damaged, agarose-embedded nucleoids (and control nucleoids from untreated cells or cells irradiated with light in the absence of the photosensitizer) with lymphocyte extract for 45 min. Also shown is the result of incubation in the absence of extract. Comet analysis revealed substantial DNA breakage after incubation of extract together with nucleoids from cells treated with Ro 19-8022 and irradiated, but low levels of damage in all other cases. Thus the extract does not contain significant non-specific nuclelease activities, but is effective at recognizing the oxidized bases introduced by light with Ro 19-8022. The enzyme FPG also causes DNA breaks in these damaged nucleoids (results not shown).

**Nicking activity is dependent on extract concentration**

Lymphocyte extract, prepared as described in Materials and methods by diluting supernatant from thawed lysed cells with buffer B (+ATP etc.), was diluted to 0.5× and 0.25× the original concentration with this reaction buffer, and incubated with nucleoid DNA for 45 min (Figure 2). Nicking activity on irradiated or untreated substrate DNA is low at all three concentrations. The activity on Ro 19-8022-treated and irradiated DNA is low with the most dilute extract, moderate with the 0.5× extract and highest with the original strength of extract.

**Reproducibility of the in vitro repair assay**

Extracts were prepared from lymphocytes collected from five volunteers and, on two occasions 1 month apart, incubated for different times with nucleoids from HeLa cells treated with Ro 19-8022 and light (Figure 3). All extracts induced strand breaks above the control level. The rate of accumulation of breaks with time of incubation varies from extract to extract,
Fig. 2. Dependence of DNA-incising activity on extract concentration. Lymphocyte extracts were incubated, at the original concentration (1.0) and diluted to 0.5 and 0.25 that concentration, for 45 min with nucleoids from HeLa cells treated with Ro 19-8022 and light (Δ). Controls: incubation of extract with nucleoids from cells treated with light only (○) or with neither light nor Ro 19-8022 (□). Lymphocyte extracts prepared from two individuals were used in several experiments. Bars indicate standard error of mean.

Fig. 3. Time-course of DNA-incising activity in lymphocyte extract. Extract from lymphocytes of five individuals (at 1X concentration) was incubated with nucleoids from HeLa cells treated with Ro 19-8022 and light, in reaction mixture containing ATP (but no ATP-regenerating system). Different lines and open symbols represent different subjects. Solid symbols represent control incubation without extract. (A) Extracts assayed within 20 days of preparation; (B) same extracts assayed 1 month later. Bars indicate range of duplicate determinations.

Fig. 4. Individual variation in DNA-incising activity. Extract from five individuals (indicated by different symbols) was prepared on two occasions, 4 months apart, and tested with nucleoids from HeLa cells treated with Ro 19-8022 and light. Lines and symbols are as in Figure 3, but initial levels of breakage have been subtracted. (A) First extracts (mean values of all data presented in Figure 3); (B) second extracts. Error bars are not shown, for the sake of clarity.

Inter-individual differences in in vitro repair

The apparent differences seen in Figure 3 are intriguing, but might have arisen through experimental variation at the extract preparation stage. Extracts were therefore prepared from the same five individuals 4 months later, and results of the tests of the two extracts are presented in Figure 4. Initial levels of DNA breakage have been subtracted so that individual repair activities can be more easily compared. There is a similarity in the plots of in vitro repair activity for each individual on the two occasions, and thus it appears that inter-individual differences are maintained over a considerable time.

Protein concentrations in the samples of lymphocytes were measured using the Lowry assay. The mean protein concentrations for the two batches of five individual samples were 21.1 and 20.7 mg/ml, with a coefficient of variation of 5%. The very low variability in protein concentration indicates that determining the extract volume on the basis of cell number is sufficiently accurate.
Cellular repair of 8-oxoguanine by mouse cells
We compared the ability of the two mouse fibroblast lines (wild-type, and derived from the Ogg1−/− knockout mouse) to repair DNA base oxidation, in two distinct ways. First, we treated cells with Ro 19-8022 and visible light to induce 8-oxoguanine, and followed repair by measuring residual FPG-sensitive sites over a period of incubation (Figure 5). Whereas the wild-type cells remove a significant proportion of FPG-sensitive sites in 4 h, cells from the knockout mouse repair few if any lesions—consistent with the absence of OGG1 protein, the mammalian equivalent of FPG.

Repair of 8-oxoguanine by mouse cells, measured with the in vitro assay
Extracts were prepared from the two mouse cell lines to test in the new in vitro assay. The experiment (Figure 6) is analogous to the tests of lymphocyte extract, and indicates an inability of the extract from the knockout mouse cell line to make breaks in the DNA substrate containing 8-oxoguanine. Thus it seems clear that the predominant activity measured by the new assay is incision carried out by OGG1 protein. The active agent in human lymphocytes is also most probably OGG1.

Discussion
Information on inter-individual differences in DNA repair capabilities is limited by methodological inadequacies. UDS can be detected as incorporation of [3H]thymidine into non-S-phase cells after treatment with UVC radiation or with chemicals that attach bulky adducts to the DNA. Attempts have been made to detect individual differences in repair capacity using UDS (Lambert et al., 1979; Hu et al., 1996). Although the repair patches inserted by nucleotide excision repair are relatively large, the doses of damage required to induce measurable UDS are so high that they are likely to saturate the repair enzyme system and obscure significant differences. In the case of base excision repair, quantitation of UDS is even more difficult, as the repair patches are much smaller. In any case, UDS is an indirect index of repair, depending on the size of the intracellular pool of dTTP, which determines the specific activity of [3H]dTTP and therefore the level of incorporation.

An alternative approach, given a sensitive assay, is to follow the removal of DNA damage with time on incubating lymphocytes in vitro following treatment with a DNA damaging agent. DNA strand breaks introduced by ionizing radiation or by H2O2 can be monitored with assays such as the comet assay. We have found very slow apparent break rejoining rates in freshly isolated lymphocytes (compared with the rapid repair kinetics of cultured cell lines), and attribute this to additional oxidative damage resulting from sudden exposure to atmospheric oxygen (Fillion et al., 1998; Torbergsen et al., 2000).

An assay based on the incorporation of 32P-labelled deoxy-ribonucleotide into plasmid DNA at the site of specific lesions has been widely used to characterize base and nucleotide excision repair at the molecular level, in extracts from wild-type and mutant cell lines (Biggerstaff et al., 1993). The assay, requiring extract from large numbers of cells, has not been applied to the measurement of individual repair capacity in humans. An alternative method is based on the detection of breakage of a 32P-labelled oligonucleotide containing a single centrally placed 8-oxoguanine residue (Roldán-Arjona et al., 1997). There are practical disadvantages in the use of 32P label. Redaelli et al. (1998) describe an assay for AP (apurinic/apyrimidinic) site endonuclease activity in crude human lymphocyte extracts, in which the accumulation of nicked circular forms of an experimentally depurinated plasmid is detected by conventional agarose gel electrophoresis; a preliminary investigation of 10 individuals revealed inter-individual differences as well as intra-individual or experimental variation.

The extraction procedure and reaction conditions employed by us are based on those employed in this last study. However, instead of measuring plasmid nicking, we use the comet assay
for estimation of strand breaks. This method has very high sensitivity; the range of detection is approximately 0.2–2 breaks per 10⁶ daltons (Collins et al., 1996). The assay is economical on material; the volume of reaction mix (operating on DNA embedded in the gel) is only 50 μl, and enough material is obtained from the lymphocytes in 10 ml blood to carry out several incubations, so this new approach should be well suited to molecular epidemiological applications.

Control experiments show that there is no non-specific endonuclease activity in the extract; experimentally induced DNA damage must be present for significant DNA breaks to accumulate in the presence of the extract. The amount of endogenous base oxidation in HeLa cell DNA is very low (Collins et al., 1997), and clearly does not provide a significant substrate in this assay. The activity shows the expected dependence on incubation time and extract concentration. Extract is stable at –80°C; aliquots of the same samples thawed at intervals over several months gave comparable results (Figure 3 and unpublished results). The failure of extract from Ogg1 mouse cells to make breaks in DNA damaged by treatment with Ro 19-8022 and light confirms the specificity of the assay, in combination with this damaged DNA, for repair of 8-oxoguanine (the main substrate for OGG1 protein).

The assay is able to discriminate between individual lymphocyte samples. The five individuals tested (Figures 3 and 4) show differences both in initial rate of repair and in the plateau level of DNA breaks reached after 40 min incubation. One individual shows a curious pattern of activity, with an initial increase in breaks followed by a fall to a minimum at 20 min and a later increase. The significance of this is not clear, but it implies an ability of this extract to carry out steps of 8-hydroxyguanine-DNA glycosylase.

References


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