Single-Cell Gel (Comet) Assay Detects Primary DNA Damage in Nonneoplastic Urothelial Cells of Smokers and Ex-smokers

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Abstract

A protocol for DNA damage assessment by the single-cell gel (SCG)/comet assay in human urinary bladder washing cells was established. Modifications of the standard alkaline protocol included an increase to 2% of sodium sarcosinate in the lysis solution, a reduction in the glass-slide area for comet analysis, and a cutoff value for comet head diameter of at least 30 μm, to exclude contaminating leukocytes. Distinguishing cell populations is crucial, because significant differential migration was demonstrated for transitional and nontransitional cells, phenomena that may confound the results. When applying the modified protocol to urinary bladder cells from smokers without urinary bladder neoplasia, it was possible to detect a significant (P = 0.03) increase in DNA damage as depicted by the tail moment (6.39 ± 3.23; mean ± 95% confidence interval; n = 18) when compared with nonsmokers (1.94 ± 1.41; n = 12). No significant differences were observed between ex-smokers and current smokers regarding comet parameters. Inflammation was not a confounding factor, but DNA migration increased significantly with age in nonsmokers (r = 0.68; P = 0.014). Thus, age matching should be a concern when transitional cells are analyzed in the SCG assay. As it is well known, DNA damage may trigger genomic instability, a crucial step in carcinogenesis. Therefore, the present data directly support the classification of individuals with smoking history as patients at high risk for urinary bladder cancer.

Introduction

It has been shown that DNA damage together with the cellular response to that damage can establish genomic instability through multiple pathways (1). Gene mutation, altered DNA repair capacity, chromosomal aberrations, clonal heterogeneity, and cellular transformation are features of genomic instability (2). Accumulation of such abnormalities in the genome is associated with cell transformation from a benign to a malignant phenotype (3, 4). These alterations have been well described during urinary bladder cancer development (5).

Urinary bladder cancer is historically the neoplasia most strongly linked to occupational and environmental exposure to chemicals (6). Smokers, together with workers exposed to organic dyes and patients previously submitted to resection of urinary bladder cancer, are considered at high risk for the development of urothelial neoplasia (7, 8). Indeed, smokers have a 2–3-fold rise in relative risk (8) and up to a 4-fold higher incidence of bladder cancer than nonsmokers (9–11). One-third of urinary bladder cancers are related to cigarette smoking (12, 13). It is well documented that DNA adduct levels (14, 15) and micronuclei formation are augmented in urothelial cells of smokers (16–18). Therefore, in a screening study, if increased DNA damage is detected in otherwise morphologically normal urothelial cells, it is of greater chance that genomic instability may arise from these damaged, although viable, cells. This may reveal an early step in urinary bladder carcinogenesis.

The SCG/comet assay allows the detection of DNA alterations of diverse kinds, such as double-strand breaks, single-strand breaks, alkali-labile sites, incomplete repair sites, and cross-links (19). In the SCG assay, cells are embedded in agarose, lysed, and electrophoresed under low voltage, so that fragmented and relaxed DNA migrate farther than intact or cross-linked DNA, resembling the image of a comet. The extent of migration of the “tail” of the comet is related to increased DNA damage. These images can be analyzed and compared in a cell-to-cell basis.

Previously, McKelvey-Martin et al. (20) analyzed cells obtained by urinary bladder washings from patients with bladder cancer; applying the SCG assay, they found increased DNA migration when compared with the controls. While running the same methodology in our laboratory, some “comets” were indeed visualized, but together with many intact transitional cells. The comets were found to be derived from leukocytes present in the urinary bladder washings, and were probably derived either from trauma attributable to the cystoscopy procedure or from concurrent cystitis. Thus, because of specific interest in transitional cells, and not in leukocytes, a modified SCG assay was established to differentiate the target cells from other cells present in the urinary bladder washings.

To test the viability of the modified protocol, the SCG

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The abbreviation used is: SCG, single cell gel.
assay was applied to otherwise cytologically normal urothelial cells from smokers, ex-smokers, and patients with no smoking history. Age and concurring inflammation were evaluated as inducing bias factors. Our hypothesis was that higher levels of DNA damage were to be found in nonneoplastic cells of patients with smoking history, in accordance with their status of high-risk individuals for urinary bladder cancer.

Patients and Methods

This study was conducted at the General Hospital of the Universidade Estadual Paulista Medical School at Botucatu, São Paulo, Brazil, and approved by the UNESP Medical School Committee for Ethics in Research. Urinary bladder washings were obtained from patients scheduled for cystoscopy to elucidate urinary complaints. Thus, these patients did not volunteer for urinary bladder washings, but did agree to participate with informed consent after a detailed description of the study, once the washings were already scheduled. Control cases had no history of smoking; the test groups (smoked >5 cigarettes/day for at least 6 months) included current smokers and ex-smokers. None of the patients had urothelial neoplasia; their final diagnosis at the Urology Service was established as “non-specific cystitis” or “negative for neoplasia.” Every patient was administered a questionnaire to determine smoking status and age (Table 1).

**Bladder Washings.** During cystoscopy, two 15-ml urinary bladder washing samples with 0.9% normal saline were obtained from patients by bladder barbotage. The first sample was submitted to the routine cytopathological analysis and the second to the SCG assay. The coded tubes containing the washings were immediately sealed, light protected, and placed at 4°C until processing, which was usually within 4 h. When stored under these conditions, viability and DNA damage are unaltered for up to 48 h, as reported by McKelvey-Martin et al.
samples. Such clouds were not analyzed in this experiment because they are by an image analysis system.

Bar, right to arrowheads, /H11569 ing DNA directly using the comet images after electrophoresis by count-

be saved for the SCG assay, viability was determined indi-

washing samples had very low cell counts, and cells should be excluded trypan blue). Afterward, because bladder i.e., trypan blue exclusion technique. Cell Viability. In preliminary studies, the viability of uri-

from controls and patients with smoking history were processed (20), albeit herein, no sample was stored over 28 h. Samples

of the small coverslip, a final layer of 120 °C to solidify the coverslip. Slides were left for 5 min at 4 °C. After lysis, slides were immersed in Tris-HCl (pH 7.5) for 5 min to remove excess salt. The following steps of alkaline unwinding (20 min), electrophoresis (0.66 V/cm for 20 min), and neutralization were performed as described by McKelvey-Martin et al. (20). After neutralization, slides were fixed with 100% ethanol and stored for staining and analysis.

A whole-blood sample from a healthy donor was assayed under these same test conditions to obtain a distribution of comet head diameter frequencies of peripheral blood leukocytes.

SCG/Comet Assay Analysis. The dried microscope slides were stained with ethidium bromide (20 µg/ml in dH2O; 50 µl/slide), covered with a coverslip, and analyzed immediately at ×200 in a fluorescence microscope under green light. An image analysis system (Comet Assay 2.2; Perceptive Instruments, Suffolk, United Kingdom) was used to determine DNA damage, as shown by the migration of DNA fragments in the agarose gel. Two parameters were estimated to indicate DNA migration: tail length (distance from the head center to the end of the tail) and tail migration (distance from the end of the head to the end of the tail), which are directly associated. Besides, the system provided the mean tail moment (product of tail DNA/total DNA by the tail center of gravity) and head diameter of each comet. Overall, at least 50 cells/patient sample were analyzed. The mean tail moment values were compared among groups by the nonparametric Mann-Whitney U test. Student’s t test was used to analyze normally distributed tail migration and head diameter. Normality was assessed by the Shapiro-Wilk’s W test (P < 0.05). Overall differences were considered significant when P < 0.05.

Immunoseparation of Urothelial Cells. To make evident the differences between the parameters of comets obtained from epithelial and nonepithelial cells, some urinary bladder washings were submitted to a cell separation procedure based on immunobiomagnetism (26). The washings were processed as described above. However, after centrifugation, the cell pellets were resuspended in 200 µl of ice-cold PBS. To this cell suspension was added 1 µl (4 × 10^5) of immunocollated metallic microspheres (Dynal, Norway). These microspheres were coated by the antibody Ber-Ep4, specific against human epithelial cells (27). Afterward, the samples were incubated for 30 min with mild agitation, to permit the antibody binding to transitional cells. Incubation at 4°C prevented unspecific adhesion of phagocytes. Cells were then exposed to a magnetic field for 1 min and the SCG assay was separately conducted with precipitated cells and with the supernatant cells, which were not attracted to the magnet (Fig. 2a). Hence, for each bladder washing sample, slides from the two aliquots were prepared. Head diameters and tail migration parameters were obtained by image analysis as mentioned before. For each group (precipitated cells and supernatant cells), the means of both parameters were compared using Student’s t test. When possible, aliquots of each patient were also compared by the same method.

Cytopathological Analysis. Samples of each one of the bladder washing specimens were also submitted to routine cytological analysis, which is part of the diagnostic procedures for urological patients. Briefly, the samples were centrifuged in

(20), albeit herein, no sample was stored over 28 h. Samples from controls and patients with smoking history were processed blindly in the same experiments.

Cell Viability. In preliminary studies, the viability of urinary bladder washing cells was first measured using the trypan blue exclusion technique (>60% of cells were viable, i.e., excluded trypan blue). Afterward, because bladder washing samples had very low cell counts, and cells should be saved for the SCG assay, viability was determined indirectly using the comet images after electrophoresis by counting DNA “clouds” in the SCG assay preparations (DNA clouds/100 comets; Ref. 21). DNA clouds, “puffs,” “tear drops,” or “balloons” are characteristic structures having no detectable head with nearly all DNA in the tail (Fig. 1). These clouds are made of very low-molecular weight DNA fragments, and the images are referred to as typical of dead cells (22, 23). The cloud counts mirrored trypan blue exclusion results with <30% (75% average viability) of comets being clouds. In this study, DNA clouds were excluded from the final quantitative analysis as suggested by Hartmann and Speit (24), who indicated that increased DNA migration in the absence of scoring clouds is more important for the evaluation of genotoxicity than an increase that depends significantly when P < 0.05.
a cytocentrifuge (1000 rpm for 5 min) so that two smears were
prepared on two glass slides. One smear was air-dried and
stained by the Giemsa method; the other smear was alcohol-
fixed and stained by the Shorr method. The proportions of
urothelial superficial and basal cells and of inflammatory cells
(mono- and polymorphonuclear cells) in each sample
were estimated semiquantitatively. The presence of RBCs was
also registered.

**Results**

**Transitional Cell Characterization in the SCG Assay Preparations.** All cells in the urinary bladder washing samples were lysed by the modified protocol in such a way that no intact urothelial cell could be visualized in the slide preparations. The lysed cells remained as agarose embedded nucleoids (i.e., the comets are electrophoresed nucleoids), which are structures formed by DNA and tightly bound proteins that occupy cavities in the agarose layer formed by the previously intact cells. Nucleoids frequently resemble in form and size the cells from which they are derived. The reduction of the area of analysis with a smaller coverslip (24 × 32 mm) optimized the visual comparison between comets because of their higher density/field (Fig. 3, a and b). A large/heterogeneous and a small/homogeneous cell comet-head population were determined; the small/homogeneous cell population being comparable with the one obtained with the whole blood leukocytes (Fig. 3c). The large nucleoids often presented odd shapes not typically round as are the ones from leukocytes (e.g., Fig. 2b). So, besides size difference, comets were also morphologically distinct among themselves. Therefore, it was hypothesized that the large/heterogeneous population, with the mean head diameter usually >30 μm, was derived from urothelial cells.

The difference between the two populations was confirmed by immunomagnetism, which efficiently separated transitional from nonepithelial cells (Fig. 2c). The cells that adhered to the microspheres (immunoprecipitated urothelial cells) had larger head diameters (38.2 ± 8.1 μm) than those which did not adhere (28.9 ± 4.6 μm), which were expected to be nonepithelial cells (P < 0.01; Student’s t test).
difference became more visible when the separated aliquots of individual samples were compared separately.\textsuperscript{4}

Because it became possible to distinguish from which cell the comet was derived according to its mean head diameter (urothelial cell comets were >30 \( \mu \)m), it was concluded that it is not necessary to use the immunomagnetism procedure or other separation method in bladder washings samples to separate urothelial cells before the SCG assay. From hereon, comets with mean head lengths <30 \( \mu \)m were excluded from the analysis, consequently enriching it with transitional cells. Fig. 4 shows where the established cutoff value is situated for all cells assayed at this stage of the study.

Moreover, transitional cells and nontransitional cells obtained by urinary bladder washings present significant differences in DNA migration (Table 2), indicating that DNA damage of inflammatory cells is not always equivalent to DNA damage of transitional cells occurring in the same sample.

**Cytopathology.** Slides prepared for routine cytopathology were analyzed to register the presence of inflammatory cells in the bladder washings. Results are presented in Table 1. It is important to note the high frequency of erythrocytes, which suggest that most of the leukocytes found in the washings originated from the blood.

**Smoking and DNA Damage in Urothelial Cells.** Data on individual cell viability is presented in Table 1. Regarding DNA damage, no significant difference in the SCG assay parameters was detectable between smokers and ex-smokers (Table 3; \( P = 0.629 \) and 0.964, for tail length and tail moment, respectively), so both were gathered together in a group of smoking history. These patients had increased DNA damage in the urinary bladder transitional cells, as depicted by the mean tail moment (6.39 \( \pm \) 3.23; mean \( \pm \) 95\% confidence interval), when compared with controls (1.94 \( \pm \) 1.41; \( P < 0.05 \); Table 3). This was also seen for tail length (44.99 \( \pm \) 10.25 against 28.7 \( \pm \) 5.8, for history of smoking patients and controls, respectively; \( P < 0.05 \)). Attempts were made to correlate DNA damage with age and inflammation. Inflammation was not correlated to any parameter (\( P > 0.10 \)). Increased DNA damage related to aging was observed for the tail length (\( r = 0.68; P = 0.014 \)). For tail moment, a positive and significant correlation (\( r = 0.80; P = 0.003 \)) was only observed when an outlier value [i.e., value that does not represent the group; larger than (mean + 2 SD)] was not considered; otherwise \( r = 0.49 \) and \( P > 0.10 \) (Table 1). There was no significant difference (\( P = 0.949 \)) in the mean age between the groups, so the comparison between them was suitable. DNA damage also correlated significantly to the number of cigarettes smoked/day in the current smoker group (\( r = 0.67 \) s.c.; \( P < 0.05 \); Table 1). A similar correlation was observed for the current nonsmoker patients (nonsmokers and ex-smokers; \( r = 0.70; P < 0.05 \); Table 1). Ex-smokers may present extensive DNA damage in urothelial cells even after 30 years since quitting smoking (Table 1).

**Discussion**

The product of bladder washings should be considered heterogeneous mixtures of cells, because blood cells can also be collected by this procedure. In the present study, leukocytes occurred in 71\% (20 of 28) of urinary bladder washings. They were assumed to be derived from inflammatory process occurring at the urinary bladder wall or from the presence of blood in the urinary bladder lumen caused by trauma. In the slides prepared for the SCG assay, it is generally difficult to discern from which type of cells the comets come. Under this circumstance, inflammatory cells may be analyzed together with target urothelial cells. This may lead to untrustworthy results once the DNA damage status may differ between those cells and urothelial cells, as demonstrated in Table 2. Additionally, different responses to mutagens and/or to the different repair capabilities of cells may lead to weak correlations when two different tissues are analyzed together (28). Hence, it should be considered a good practice to perform a SCG assay only when transitional cells are exclusively analyzed. Although handled differently, other researchers encountered similar problems with leukocyte contamination of in vivo specimens when assaying cell suspensions rich in contaminant leukocytes obtained by stomach biopsies (29).

Herein, we proposed modifications to the SCG assay pro-

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**Table 2** Tail migration of comets (\( \mu \)m; mean \( \pm \) SD) of immunomagnetically precipitated and supernatant cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Supernatant cells</th>
<th>Precipitated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64.91 ( \pm ) 38.18</td>
<td>32.00 ( \pm ) 33.61</td>
</tr>
<tr>
<td>2</td>
<td>14.95 ( \pm ) 19.49</td>
<td>31.46 ( \pm ) 24.31\textsuperscript{a}</td>
</tr>
<tr>
<td>3</td>
<td>70.77 ( \pm ) 47.30</td>
<td>61.67 ( \pm ) 32.10</td>
</tr>
<tr>
<td>4</td>
<td>67.10 ( \pm ) 36.67</td>
<td>79.82 ( \pm ) 32.40\textsuperscript{a}</td>
</tr>
<tr>
<td>5</td>
<td>104.76 ( \pm ) 31.66</td>
<td>114.77 ( \pm ) 51.82</td>
</tr>
<tr>
<td>6</td>
<td>123.71 ( \pm ) 12.45</td>
<td>68.21 ( \pm ) 51.70\textsuperscript{a}</td>
</tr>
<tr>
<td>7</td>
<td>4.83 ( \pm ) 5.70</td>
<td>16.60 ( \pm ) 24.22\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} \( P < 0.01 \).  
\textsuperscript{b} \( P < 0.05 \).  
\textsuperscript{c} \( P < 0.001 \); Student’s \textit{t} test.

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\textsuperscript{4} Unpublished observations.

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**Table 3** Age (mean \( \pm \) SD) and SCG assay parameters (mean \( \pm \) 95\% confidence interval) of transitional cells obtained by bladder washings

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Ex-smokers</th>
<th>Current smokers</th>
<th>( P )\textsuperscript{a}</th>
<th>( P )\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>(n = 12)</td>
<td>(n = 11)</td>
<td>(n = 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs.)</td>
<td>53.1 ( \pm ) 20.2</td>
<td>57.6 ( \pm ) 15.5</td>
<td>47.1 ( \pm ) 24.2</td>
<td>0.276\textsuperscript{c}</td>
<td>0.949\textsuperscript{c}</td>
</tr>
<tr>
<td>Tail length (( \mu )m)</td>
<td>28.7 ( \pm ) 5.8</td>
<td>47.1 ( \pm ) 13.5</td>
<td>41.7 ( \pm ) 16.6</td>
<td>0.629\textsuperscript{c}</td>
<td>0.012\textsuperscript{d}</td>
</tr>
<tr>
<td>Tail moment</td>
<td>1.9 ( \pm ) 1.4</td>
<td>6.7 ( \pm ) 4.6</td>
<td>5.9 ( \pm ) 4.5</td>
<td>0.964\textsuperscript{c}</td>
<td>0.034\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Ex-smokers versus current smokers.  
\textsuperscript{b} Nonsmokers versus history of smoking (ex-smokers and current smokers).  
\textsuperscript{c} Student’s \textit{t} test.  
\textsuperscript{d} Non-parametric Mann-Whitney \textit{U} test.
tocol described by McKelvey-Martin et al. (20) for urothelial cells collected by urinary bladder washings. Foremost, transitional cells obtained by bladder washings could only be lysed when a 2% sodium sarcosinate detergent was added to the standard lysis solution. Before that, the obtained comets were mostly derived from leukocytes, whereas transitional cells remained intact, even after extended lysis. Such resistance to lysis is expected to be caused by the specialized cytoskeleton of those epithelial cells. Sarcosinate is an anionic detergent widely used to disrupt cell walls, and it can make the urothelial cell lysis possible by denaturing its extensive intermediate filament network. Similar resistance to standard lysis solutions was observed in our laboratory for other epithelial cell types, in vivo, as hair follicle keratinocytes and cervical cells.5 After 1 h in the modified lysis solution, no additional step for enzymatic digestion (e.g., proteinase K) of urothelial cells was necessary. Advantage can be taken of such lysis procedures, once DNA damage of inflammatory cells of patients with cystitis can sometimes outnumber urothelial cells in bladder washings (Table 1). Under these conditions, the analysis focused as much as possible on urinary bladder transitional cells. Not many SCG studies have attempted to recognize subtype cells within mixed populations. Yet, cell typing by head diameter has been reported for cells other than urothelial. The random measurement of head diameters of liver cells obtained by mincing leads to a bimodal distribution characteristic of parenchymal and nonparenchymal cells with peaks at 45 and 30 μm, respectively.6 Haploid and euploid male germ cells are also differentiable by head diameter (see Ref. 2). Thus, comet head diameter is a seemingly reliable, fast, and inexpensive approach to cell-typing in the SCG assay.

Although reduced cancer risk of ex-smokers is controversial when compared with current smokers (30, 31), in the present study increased DNA damage was noticeable in urothelial cells of individuals who either had quit smoking or were currently smoking. Indeed, data accumulates which indicate that changes caused by smoking are long-lasting, and recovery after smoking cessation, reflected by a decreased risk of lower urinary tract symptoms, is a likewise delayed process (31, 32). Herein, individual data indicates appreciably different DNA susceptibility to the number of cigarettes smoked/day. Patients who had quit smoking for ~30 years, but who have smoked ~40 cigarettes/day for at least 6 months, still had expressive DNA damage (Table 1). On the other hand, a patient (patient 22) who had smoked 30 cigarettes/day and had quit for 5 years presented basal levels of DNA damage (Table 1). Albeit 97% (29 of 30) of the patients in this study were Caucasians, variability of this kind is expected and can be partially explained by factors which were not considered in this study, such as the genetic polymorphisms of xenobiotic metabolizing enzymes (33). Nevertheless, DNA damage correlated positively and significantly with the quantity of cigarettes smoked/day for both smokers and ex-smokers (Table 1). Interestingly, and in the same line of observation, no significant differences between the frequencies of micronucleated cells (which are used as index of chromosomal damage) of smokers and ex-smokers were registered in urothelial tissues (17). Cells with high levels of DNA lesions, detected by the SCG assay in smokers and ex-smokers, should be much more susceptible to chromosome aberrations and micronuclei formation (1). Our data suggest that smoking is associated with the development of persistent clones of DNA-damaged cells in the urothelium and partially explain the continuous occurrence of micronucleated cells in that mucosa (17). It is tempting to discuss how increased DNA damage may prowl around the urothelium of long-time ex-smokers, once no noxious stimuli is detectable. A history of smoking is associated with increased p53 mutations in the bladder (34–36). Indeed, p53 is overexpressed in the normal urothelial cells of smokers and seemingly in that of past-smokers (37). The presence of mutant p53 clones in ex-smokers suffices to lay the foundations for an increase in endogenous damage. This is possible once mutant p53 clones tend to escape protective apoptosis and fail to modulate DNA repair after genotoxic stress (38). In fact, significantly reduced DNA repair capacity was observed for cultured lymphocytes of individuals with history of smoking (39, 40). Thus, the perpetuation of mutant cells in the urothelium (17) caused by a history of active smoking may explain the occurrence of highly damaged urothelial cells in former heavy-smokers.

It is important to notice that the increased DNA damage observed in the transitional cells of smokers and ex-smokers by means of the SCG assay occurred in patients free of neoplasia and with normal urinary bladder cell cytology. The exclusion of DNA clouds from analyses mostly ruled out the possibility that the higher DNA damage was attributable to cytotoxicity. The influence of other possible confounding factors, such as age and concurrent local inflammation, were also excluded. In this way, the increased DNA damage observed in transitional cells of smokers and ex-smokers should be attributed to smoking-associated genotoxicity.

Recently, Møller et al. (41) reviewed the SCG assay as a test for DNA-damaging potential in biomonitoring studies and the effect of confounding factors. Age, inflammation, and smoking status are potential confounding factors when blood samples are evaluated by the SCG assay, despite that it is still not known which factors are indeed relevant for leukocytes (41). Therefore, as confounding factors are still even more obscure for urothelial cells, the present study should help clarify that at least smoking status, age, and cell-type heterogeneity may influence the outcome of the SCG assay when using human urothelial cells.

In conclusion, a modified SCG/Comet assay was developed to evaluate DNA damage of human transitional cells in vivo obtained by urinary bladder washings. Smoking was significantly associated with high levels of DNA damage in transitional cells, where no significant differences were observed in the SCG assay results between ex-smokers and current smokers. Our data suggest that aging is associated with an increase in DNA damage in nonsmokers. The present data indicate that smoking is associated with the development of persistent clones of DNA-damaged epithelial cells in the urinary bladder mucosa, which may explain the higher risk for urinary bladder cancer associated with this habit.

Acknowledgments

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5. Å. M. de M. C. Gontijo and D. M. F. Salvadori, unpublished observations.
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