Reference cells and ploidy in the comet assay

Gunnar Brunborg1 *, Andrew Collins2, Anne Graupner1, Kristine B. Gutzkow1 and Ann-Karin Olsen1

1 Department of Chemicals and Radiation, Division of Environmental Medicine, Norwegian Institute of Public Health, Oslo, Norway
2 Department of Nutrition, University of Oslo, Oslo, Norway

*Correspondence: Gunnar Brunborg, Department of Chemicals and Radiation, Division of Environmental Medicine, Norwegian Institute of Public Health, PO.Box 4404 Nydalen, N-0403 Oslo, Norway e-mail: gunnar.brunborg@fhi.no

INTRODUCTION

In our past studies of genotoxicity in mixtures of primary cultures of testicular cells from rats and humans (Bjorge et al., 1996a), there was a need to characterize subpopulations of spermatogenic cells. Flow cytometric analysis of DNA-stained cells was used for this purpose. We applied alkaline filter elution to measure DNA damage in testicular cell populations partly purified by means of centrifugal elutriation. We subsequently found that different testicular cell types could be identified when using the comet assay, which – unlike alkaline elution – allows measurement of DNA damage in individual cells (Bjorge et al., 1996b). This was possible since the fluorescent signal in the comet assay is related to the amount of DNA, which depends on cell ploidy. Testicular cell suspensions contain spermatogonia and secondary spermatocytes (but also Sertoli and Leydig somatic cells; 2n), primary spermatocytes (4c), secondary spermatocytes after first meiotic cleavage (2C), and spermats and spermatozoa at different stages of differentiation and maturation (1n). The relative proportions of these cell types are specific for human and rat testicular cells (Bjorge et al., 1996a; Olsen et al., 2003). Provided that scoring conditions (light intensity and staining) are standardized, the response of cell populations may be compared in different comet assay experiments. We used these approaches to estimate DNA damage induction and its repair in spermatogenic cells from mixed testicular cell populations (Olsen et al., 2001, 2003).

Cell-specific fluorescence can also be used as a basis for reference cells in the comet assay, as will be shown here. Reference cells are useful in standardization of conditions during various stages of the experimental protocol. Unexpected variations in measurement of specific DNA lesions occur between laboratories (Forchhammer et al., 2010), even for experienced comet assay users. Calibration trials, validation efforts, standardization of methods, and comparison of comet results between experiments and laboratories should profit from reference cells which are useful in standardization of conditions during measurement of specific DNA lesions occur between laboratories (Forchhammer et al., 2010), even for experienced comet assay users. Calibration trials, validation efforts, standardization of methods, and comparison of comet results between experiments and laboratories should profit from reference cells which could be analyzed in parallel with the sample cells. Reference cells could be in neighboring gel samples, or – preferably – mixed with the samples cells before the comet analysis and therefore subjected to exactly the same treatment conditions at all steps of the comet assay protocol (the only requirement being that the reference comets should be distinguishable from the comets from sample cells). This should also allow a better control of local variations in electrophoresis conditions. Zainol et al. (2009) developed a comet assay using internal bromodeoxyuridine-prelabelled reference cells that were identified on the basis of in situ immunostaining before scoring. Such cells can be mixed with unlabelled cells. This very useful approach, however, involves extra treatment steps and – compared with a method based on differential DNA content – is more costly because the method relies on antibodies.

We describe how DNA content-specific fluorescence intensity in the comet assay can be used, (i) for characterization of testicular cell populations; and (ii) as a convenient and low-cost system for...
internal reference cells taking advantage of the lower DNA content of some species of fish.

**MATERIALS AND METHODS**

Human testicular biopsies were obtained from organ donors, and single-cell suspensions of testicular cells were prepared as described (Bjorge et al., 1996a). Cells (unfrozen) were processed for comet assay analysis, stained with ethidium bromide, and analyzed using the Fenestra Comet image analysis system (Kinetic Imaging LTD, Liverpool, UK; Bjorge et al., 1996b). Comet Tail Moments (TM; these experiments were partly done in the early days of the comet assay, when TM was often used) and total fluorescence intensity (TFI) were recorded for each cell; these parameters are already integrated in Fenestra. During scoring no cells were excluded on the basis of either a very strong or a very weak fluorescence signal.

Blood (1–2 mL) was drawn using a 2 mL syringe from the tail vein of a turbot (Scophthalmus maximus) weighing about 600 g, kept in an indoor aquarium (about 50 m³, 8°C, continuously flushed with fresh sea water from the Oslo fjord at Solbergstrand, Drøbak, 40 km south of Oslo). The blood was diluted 1:10 in ice-cold phosphate buffered saline (PBS; 10 mM PO₄³⁻, 137 mM NaCl, and 2.7 mM KCl) with 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4; or in RPMI1640 medium (w/Hepes and glutamine; pH 7.4); and transported on ice to the laboratory in Oslo. After microscopic examination and counting, samples of the fish erythrocytes (FE), which are nucleated, were diluted once more 1:10 in RPMI1640 but now containing 10% dimethyl sulfoxide (DMSO) and 20% foetal calf serum (FCS), and 1 mL aliquots were frozen slowly to −80°C while placed in a Mister Frosty™ Freezing Container (Thermo Scientific, Oslo, Norway) unit containing isopropanol, for slow freezing of biological samples (the temperature is reduced at a rate of approximately 1°C per minute). For use, samples were thawed by warming for a few seconds to allow the frozen ice to be transferred into a tube containing 10 mL RPMI medium followed by centrifugation for 10 min at 400 × g and washing in the same medium. Suitable aliquots were mixed 1:10 in LMP agarose (0.7%) and analyzed according to our protocol for 96 minigels and electrophoresis with circulation (Gutzkow et al., 2013). Fresh (unfrozen) samples were diluted in RPMI medium (without DMSO/serum), mixed with agarose, and analyzed in the same way. The FE were added to GelBond™ films either as independent samples, or they were mixed 1:1 with human peripheral blood mononuclear cells (PBMN), which had been previously frozen, before embedding in agarose on GelBond™ films (for details, see Gutzkow et al., 2013). Photomicrographs of stained cell samples were recorded using Comet IV (see below). In some experiments, samples were irradiated before analysis with defined doses of X-rays (260 KeV, filtered through 0.5 mm Cu, dose rate 10 G/min) on ice.

For scoring of FE and PBMN comets, dried GelBond films were rehydrated and stained with SYBRGold (Life Technologies Ltd, Paisley, UK; 2 μL in 25 mL TE buffer pH 7.4, 20 min at RT), rinsed in water, and analyzed with Comet IV (Perceptive Instruments Ltd, Bury St. Edmunds, UK). An Olympus BX51 fluorescence microscope with CCD camera was used. Tail%DNA (TD) and TFI were recorded for each cell; these parameters are already integrated in Comet IV. During scoring no cells were excluded on the basis of either a very strong or a very weak fluorescence signal.

Statistics: Means and frequency distributions were calculated using Microsoft Excel.

**RESULTS**

**COMET ANALYSIS OF TESTICULAR CELL SUSPENSIONS**

The results shown in Figure 1 were obtained by pooling all data from an analysis of 16 samples of unexposed (control) fresh human testicular cells (from one donor). The data were sorted according to TFI values and they fell into three distinct classes. Based on the distribution of the total intensities, thresholds were sought for separation of the three populations of cells (vertical lines in Figure 1). The profile resembles the graphs obtained with flow cytometric analysis of testicular cells (Bjorge et al., 1996b). The mean fluorescence intensities (∼DNA content) are given in the legend. These cells were not subjected to genotoxic treatment and the levels of DNA damage in the three classes were low and very similar.

**REFERENCE CELLS FROM FISH WITH LOW GENOME SIZE**

In initial experiments, we tried fish cells from various genera. The polar cod, Boreogadus saida, has a suitable genome size (0.88 pg haploid genome; http://www.genomesize.com/), but the intrinsic level of DNA damage in frozen cells was high and variable. Rainbow trout (Oncorhynchus mykiss) had less DNA damage but its genome size (2.4–2.7 pg) is quite similar to that of Homo sapiens (3.50 pg); this was obvious in scatter plots similar to Figure 3 (see below) but without the two distinct populations (data not shown). In the subsequent experiments we used the turbot (Scophthalmus maximus; 0.86 pg).

Figure 2 illustrates the appearance in fluorescence microscopy of the different control (unexposed) cells of HPBL and FE, embedded either in separate gels or mixed 1:1 in the same gel before analysis with the comet assay. As with the testicular cells, the mixed cell population of control cells (HPBL and FE) belong to distinct classes but in this case there are only two classes (Figure 3A).

The mean TFI for turbot FE in Figure 3A is approximately 3–4 times lower than for HPBL, as expected from the genome size differences (see also Figure 4). It is apparent from the figure that there are no cells with TFI below 70,000 and 100,000. The absolute threshold value is subject to inter-experimental variations depending on the intensity of the lamp and the staining of DNA. Traditional fluorescent light sources (Mercury Xenon) produce less light with time of use, but newer technologies (liquid light guide combined with metal halide or LED light) solve this latter problem.

Other FE and HPBL samples were exposed to X-rays (0, 1.5, 3, 6, 8, 10, or 15 Gy) on ice and analyzed quickly to prevent repair of DNA strand breaks. The graphs in Figure 3 show scatter plots of DNA damage (TD) of the cell populations. The TD values may be discriminated and analyzed separately for the two populations (i.e., intensity levels either below 70,000 or above 100,000). Mean TD values were calculated for each population in Figure 3 and used to construct the dose response curves for both cell types presented in Figure 4. DNA damage increases linearly with the radiation
FIGURE 1 | Scatter plots of DNA damage of cells of different ploidy. Each single cell (with varying tail size) is scored for DNA damage together with its total fluorescence intensity (TFI). DNA damage (left vertical axis) and TFI (horizontal axis) were determined for 1600 control cells (dots) prepared from a human testicular biopsy. The stippled vertical lines indicate the partitions of three different populations of cells, of ploidy 1n, 2n, and 4c (discriminated at fluorescence intensity 350 000, 600 000, and 950 000, respectively). The mean intensities of cells within these classes are 255 000, 471 000, and 761 000 for 1n, 2n, and 4c, respectively. Mean levels of DNA damage (TM) are in the range 2.2–2.5 for the three classes and are not significantly different. Plotted line: mean frequency (%), right vertical axis) of DNA damage at each intensity level.

FIGURE 2 | Micrographs of control (unirradiated) erythrocytes from the turbot fish and human blood mononuclear cells. Left: human lymphocytes; center: fish erythrocytes (FE); right: 1:1 mixture of the two cell types. Representative microscopic fields are shown. Size bar: 50 μm for all micrographs. See Materials and Methods for further details.

dose for both fish and human cells, except at the highest level of damage (TD > 80%) induced by 15 Gy which is beyond the dynamic range of the assay. In contrast to the DNA damage, the mean TFI does not change much with radiation dose. The slopes of the two dose-response curves are different, i.e., the comet assay indicates that less DNA damage is induced per unit radiation dose in fish than in human cells. This may be related to the lower DNA content of turbot cells, implying a smaller target size for ionizing radiation.

DISCUSSION
With the testicular cell suspensions, the comets giving strongest fluorescence represent 4c cells (i.e., premeiotic spermatocytes). This is a relatively small population (17.2% of total), compared to the larger proportion of 59.4% 1n cells (post-miotic haploid spermatids) and 23.5% 2n (2C secondary spermatocytes plus 2n Leydig/Sertoli somatic cells); these data are all derived from Figure 1. In a normal experiment, the standard deviation of the small population (low numbers) of 4c cells is higher than for the other populations. We have in some cases selectively scored strongly fluorescing cells, in order to measure DNA damage in this class of 4c cells with higher precision. With automated imaging (e.g., IMSTAR™ Pathfinder, Paris, France), larger numbers of cells may be scored. To estimate the ratio of 2C spermatocytes vs. 2n somatic cells, flow cytometric analysis of vimentin-stained cells may be used.
FIGURE 3 | (A–G) Mixtures of fish erythrocytes and human PBMN cells exposed to ionizing radiation. Scatter plots are shown for DNA damage (Tail %DNA, y-axis) vs. Total Fluorescence Intensity (arbitrary units, x-axis). X-irradiation doses are indicated; x- and y-axis and symbols are equal for A–G.
Fish erythrocytes from the turbot fish have significantly lower genome size than human cells, but there is sufficient DNA in the fish cell to produce a good fluorescent signal (Figure 2). Unlike the polar cod, fresh samples of the turbot showed very low background levels of DNA damage. However, turbot FE that had been frozen and stored for a few weeks at $-80^\circ C$ before thawing and analysis, expressed significantly elevated levels of DNA damage which also increased with longer periods of storage (data not shown). Further optimization should be performed to improve the integrity of turbot cell DNA (either FE or other cell types) upon storage. It is well known that human lymphocytes can keep their DNA integrity during freezing and storage for months, both as control and irradiated samples. The ultimate aim would be to prepare and distribute frozen samples of fish cells, either untreated or treated with defined doses of ionizing radiation inducing known levels of DNA damage. Such cells can be used as standards for calculation of frequencies of induction of DNA lesions in sample cells treated with genotoxics. As an alternative to frozen cells, fresh blood samples may be obtained cheaply and reproducibly from fish living in an aquarium and the same fish may be sampled many times. Collection of blood from the tail vein is not known to harm the fish or to induce disease or pathological changes. The trivial fact that the turbot is a flatfish contributes to easy handling and blood collection, even for untrained personnel. One milliliter of peripheral blood contains sufficient numbers of erythrocytes for 100s of experiments.

The fluorochrome used in these analyses does not seem to be crucial, since we obtained consistent results with both ethidium bromide and SYBRGold. However, care should be taken to avoid saturation of the light signal, since the quantitative relationship with DNA content would then be distorted.

Cells of different ploidy, or in different stages of mitosis and meiosis, may be analyzed using the methodologies described here, and this could represent a cheap (although much more time-consuming) alternative to staining and analysis of the cell cycle distribution of a cell population, or of mixed cell populations, by flow cytometry. Furthermore, very few (as low as 100) cells are needed. Kruszewski et al. (2012) recently used the same methodology to show that the fluorescence intensity corresponds to the position in the cell cycle of dividing cultures. The effect of the cell cycle on induction and repair of DNA damage may this be analyzed. In conclusion, the methods described here represent new applications of the comet assay. Some further validation should be useful.

ACKNOWLEDGMENTS

This work was financed by the European Commission via the project Comet assay and cell array for fast and efficient genotoxicity testing (COMICS; contract no. LSHB-CT-2006–037575).

REFERENCES

Bjorge, C., Brunborg, G., Wiger, R., Holme, J. A., Scholz, T., Dybing, E., et al. (1996a). A comparative study of chemically induced DNA damage in isolated human and rat testicular cells. Reprod. Toxicol. 10, 509–519. doi: 10.1016/S0890-6238(96)00138-4

Bjorge, C., Wiger, R., Holme, J. A., Brunborg, G., Scholz, T., Dybing, E., et al. (1996b). DNA strand breaks in testicular cells from humans and rats following in vitro exposure to 1,2-dibromo-3-chloropropane (DBCP). Reprod. Toxicol. 10, 51–59. doi: 10.1016/0890-6238(95)02018-7
Forchhammer, L., Johansson, C., Loft, S., Moller, L., Godschalk, R. W., Langie, S. A., et al. (2010). Variation in the measurement of DNA damage by comet assay measured by the ECVAG inter-laboratory validation trial. *Mutagenesis* 25, 113–123. doi: 10.1093/mutage/gep048

Gutzkow, K. B., Langleite, T. M., Meier, S., Graupner, A., Collins, A. R., and Brunborg, G. (2013). High-throughput comet assay using 96 minigels. *Mutagenesis* 28, 333–340. doi: 10.1093/mutage/get012

Kruszewski, M., Iwanenko, T., Machaj, E. K., Oldak, T., Wojewodzka, M., Kapka-Skrzypczak, L., et al. (2012). Direct use of the comet assay to study cell cycle distribution and its application to study cell cycle-dependent DNA damage formation. *Mutagenesis* 27, 551–558. doi: 10.1093/mutage/get018

Olsen, A. K., Bjortufi, H., Wiger, R., Holme, J., Seeberg, E., Bjoras, M., et al. (2001). Highly efficient base excision repair (BER) in human and rat male germ cells. *Nucleic Acids Res.* 29, 1781–1790. doi: 10.1093/nar/29.8.1781

Olsen, A. K., Duale, N., Bjoras, M., Larsen, C. T., Wiger, R., Holme, J. A., et al. (2003). Limited repair of 8-hydroxy-7,8-dihydroguanine residues in human testicular cells. *Nucleic Acids Res.* 31, 1351–1363. doi: 10.1093/nar/gkg216

Zainol, M., Stoute, J., Almeida, G. M., Rapp, A., Bowman, K. J., and Jones, G. D. (2009). Introducing a true internal standard for the Comet assay to minimize intra- and inter-experiment variability in measures of DNA damage and repair. *Nucleic Acids Res.* 37:e130. doi: 10.1093/nar/gkp826

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 July 2014; accepted: 08 February 2015; published online: 27 February 2015.

Citation: Brunborg G, Collins A, Graupner A, Gutzkow KB and Olsen A-K (2015) Reference cells and ploidy in the comet assay. *Front. Genet.* 6:61. doi: 10.3389/fgene.2015.00061

This article was submitted to Genomic Assay Technology, a section of the journal *Frontiers in Genetics*.

Copyright © 2015 Brunborg, Collins, Graupner, Gutzkow and Olsen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.