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Review

Physical principles and new applications of comet assay

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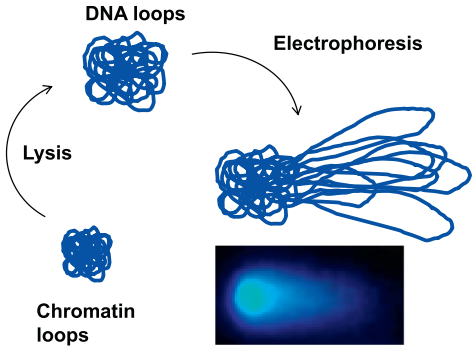
HIGHLIGHTS

- The electrophoretic track in the comet assay is formed by extended DNA loops
- These loops are about the same as chromatin loops in the cell nuclei
- Kinetics of the track formation may be used to investigate the loop organization.

GRAPHICAL ABSTRACT



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ABSTRACT

The comet assay is a sensitive method to assess DNA damages in single cells. The approach consists of an analysis of electrophoretic migration of DNA from nucleoids obtained after cell lysis in a thin layer of agarose. Although the method is widely used the physical mechanisms of DNA track formation remained to be rather elusive for a long time. This review is devoted to our recent results pertaining to this subject, using an original approach based on the kinetic measurements of the comet formation. We argue that linear DNA fragments give an essential contribution into the tail formation in the alkaline conditions and, at neutral pH, when the level of DNA damages is very high. On the other hand, in the neutral comet assay at low levels of DNA damages (and also in the case of undamaged cells) the tail is formed by extended DNA loops. These loops are about the same as chromatin loops in the cell nuclei. Kinetic measurements in the comet assay give an opportunity to investigate the topology of the loops and large-scale features of the loop domain organization (and re-organization) in nucleoids obtained from different cell types.

1. Introduction

Single-cell gel electrophoresis (the comet assay) was initially developed as a technique that helps to evaluate the rate of DNA damages at the level of individual cells [1–3]. The assay, which combines gel-electrophoresis and fluorescent microscopy, usually starts with the preparation of microscopic slides with cells embedded in a thin layer of agarose. At the next step the immobilized cells are lysed with detergents and high ionic strength to produce so-called nucleoids that consist of DNA attached to some residual nuclear proteins that are insensitive to the lysis treatment. The slides are then electrophoresed: under the electric field DNA migrates from the nucleoid to the anode and forms an electrophoretic track, which, after staining with a fluorescent dye, looks like a comet tail [1–5]. The electrophoresis may be performed at neutral pH (the neutral comet assay) or in alkaline conditions at pH $>$ 13 (the alkaline comet assay) [3–7]. It was shown in numerous experiments

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that the comet formation appears to be very sensitive to even low mutagenic exposure: the DNA migration is facilitated if damages (predominantly single- and double-strand breaks) are present in DNA [1–3,8–10]. Among other techniques developed for evaluation of genome damages, the comet assay has become very popular due to its simplicity and sensitivity. The main advantages of the assay involve the possibility to work with small number of cells without preliminary mitotic stimulation and thus to test different types of cells [3–7,10].

Although the method is widely used the physical mechanisms of DNA track formation remained to be not completely understood for a long time [5,7,11,12]. Two different hypotheses were proposed to explain how the comet tail is formed. The first one was based on the observation that the comet formation is significantly facilitated after cell treatments which induce DNA strand breaks (ionizing irradiation, free radicals etc.). The explanation which seems to be straightforward is that the comet tail is formed by linear DNA fragments that either keep (with one of its ends) connection with the residual nuclear proteins or lose it. The fragments are single stranded and double stranded in, respectively, alkaline and neutral conditions. Therefore, it is very often assumed that the neutral comet assay detects double-strand breaks while the alkaline assay is sensitive also to single-strand breaks [2–5,11–13].

Another hypothesis was based on the fact that the nucleoid consists of negatively supercoiled DNA loops attached to the residual nucleoid proteins [14,15]. It has been suggested that, in the neutral comet assay, the tail contains extended DNA loops [1]. A negative supercoiling appearing in the loops after nucleosome removal during lysis should maintain a compact state of the loops and make their exit to be hampered. At least one single-strand break should relax the loop: the supercoiling should disappear and it would be easier for DNA to migrate. It was proposed that the same mechanism of the loop relaxation works also in the case of the alkaline comet assay [5,16].

In this review we will focus on physical mechanisms of the comet tail formation. We will argue that the two views, mentioned above, are not mutually exclusive. DNA fragments give an essential contribution into the tail formation in the alkaline conditions and, at neutral pH, when the level of DNA damages is very high. On the other hand, in the neutral comet assay at low levels of DNA damages (and also in the case of undamaged cells) the tail is formed by extended DNA loops. These conclusions follow, in particular, from our recent results using an original approach based on the kinetic measurements of the comet formation. This approach opens a new perspective, which also will be discussed: to investigate the DNA loop domain organization using the comet assay.

2. Principles of the comet assay

The comet assay protocol was described for the first time by Östling and Johanson in 1984 [1]. The approach consisted of analyzing the DNA migration from cells embedded in agarose on a glass slide after their lysis in neutral detergent solution to remove cellular and nuclear membranes and proteins (Fig. 1). Under such conditions a small fraction of genome DNA extends towards the anode and forms the electrophoretic track (the tail) that can be visualized with fluorescence dyes. The rate of DNA migration was estimated as the relative fluorescence intensity in the tail. This relative amount of DNA in the tail was proportional to the dose of ionizing radiation. Thus, the level of DNA exit appeared to be a measure of DNA damages in individual cell.

A modification of this microgel electrophoresis (which is applied more often now) was proposed by Singh and colleagues in 1988 [2]. In contrast to the original protocol, the lysis was performed with detergents and high ionic strength; electrophoresis was done under alkaline conditions (pH > 13) and the slides were neutralized before staining. The same tendency was observed: the level of the DNA exit depended on the dose of mutagens that induced DNA strand breaks. The authors reasoned that the alkaline electrophoresis, when the migration of single stranded (denaturated) DNA occurs, would increase the sensitivity to more types of DNA damages (see below). Moreover, the cell lysis with detergents and high salt ensured more complete protein removal. In fact, such lysis procedure was used for many years for obtaining nucleoids, the structures formed by DNA loop domains that lost most of chromatin proteins but remained to be attached at their ends to nuclear elements being insensitive to lysis [14,15]. The lysis with detergents and high salt has become a standard procedure in the comet assay (including its neutral variant) [3–10].

The final step of the assay is an analysis of slides after electrophoresis and staining. The simplest way to estimate the efficiency of DNA exit from nucleoids is numbering the comets, i.e. the nucleoids with electrophoretic tracks, by visual inspection. Such inspection may include classifying the comets into five categories according to the tail appearance [4,5]. The comets of the class that is referred to as C_0 lack the tail at all; the C_1–C_3 classes represent the comets with increasing tail intensities; the C_4 class includes the comets with highly fragmented DNA (e. g. apoptotic cells) when almost all the DNA migrates into the comet tail and forms a cloud, rather than a tail, which loses its connection with the comet head. Obviously, any visual scoring is subjective. More precise quantitative estimation of the DNA exit efficiency is possible due to special softwares that enables to analyze digital comet images. The two parameters are usually measured: the relative amount of DNA in the tails and the tail length [17–19]. The DNA amount in the tail is defined as the ratio of the tail fluorescence intensity to the total intensity of the comet, while the tail length – as the distance from the center of mass of the head to distal end of the tail. Additionally, the product of these two parameters, the tail moment, is used in some studies [17,20].

3. Common applications of the comet assay

Both variants of single cell gel electrophoresis, the neutral and the alkaline, rapidly became very popular because of their simplicity and availability. Small number of cells needed for analysis, the possibility to work with cells of almost any type, ability to measure various DNA damages with high accuracy ensured the indispensability of the comet assay in biomonitoring and clinical studies. A great number of chemicals, physical factors, nanoparticles, drugs etc. were tested for mutagenicity with this technique [19,21,22].

The fact that the presence of DNA strand breaks significantly facilitates the formation of the electrophoretic track indicates that this particular type of genomic lesions can be detected using the comet assay. It was widely thought for a long time that the neutral variant of the electrophoresis protocol, when the migration of double stranded molecules occurs, allows one to estimate the double-stranded breaks only, while the alkaline electrophoresis, since DNA is denatured, is applicable for estimation of both single- and double-strand breaks [3,8]. This simplified view is not exactly true (see below), and there is much evidence that the neutral comet assay is just as good for single-strand break detections [5,23,24]. It has to be mentioned, however, that the alkaline comet assay is also sensitive to alkali labile sites – apurinic/

Fig. 1. Main steps of the general procedure of the comet assay: cells are embedded in agarose on the surface of a microscopic slide (A), lysed to produce nucleoids (B) and electrophoresed to obtain comets (C).