

Potential of Single Cell Gel Electrophoresis Assay (Comet Assay) in Heavy Ion Radiation Biology

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ABSTRACT DNA damage and repair following heavy ion radiation have been studied using various techniques like pulse field gel electrophoresis, sedimentation, and neutral/alkali elution. These techniques are cumbersome, time consuming and need a substantial cell-sample size. In addition, these techniques are unable to identify differentially, highly damaged or apoptotic subpopulation of cells, a characteristic result of exposure of heavy ion radiation, since the results are based on a pooled population of cells. Most of these difficulties can be overcome by using single cell gel electrophoresis assay, commonly known as comet assay. For this assay, a small cell-sample size (a few thousand cells) is required, and the results are available in a short time (5-6 hours). Since, the information on DNA damage and repair is available on individual cells, any subpopulation of cells with a different response like high/low DNA damage or apoptosis can be identified. Moreover, this assay is adept in evaluating SSBs, DSBs, alkali labile sites, adduct formation, cross-linking (DNA-DNA or DNA-protein) and base damage. Not only that, damage to specific region of chromosome may be detected using FISH in combination with comet assay. Hence, the use of comet assay may have potential in the estimation of DNA damage and repair in heavy ion radiation biology.

INTRODUCTION

There is a growing interest in high linear energy transfer (LET) radiation due to its application in radiotherapy of cancer and its importance in radiation protection especially in manned space flights (Blakely and Kronberg 1998). Ionizing radiation produces DNA damage by direct hits. High LET radiation tracks produce highly localized clustered damage within the DNA and also spatially separated sites of damage along the path of radiation track (Anderson et al. 2000). Also, free radical production is an important aspect of ionizing radiation. In addition to other effects, free radicals also cause damage to DNA.

Many types of DNA lesions are produced by ionizing radiation; the lethal lesion is probably an unrepaired or misrepaired double-strand break (Olive 1998).

Radiation may cause single-strand breaks (SSBs), double-strand breaks (DSBs), DNA-DNA as well as DNA-protein crosslinks and damage to bases (Plappert et al. 1995). However, the quantity and quality of DNA damage varies from cell to cell. The heterogeneity in DNA damage and repair caused by ionising radiation is of importance. A variety of assays have been used to understand and analyse the basic mechanism of ionising radiation-induced DNA lesions (Radford 1988). These include sedimentation, neutral/alkali elution, pulse field gel electrophoresis (PFGE) and unscheduled DNA synthesis (UDS). These assays provide information only on the average DNA damage. However, in heterogeneous cell populations, averages are not adequate to describe DNA damage. To the list of the assays mentioned above, more recently, another assay called single cell gel electrophoresis (comet) assay has been added. The comet assay, a technique capable of detecting DNA damage and repair in individual cells, is a valuable approach for human biomonitoring studies (Kassie et al. 2000). The technique can be used for biomonitoring humans exposed to ionising radiation as well as for in vitro radiation exposed cells.

PRINCIPLE BEHIND COMET ASSAY

A single cell suspension is necessary for comet assay. For this reason, peripheral blood leukocytes, which are easily available, are quite suitable for operation. However, any animal or plant tissue that can be rendered into a single cell suspension, can be used. The cells embedded in agarose gel are layered on a microscope

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slide. They are lysed in detergents with high salt concentrations and liberated DNA electrophoresed. Since DNA is negatively charged, nucleoids with damage display migration of DNA towards anode. The slides are stained with a fluorescent dye, like ethidium bromide, and examined under a fluorescent microscope for quantification of DNA damage in each cell. Non-fluorescent staining technique for DNA, though somewhat cumbersome, is also available, which gives equally satisfactory results (Ahuja and Saran 1999).

There are two major versions of comet assay. The first one is after Singh et al. (1988) who used alkaline lysing solution (pH~10) and alkaline electrophoresis buffer (pH>13). This version is capable of detecting single-strand breaks and alkali-labile lesions in the DNA of individual cells. This technique was called single cell gel (SCG) assay by Singh et al. (1998), although commonly referred to as comet assay. The second version is after Olive and her coworkers who used mildly alkaline (pH 8) lysis followed either by electrophoresis at neutral (Olive et al. 1990a) or alkaline (pH 12.3) conditions (Olive et al. 1990b) to detect double or single strand breaks respectively. However, based on the respective ability of these two versions to detect DNA damage induced by ionizing radiation, the technique of Singh et al. (1998) is at least an order or two of magnitude more sensitive (Tice and Strauss 1995).

Sensitivity of the comet assay has been improved by various modifications. For example, addition of antioxidant to the lysing/electrophoretic buffer, DNA precipitation with ethanol, and use of sensitive dye (e.g., YOYO-1) have enhanced the sensitivity of the alkaline technique to screen for low level of DNA damage in a variety of cells (Singh 1996). To attain sensitivity under neutral microgel electrophoresis condition, relatively pure DNA (free from RNA and proteins) in microgels is a prerequisite. This could be achieved by treating the microgels on slides with the enzyme ribonuclease-A and proteinase-K (Singh and Khan 1995).

ESTIMATION OF DNA DAMAGE

The simplest method for collecting SCGE

data is based on determining the proportion of cells with damage (i.e., those exhibiting the damage vs those without it). However, this approach fails to provide information about the extent of damage. Another approach subclassifies damaged cells into the ones having various degree of DNA migration (Anderson et al. 1994). Since the classification in this approach is arbitrary, it is highly subjective. The parameter most commonly used is the length of DNA migration. Migration length is related directly to fragment size and is expected to be proportional to the extent of DNA damage. Furthermore, some investigators have used the ratio of length/width or width/length. Olive et al. (1990a) discounted the utility of DNA migration as a parameter for DNA damage based on the observation that with increasing dose of many mutagens the length of DNA migration reached a plateau while the percentage of migrated DNA continued to increase. The concept of tail moment (tail length x tail intensity or percentage migrated DNA) as a parameter for DNA damage was introduced. Whereas the tail length can be measured with the help of an ocular micrometer fixed in the eyepiece of the microscope, for the estimation of tail moment an image analysis system is required.

DETECTION OF CELLULAR HETEROGENEITY

In human biomonitoring studies, the major source of cells for analysis is blood. Leukocytes are a heterogeneous mixture of monocytes (2-10%), lymphocytes (20-40%), neutrophils (50-70%), eosinophils (1-4%) and basophils (0-0.5%). Lymphocytes consist of T (80%) and B (20%) cells. The last three cell types comprise the granulocyte population. Strauss et al. (1994) introduced an interesting immunological typing technique, which circumvents the need for cell sorting by allowing for the identification of subtypes of blood leukocytes in the gel matrix. In this method, specific cell types are individually recognized in the agarose gel at the time of analysis by the co-presence of immunogenetic beads which had adhered to selected membrane markers prior to processing. This approach has been used to demonstrate comparable DNA damage

and repair kinetics in irradiated B, T, T-helper, T-suppressor cells and granulocytes. Monitoring of DNA from cells other than lymphocytes may show less heterogeneity.

Differences in the radiation response of tumors of the same type, size and grade has generally been attributed to tumor cell heterogeneity and in particular to differences in intrinsic radiosensitivity, tumor growth kinetics and the presence of resistant subpopulation such as hypoxic cells. Olive et al. (1993) also reported a successful application of comet assay to the identification of hypoxic, DNA damage-resistant cells in needle biopsy material from irradiated human breast cancers. This study has merit in human tumor types where hypoxic cells may limit radiocurability, with the potential for selective use of hypoxic sensitizers.

DETECTION OF BASE DAMAGE

Ionizing radiation is able to break the DNA directly by deposition of energy in the deoxyribosephosphate backbone, thereby producing single-strand breaks and double-strand breaks. However, most of the energy is deposited in water leading to free radicals which can subsequently react with bases and sugars in DNA, producing base modifications, sites of base loss (AP sites) and strand breaks. Collins et al. (1993) reported the use of endonuclease III (Endo III) and fapy-DNA glycosylase to probe for base damage to produce single strand breaks.

DETECTION OF CROSS-LINKS

Free-radical production is an important effect of ionizing radiation, which are known to cause DNA damage. It is also known that free radicals can cause DNA crosslink (DNA-DNA and DNA-protein) formation (Lloyd et al. 1997). DNA-protein crosslinks may be evaluated by treating the cells with proteinase-K, which will remove the crosslink and release the DNA fragments held by crosslink, enhancing the DNA migration (Singh and Lai 1998). To study the DNA-DNA crosslinks produced by high LET, cells may subsequently be treated with low LET. If there is retardation in DNA migration as compared to the low LET treatment alone it would

indicate DNA-DNA crosslinks.

DETECTION OF APOPTOTIC CELLS

DSBs have often been cited as the critical lesion produced by ionizing radiation (Ostling and Johanson 1984). If unrepaired, or incorrectly repaired, DSBs can lead to chromosome damage and cell death.

The aim of radiotherapy is to kill the cancer cells. Cells undergoing active cell death or apoptosis demonstrate highly fragmented DNA. Apoptosis results in the extensive formation of double-strand breaks and is readily detected using either neutral or alkaline electrophoretic conditions. When viewed using the comet assay, only a small percentage of DNA of an apoptotic cell remains associated with the comet head (Olive et al. 1993; Gopalkrishna and Khar 1995). Recently, Singh (2000) has published a modified version of this assay, to study apoptosis.

EFFECT OF ANTIOXIDANTS/FREE-RADICAL SCAVENGERS

Experiments with the comet assay suggest that diet could affect human sensitivity to ionizing radiation damage. Effect of some antioxidants/free radical scavengers like vitamins C, E and beta-carotene, and a pineal gland hormone, melatonin, have been studied on the radiation-induced DNA damage. Blood samples were taken both before and after volunteers ate breakfast and took vitamin C tablets. The samples were irradiated and the comet assay carried out. Those samples taken after breakfast and vitamin C administration showed substantially less damage (Green et al. 1994).

In another experiment, when vitamins, C, E and beta-carotene, were administered before irradiation, a protective effect on DNA damage was seen and it can be ascribed to their antioxidant properties. However, it was seen that vitamins also greatly reduce radiation-induced damage when administered immediately after radiation exposure. In this situation radioprotection by vitamins must be related to another mechanism, probably the process of DNA repair (Konpacka et al. 1998).

Radioprotective effect of melatonin has been

reported by Vijayalaxmi et al. (1998). Irradiated lymphocytes from all volunteers, which were collected after melatonin ingestion exhibited a significantly decreased extent of primary DNA damage as compared with irradiated cells collected before the oral dose of melatonin.

EFFECT OF CELL CYCLE

Sensitivity for detecting both single and double-strand breaks is reduced in populations containing cells in S-phase. Cells in the process of synthesizing DNA contain replication forks and bubbles. Each replication fork behaves as a single strand break when exposed to alkali, thus increasing the background damage in S-phase cells. Under neutral conditions, replication structures inhibit migration during gel electrophoresis so that the shape of the dose-response curve is reduced about 2-3 fold in S-phase cells (Olive 1999).

SENSITIVITY OF COMET ASSAY

Olive et al. (1990a, 1990b) have shown that the sensitivity for detecting damage in single cells analysed in the comet assay is comparable to the sensitivity of other methods which measure average damage to a population of cells. Sensitivity of comet assay has allowed detection of very low levels of DNA strand breaks induced by X-rays. It is possible to detect one single-strand break/ 2×10^{10} Dalton of DNA at a dose of 3.2 rads of X-rays (Singh et al. 1994).

NATURE OF COMETS

The nature of comets, and the physical events underlying their formation, are not fully understood or agreed upon. According to Singh and Stephens (1997), during neutral electrophoresis, individual DNA molecules behave as if anchored at one end while the other end is free to migrate in response to the electric field. In other words, it gives the impression that each strand belongs to single chromosome to which tightly bound proteins are attached at regular intervals. The single-stranded DNA after alkaline electrophoresis breaks down into small units (possibly of 4 to $10 \mu\text{m}$) by the dehydrating and precipitating effect of ethanol. Once broken, these pieces seem

to curl upon them selves and fluoresce as bright particles.

LOW-LET vs HIGH-LET RADIATION

The relative biological effect (RBE) for cell killing increases as LET increases, reaching a maximum around $100 \text{keV}/\mu\text{m}$. The lesions produced by high-LET radiation are more complex because of the larger number and size of multiply damaged sites (Olive 1999). Densely ionizing radiations (high LET) are relatively more effective in producing multiple ionizations in spurs, blobs and short tracks (Goodhead et al. 1993). The spatial distribution of these lesions produced along the DNA molecule is a major significant factor for the observed biological effects. When cells are exposed to low dose (0.39 Gy) radon, there is a finite probability that a specific cell will not be hit by an alpha particle. The comet assay can identify cells that are hit from those that escape damage (Jostes et al. 1993).

ADVANGAGES AND LIMITATIONS OF COMET ASSAY

The comet assay is a particularly valuable technique in that it allows the detection of inter-cellular differences in DNA damage and repair in virtually any eukaryotic cell population that can be obtained in a single cell suspension; it requires only extremely small cell-samples (about 10,000 cells) and results can be obtained in a single day.

An optimal technique for radiation biomonitoring studies should be sensitive, quantitative, specific for radiation-induced DNA damage, and applicable to diverse cell populations. The comet assay satisfies many of these criteria. Due to its simplicity the comet assay can easily be used to evaluate the ability of virtually any type of eukaryotic cells to repair different kind of DNA damage, including double and single strand breaks, base damage, and DNA crosslinks.

With all the advantages of comet assay mentioned above there are a few limitations also: (1) The comet assay can determine whether an agent produces DNA strand breaks but cannot measure the fidelity of repair of those breaks

(Fairbairn et al. 1995). (2) There is lack of specificity for DNA damage and the possible effect DNA repair has on detection sensitivity (Tice and Strauss (1995), (3) The necessity for single cell suspension.

FUTURE PROSPECTS

A new exciting approach has been introduced in comet assay: Fluorescent-in-situ hybridization (FISH) has been combined with it (Santos et al. 1997; McKelvey-Martin et al. 1998). It has been suggested that damage or repair of specific chromosome regions might be examined by applying this approach. The applicability of the system mentioned here is vast. Further studies will probably offer more insight into the nuclear ultrastructure and nuclear changes as a function of the cell cycle, cell type and cell differentiation state (Singh and Stephens 1997).

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