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Measurement of DNA damage in rat urinary bladder transitional cells: Improved selective harvest of transitional cells and detailed Comet assay protocols

Amy Wang [a,*], John L. Robertson [a], Steven D. Holladay [a], Alan H. Tennant [b], Andrea J. Lengi [c], S. Ansar Ahmed [a], William R. Huckle [a], Andrew D. Kligerman [b]

[a] *Department of Biomedical Sciences and Pathobiology, Virginia Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University (Virginia Tech), Phase II, Duckpond Drive, Blacksburg, VA 24061-0442, USA*
[b] *Environmental Carcinogenesis Division, National Health and Environmental Effects Research Laboratory, B143-06, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA*
[c] *Department of Dairy Science, Virginia Tech, 2760 Litton Reaves Hall, Blacksburg, VA 24061-0315, USA*

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Abstract

Urinary bladder transitional epithelium is the main site of bladder cancer, and the use of transitional cells to study carcinogenesis/genotoxicity is recommended over the use of whole bladders. Because the transitional epithelium is only a small fraction of the whole bladder, the alkaline single cell gel electrophoresis assay (Comet assay), which requires only a small number of cells per sample, is especially suitable for measuring DNA damage in transitional cells. However, existed procedures of cell collection did not yield transitional cells with a high purity, and pooling of samples was needed for Comet assay. The goal of this study was to develop an optimized protocol to evaluate DNA damage in the urinary bladder transitional epithelium. This was achieved by an enzymatic stripping method (trypsin–EDTA incubation plus gentle scraping) to selectively harvest transitional cells from rat bladders, and the use of the alkaline Comet assay to detect DNA strand breaks, alkaline labile sites, and DNA–protein crosslinks. Step by step procedures are reported here. Cells collected from a single rat bladder were sufficient for multiple Comet assays. With this new protocol, increases in DNA damage were detected in transitional cells after *in vitro* exposure to the positive control agents, hydrogen peroxide or formaldehyde. Repair of the induced DNA damage occurred within 4 h. This indicated the capacity for DNA repair was maintained in the harvested cells. The new protocol provides a simple and inexpensive method to detect various types of DNA damage and to measure DNA damage repair in urinary bladder transitional cells.

Keywords: Comet assay; Urinary bladder; Transitional epithelium

1. Introduction

Urinary bladder cancer is one of the five most common cancers in Europe and the United States of America [1], and more than 90% of malignant bladder cancer is transitional cell carcinoma [2]. The urinary

* Corresponding author. Tel.: +1 540 231 3372; fax: +1 540 231 6033.
E-mail address: amywang@vt.edu (A. Wang).

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bladder consists of three layers of tissues: inner epithelium (urothelium), submucosa (connective tissue), and smooth muscle. The urothelium consists of transitional cells which are constantly exposed to metabolic wastes and other chemicals through contact with the urine [3]. Laboratory research addressing the mechanisms of urinary bladder carcinogenesis should ideally be conducted using pure transitional cells instead of a mixture of separate cell types from whole bladders [4,5] to avoid dilution or misleading responses [5]. Because transitional urothelium comprises only a small fraction of total bladder tissue, assays utilizing transitional cells must make use of very small tissue samples or sample pooling may be needed.

Genotoxicity is important for characterizing the mode of action for carcinogens, including bladder carcinogens [4]. An assay for genotoxicity that is gaining widespread use is the single cell gel electrophoresis assay, commonly called the Comet assay. This assay has the advantages of requiring a small number of cells per sample (as few as 10^5), being amenable to almost all cell types whether actively dividing or quiescent, and being relatively low cost requiring only basic equipment [6–8]. In the Comet assay, cells are embedded in agarose on a glass slide, lysed to break cell and nuclear membranes, and unwound in an alkaline solution to yield single stranded DNA from liberated double-stranded DNA. Following electrophoresis, the sample is stained with a DNA-binding dye and viewed under a microscope. Short strands of DNA generated from DNA strand breaks and/or alkaline labile sites (depending on pH of the electrophoresis solution) migrate further than intact DNA during electrophoresis and form the "tail" of the "Comet". In the presence of DNA–protein crosslinks or rare DNA–DNA crosslinks, DNA migration is inhibited by crosslinks. The degree of DNA migration is usually presented as percentages of DNA in the tail (by image analysis software) or morphological classes of Comets measured visually [9]. When compared to control samples, a significant increase in DNA migration indicates the presence of increased levels of strand breaks, alkali labile sites, or incomplete excision repair sites, whereas a significant decrease indicates the presence of DNA–protein or DNA–DNA crosslinks [6–8].

The ability to conclude that altered DNA migration is due to genotoxicity generally requires samples with a minimum of 70–75% viability as measured by the trypan blue exclusion assay [10,11]. Concurrent measurements of cell viability/cytotoxicity in samples subjected to the Comet assay are important because cytotoxicity can cause artifactual changes in DNA migration [12]. For example, apoptotic and necrotic cells that contain highly degraded DNA can increase mean DNA migration of the samples [6]. On the other hand, at cytotoxic doses loss of heavily damaged or dying cells during sample processing and electrophoresis can decrease the recorded mean DNA migration [12].

While the Comet assay has already been recommended [13] and used on urinary bladder cells [14–18], previously published studies using the Comet assay have several drawbacks, mainly regarding collection of target cells. These drawbacks include: (1) use of the nucleus instead of whole cells from scraped bladder mucosa without information on cell viability [14], (2) use of minced bladder tissue containing a mixture of different cell types with low cell viability [15], and (3) difficulty in collecting viable cells through scraping bladder mucosa and resuspending the cells in Merchant's solution[1] [16]. Robbiano et al. [18] reported a selective harvest of bladder transitional cells with 95–98% viability by scraping trypsin and ethylenediaminetetraacetic acid (EDTA)-treated bladders that is based on a method originally developed to establish a primary cell line [17]. However, each sample for the Comet assay required pooling cells collected from three bladders [18], and the pooling greatly increased the number of animals needed for in vivo studies.

Here we present a method, modified from the previously published method using trypsin and EDTA [17,18], to selectively harvest urinary bladder transitional cells suitable for the Comet assay. This new method requires no pooling of samples. Sufficient cells for six samples were collected from each bladder, which can simultaneously be used for performing Comet assays with and without enzymes, or studying DNA repair over time while still allowing sufficient samples for positive and negative experimental controls. Detailed Comet protocols for the alkaline Comet assay and the detection of crosslinks in rat bladder transitional epithelial cells are presented below.

2. Materials and methods

2.1. Chemicals

Isoflurane (CAS no. 26675-46-7) was purchased from Abbott Animal Health, North Chicago, Illinois. Beuthanasia [390 mg pentobarbital sodium (barbituric acid derivative) and 50 mg phenytoin sodium per ml of Beuthanasia] (CAS no. 8024-20-2) was from Schering-Plough Animal Health Corporation, Union, NJ. Fetal bovine serum (FBS) was from

[1] Merchant's solution is 0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8 mM Na_2HPO_4, 0.53 mM Na_2EDTA, pH 7.4.