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REVIEW

The comet assay: topical issues

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The comet assay is a versatile and sensitive method for measuring single- and double-strand breaks in DNA. The mechanism of formation of comets (under neutral or alkaline conditions) is best understood by analogy with nucleoids, in which relaxation of DNA supercoiling in a structural loop of DNA by a single DNA break releases that loop to extend into a halo—or, in the case of the comet assay, to be pulled towards the anode under the electrophoretic field. A consideration of the simple physics underlying electrophoresis leads to a better understanding of the assay. The sensitivity of the assay is only fully appreciated when it is calibrated: between one hundred and several thousand breaks per cell can be determined. By including lesion-specific enzymes in the assay, its range and sensitivity are greatly increased, but it is important to bear in mind that their specificity is not absolute. Different approaches to quantitation of the comet assay are discussed. Arguments are presented against trying to apply the comet assay to the study of apoptosis. Finally, some of the advantages and disadvantages of using the comet assay on lymphocyte samples collected in human studies are rehearsed.

Introduction

The comet assay, even after 20 years, is still in the growth phase, with many new users each year. At the biennial Comet Assay Workshop, many questions are repeatedly raised, that may seem to many of us to have self-evident answers. But clearly, it is necessary to reiterate them for the benefit of the new audience, and sometimes being forced again to think about old topics can shed new light. So we have written this review not only for the beginner but also for the experienced users.

We start with the issue of how comets are formed—surprisingly, still a controversial topic. Then come considerations of size and sensitivity. Some understanding of the simple physics underlying electrophoresis helps to define the factors that we really need to think about when filling the tank. The sensitivity and selectivity of the assay can be improved if lesion-specific enzymes are used to convert damaged bases to DNA breaks. Some recommendations on the scoring of comets follow. Finally, we deal with some biological matters, including viability testing, and the advantages and disadvantages of lymphocytes as biomonitoring material and in experimentation.

How are comets formed?

Statements like 'The neutral comet assay was used to measure double-strand breaks' appear all too often in publications and presentations. There is a belief that alkaline conditions are required to reveal single-strand (SS) breaks, and that therefore using a near-neutral pH ensures that only double-strand (DS) breaks are picked up. This belief probably arises from a false comparison of the comet assay with other common or historical methods for measuring DNA breaks, namely alkaline sucrose gradient sedimentation, alkaline elution and alkaline unwinding. In these, a period of alkaline treatment allows the DNA strands to separate, starting from the sites of breaks, which can be either SS or DS. In the case of alkaline elution, the pieces of unwound DNA are more likely to pass through the filter the smaller they are (like spaghetti through a sieve), and so the rate of elution of the DNA is determined by the frequency of breaks. There is a corresponding neutral elution method, without denaturation, where the rate of elution depends on the frequency of DS breaks and is not affected by SS breaks. The alkaline unwinding method applies a high pH for a relatively short time, so that the DNA is partially unwound, the extent of strand separation depending on how many breaks are present. On neutralization, a mixture of SS and DS DNA is formed, the proportion of SS DNA reflecting the break frequency. The procedure for alkaline sucrose gradient sedimentation (suitable for the analysis of relatively small-sized DNA fragments, and rarely used nowadays) includes extensive alkaline incubation before centrifugation, producing a lysate which consists of pieces of SS DNA of varying lengths which then sediment at different rates.

That the comet assay is not based on a similar requirement for high pH to reveal SS breaks should be clear from a comparison of the two earliest versions of the assay. Östling and Johanson (1) first described the behaviour of DNA from single cells under an electric field, detecting breaks introduced by ionizing radiation. They employed a pH of 10—well below that required to unwind DNA. A few years later, Singh *et al.* (2) described a similar method, but using alkaline conditions. It is notable that both methods were able to detect the same low doses of radiation—well below 1 Gy. This would be hard to explain if only DS breaks were detected at near-neutral pH, since ionizing radiation produces predominantly SS breaks: for low-linear energy transfer radiation such as X-rays and

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γ-rays, the yield of DS breaks is around one-twentieth of that of SS breaks (3). Östling and Johanson (1) had an explanation, in terms of nucleoids—protein-depleted nuclei in which the DNA is still constrained as supercoiled loops (4), probably via attachment to a nuclear framework or matrix. In the living cell, the DNA is organized in nucleosomes, and it is the winding of the DNA around the histone core that creates the (negative) supercoiling; the double helix is underwound, with fewer than the 10 bases per turn found in a relaxed molecule. After lysis in 2.5 M NaCl, most histones are removed, and nucleosomes are disrupted, but the DNA remains supercoiled. The loop can be regarded as an independent topological unit. Thus, one SS break will relax the supercoiling in the loop in which it occurs, allowing that loop to extend under the electrophoretic field. Clearly, the relaxation of supercoils and extension of DNA loops can occur under either 'neutral' or alkaline conditions.

Of course, under alkaline conditions, unwinding of the two strands does occur, and it is not surprising that neutral and alkaline comet tails differ in detail. Compared with the homogeneous staining of neutral comets, the DNA in the tail of an alkaline comet appears granular, as if DNA fragments are present. Fragments will result if two breaks occur within one strand of a loop (a possibility at high levels of damage). Also, it is possible that DNA detaches from the matrix at high pH and coalesces on neutralization. However, the essential factor determining whether a segment of DNA appears in the tail rather than the head of the comet is the relaxation of supercoiling, which simply depends on a break and occurs regardless of pH. Thus, it is a matter of everyday observation that, as the level of damage increases, it is the relative intensity of staining of DNA in the tail that increases, rather than tail length—entirely consistent with an increasing number of loops becoming relaxed.

Calibration and the importance of size

How big is a DNA loop? Cook and Brazell (5) estimated the average loop length as 2.2×10^5 bp, equivalent to 75 μm, which is quite consistent with the typical length of a comet tail.

It is tempting to refer to a comet in which most of the DNA is in the tail as representing a highly damaged cell. At the same time, we regard the comet assay as a very sensitive assay, and so 'highly damaged' is very much a relative term. To gain a proper perspective, we need to calibrate the assay, and this is normally done by measuring damage in cells following a range of doses of ionizing radiation, so that comet scores can be expressed as Gy equivalents. Many years ago, using alkaline sucrose sedimentation, it was ascertained that 1 Gy of X- or γ-irradiation introduced 0.31 breaks per 10^9 Da of cellular DNA, which is close to 1000 breaks per diploid mammalian cell (6). Figure 1 shows a selection of calibration curves for the alkaline comet assay with damage dose expressed in terms of Gy. As the percentage of DNA in the tail approaches its maximum, a saturation effect is seen, and further damage cannot accurately be measured.

The calibration curves are not all in agreement. While it has been accepted for some years that the maximum dose detected with the assay is ~10 Gy equivalents, some published calibration curves (e.g. Figure 1f) have a more gradual dose–response slope. Such discrepancies might arise from subtle variations in protocols, or from differences in yield of breaks arising from the use of radiation sources differing in type, energy or quality. It has been recognized for some time that, since SS breaks are readily repaired, it is important to carry out irradiation on ice and to keep the cells cold and for the minimum time before lysis (which stops all repair). The simplest way to ensure this is to carry out irradiation after embedding cells in the gel. Figure 1d illustrates this point.

As a result of discussions at comet assay workshops, an interlaboratory trial has been set up to carry out rigorously controlled calibration experiments. Meanwhile, for the sake of the present argument, let us accept that the range of damage detectable with the comet assay is, roughly, from 0.2 up to 10 Gy equivalents, or from 0.06 to 3 breaks per 10^9 Da. Thus, from about one hundred to several thousand breaks per (human) cell can be detected. This conveniently encompasses the level of damage likely to be found in control cells as a normal background, as well as damage that is inflicted experimentally without killing the cells. But it is important to appreciate that we are dealing with pieces of DNA that are—to quote from Östling and Johanson (1)—'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'.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) has a size of 16569 bp or 11×10^6 Da, which is much less than the size of the 'fragments' that make up the comet tail. Furthermore, mtDNA is not linked in any way to the nuclear matrix. Nevertheless, the question is occasionally asked, whether damage to mtDNA can be detected using the comet assay. To confirm the seemingly obvious answer, that mtDNA is simply too small to be detected with this technique, we carried out a fluorescent *in situ* hybridization experiment, using so-called padlock probes to identify mtDNA at different stages in the comet assay procedure (7). The padlock probe is designed with the two ends complementary to adjacent regions of the target DNA, but in opposite orientations, so that when the probe is hybridized, the two ends are next to each other. A ligation reaction locks the probe onto the target DNA molecule, and it is not dislodged by the most stringent washes; thus, a very clean image, free of interference from non-specific background, is obtained. The results of our experiment are shown in Figure 2. Just after embedding cells in agarose, mtDNA is clearly visible around the nucleus. Soon after lysis has begun, mtDNA starts to disperse, and after 20 min, the signals are randomly spread over the gel. After alkaline electrophoresis, very few signals remain in the gel. In contrast, padlock probes designed for Alu sequences within the genomic DNA give strong signals over the whole comet (not shown).

Apoptosis

It is even less likely that the comet assay could detect the fragments of DNA that occur in apoptosis. During this process, the DNA is broken down ultimately into nucleosome-sized pieces. Yet, the idea has taken hold that comets with almost all DNA in the tail and a small head (so-called 'hedgehog' comets, after their resemblance to the spiny mammal, equivalent to 'class 4' comets according to visual scoring: see Figure 3) represent apoptotic cells. Some researchers exclude these comets from their scoring. But such comets are regularly seen after, for example, treating cells with a non-lethal dose of damaging agent, such as H_2O_2. If the treated cells are incubated

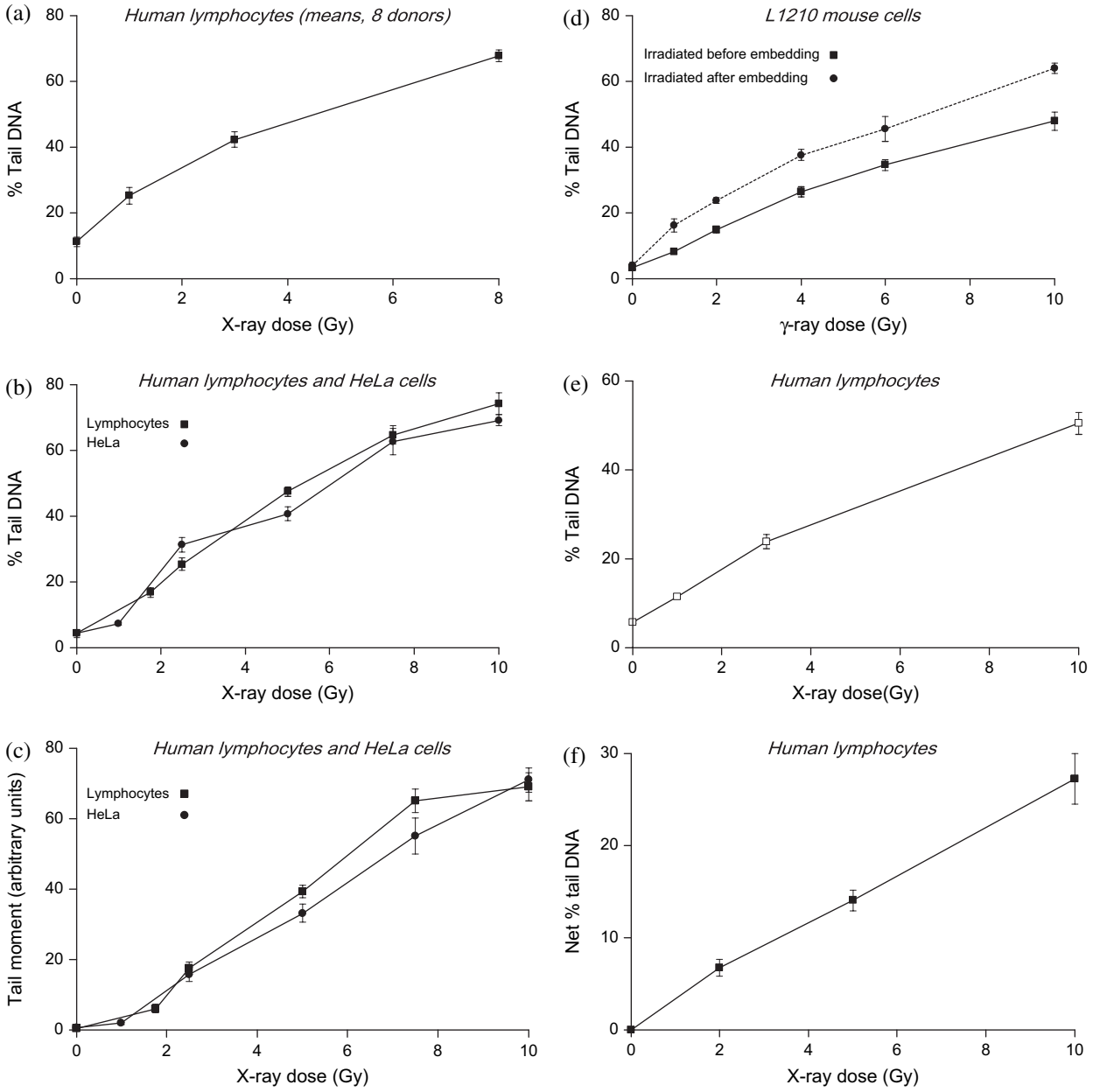

Fig. 1. Comet assay calibration curves obtained in different laboratories using ionizing radiation to induce DNA breaks. Details of irradiation are given in parenthesis for each laboratory. (**a**) Human lymphocytes were X irradiated on ice after embedding in agarose. Mean values from eight donors are shown; bars indicate standard error of mean (SEM) (0.8 Gy/min, 180 keV, filtered through 1.5 mm Cu) (data from M. Kruszewski). (**b, c**) Human lymphocytes and HeLa (human transformed endothelial) cells were X irradiated on ice after embedding in agarose. Results are expressed as either % tail DNA (b) or tail moment (c) (data from R. Štětina). (**d**) L1210 mouse cells (an established cell line) were irradiated on ice before (solid line) or after (broken line) embedding in agarose. Means ± SEM are shown, from three experiments with duplicate cell samples (γ-ray source: Cæsium 137, 0.45 Gy/min) (data from C. C. Smith). (**e**) Human lymphocytes were X irradiated in suspension at 4°C, and held for up to 30 min before embedding in agarose. Data shown are means of four replicate cell samples (from single donor) ± SEM (10 Gy/min, 260 keV, filtered through 0.5 mm Cu) (data from G. Brunborg). (**f**) Human lymphocytes from two donors (duplicate experiments) were X irradiated after embedding in agarose. Means ± SEM are shown (high energy X-rays from a linear accelerator; 2 Gy/min, 6 MeV) (data from L. Giovannelli, redrawn from (33)).

for 30 min and comets are again prepared, these class 4 comets are no longer seen, because the cells have repaired the breaks. Clearly, we are not observing apoptosis: first, because apoptosis is not an immediate consequence of a severe assault on the cell, but takes time to develop; second, because the breaks are repaired, and by definition the breakage that occurs under apoptosis is irreversible. At best, if we are looking at cells several hours after an apoptosis-inducing treatment, the breaks revealed using the comet assay may represent the earliest stages of apoptosis, but hedgehog comets cannot be used as a specific indicator of apoptosis.

A recent paper from Morley *et al*. (8) describes an interesting and revealing experimental approach: after embedding cells in agarose, but before lysis, apoptotic cells were identified using an Annexin-V fluorescence staining method, based on the binding of Annexin-V to phosphatidyl serine which is translocated to the outer cell membrane at an early stage of apoptosis. The positions of the cells were marked so that they

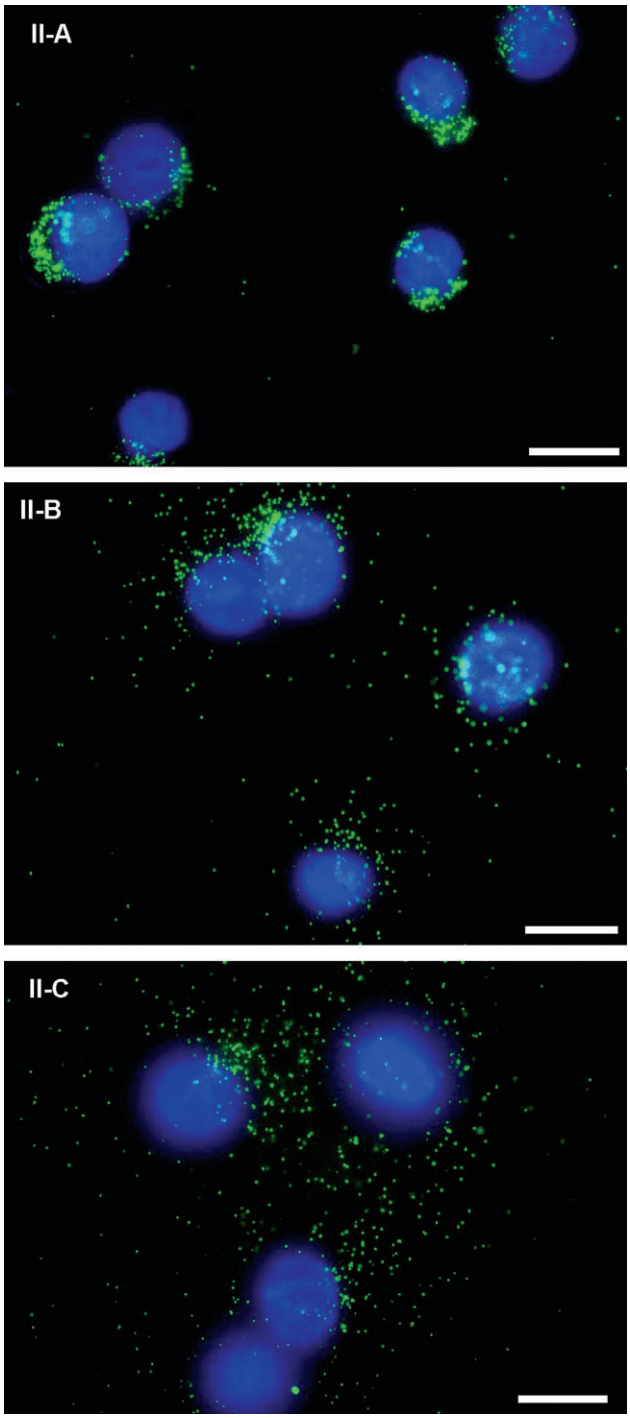

Fig. 2. Detection of mtDNA by fluorescent *in situ* hybridization to DNA of 143B (human osteosarcoma) cells using a padlock probe. Nuclear DNA is counterstained with DAPI. Top: immediately after embedding in agarose. Middle: after 150 s in lysis solution. Bottom: after 20 min of lysis. Bars represent 20 μm. From (7), by permission of Oxford University Press.

could be relocated after carrying out the comet assay, and the images produced from the Annexin-V staining and from comets could be overlaid. Heat treatment was used to induce apoptosis and also to cause DNA breakage. Four hours after this treatment, a mixture of Annexin-V positive and negative cells was seen. In the case of the Annexin-V positive cells, the resulting comets were barely detectable, and are described as 'ghost comets'—consistent with the complete migration of the bulk of the (highly fragmented) DNA.

Electrophoresis

Published comet assay results often vary considerably, even after a calibrated fixed dose of ionizing radiation. Lack of standardization of protocols may explain some variations. One protocol step which is often insufficiently described is electrophoresis and some basic considerations will therefore be presented here. It is usual to perform electrophoresis at a fixed voltage of 25 V, and a current of (most commonly) 300 mA is achieved by adjusting the total volume of electrophoresis buffer. In most tanks, this leaves a rather thin layer of just a few millimeters of buffer above the platform where the slides are placed for electrophoresis. Most of the total voltage drop would be across this area because of its high ohmic resistance. Any slight variation in this buffer depth along the surface may have significant effects on the voltage, and also on local temperature and pH. It should be emphasized that the total applied voltage and also the current are in theory irrelevant, since it is the voltage across the gel (approximated by the V/cm on the platform) which is the driving force for electrophoresis of the charged DNA molecule. A higher current would hence be preferable, provided that the power supply can provide it. [Adding more buffer will in fact lead to a lower V/cm across the platform unless the total (applied) voltage is similarly increased.] It should be noted that in the original protocol of Singh *et al.* (2), a thin layer above the gels is specified, together with a total voltage of 25 V. There is no mention of a particular current. The habit of working with 300 mA has probably arisen because power supplies producing higher currents are costly. A very good alternative is in fact to use two car batteries coupled in series, producing several amperes of current at a fairly constant voltage of $\sim$ 25 V, allowing a greater depth of buffer, a higher current, and hence a more homogeneous electric field. These simple physical considerations should be taken into account when choosing electrophoresis tanks and power supply. In a description of electrophoresis conditions in publications, the only relevant parameter that always should be specified is V/cm across the platform.

A simple spreadsheet is available through the EC FP6 specific-targeted project COMICS (LSHB-CT-2006-037575) (http:/comics.vitamib.com/electrophoresis-physics). Upon filling in physical dimensions and some other parameters, the spreadsheet will return the information most relevant for comet assay electrophoresis.

Lesion-specific endonucleases

The standard alkaline comet assay detects strand breaks and alkali-labile sites. These latter include apurinic and apyrimidinic sites, or AP sites, which arise from the loss of a damaged base, leaving a base-less sugar in the backbone. AP sites occur as intermediates during base excision repair (BER) and may also arise spontaneously owing to altered chemical stability resulting from changes in bases or sugars. It appears that pH $>$ 13 (corresponding to 0.3 M NaOH as in the usual comet assay protocol) is sufficient to convert AP sites to breaks, whereas pH $\sim$ 12 (reached with 0.03 M NaOH, less commonly employed) is not. Performing unwinding and electrophoresis in parallel at these two pHs distinguishes agents that induce just strand breaks from those that produce AP sites (9). However, it has

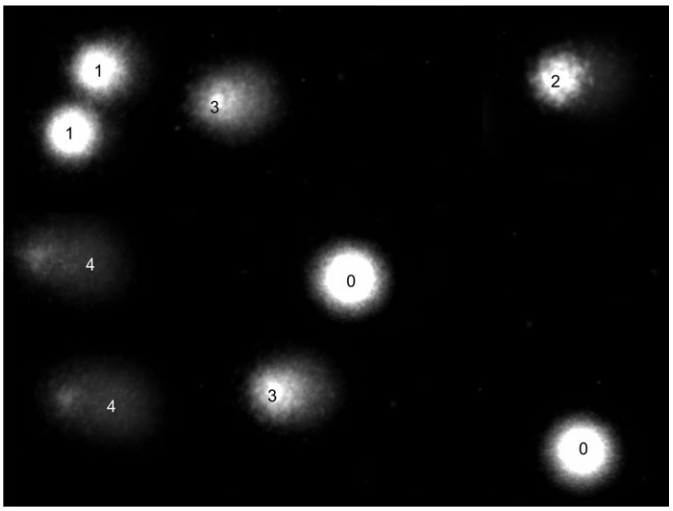

Fig. 3. Examples of comets, from human lymphocytes. Numbers indicate the scores assigned by visual scoring.

not been rigorously established that 40-min incubation in 0.3 M NaOH at 4°C is really sufficient to convert all AP sites, and nor is it clear what the background level of AP sites (which probably varies among cell types) actually is.

In normal cells, strand breaks (and AP sites) are not the only kind of damage. Oxidized bases are present in at least as great a number. They can be readily detected with the comet assay, by incorporating an additional step: after lysis of agarose-embedded cells, the DNA is digested with a lesion-specific endonuclease (10,11). Formamidopyrimidine DNA glycosylase (FPG) recognizes the common oxidized purine 8-oxoGua, and also ring-opened purines, or formamidopyrimidines (Fapy). Endonuclease III converts oxidized pyrimidines to strand breaks. In parallel with the enzyme incubation, a slide is incubated with buffer alone, and the score from this control slide is subtracted from the '+enzyme' score to give 'net enzyme-sensitive sites'. These enzymes have been widely used to examine the effect of antioxidant supplementation in human volunteers (typically a decrease in endogenous base oxidation) (12). FPG and the comet assay were used to determine the basal level of 8-oxoGua in human cells, in a multicentre collaboration, the European Standards Committee on Oxidative DNA Damage (ESCODD). The enzyme-based approach gave a mean value for background oxidation of 0.3 8-oxoGua per 10^6 Gua (in lymphocyte DNA), at least 10 times lower than the median or mean values obtained with chromatographic techniques (13): the latter suffer from oxidation of DNA during sample preparation, while the comet assay, requiring much less sample manipulation, is relatively free of this artefact.

If some lesions are inaccessible to the enzyme, or if they occur in clusters (i.e. several lesions in one DNA loop), 8-oxoGua will be underestimated. On the other hand, the level of damage detected with FPG may in fact be an over estimate of 8-oxoGua, since it is possible that formamidopyrimidines are also present. Since both FPG and endonuclease III have an associated AP lyase (nicking 3′ to a baseless sugar), they will also detect AP sites, but we assume—perhaps wrongly—that the AP sites, being alkali-labile, are included in the breaks detected on the control slide, so that net enzyme-sensitive sites include only the altered bases. Finally, the accuracy of our estimates of 8-oxoGua depends on the calibration of the assay.

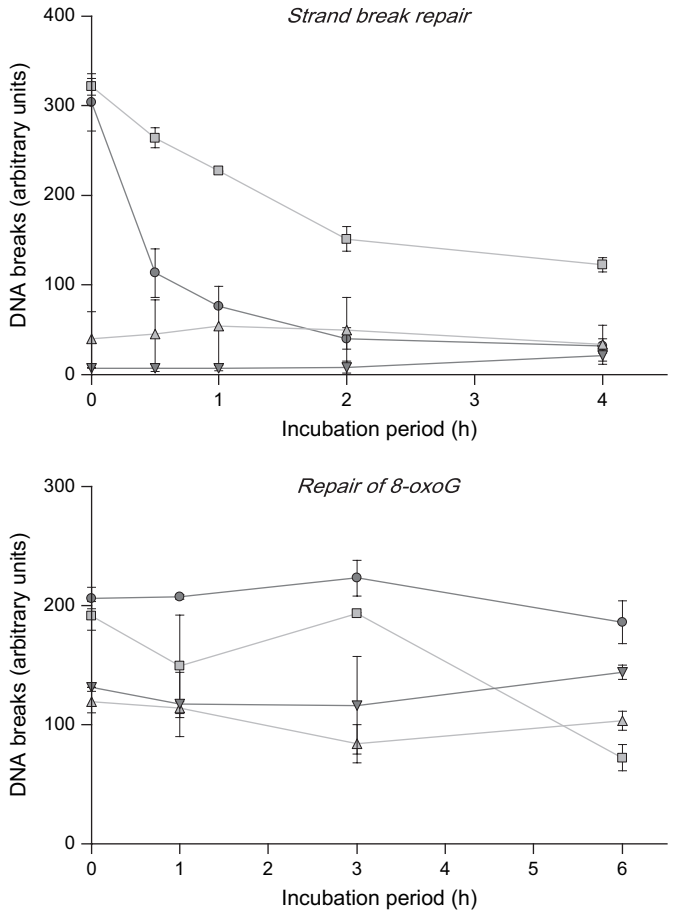

Fig. 4. DNA repair by lymphocytes; apparent effect of adaptation to *in vitro* culture conditions (data from A.A.O., I.G. and A.R.C.). Human lymphocytes, isolated from venous blood, were used either at once (■, ▲) or after culture in medium at 37°C, 5% CO_2 for 2 days (●, ▼). They were treated with 50 μM H_2O_2 for 5 min on ice to cause (mostly) SS breaks (top panel), or with photosensitiser Ro 19-8022 + visible light to induce 8-oxoguanine (lower panel) (■, ●, solid lines), and then incubated at 37°C to allow rejoining of strand breaks or removal of 8-oxoguanine. Control cultures (▲, ▼, broken lines) were not subjected to damaging treatment. Strand breaks were monitored using the standard comet assay. 8-oxoGua was measured using FPG with the comet assay.

An indication that these complications do not materially affect the determination of 8-oxoGua (or that they cancel each other out) comes from experiments where HPLC- and the FPG-based methods are used in parallel to measure 8-oxoGua experimentally induced by treating cells with a photosensitiser plus visible light (14). The dose–response slopes do not differ significantly between the two approaches, implying identical efficiency of detection; it is in the estimates of background levels of damage that a great difference is seen.

There are situations when oxidative DNA lesions are probably not detected quantitatively by the enzyme. Shortly after ionizing irradiation, it is our experience that hardly any additional lesions are detected with FPG, although it is known from other methods that the number of oxidative lesions induced is initially about the same as the number of single-strand breaks. After repair incubation for 30 min, the large majority of lesions found are FPG sensitive. This phenomenon is most probably related to the tendency of X-ray-induced lesions to be clustered, with a mixture of strand breaks and oxidized bases in close proximity. Only when the strand breaks

have been repaired will the oxidized bases be recognized as such (15,16).

Other enzymes can be used. The human analogue of FPG, hOGG1, was shown to be more specific than FPG, recognizing only oxidation products, 8-oxoGua and methyl FapyGua, whereas FPG also recognized some alkylation damage (17). AlkA detects 3-methyladenine (18), but should be used with care, as it is rather non-specific, and at high concentration will even nick undamaged DNA (19). T4 pyrimidine dimer glycosylase, which converts cyclobutane pyrimidine dimers to breaks, is useful for detecting the damage induced by UV light (20). Misincorporated uracil in DNA (a metabolic mistake, rather than DNA damage—perhaps a consequence of folate deficiency) can be recognized using uracil DNA glycosylase (21).

The substrate requirements (and the ability to cleave-specific adducts) may be different *in vitro* and *in vivo*. Care should be taken to prepare suitable positive controls treated in such a way as to produce the lesions that one is interested in. To be sure that the lesions of interest are quantitatively detected, and that non-specific breakage is not occurring, the optimal reaction conditions (for each batch of enzyme) need to be established by titration, varying concentration of enzyme and/or time of incubation.

Scoring comets

The different approaches to analysis of comets include the following:

1. Measuring the length of the comet tail on a photomicrograph or using a graticule—a laborious method, which gives limited information, as the tail length increases only at the lowest levels of damage and soon becomes maximal, so reducing the useful range of the assay.
2. Classifying comets by visual inspection, typically into five categories: 0 representing undamaged cells (comets with no or barely detectable tails) and 1–4 representing increasing relative tail intensities (Figure 3). Summing the scores (0–4) of 100 comets gives an overall score of between 0 and 400 arbitrary units.
3. Image analysis, with a charge-coupled device camera linked to a computer with appropriate software, available commercially or from the Internet. Comet images are selected by the operator.
4. Automated systems, which search for comets and carry out the analysis with minimal human intervention.

There are several observations to be made. First, visual inspection is quite respectable! Certainly, if you are just setting up the assay, you should start by classifying comets by eye, before committing yourself to expensive solutions. Visual scoring has been correlated with computer-based image analysis (22); there is a good, though not perfectly linear, correlation between visual score and image analysis parameters (relative tail intensity or tail moment), and radiation-based calibration can of course be applied to comets that have been scored visually, just as to computer-based scores. Visual scoring, by an experienced operator, tends to be faster than image analysis with computer. It continues to be the method of choice for laboratories involved in the analysis of large numbers of samples, for instance from biomonitoring studies. Obviously, there can be differences between different scorers; they can be trained so that their results are comparable, but it is still safest to have a single scorer deal with all samples from a given project or experiment.

Second, image analysis can provide a surfeit of information. As a minimum, expect a commercial system to give you the total intensity of each comet, its tail length, % DNA in head, % DNA in tail and tail moment. Tail moment is, roughly, the product of tail length and % tail DNA. Which to choose? It may be that, when very low levels of damage are present, tail length is most informative. But generally, % tail DNA covers the widest range of damage, and it is linearly related to break frequency over most of this range (Figure 1b) (% DNA in head, as the complement of % tail DNA, shows a decrease with increasing damage, which is not very helpful). Tail moment has been promoted as a way of expressing both tail length and tail intensity in a single value. The advantage of doing so is not so clear, as it may make the dose–response curve deviate from linearity at low doses (Figure 1c). A more serious disadvantage of tail moment, however, is that it can be calculated in different ways, and it does not have standard units [unless the system is calibrated for the particular optical system using a graticule, in which case the unit is length (in micrometres)]. Values of tail moment given in a research publication are therefore difficult to interpret as representing any particular kinds of comets, and so we are unable to visualize the comets or to evaluate the degree of damage being described. In contrast, given a relative tail intensity of, say, 25 or 50%, we are immediately able to visualize the comets concerned. Cells from different tissues or different species can differ substantially in tail length. Thus, since tail length is a factor in calculating tail moment, similar amounts of damage can give rise to different values of tail moment—a further reason to prefer % tail DNA, which is insensitive to this effect. % DNA in tail is, therefore, strongly recommended as the parameter of choice. It is of course also possible to calibrate with ionizing radiation and to express all data as 'Gy equivalents' which may then be converted into lesion frequencies per 10^9 Da.

Whichever method is chosen, scoring takes time, and becomes the limiting factor when large numbers of samples have to be analysed. The automated systems so far available still rely on the analysis of individual comets. The search is on, for example, through the COMICS project (*op. cit.*), for a staining method that will allow determination of relative tail intensity in the comet population as a whole, which could make scoring virtually instantaneous.

The assay has a limited dynamic range, saturating when most of the DNA is in the tail. This is not a problem with the low levels of background damage in normal cells. However, when using lesion-specific endonucleases to measure additional base damage, total damage scores may approach the saturation limit, and net enzyme-sensitive sites are likely to be underestimated unless care is taken to calibrate the assay.

Various stains have been used to visualize comets. Ethidium bromide and DAPI are the most commonly employed fluorochromes. They bind in different ways: ethidium bromide intercalates between base pairs and DAPI binds in the minor groove. This means that, in theory, fluorescence should be stronger when the DNA has double helical structure. In practice, tail and head of comets show similar intensity of staining—perhaps reflecting the presence of renatured DNA in the tail, or the use of relatively high stain concentrations, or our ignorance of the precise mechanism of binding of these stains. There are a number of alternative stains, including propidium iodide, YOYO-1™, SybrGold ™, SybrGreen™, TOTO™ and silver (for non-fluorescent staining).

Viability

It is common for a 'viability check' to be carried out on cell samples before they undergo the comet assay, and the most usual test is the trypan blue exclusion test. Dead cells take up the dye and appear blue. However, a cell does not have to be dead to take up trypan blue; the dye simply tests membrane integrity. Some researchers apply a criterion for rejecting samples, based on their trypan blue score, assuming that the positive cells are non-viable. We once had occasion to compare HeLa cells removed from the culture dish either by trypsinisation or by scraping with a silicone rubber scraper. According to the trypan blue test, >80% of the scraped cells were dead, while the trypsinised cells showed >80% viability. The comet assay gave very similar, low levels of strand breaks in both scraped and trypsinised samples. We took similarly treated (scraped or trypsinised) samples of HeLa cells and replated them to allow viable cells to reattach, and the next day recovered and counted the attached cells: there was no difference in the number of cells between the scraped and trypsinised samples (C. M. Gedik and A. R. Collins, unpublished data). Thus, in spite of their high trypan blue score, the scraped cells were as viable as the trypsinised cells.

The trypan blue exclusion assay and other so-called viability tests are not reliable tests of cell survival capacity but measure some aspect of cellular dysfunction. Fellows and O'Donovan (23) have recently drawn attention to the deficiencies of these tests in comparison with true indices of survival, i.e. relative cloning efficiency or proliferation assays. Probably a low comet assay DNA damage score would be a better indicator that the cells were alive than the common cytotoxicity tests.

At the low concentrations of genotoxic agents that it is possible to study in *in vivo* animal experiments—given the high sensitivity of the comet assay—it is unlikely that significant cytotoxicity will occur. So a poor result in the trypan blue (or other) cytotoxicity test is more likely to be caused by faulty cell preparation procedures (for example, centrifugation at excessive *g*). The same applies to human lymphocytes taken in population studies; reduced 'viability' is less likely to result from low-level occupational or environmental exposure to genotoxins than from poor handling of cells. In the case of cell cultures treated with genotoxins, relatively high concentrations of chemicals are often used, and cytotoxicity may then be a consequence to be wary of.

The comet assay is often used with nuclei rather than cells, derived from tissues of exposed animals or from biopsies. It is also possible to measure DNA lesions induced in embedded cells after lysis. 'Live' in this context is not a meaningful concept; nuclei are unable to divide and they are 'dead' in any viability test, but their DNA may be of high molecular weight provided that unspecific nuclease degradation is avoided.

Lymphocytes: pros and cons

Human blood is a very convenient source of cells, either for biomonitoring purposes or for experimentation. Leukocytes are generally the only cells that are available for biomonitoring, and it is important to realize their limitations, as well as their advantages. The advantages are clear; they are easily obtained, in large numbers, and do not require cell culture facilities; they are diploid and are almost all in the same phase of the cell cycle (G0). Lymphocytes are isolated by centrifugation over a layer of Lymphoprep™ (a solution containing sodium diatrizoate and polysaccharide), or a similar preparation, which retains lymphocytes while other white blood cells and erythrocytes pass through. Although lymphocytes are, like all tissues, highly specialized, they can be seen as reflecting the overall state of the organism, insofar as they circulate through the whole body. For example, measuring oxidized bases in lymphocyte DNA using FPG or endonuclease III provides an index of oxidative stress in the body as a whole. However, often it is important to consider the level of damage in a particular organ (with its specific metabolism), in which case lymphocytes may not be a good guide. A further limitation is that lymphocytes show very limited survival *in vitro*, unless stimulated by a mitogen such as phytohaemagglutinin. The fact that they show phenotypic variation from individual to individual, though valuable from the point of view of biomonitoring, means that they are not as suitable as standard permanent cell lines for many experimental purposes.

It is instructive to look at the potential use of lymphocytes in DNA repair studies. There are various DNA repair pathways; we will consider SS break repair and BER, both of which can be studied using the comet assay. First, SS break repair, which applies to the rejoining of breaks induced by ionizing radiation or agents such as H_2O_2: in most normal cell lines, this is a rapid process, with a $t_{½}$ of only a few minutes. But freshly isolated lymphocytes can take several hours to repair H_2O_2-induced damage (24). We surmised that, immediately after isolation, lymphocytes suffer oxidative damage from sudden exposure to the high concentration of O_2 in the atmosphere, compared with blood, and so the apparent slow repair of H_2O_2-induced breaks is partly accounted for by a continuous input of oxidative damage, and additional strand breaks, while repair is proceeding. A transient increase in DNA breaks can be seen in control cells (without H_2O_2 treatment), supporting this idea (25). However, it is puzzling that X-ray-induced breaks are rapidly repaired in fresh lymphocytes (26). We have now examined whether lymphocytes become repair competent when incubated after isolation (without mitogen stimulation). Figure 4 shows that, after 48 h, lymphocytes rejoin H_2O_2-induced breaks to the extent of >50% in 30 min. Perhaps, then, this simply reflects adaptation to the high O_2 level.

Lymphocytes are also relatively slow at BER of FPG-sensitive sites (8-oxoGua) induced by visible light in the presence of the photosensitiser Ro 19-8022 (F. Hoffmann-La Roche). In this case, there was no indication of faster repair after incubating the cells for 48 h before inducing the damage (Figure 4).

Because of the apparent unreliability of lymphocytes undergoing cellular DNA repair, we developed an alternative *in vitro* approach. A simple whole-cell extract is prepared from lymphocytes, by freeze thawing and lysis with Triton X-100. The substrate for the reaction consists of agarose-embedded nucleoids containing an excess of a particular kind of DNA damage. In the first version of this assay (27), which was designed to measure BER of oxidative DNA damage, the substrate cells were first treated with Ro 19-8022 plus light to induce 8-oxoGua before embedding in agarose. Incubation of substrate with extract results in the production of strand breaks in the substrate DNA, reflecting the capacity of the cells to carry out the initial step of repair of oxidized bases, i.e. removal of the bases by glycosylase, followed by cleavage of the resulting AP site—either by AP lyase/endonuclease in the extract or by the subsequent alkaline lysis. Lymphocytes have measurable activity, and this parameter of repair capacity has

been used in biomonitoring studies (28–30). Recently, the assay was modified for measuring nucleotide excision repair (NER) capacity, using a DNA substrate damaged by benzo[*a*]pyrene diolepoxide (31) or nucleoids from cells pretreated with UV (32). Looking at *in vitro* repair in a group of >30 normal individuals, we found that BER rates differ over a 4-fold range, while there appears to be a wider variation—up to 10-fold—in NER activities.

Several important questions remain concerning the measurement of DNA repair as a biomarker assay. Do individuals with a high BER rate *in vitro* also show relatively faster removal of FPG-sensitive sites? Is a high NER rate *in vitro* reflected in a relatively high level of incision events, i.e. breaks accumulated during incubation of UV-irradiated cells? Does *in vitro* repair in lymphocyte extracts reflect repair capacity in other tissues of the individual? Whether and how DNA repair can be used as a valid biomarker in population studies are matters for continuing investigation and discussion.

Conclusions

In this review, we have tried to deal with issues of theoretical and practical importance to the comet assay community. At a basic practical level, regarding electrophoresis, it is important to appreciate that we should be more concerned with the voltage drop across the gel than with total voltage or current. Electrophoresis can be carried out at either alkaline or neutral pH: whether, under neutral conditions, comets reflect SS and/or DS breaks makes an enormous difference to the interpretation of experiments. The biological significance of DNA damage in the cell can only be fully appreciated if we express results in real units, such as breaks per cell or per 10^9 Da, or Gy equivalents, and so calibration is important—but usually neglected. The way in which comets are scored is not critical: visual scoring and computer-based image analysis give comparable results. The comet assay has its limitations. Only a fairly narrow range of break frequencies (happily coinciding with the levels of DNA lesions seen in normal cells, or cells treated with sublethal doses of damaging agent) can be measured: the DNA fragments produced during apoptosis are much too small to be detected, but—like mtDNA—will diffuse away even before electrophoresis starts. However, it remains a highly versatile and adaptable assay, capable of giving information about the different kinds of damage present in a cell's DNA, and also about the cell's ability to repair the damage. Its application in the genotoxicity testing of chemicals and human biomonitoring seems to increase with the imminent development of high throughput comet assay methods and fully automated comet analysis.

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