The Comet Assay for DNA Damage and Repair

Principles, Applications, and Limitations

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Abstract

The comet assay (single-cell gel electrophoresis) is a simple method for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. Cells embedded in agarose on a microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks. The likely basis for this is that loops containing a break lose their supercoiling and become free to extend toward the anode. The assay has applications in testing novel chemicals for genotoxicity, monitoring environmental contamination with genotoxins, human biomonitoring and molecular epidemiology, and fundamental research in DNA damage and repair. The sensitivity and specificity of the assay are greatly enhanced if the nucleoids are incubated with bacterial repair endonucleases that recognize specific kinds of damage in the DNA and convert lesions to DNA breaks, increasing the amount of DNA in the comet tail. DNA repair can be monitored by incubating cells after treatment with damaging agent and measuring the damage remaining at intervals. Alternatively, the repair activity in a cell extract can be measured by incubating it with nucleoids containing specific damage.

Index Entries: Comet assay; single-cell gel electrophoresis; DNA damage; DNA repair; genotoxicity testing; molecular epidemiology.

1. Introduction

Over the past decade, the comet assay, or single-cell gel electrophoresis (SCGE) has become one of the standard methods for assessing DNA damage, with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology, as well as fundamental research in DNA damage and repair. The assay attracts adherents by its simplicity, sensitivity, versatility, speed, and economy, and the number of publications it spawns rises each year. It is sometimes used without too much thought regarding how it works or what sort of information it provides; the fact that it is so successful at demonstrating DNA damage is enough to justify its use. This is a shame, as it is capable of subtle manipulation to tell us not just how much damage is present in cells, but what form it takes. Although it is essentially a method for measuring DNA breaks, the introduction of lesion-specific endonucleases allows detection of, for example, ultraviolet (UV)-induced pyrimidine dimers, oxidized bases, and alkylation damage.

The purpose of this review is to clear up some misconceptions about the comet assay and to show how its special features can best be exploited.

2. How the Comet Assay Works and What It Measures

2.1. Background

In the 1970s, Peter Cook and colleagues *(1)* developed an approach to investigating nuclear structure based on the lysis of cells with nonionic detergent and high-molarity sodium chloride. This

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treatment removes membranes, cytoplasm, and nucleoplasm, and disrupts nucleosomes, almost all histones being solubilized by the high salt. What is left is the nucleoid, consisting of a nuclear matrix or scaffold composed of ribonucleic acid (RNA) and proteins, together with the DNA, which is negatively supercoiled as a consequence of the turns made by the double helix around the histones of the nucleosome. The survival of the supercoils implies that free rotation of the DNA is not possible; Cook et al. proposed a model with the DNA attached at intervals to the matrix so that it is effectively arranged as a series of loops, rather than as a linear molecule. When the negative supercoiling was unwound by adding the intercalating agent ethidium bromide, the loops expanded out from the nucleoid core to form a "halo." A similar effect was seen when ionizing radiation was used to relax the loops—one single-strand break being sufficient to relax the supercoiling in that loop.

The comet assay, too, in its most commonly used form, involves lysis with detergent and high salt—after embedding cells in agarose so that the DNA is immobilized for subsequent electrophoresis. The first demonstration of "comets" (though they did not use the word) was by Östling and Johanson *(2)*, who described the tails in terms of DNA with relaxed supercoiling and referred to the nucleoid model of Cook et al. Essentially, the comet tail seems to be simply a halo of relaxed loops pulled to one side by the electrophoretic field. Östling and Johanson employed a pH of less than 10. It needs to be clearly understood that although the most common variant now employed is alkaline SCGE, a high pH is not essential to detect single-strand breaks.

The comet assay is most commonly applied to animal cells, whether in culture or isolated from the organism (e.g., lymphocytes separated from blood, or cells from disaggregated tissues). However, methods have also been developed to examine damage in the DNA of plant cells. The cellulose plant cell wall presents a barrier to the release of DNA to form a comet tail, but physically chopping up the tissue with a knife releases nuclei that can then be embedded in agarose *(3)*.

2.2. Common Comet Assay Variants

2.2.1. Alkaline Single-Cell Gel Electrophoresis

The procedure of Östling and Johanson was not widely adopted. A few years later, two research groups independently developed procedures involving treatment at high pH. Singh et al. *(4)* lysed cells at pH 10 with 2.5M NaCl, Triton X-100, and Sarkosyl for 1 h, following this with a treatment with alkali (0.3M NaOH) and electrophoresis at the resulting high pH (>13). Olive et al. *(5)* simply lysed cells in weak alkali (0.03M NaOH) for 1 h before electrophoresis. Thus the idea has grown up that the comet assay is in the same category as alkaline unwinding, alkaline elution, or alkaline sucrose sedimentation, where separation of two DNA strands around a break by alkaline denaturation is essential to reveal the break. For the reasons stated in **Subheading 2.1.**, I believe this to be a profound misunderstanding. The use of alkali makes comet tails more pronounced and extends the useful range of damage that can be detected *(6)*, but it does not increase the sensitivity (i.e., the lowest dose of damage detected). Östling and Johanson *(2)* detected the effect of ionizing radiation from a fraction of a Gy up to 3 Gy; Singh et al. *(4)* reported tails increasing in length over the range of 0.25 to 2 Gy.

The protocol introduced by Singh et al. has been simplified *(7)*. It is now common practice to embed cells in a single layer of agarose on a plain glass slide precoated with agarose and dried (in the original method, the cells were in the middle of three agarose layers on a frosted glass slide). Sarkosyl is frequently omitted from the lysis solution.

2.2.2. Neutral Single-Cell Gel Electrophoresis

Following the example of Östling and Johanson *(2)*, we demonstrated the ability of a neutral procedure to detect low levels of DNA breaks *(6)*. We later used a variant in which, after a period of alkaline treatment, conditions were restored to neutral for the electrophoresis *(8)*. This modification decreased the sensitivity and extended the useful range of the assay, but it was clear (from the kind of damaging agent used) that we were still detecting single-stranded breaks.

A radically different kind of neutral comet assay was developed earlier by Olive et al. *(9)* to facilitate detection of double-stranded breaks without interference from single-strand breaks. Their procedure employed extended treatment of lysed cells in agarose at 50°C, and under these conditions it is likely that the nuclear matrix is disrupted so that we are truly looking at the behavior of double-stranded pieces of DNA (or the free ends of these fragments).

2.2.3. Use of Lesion-Specific Enzymes

Measuring DNA strand breaks gives limited information. Breaks may represent the direct effect of some damaging agent, but they are generally quickly rejoined. They may in fact be apurinic/apyrimidinic sites (i.e., AP sites or baseless sugars), which are alkali labile and therefore appear as breaks. Or they may be intermediates in cellular repair, because both nucleotide and base excision-repair processes cut out damage and replace it with sound nucleotides.

To make the assay more specific as well as more sensitive, we introduced the extra step of digesting the nucleoids with an enzyme that recognizes a particular kind of damage and creates a break. Thus endonuclease III is used to detect oxidized pyrimidines *(10)*, formamidopyrimidine DNA glyco–sylase (FPG) to detect the major purine oxidation product 8-oxoguanine as well as other altered purines *(11)*, T4 endonuclease V to recognize UV-induced cyclobutane pyrimidine dimers *(12)*, and Alk A incises DNA at 3-methyladenines *(13)*. In each case, the enzyme-sensitive sites converted to additional DNA breaks increase tail intensity.

Oxidized bases are detected with endonuclease III or FPG in cells that have been treated with H_2O_2, or with photosensitizer plus visible light. They are also present in significant numbers in normal human lymphocytes (*see* **Subheading 4.2.4.**).

2.3. Some Less Common Variants

2.3.1. Bromodeoxyuridine Labeling to Detect Replicating DNA

DNA breaks associated with replicating DNA would be expected to give rise to comet tails. However, it is normally impossible to distinguish S-phase from non-S-phase cells in this way—perhaps because the amount of DNA taking part in replication at any one time is exceedingly small, or because the replication apparatus stabilizes the replicating fork in some way so that the break does not behave as a normal damage break. If cells are labeled during replication with bromodeoxyuri–dine (BUdR), which is then visualized with anti-BUdR antibody, labeled comet tails are seen; maturation of replicating DNA during a post-BUdR-labeling chase results in "retreat" of the labeled material into the head *(14)*.

2.3.2. Detecting Intermediates in DNA Repair

The breaks that occur as intermediates in nucleotide excision repair of UV-induced damage or bulky adducts are normally short-lived—at least in proliferating cells. Incubation of UV-irradiated cells with DNA synthesis inhibitors hydroxyurea, cytosine arabinoside, or aphidicolin blocks repair patch synthesis and causes incision breaks to accumulate, and this provides a sensitive way to detect the effects of the damaging treatment *(15)*. In non-dividing cells, such as peripheral lymphocytes, incision breaks accumulate without inhibitors, as the rate of religation is limited by the poor supply of deoxyribonucleoside triphosphates *(16,17)*.

2.3.3. FISH Comets

The appearance of a comet reflects damage in the cellular DNA overall. It would be extremely informative to locate specific chromosomes, or regions of chromosomes, classes of DNA or specific genes within the comet. Fluorescent *in situ* hybridization (FISH), using probes of cDNA or oligonucleotides to recognize the sequences of interest is the normal approach to this, but hybridization to DNA with the fine structure of comets in a slide-mounted agarose matrix that melts at normal hybridization temperatures presents technical difficulties. These have been solved, and it is possible to identify DNA of a particular chromosome, telomeric DNA, centromeric DNA, and single copy genes *(18–20)*. Little by way of useful information has emerged so far (though the pictures are pretty), but the approach is promising. For example, it is possible to measure gene-spe-

cific repair rates after low doses of DNA-damaging agent *(21)*.

2.4. Does the Comet Assay Detect Apoptosis?

When almost all the DNA is in the tail of a comet, the head is reduced in size, and the image has fancifully been referred to as a "hedgehog" comet. For some reason, the idea has grown that hedgehog comets represent apoptotic cells. It is possible, of course, that some of these relatively severely damaged cells will subsequently go through programmed cell death, but they cannot be described as apoptotic, for two reasons:

1. Apoptosis is irreversible, but cells with damage revealed as hedgehog comets (e.g., after treatment with H_2O_2 at sublethal concentrations) can repair their damage so that hedgehogs are no longer seen *(17)*. The explanation is not that the damaged cells die and disappear from the experiment, as the unaltered density of comets in the gel indicates that all cells are still present.
2. Apoptosis is characterized by fragmentation of DNA to the size of nucleosome oligomers. Such small pieces of DNA would certainly disappear during lysis or electrophoresis. The "ghosts" of comets that are sometimes seen, with a small percentage of normal DNA fluorescence, may represent a residue of high-molecular-weight DNA in apoptotic cells.

Singh *(22)* has described a visualization method for apoptotic cells in which cells are embedded in agarose and lysed as for the normal comet assay, and then—instead of electrophoresis—the DNA is precipitated with ethanol. Cells treated with an agent known to induce apoptosis appeared with a halo of granular DNA and a hazy outer boundary—as one would expect from diffusing small fragments.

2.5. Are AP Sites Seen as DNA Breaks in the Comet Assay?

AP sites are alkali labile—but are the pH and treatment time commonly used for alkaline SCGE enough to convert some or all of them to breaks? It seems likely that the methods employing strong alkali (0.3M NaOH) convert more AP sites to breaks than do the methods employing only 0.03M

NaOH, and this may account in part for the apparently lower sensitivity of the mild alkali methods, but pH is not the only variable, and a direct comparison of methods is difficult.

It should be possible to use AP endonucleases to clear up this question; under specific conditions of pH, and after treatment of cells with an agent known to induce base loss from DNA, does digestion of nucleoids with AP endonuclease increase the yield of breaks? However, AP endonucleases are often associated with glycosylases that remove particular damaged bases, producing more AP sites, and there is also the possibility of nonspecific contaminating nuclease activity, so attempts to give decisive answers often leave a suspicion of ambiguity. Experiments with methyl methane–sulfonate-treated Vicia faba root tip cells indicated that there certainly are some AP endonuclease-sensitive sites that are not converted to breaks under strongly alkaline conditions (0.3M NaOH) *(8)*.

3. Quantitation

3.1. Visualizing Comets

DNA is visualized by fluorescence microscopy after staining with a DNA-binding dye. Ethidium bromide (EB) is probably most commonly used, followed by 4,6-diamidino-2-phenylindole (DAPI). EB is an intercalating dye that binds more efficiently to double-stranded DNA than to single-stranded DNA. DAPI binds to the major groove, and fluorescence should therefore be dependent on double-stranded structure. DNA strand separation occurs during alkaline treatment, and even if supercoiled intact loops of DNA in the head renature readily on neutralization, at least the DNA in the tail should be thoroughly single stranded. Staining with acridine range (AO) confirms this. AO gives red fluorescence with single-stranded nucleic acid and yellow–green fluorescence with double-stranded DNA. We found *(6)* that AO staining produced red comet tails and predominantly yellow–green heads. So what can we make of the EB and DAPI fluorescence? It should, at least, be less intense with single-stranded DNA compared with double-stranded DNA, so the higher the proportion of DNA in the tail, the less the total fluorescence detected in the whole comet should be. We measured total fluorescence (with
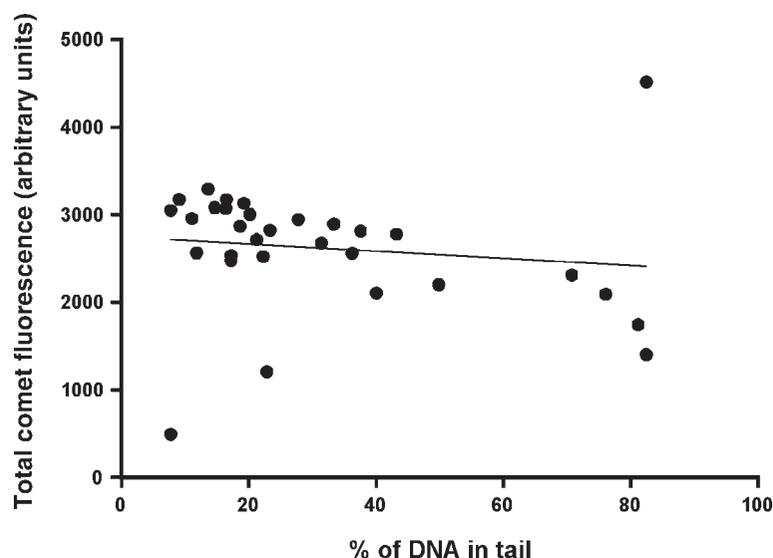
Fig. 1. Relationship between total DAPI fluorescence and percentage of DNA in the tail. Typical results, with comets from H_2O_2-treated lymphocytes. Data were obtained by Umit Türkoğlu.

DAPI staining) in comets showing different levels of damage and found only a slight decrease in total fluorescence with an increasing fraction of DNA in the tail (**Fig. 1**). Perhaps there is a fortuitous compensation—self-quenching of fluorescence in the densely packed DNA of the head detracting from the more efficient staining of double-stranded DNA. Whatever the reason, it allows us to obtain additional information from comets derived from a proliferating cell population. Total fluorescence reflects DNA content, and a cell in G_2 should give rise to a comet with more fluorescence than a cell in G_1. Thus levels of damage in individual comets from a cell population can be related to cell cycle phase *(23)*. In effect, comets are sorted according to DNA content in the same way as in flow cytometry.

3.2. Measuring Comets: Image Analysis

There are numerous software packages to choose from that will compute fluorescence parameters for comets selected by the operator. The most commonly used parameters are tail length, relative fluorescence intensity of head and tail (normally expressed as a percentage of DNA in tail), and tail moment. Mean tail length, in my experience, is not very useful, as it increases only while tails are first becoming established, at relatively low dam-

age levels. Subsequently, the tail increases in intensity but not in length as the dose of damage increases. Tail length is also sensitive to the background or threshold setting of the image analysis program, as the end of the tail is defined by a certain excess of fluorescence over background. Relative tail intensity is the most useful parameter, as it bears a linear relationship to break frequency (*see* **Subheading 3.5.**), is relatively unaffected by threshold settings, and allows discrimination of damage over the widest possible range (in theory, from 0 to 100% DNA in tail). It also gives a very clear indication of what the comets actually looked like. In contrast, the third parameter, tail moment (essentially the product of tail length and tail intensity) is not linear with respect to dose and does not give any impression of the comet's appearance. It is hard to understand why this method of quantitation is so popular. Analysis of 50 comets per slide is recommended.

3.3. Measuring Comets: Visual Scoring

It is possible to compute DNA damage from comets without sophisticated image analysis programs. The human eye is easily trained to discriminate degrees of damage according to comet appearance (**Fig. 2**). We find that 5 classes, from 0 (no tail) to 4 (almost all DNA in tail) give sufficient

resolution. If 100 comets are scored, and each comet assigned a value of 0 to 4 according to its class, the total score for the sample gel will be between 0 and 400 "arbitrary units." Visual scoring is rapid as well as simple and should appeal to those exploring the usefulness of the technique without wanting to invest in expensive analytical equipment. There is a very close agreement between the two methods, demonstrated in **Fig. 3**, in which slides prepared from a large number of human subjects were scored independently in both ways.

3.4. Selection of Comets

This is an issue for both computer-based analysis and visual scoring. Comets must be selected without bias and must represent the whole gel, so it is necessary to scan the gel in a systematic way. The edges, as well as areas around air bubbles, should be avoided, as they often display comets with anomalously high levels of damage. Analysis of overlapping comets is impossible, at least with computer analysis, but the most likely comets to overlap are those with big tails. If too many large overlapping comets are rejected, there may be a significant bias toward undamaged, tailless comets. It is important not to have gels too densely packed with cells; the recommended number of cells in one gel is around 2×10^4.

3.5. Calibration

The standard method of calibration (though it is performed more rarely than it should be) is to irradiate samples of cells with γ- or X-rays to induce known numbers of single-stranded breaks in DNA. The breaks tend to be rapidly repaired, so cells should be irradiated on ice—preferably already embedded in agarose to minimize postirradiation handling.

Comets show a linear increase in percentage of DNA in tail (or in visual score) over a range of 0 to 8 Gy *(24)*, that corresponds to DNA damage up to about 2.5 breaks per 10^9 Dalton. It is important to keep this figure in mind when considering the behavior of DNA under SCGE conditions: It corresponds to a "fragment" length of about 160 μ*M*—far longer than the comet tail itself—and so it is clearly nonsense to represent the tail as made

up of DNA fragments migrating according to their size. This point was recognized by Östling and Johanson *(2)* who stated that the molecular weight of DNA is "certainly many magnitudes higher than the molecular weight of DNA used in conventional electrophoretic separations and any comparison with separation of DNA of 10^9 Daltons or less is not relevant."

4. Applications
4.1. Genotoxicity Testing

The comet assay has achieved the status of a standard test in the battery of tests used to assess the safety of novel pharmaceuticals or other chemicals *(25)*. It is readily applied to in vivo experiments; tissues that can be disaggregated to single-cell suspensions, as well as white blood cells, provide the material. The assay is normally used in its simple form to measure strand breaks, but increased sensitivity, as well as additional information on mechanisms of action, would accrue from inclusion of repair endonucleases to measure specific types of lesions.

Genotoxicity is also assessed in cell culture systems, on their own or in conjunction with the microsomal "S9" fraction from liver, that provides enzymes to metabolize chemicals to more reactive forms *(26)*.

Chemoprotection is the other side of the coin: The comet assay is eminently suitable for assessing the ability of phytochemicals, for example, to protect cells against genotoxic insult *(27)*.

4.2. Ecological Monitoring

Suitable organisms can be used in combination with the comet assay as biosensors for contamination of the environment with genotoxins. This work is at an early stage, but promising results have been reported. Mussels are the favorite organism for assessing contamination in the marine environment *(28)*. On land, earthworm coelomo–cytes have been used successfully to detect geno–toxic compounds in soil *(29)*, and small rodents living around waste sites have been shown to have elevated levels of DNA damage in lymphocytes compared with animals living on clean land (E. Delgado Sureda, personal communication).
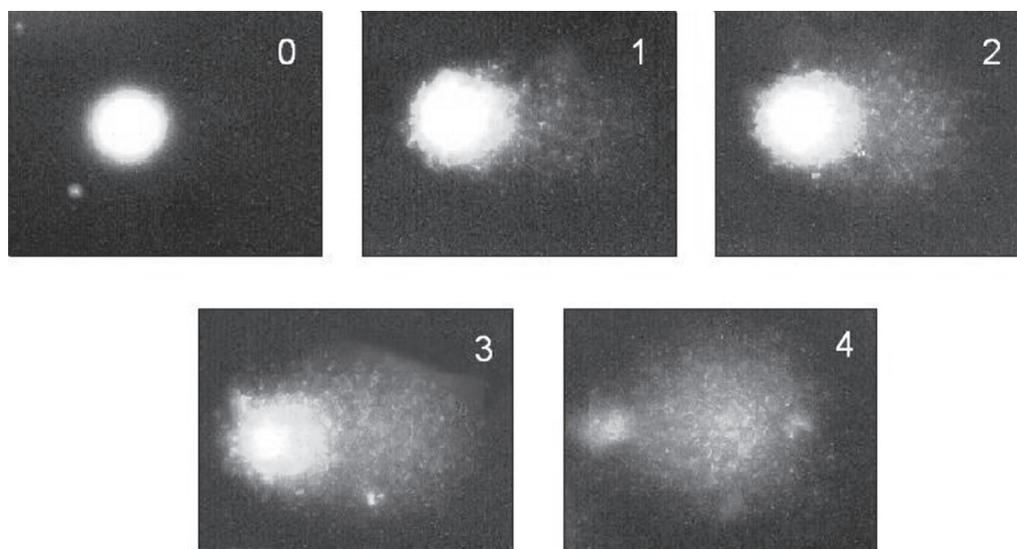
Fig. 2. Images of comets (from lymphocytes), stained with DAPI. They represent classes 0–4 as used for visual scoring.
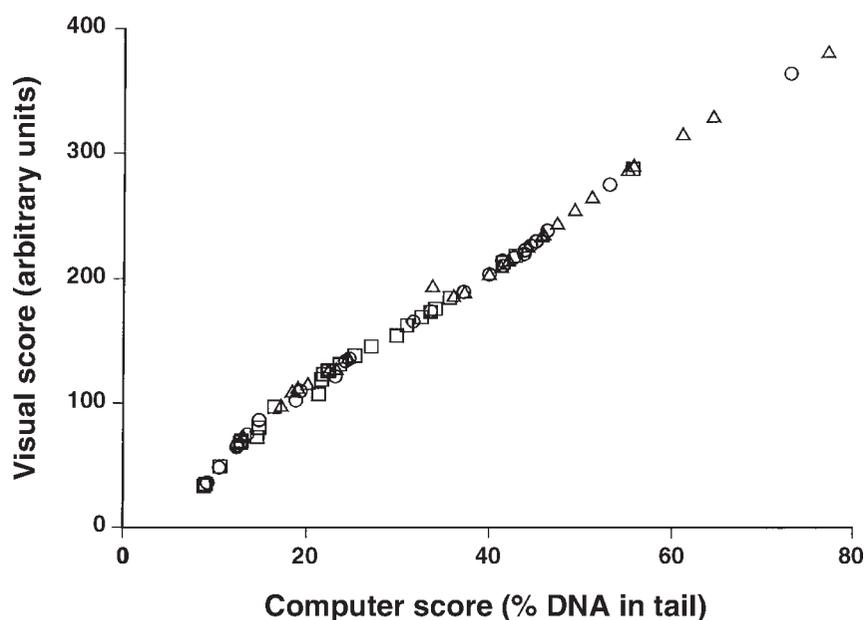

Fig. 3. Correlation between computer image analysis (percentage DNA in tail) and visual scoring (arbitrary units) on the same samples of human lymphocytes. Reprinted with permission from **ref. 45**.

4.3. Human Studies

The comet assay is ideally suited for human investigations because it requires no prelabeling with radioactivity or other harmful procedures and can be applied to easily obtainable cells. Nor-mally, white blood cells are used, as they are obtained in a relatively noninvasive way, do not require tissue disaggregation, and behave well in the comet assay. They do, however, have disadvantages. They are not a target tissue for cancer, and

it is not clear that the damage detected in white blood cells reflects the damage in actual target tissues. Sometimes human tissue removed at surgery can be investigated for elevated levels of damage; but the necessary control tissue from healthy individuals is harder to obtain. Efforts have been made to use other cells, such as buccal epithelial cells, or urothelial cells centrifuged from urine, but it seems that the cytostructure of epithelial cells impedes release of DNA into comets, and extensive lysis and digestion with proteases is necessary.

4.3.1. Biomonitoring

Applications include monitoring occupational exposure to genotoxic chemicals or radiation *(30)*, assessment of oxidative stress associated with various human diseases *(31)*, and detection of DNA damage associated with smoking *(32)*. In general, although significant differences are seen between exposed and control groups of subjects, there is a wide interindividual range of damage levels within any group—as well as intraindividual variability resulting from changes in diet, stress, or infection—and attempts to assess individual risk of diseases such as cancer from comet assay results would be premature and unjustifiable.

4.3.2. Nutritional Studies

The comet assay is ideal for investigating nutrient or micronutrient effects at the level of DNA damage in humans. Diets differing in lipid content lead to changes in oxidative DNA damage in lymphocytes—polyunsaturated fatty acids apparently causing an increase *(33)*. On the other hand, the protective effects of in vivo supplements of antioxidants, or of foods rich in antioxidants, are very readily demonstrated in lymphocytes as either a decrease in endogenous base oxidation (measured with endonuclease III or FPG) or a decreased sensitivity to H_2O_2-induced damage in vitro *(34,35)*.

4.3.3. Diagnosis

In a few cases, it may be possible to use the comet assay as an aid to diagnosis. The Nijmegen breakage syndrome is an autosomal recessive condition associated with genetic instability and cancer proneness. Heterozygote carriers are identifiable by an abnormally high level of strand breakage in lymphocyte comets *(36)*.

Lymphocytes from patients with xeroderma pigmentosum, with a defect in the early stages of nucleotide excision repair, do not show the normal UV-induced accumulation of DNA strand breaks as repair intermediates (*see* **Subheading 2.3.2.**), and this provides a simple way to identify the disease using the comet assay *(37)*.

4.3.4. Assessing Background Levels of Damage

Oxidative base damage in DNA is a possible factor in cancer etiology, and yet we do not know how much damage is present. Estimates of the content of 8-oxoguanine in human DNA vary over three or four orders of magnitude. High values provided by gas chromotography-mass spectrometry (GC-MS) or high-pressure (performance) liquid chromatography (HPLC) with electrochemical detection are affected by the serious artefact of oxidation of guanine during sample preparation; limiting the possibilities for oxidation has brought estimates down. The comet assay, used in a quantitative way with FPG, indicates that the frequency of 8-oxoguanine in cultured human cells (HeLa) or human lymphocytes is less than one per 10^6 guanines—several times less than the lowest of the HPLC estimates *(38,39)*.

4.4. Measuring DNA Repair

We know surprisingly little about the variation in capacity for DNA repair between individuals, even though this is likely to be an important determinant of individual susceptibility to cancer. A suitably robust and sensitive assay has not been available, but the comet assay has the potential to fill this gap.

4.4.1. Cellular Repair

Theoretically, a sound approach to measuring repair capacity is to inflict DNA damage on cells and to monitor the speed with which they remove the lesions. Thus, lymphocytes can be treated with ionizing radiation, or H_2O_2, and rejoining of breaks followed; or, after treatment with base-damaging chemicals, and incubation, the remaining lesions can be assayed by use of an appropriate endonuclease on the gel.

Rejoining of DNA strand breaks by most cell types is known to be a rapid process, with a half-time of a few minutes *(40)*, and these kinetics are seen with the comet assay, too. However, freshly isolated lymphocytes, in our hands, appear to repair H_2O_2-induced breaks very slowly. This may be because they suffer additional DNA breakage as a result of sudden exposure to atmospheric oxygen during the repair incubation *(41)*.

Repair of endonuclease III- or FPG-sensitive sites (i.e., oxidized purines and oxidized pyrimidines), by base excision repair, is a slower process, requiring a few hours *(42)*. Repair of UV-induced cyclobutane pyrimidine dimers by nucleotide excision repair can be detected with the enzyme endonuclease V. This, too, is a relatively slow process *(12)*.

4.4.2. An In Vitro Repair Assay

As an alternative to following cellular repair, the repair activity of a cell extract can be assessed in an in vitro assay *(43)*, which is the converse of the normal enzyme-linked comet assay. A simple extract is prepared from cells, such as lymphocytes, by freezing, thawing, adding Triton X-100, and centrifuging to remove cell debris. A damaged DNA substrate is prepared in the form of gel-embedded nucleoids from (cultured) cells treated with whatever damaging agent is appropriate for the repair being measured. The substrate is then presented to the extract, and the rate at which breaks accumulate indicates the capacity of the extract to incise at damage sites. Incision is normally regarded as the rate-limiting step of repair. This method was developed for measuring repair of oxidized bases by the 8-oxoguanine DNA glycosylase, Ogg1 (the eukaryotic counterpart of the bacterial FPG). The substrate is prepared by treating cells with the photosensitizer Ro 19-8022 plus visible light, to induce base oxidation, principally 8-oxoguanine. The extract is remarkably free of nonspecific nuclease activities.

Ogg1 activity measured in this way shows considerable variation between individuals, and it can be modulated. In a recent human intervention trial, we found not only a decrease in endogenous DNA oxidation, and increased antioxidant status of lymphocytes, but also an enhanced DNA repair activity after supplementation of the diet with kiwifruit *(44)* (*see* **Fig. 4**).

4.5. Practical Considerations for the Use of the Comet Assay in Human Population Studies and in Animal Trials

The comet assay is eminently suited for use in molecular epidemiology and animal experiments, but care must be taken to ensure its reliability and to make it truly quantitative.

4.5.1. Study Design

Human studies should be designed according to standard epidemiological considerations. Power calculations are required to establish the size of groups to be studied; a pilot study may be necessary to estimate the range of intra- and interindividual variation in the kind of damage being measured.

4.5.2. Standardization of the Comet Assay

Standard cells should be included in the analysis. For example, lymphocytes prepared from several individuals can be pooled and frozen as aliquots, to be thawed as needed. The standard should give a similar measure of damage each time, and deviation or drift gives warning that some aspect of the assay has changed.

4.5.3. Storage of Lymphocytes

It is often useful to be able to freeze and store cells (for example, from blood samples taken during a human population study) so that they can be analyzed with the comet assay at a later date. Conventionally, lymphocytes are frozen slowly to –80°C in medium rich in fetal calf serum and containing 10% dimethyl sulfoxide (DMSO) to preserve viability. However, the main requirement for the comet assay is DNA integrity (i.e., intact loops) rather than an ability to proliferate. Lymphocytes in medium or phosphate-buffered saline (PBS) without serum but with 10% DMSO can be slowly frozen then stored at –80°C, or they can be stored in liquid nitrogen for months or even years. For use they are thawed quickly, diluted with PBS, and immediately centrifuged to remove them from the DMSO.
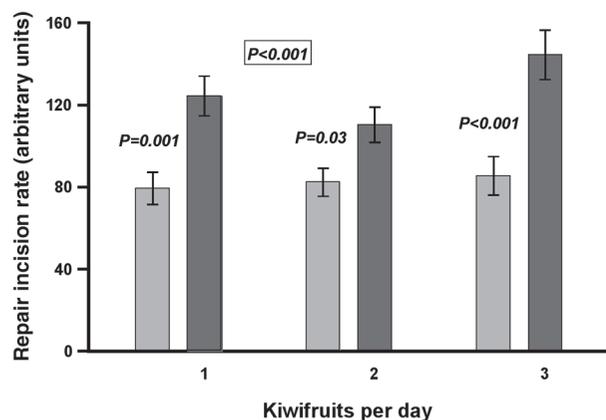
Fig. 4. Enhancement of in vitro DNA repair capacity of extracts from lymphocytes taken from volunteers before (*light-shaded bars*) and after (*dark-shaded bars*) supplementation with different doses of kiwifruit, 1, 2, or 3 per day, for 3 wk. Repair was measured as incision activity on a substrate containing 8-oxo–guanine. Reprinted with permission from **ref. *44***.

4.5.4. Checking Cell "Viability"

In many laboratories, it is routine to test the "viability" of cells by adding trypan blue to the cell suspension. Cells that take up the dye are pronounced nonviable, whereas those that do not are viable. A high viability is regarded by many as a prerequisite for the comet assay. However, Trypan blue does not measure viability, but simply indicates whether cell membranes are intact. Cells with damaged membranes (e.g., after harvesting by scraping from a plastic culture dish) are trypan blue-positive, but recover and survive (C. M. Gedik, personal communication). Thus, by definition, they must be viable. Second, as already noted, viability *per se* is not necessary for sound comets. The best test of whether cells are in a satisfactory condition for comet assay analysis is that control, untreated cells should give comets with a background level of breaks (i.e., mostly class 0, or around 10% of DNA in the tail).

4.5.5. Storage of Gels After Electrophoresis

It is often impracticable to score slides immediately after performing the comet assay. If the gels are prepared on ordinary glass slides, they can be dried and stored indefinitely. Dried gels are easier to score than fresh ones, as all comets are in the same plane and there is no need for constant refocusing. Gels on frosted slides cannot be stored in this way, as the comets after drying are too close to the frosting, which interferes with the optics.

4.5.6. Statistical Analysis

This can be carried out at different levels. For instance, a single gel might be analyzed in terms of the levels of damage recorded in different comets, and the way in which they are distributed; or we might be interested in the coefficient of variance (CV) for replicate determinations of the same sample. But when we are analyzing differences between groups in human or animal experiments, or effects of treatment, all we are interested in is the overall mean comet score for each individual (whether it is expressed as a percentage of DNA in tail, or arbitrary units by visual examination). A false idea of precision is conveyed by quoting the standard error of the mean comet score of an individual sample.

4.5.7. Assay Saturation

DNA break frequency is linearly related to the percentage of tail DNA—up to a certain level. Saturation obviously occurs once all the DNA is in the tail, and a deviation from linearity is seen as this level of damage is approached. Particular care is needed when the sites revealed with lesion-specific endonucleases are superimposed on already high levels of strand breakage; there is then a tendency for the damaged bases to be underestimated.

Figure 5 is an example of an experiment in which we checked for this saturation effect. Cells were treated with different concentrations of photosensitizer and irradiated with visible light to induce increasing amounts of 8-oxoguanine, measured with FPG. Even at the highest concentration, there were very few class 4 comets, and so we could safely assume a linearity of response.

5. Conclusions

The comet assay, in various guises, is now well established as a sensitive method for detecting strand breaks in the DNA of single cells. Once calibrated, it can be used in a quantitative way.
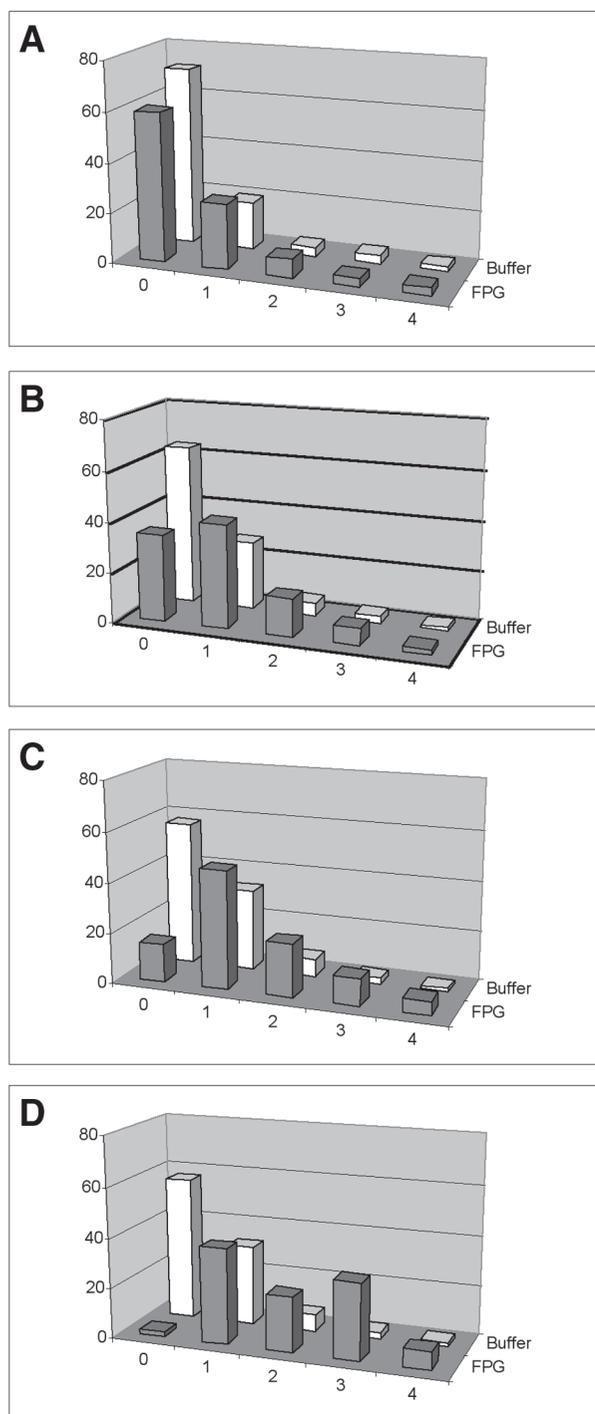
Lesions other than strand breaks can be detected with the inclusion of a step in which nucleoid DNA is incubated with a lesion-specific endonuclease. The comet assay promises to provide answers to important questions concerning, for example, background levels of DNA damage in normal cells, the variation in DNA repair capacity within human populations, and the regulation of DNA repair at the molecular level within the nucleus.

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Fig. 5. Test of ability of comet assay to detect FPG-sensitive sites quantitatively over a range of concentrations of 8-oxoguanine in the DNA. HeLa cells were untreated (**A**), or irradiated with visible light (**B**), or treated with light and 0.2 µ*M* Ro 19-8022 (**C**), or treated with light and 0.4 µ*M* Ro 19-8022 (**D**). Ro 19-8022

Fig. 5. *(continued)* is a photosensitizer that, in combination with light, induces oxidation of guanine in DNA. Incubation of nucleoids with buffer alone shows DNA strand breaks (*white bars*); incubation with FPG (*dark bars*) reveals additional sites of damage (8-oxoguanine). Results are displayed as frequencies of comets (of 100 scored) in each class (*see* **Fig. 2**). The figure is redrawn from data in *(38)*, which contains full details of the experiment.

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