Modulation of novel DNA adducts during human uterine cervix cancer progression

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Abstract. Progressive accumulation of DNA lesions leads to genetic mutations that are central to the process of tumorigenesis. Human cervix provides an ideal system to determine progressive accumulation of DNA adducts in the target tissue because of its accessibility during routine diagnostic checkups. Uterine cervix samples from various pathologies, i.e. normal (n=13), inflammation (n=9), dysplasia (n=5) and different stages of invasive cancer (n=47), were analyzed for DNA adduct burden by modified 32P-postlabeling/TLC systems. Six subgroups of adducts were detected in the following descending order of polarities: P-1, P-2, PL-1, PL-2, L-1 and L-2 (P, polar; L, lipophilic; PL, between polar and lipophilic). No qualitative differences were observed in adduct profiles in the various cervix pathologies analyzed. However, significant quantitative differences were found. Previously known lipophilic adducts increased significantly from normal to cancer (144±61 to 503±51 adducts/10^9 nucleotides). Interestingly, the newly discovered polar adducts were present at 61- to 527-fold higher levels than lipophilic adducts. Of all the polar adducts, the known mutagenic lesion, 8-oxodeoxyguanosine, predominated in all cervix conditions. Notably, this lesion was elevated 27-fold in inflammation compared with normal cervix (51,058±9,863 versus 1,886±507 adducts/10^9 nucleotides). The P-1, PL-1, PL-2 and L-1 adducts were elevated 3- to 13-fold in inflammation compared with normal cervix, and were also higher in dysplasia and cancer. Our data suggest that inflammation may be involved in directing the course of disease progression by accumulating higher levels of DNA lesions. The data further suggest the biomarker potential of the newly detected array of DNA adducts.

Introduction

During the normal metabolic process, human cells are exposed to endogenous, DNA-reactive substances such as hydroxyl radicals, and lipid oxidation products such as malondialdehyde, and α, β-unsaturated aldehydes (enal)s (reviewed in ref. 1). Nitric oxide, a free radical produced by phagocytes, and by the induction of NO synthase by inflammatory cytokines (2), undergoes mutual reaction to produce peroxynitrite, which also generates hydroxyl radicals that oxidize or deaminate DNA (3). Hydroxyl radicals can produce over 20 oxidized DNA bases (4). Endogenous DNA adducts have been estimated at >10,000 per 10^9 nucleotides (5). 8-Oxodeoxyguanosine (8-OxodG), thymidine glycol and 5-hydroxymethyl-dU are the commonly observed lesions in mammalian DNA. 8-OxodG is highly mutagenic, producing G→T transversions, while thymidine glycol (T→C transitions) and 5-hydroxymethyl-dU (C→T transitions) are weakly mutagenic (6). Other adducts from reactive intermediates include cyclic adducts that are formed from lipid peroxidation products. Cyclic adducts also induce transversion and transition mutations, depending upon the type of adducts (7-9).

The novel 32P-postlabeling/TLC systems, developed recently in our laboratory, have been employed to measure collectively a host of DNA adducts encompassing high polarity to high lipophilicity (Gupta RC et al, manuscript in preparation). This technology, termed ‘adductomics’ allows global detection of DNA adducts. We set out to test this new technology in diseases that have clearly defined stages of progression, and the target tissue is easily accessible. Human uterine cervical lesions progress from low-grade to high-grade intra-epithelial lesions to invasive carcinoma. Procurement of the samples by non-invasive methods during routine annual checkups, and the ability of the Pap smear to detect the progression of the disease make the cervix one of the best models to study the process of human carcinogenesis.
and evaluate the biomarker potential of newly developed technologies besides diagnostic and therapeutic interventions (10).

Cancer of the cervix uteri is the second most common form of cancer in women worldwide, with low- and medium-resource countries contributing to approximately 80% of the burden. In the United States, approximately 9,710 new cases are expected to be diagnosed and approximately 3,700 women are expected to die due to this cancer in 2006 (11). The incidence rate of invasive cervical cancer in Kentucky is 40% higher than the SEER rate during 1991-1998 (12); it ranks higher in incidence with age-adjusted rate (11.1/100,000), which is higher than the national average (8.4/100,000).

Epidemiological studies confirm a causal role of human papillomaviruses (HPV) infection in cervical carcinogenesis. However, while >99% of the invasive cancers are HPV-positive (13), it is important to acknowledge that only <5% of all HPV-positive cases progress to develop invasive cancer (14). Therefore, attention has been given to several co-factors that influence the outcome of a cervix infection, including tobacco smoking, oral contraceptive use and parity (15). In addition, an association was found between cervical inflammation and high-grade lesions in women infected with HPV (16). Elevated levels of inflammatory cytokines IL-6 and IL-8 in cervicovaginal secretions (17) and in cervical cancers (18,19) have been reported. In this study, we examined the influence of cervical inflammation and other forms of cervix pathogenesis on the accumulation of DNA adducts.

Materials and methods

Micrococcal nuclease (MN) (235 U/mg protein), apyrase, RNase A (type III-A) and RNase T1 (500,000 U/mg) were purchased from Sigma Chemical Co., (St. Louis, MO); nuclease P1 (389 U/mg dry weight) from Calbiochem (San Diego, CA); proteinase K from Roche (Indianapolis, IN); spleen phosphodiesterase (SPD) (2.45 U/mg) from Worthington Biochemical Corp (Lakewood, NJ); and cloned T4 poly-nucleotide kinase (10 U/μl) from New England Bio Labs, Inc. (Beverley, MA). [γ-32P]ATP (6,000 Ci/mmol; 80 μCi; 13.3 pmol). Labeled digests were resolved by 2-directional polyethyleneimine (PEI)-cellulose TLC (20x10 cm) in 1 M formic acid containing varying concentrations of sodium phosphate in direction 1, followed by development in direction 2 in isopropl alcohol:dilute ammonium hydroxide, as described in the Legend of Fig. 1. 8-OxodGp was enriched by PEI-cellulose TLC, [32P]-labeled and resolved by 2-directional TLC as described (21). Normal nucleotides were labeled in parallel with adducts and resolved by 1-directional PEI-cellulose TLC. Adduct and normal nucleotides were detected and quantitated by Packard InstantImager, and adduct levels were calculated as relative adduct labeling (RAL) as cpm in adduct(s)/cpm in normal nucleotides x 1/dilution factor.

Analysis of DNA adducts by modified 32P-postlabeling/TLC systems. DNA samples were analyzed by 32P-postlabeling method after enrichment of adducts with nuclease P1 (20), with modifications: briefly, DNA was digested with micrococcal nuclease and spleen phosphodiesterase (enzyme: substrate, 1:5) for 5 h at 37°C. An aliquot was removed and used for evaluation of normal nucleotides and the remaining digest was treated with nuclease P1 (enzyme:substrate, 1:2.5) for 1 h at 37°C. Enriched digests were labeled with T4 polynucleotide kinase-catalyzed phosphorylation in the presence of [γ-32P]ATP (6,000 Ci/mmol; 80 μCi; 13.3 pmol). Labeled digests were resolved by 2-directional polyethyleneimine (PEI)-cellulose TLC (20x10 cm) in 1 M formic acid containing varying concentrations of sodium phosphate in direction 1, followed by development in direction 2 in isopropl alcohol:dilute ammonium hydroxide, as described in the Legend of Fig. 1. 8-OxodGp was enriched by PEI-cellulose TLC, [32P]-labeled and resolved by 2-directional TLC as described (21). Normal nucleotides were labeled in parallel with adducts and resolved by 1-directional PEI-cellulose TLC. Adduct and normal nucleotides were detected and quantitated by Packard InstantImager, and adduct levels were calculated as relative adduct labeling (RAL) as cpm in adduct(s)/cpm in normal nucleotides x 1/dilution factor.

Statistical analysis. The analysis of data on DNA adducts was carried out in the context of Generalized Linear Models described by McCullagh (22). The data was analyzed using the Negative Binomial Regression model with logarithm as link function to study the dependence of the adduct levels on the type of subject (or the stage of subject). The parameters in the model were estimated using the method of maximum likelihood and the over-dispersion was accounted for by the scaled Pearson Chi-square. Statistical differences in adduct
burden among different groups were calculated using Wald's asymptotic test procedures. A p-value of <0.05 was considered significant. The data analysis was carried out with the aid of SAS software version 8.

**Results**

Over 60 adducts were detected in each DNA. Based on the elution profiles, six groups of adducts were detected which are designated as P-1, P-2, PL-1, PL-2, L-1 and L-2, where P stands for polarity and L for lipophilicity; the PL denotes adducts that are neither very polar nor lipophilic (Fig. 1). The adducts eluted in the following descending order of polarities: P-1, P-2, PL-1, PL-2, L-1 and L-2. The P-2 adducts were further classified as P-2a containing multiple adducts and P-2b which represented a single entity, namely 8-oxodG, based on previous identification (21). With the exception of the P-2b adduct and L-2 adducts, all other groups of adducts detected have not been reported previously. Based on chromatographic mobility of \( ^{32}\text{P} \text{p8-oxodGp} \), which resembled the mobility of the P-2a adducts, the 8-oxodG adduct was coupled with P-2 adducts. Each group of adducts comprised of multiple adducts: at least 16 adducts in the P-1 subgroup, 11 adducts in the P-2a, and 3 and 4 adducts in the PL-1 and PL-2 classes, respectively. The L-1 and L-2 adducts were poorly resolved or unresolved. In some analyses, however, the L-1 adducts separated in the form of beads of a string accounting for up to 25 adducts. A cluster of unresolved L-2 adducts migrated in the form of a diagonal radioactive zone (DRZ). This DRZ was embedded with at least five distinct adduct spots (Fig. 1). The L-2 adducts that are most lipophilic are detected with high-salt, high-urea solvents (23).

No qualitative differences were found in DNA adduct profiles from the four types of cervix specimens analyzed; i.e. normal, inflammation, dysplasia and invasive cancer. However, significant quantitative differences were found (Table I). The most pronounced adduct was 8-oxodG (P-2b) which represented 9%, 57%, 41% and 18% of the total adduct burden in the normal, inflammation, dysplasia and invasive cancer specimens, respectively. This adduct was elevated 27-fold in cervix with inflammation (51,058±9,863 adducts/10^9 nucleotides) compared with normal cervix (1,886±507 adducts/10^9 nucleotides). This lesion was also significantly higher in the dysplasia (30,362±10,354 adducts/10^9 nucleotides) and invasive cancer (5,659±1,371 adducts/10^9 nucleotides). The P-1 adducts in cervix with inflammation were higher by 9-fold than that in normal cervix and remained at this level in the dysplasia group, but the increase was less pronounced (5-fold) in invasive cancer. The P-2a adduct levels in normal cervix did not differ from the inflammation and dysplasia groups. In fact, these adducts declined by approximately 70% in invasive cancer. The PL-1 and PL-2 adducts in cervix with inflammation were also elevated, by 6- and 13-fold, respectively, and these levels remained high through dysplasia and invasive cancer conditions. The L-1 adducts in the inflammation group were higher by 3-fold and remained practically constant through

<table>
<thead>
<tr>
<th>Tissue</th>
<th>P-1</th>
<th>P-2a</th>
<th>P-2b (8-oxodG)</th>
<th>PL-1</th>
<th>PL-2</th>
<th>L-1</th>
<th>L-2</th>
<th>Total</th>
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</thead>
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<tr>
<td>Normal (n=13)</td>
<td>1,343±432</td>
<td>13,490±3,411</td>
<td>1,886±507</td>
<td>376±68</td>
<td>70±22</td>
<td>3,820±1,211</td>
<td>144±61</td>
<td>22,029±5,918</td>
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<td>Inflammation (n=9)</td>
<td>12,226±5,445</td>
<td>10,708±3,209</td>
<td>51,058±9,863</td>
<td>2,380±436</td>
<td>911±321</td>
<td>11,902±2,073</td>
<td>170±56</td>
<td>89,684±13,755</td>
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<td>p-value</td>
<td>&lt;0.0001</td>
<td>0.58</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0008</td>
<td>0.669</td>
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<tr>
<td>Dysplasia (n=5)</td>
<td>12,369±9,413</td>
<td>3,332±14,811</td>
<td>30,362±10,354</td>
<td>2,221±735</td>
<td>458±388</td>
<td>10,768±2,837</td>
<td>424±152</td>
<td>74,836±25,220</td>
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<tr>
<td>p-value</td>
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<td>0.9807</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0137</td>
<td>0.0119</td>
<td>0.0224</td>
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<tr>
<td>Cancer stage I (n=12)</td>
<td>4,471±785</td>
<td>3,592±502</td>
<td>12,371±4,184</td>
<td>1,088±210</td>
<td>327±62</td>
<td>9,904±1,630</td>
<td>263±47</td>
<td>30,825±5,133</td>
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<td>&lt;0.0001</td>
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<tr>
<td>Cancer stage II (n=22)</td>
<td>5,974±1,062</td>
<td>4,436±1,024</td>
<td>2,838±552</td>
<td>1,789±215</td>
<td>1,469±439</td>
<td>17,132±2,445</td>
<td>579±72</td>
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<tr>
<td>Cancer stage III (n=13)</td>
<td>9,351±2,769</td>
<td>2,636±322</td>
<td>3,916±436</td>
<td>1,755±172</td>
<td>580±61</td>
<td>7,706±353</td>
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<td>Cancer all stages (n=47)</td>
<td>6,681±977</td>
<td>3,723±509</td>
<td>5,659±1,371</td>
<td>1,600±129</td>
<td>932±218</td>
<td>12,680±1,357</td>
<td>503±51</td>
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Values are represented as mean ± SEM. P-values were calculated by comparison with normal group using Wald's asymptotic test procedures. A p-value of <0.05 was considered significant.
dysplasia and invasive cancer. The levels of the highly lipophilic L-2 adducts, in the normal and inflammation groups were not different, but the levels then increased steadily, reaching 3-fold higher in the invasive cancer. When comparing total adduct burden, the levels in both inflammation and dysplasia groups were significantly higher than in normal cervix. In the invasive cancer, the total adduct burden was also higher than in normal cervix but the increase was statistically insignificant. It may be noted that the burden of some subgroups of adducts in the inflammation, dysplasia and invasive cancer were much more elevated than the total adduct burden (Table I).

The modulation of individual adducts in all subgroups largely reflected the modulation of total adducts. Therefore, we have described the modulation of total adducts under each subgroup. To determine if adduct burden varied during the invasiveness of the cancer, we compared the adduct profiles from stage I versus stages II and III. Again, no qualitative differences were noticed, but significant quantitative differences were found. For instance, the 8-oxoG levels declined to one third from stage I to stage II and then no further decline occurred, but the levels of PL-1, PL-2, L-1 and L-2 all increased by 2- to 5-fold from stage I to stage II and then in stage III the adduct levels either remained unchanged or declined (Table I).

Data presented in Table I also reveal that the previously known lipophilic adducts (the L-2 adducts) represent only a very small fraction (0.1-0.5%) of the total adduct burden.
The changes in adduct profiles that occur during the progression of cervical cancer are represented, for simplicity, by stacking the levels of each group of adducts (Fig. 2). Clearly, adducts, particularly in the P-1, P-2b and L-1 groups, accumulate the most in cervix with inflammation. The interindividual differences observed in the levels of each group of adducts varied depending upon the diseased state of the cervix, as illustrated by the scatter plots (Fig. 3). While there was a 3- to 5-fold variability in the total adduct burden in inflammation, dysplasia and invasive cancer groups, the variability was up to 27-fold in the normal individuals. This could be indicative of the susceptibility of some of these individuals to accumulate more damage and warrants a longitudinal study to better understand the biomarker potential of this newly identified group of adducts.

We also tested 5 residual negative cytology specimens obtained from the University of Kentucky Hospital, and found that the DNA recovery (mean value = 70±39 μg) and adduct profiles (data not shown) were essentially the same as in the Chennai samples.

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**Discussion**

Techniques used to detect and identify endogenous DNA adducts include immunoassay, gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry and 32P-postlabeling. The former three techniques have been valuable in identifying many endogenous DNA adducts, however, they either apply to targeted adducts (immunoassay), or they require relatively large quantities of DNA (100-500 μg) (spectrometric techniques). The 32P-postlabeling assay, a standard in the field for adducts from exogenous carcinogens, requires relatively small quantities of DNA (<10 μg) and can detect <1 adduct in 10^9 nucleotides. It, however, does not allow adduct identification without authentic standards. Only a few known oxidative adducts have been measured by this assay. No attempt has been made so far to detect the numerous structurally diverse endogenous DNA adducts in tissues during the progression of human cancer.

With the exception of 8-oxodG (P-2b), all other adducts remain as yet unidentified. It may be noted that the newly discovered array of adducts were present at 61- to 527-fold higher levels than the previously reported highly lipophilic (L-2) adducts (Table I). Interestingly, data indicating that 8-oxodG levels in cervical cancer are 3-fold higher compared with normal cervix, and that cervix with inflammation has dramatically (27-fold) elevated levels of this oxidative lesion than normal cervix have not been reported previously.

A causal relationship between inflammation and cancer was speculated as early as 1863 by Rudolph Virchow (cited in ref. 24). Development of several cancers has been attributed to chronic inflammatory diseases (reviewed in ref. 25). Lately, the association between inflammation and cancer is receiving greater attention (26-29). We found a parallel increase of the inflammatory cytokines IL-6 and IL-8, neutrophils and macrophages and several novel adducts, suggesting a possibility of inter-relationship (unpublished data). Rapidly dividing cells are most prone to errors in DNA replication and repair. Besides, hot spots for damage could more often be cold spots for repair (30). The distribution of many polar adducts discovered in this study may influence the disease status as it progresses from normal cervix through inflammation by accruing mutations.

Several studies have shown an association of smoking with the known highly lipophilic group of adducts (the DRZ) in the cervix (31-33). But there are no studies that have analyzed DNA adducts at different stages as the disease progresses to malignancy. Smoking was found to increase the risk of cervical cancer among HPV-positive women in a multicentric case-control study (32). Benzo[a]pyrene diol epoxide-DNA adducts were reported in the cervical epithelium of smokers and the role of polycyclic aromatic hydrocarbons and their carcinogenic metabolites in cervical cancer has been described by Melikian et al (33). A progressive increase in the accumulation of adducts that migrate in the form of DRZ on the chromatograms suggests a positive role of these modifications in cervical carcinogenesis. Although passive smoking and tobacco chewing is widespread, smoking is not a common practice among women in the region where these samples were collected. It is therefore quite possible that the...
by the scatter plots (Fig. 3), suggestive of susceptibility and/or variability. Analysis of the individual adduct levels shows a heterogeneous pattern of adduct distribution as shown for those on the inactive X chromosome and parentally imprinted genes (34). Global hypomethylation has been implicated in oncogenic transformations (35,36).

The levels of P-2a adducts are lower in cancer tissues than in normal cervix or cervix with inflammation and dysplasia. It remains to be determined if these adducts may represent some kind of essential modifications required for the normal functioning of cellular activities. One such example is 5-methylcytosine in DNA. Certain regions of the genome defined as CpG islands contain a predicted frequency of CpG dinucleotides that are unusually unmethylated, except for those on the inactive X chromosome and parentally imprinted genes (34). Global hypomethylation has been implicated in oncogenic transformations (35,36).

Data from Table I clearly indicate a significant increase in the various adduct groups from normal to cancer except P-2a where a significant decrease was observed. This skews the total adduct level and hence a statistically significant change was not observed. It may therefore not be appropriate to compare the total adduct burden.

The profiles of newly detected adducts are qualitatively similar to the patterns observed in several other organs in normal and disease status in humans (unpublished data). The similarity in adduct profiles in the Chennai cervix samples and the Kentucky cervix samples suggest that these adducts may be derived from endogenous factors and may not be specific to any ethnic group or environmental factors. This exemplifies the qualitative similarity and quantitative differences in the levels of these new sets of adducts in different cervix pathology, and their implications in the progression of cancer. The new group of adducts also showed substantial inter-individual variability. Analysis of the individual adduct levels shows a heterogeneous pattern of adduct distribution as shown by the scatter plots (Fig. 3), suggestive of susceptibility differences among individuals in accumulating and repairing these lesions. A possible explanation may be the difference in their repair capacity due to polymorphisms in select DNA repair genes. This inter-individual variability may facilitate the identification of high-risk populations and planning of intervention strategies.

Our findings support the hypothesis that infections of the cervix lead to inflammation, and the release of ROS by endogenous factors leads to high oxidative damage to the DNA. Some of the modifications thus accumulated may lead to irreversible changes in the genome and initiate cancer. Several studies have linked smoking and cervical carcinogenesis. Possible mechanisms of smoking-related cervical carcinogenesis include suppression of DNA repair capacity (37) leading to accumulation of DNA damage and induction of further DNA damage by the smoke constituents (reviewed in ref. 38). We therefore propose a scheme for cervix carcinogenesis (Fig. 4). HPV infection is assumed to be a necessary but insufficient step for carcinoma development. Many, if not the majority of women, are exposed to HPV at a young age, but only a few women develop cancer. Persistent infection (>1-2 years) with a high-risk HPV type increases the risk status of a woman. Our focus in this scheme is on the role of inflammation and associated factors like cigarette smoking, that promote the progression of a HPV-infected cervix to cancer. Since there are no appropriate markers with which to monitor disease during progression, high-grade intraepithelial lesions are often treated by a surgical resection or loop excision, which may be an over-treatment for several patients. Identification of biomarkers that predict tumor formation and progression would improve drastically the current clinical scenario. Although these novel adducts detected are indicative of an association between inflammation and cancer, it may be noted that this is a cross-sectional study. Further evaluation of these adducts that show a significant quantitative change in disease progression in a longitudinal study may prove them to be suitable markers of risk progression for the identification of high-risk subjects.

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References