Epstein–Barr virus episomes as targets for cigarette smoke- and γ-irradiation-induced DNA damage: studies on the EBNA-1 region by a new gene-specific technique

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Following our demonstration of cytochrome P450-independent DNA damage induced by aqueous solutions of cigarette smoke in human mucosal cells in vivo, and in a lymphoblastoid cell line, we have developed a new technique to demonstrate gene-region specific DNA damage, with the EBNA-1 gene present in multiple nuclear matrix-attached episomes in Raji cells serving as an amplified target. DNA was extracted from Raji cells treated by γ-irradiation or aqueous solutions of cigarette smoke; adducted bases or other damage were removed chemically by depurination/alkali treatment. Single-strand breaks induced directly by cigarette smoke as well as DNA cleaved at the site of alkali treatment. DNA was purified by extraction with phenol/chloroform/isoamyl aqueous solutions of cigarette smoke; adducted bases or other damage were removed chemically by depurination/alkali treatment. Single-strand breaks induced directly by cigarette smoke as well as DNA cleaved at the site of alkali treatment. DNA was purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and the aqueous phase extracted with diethyl ether to remove residual phenol. RNA was digested with 1 µg/ml DNase-free RNase for 1 h at 37°C. To avoid ethanol precipitation, the DNA was dialysed overnight against an excess of TE buffer. Approximately 100 µg DNA per sample was obtained.

Depurination and cleavage

Neutral depurination and alkaline hydrolysis were accomplished according to Link et al. (33), by taking 10-µg aliquots of DNA from each sample and heating them to 70°C for 30 min in a citrate-phosphate buffer, pH 7.0, to maximize the conversion of all heat-labile alkylated bases to apurinic (AP) sites. Finally, the samples were treated with NaOH (0.1 M) at 37°C for 30 minutes. DNA was precipitated and purified for the following experiments.

Materials and methods

Cells

All experiments were performed with Raji cells, kindly provided by Dr A. Polack, GSF Munich. The cells were maintained in Roswell Park Memorial Institute 1640 (Gibco, Eggenstein, Germany) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine, and kept at 5% CO2/37°C.

Induction of DNA damage and DNA extraction

A total of 5×10^7 Raji cells per sample (in duplicate) were exposed to either 22.4 Gy of γ-irradiation or to different concentrations of an aqueous solution of cigarette smoke (CS) for 10 min, as described elsewhere (Yang et al., in preparation). After washing the cells, DNA was isolated according to Ausubel et al. (32). Briefly, the cells were extracted by incubation in 300 µl digestion buffer (containing 100 mM NaCl, 10 mM Tris–HCl, pH 7.9, 25 mM EDTA, 0.5% sodium dodecyl sulphate (SDS), and 0.3 mg/ml proteinase K) for 18 h at 50°C. DNA was purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and the aqueous phase extracted with diethyl ether to remove residual phenol. RNA was digested with 1 µg/ml DNase-free RNase for 1 h at 37°C. To avoid ethanol precipitation, the DNA was dialysed overnight against an excess of TE buffer. Approximately 100 µg DNA per sample was obtained.

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Introduction

Carcinogenic and toxic compounds in tobacco smoke (1–4) or in snuff (5) are thought to be responsible for the damage to target cell DNA that leads to mutations in cancer-related genes (6,7) and chromosomal rearrangements (8,9) that are observed in head and neck (HN+) tumours and derived cell lines. However, neither the full spectrum of tobacco smoke-derived adducts/DNA damage nor their relative importance in causing mutational or chromosomal changes in cancer-relevant genes are yet known (10). In particular, to our knowledge no method to detect gene-specific DNA damage and its repair has been applied to human H/N tissues.

The Epstein–Barr virus (EBV) genome is present in >90% of healthy carriers in B-cells of the oropharynx, the tonsils and sometimes also in the parotid glands (11–13). It is, thus, exposed to carcinogens and toxic compounds from tobacco smoke and other environmental sources, and their DNA-binding metabolites. A positive association has been described between cigarette smoking, especially in heavy smokers, exposure to smoke fumes and nasopharyngeal carcinoma, a cancer in which active EBV genes seem to play a major role (14–19). Activated EBV associated with smoking was found in rare polyclonal B-cell lymphocytosis in women (20,21). Non-random point mutations, deletions and insertions have recently been described in the transforming EBV−LMP1 gene in nasopharyngeal carcinoma (22–24), in EBV-containing Hodgkin’s lymphomas and other tumours (25–27). However, they were also frequently discovered in a group of juvenile Japanese patients with benign EBV-associated diseases and one with a malignant EBV-associated tumour, and in all three healthy controls (28). In other studies, mutations were also reported in the EBNA-1 gene (29,30), which may have oncogenic properties (31). Some of these mutations may be de novo mutations. Accordingly, research is warranted to verify a possible link between environmental risk factors and mutations in transforming genes of EBV. This paper describes a new approach for studying this problem in Raji cells, which could possibly be extended to single-copy cancer-relevant genes in other cell types.

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*Abbreviations: AP sites, apurinic/apyrimidinic sites in DNA; CS, aqueous solution of cigarette smoke; EBV, Epstein−Barr virus; EBNA-1, Epstein−Barr nuclear antigen 1; H/N, head and neck; PCR, polymerase chain reaction; streptavidin-coated magnetic beads (Dynal); TE buffer, Tris–EDTA electrophoresis buffer; SDS, sodium dodecyl sulphate.
min to cleave the AP sites and the pH was adjusted to 7.0. This DNA was used for end-labelling.

**End-labelling**

First, the relationship between CS concentration and 3′-OH ends in DNA was determined by end-labelling with α-[32P]dCTP (Amersham, Braunschweig, Germany). Aliquots of 5 µg DNA (in duplicate) were incubated in 25 µl of end-labelling buffer consisting of 50 nM Tris–HCl, pH 8.0 at 25°C, 5 mM MgCl₂, 1 mM DTT, 1 unit Klenow fragment (MBI Fermentas, St Leon-Rot, Germany), 0.5 mM each of dATP, dGTP and dTTP (Boehringer, Mannheim, Germany) and 0.5 µCi[32P]dCTP, overnight at room temperature. The reaction was stopped by addition of an equal volume of 12.5 mM EDTA. Samples (in duplicate) were subsequently applied onto Whatman DE-81 ion exchange filters (Herolab, Wiesioch, Germany) and washed in 0.5 M sodium phosphate buffer (pH 6.8) to remove unincorporated precursors. The radioactivity remaining on the filters was measured in a Packard Tricarb 2200CA liquid scintillation analyser. Results are expressed as c.p.m. dCTP incorporated/µg DNA.

Second, DNA 3′-ends prepared as above were elongated by end-labelling with 0.5 mM biotin-16-dUTP (Boehringer Mannheim, Germany) under the same conditions as above. Unincorporated biotinylated nucleotides and the Klenow enzyme were then removed by spinning over a size-exclusion column (Clontech, ICT Biotechnology, Heidelberg, Germany). All of the recovered DNA was used as the next step.

**DNA cleavage by restriction enzymes**

Labelled and unlabelled DNA was cleaved by a combination of restriction enzymes Bam HI and Hind III in Bam HI buffer for 3 h at 37°C. DNA fragments were separated in 0.8% agarose gel/TE buffer at 50 V for 1 h and bands in the region of the 2932 bp band for EBV were cut out and extracted from agarose gel with a QIA quick Gel Extraction Kit (QIAGEN, Hilden, Germany).

**Binding of biotin-DNA to streptavidin-coated magnetic beads**

Biotin-labelled and unlabelled DNA were incubated with 20 µl of streptavidin Dynabeads M 280 (DYNAL, Hamburg, Germany) according to Kotsopoulos et al. (34). Briefly, DNA was bound to the beads for 30 min at 50°C under rolling in a high-salt binding buffer. After washing away biotin-free DNA 20 µl of bead suspension was obtained.

**Polymerase chain reaction**

Bound DNA was subjected to polymerase chain reaction (PCR) using EBNA-1-specific primers as described by Ambinder et al. (35). After this step, 20 µl of DNA per sample was obtained. PCR for EBNA-1 was carried out using a Perkin-Elmer automated thermocycler for 40 cycles with denaturation, annealing/extension times and temperatures of 94°C for 2 min; 52°C for 2 min and 72°C for 3 min, respectively. The 50 µl reaction mixture included 1 mM primer, 200 µM dNTPs, 10 mM Tris–HCl (pH 8.1), 1.5 mM MgCl₂, 50 mM KCl and 10 µl DNA samples. All of the components were added and denatured at 98°C for 5 min and cooled to 95°C, then 2.5 units Taq DNA Polymerase (Boehringer Mannheim, Germany) were added and the vials proceeded immediately to the thermocycler.

A aliquots of 5 µl of amplification products were electrophoresed on 2% agarose gels. Gels were stained with ethidium bromide and photographed.

**Sequencing of the PCR product**

Direct sequencing of PCR products by the dideoxy chain termination method of Sanger was as described in Lehman et al. (36), with minor modifications. In brief, after denaturation, the samples were moved to 40°C for 2 min, for annealing of template and primers, and then incubated at 40°C for 5 min in labelling buffer that contained [35S]-labelled nucleotide analogues. The reaction was stopped by the addition of 2.5 µl stop solution and the samples were run on a sequencing gel.

**Results and discussion**

Conditions which aim at determining gene-specific DNA damage induced by tobacco smoke, and its repair or persistence, have to fulfil two requirements: (i) the genomic region investigated has to be sufficiently large so that damage may be observed at relevant levels of exposure, and (ii) the method has to be relatively insensitive to the type of adduct or DNA damage, in view of the wide spectrum of DNA changes to be expected from such a complex mixture (1–4). In a previous study in this laboratory, cigarette smoke (CS) was shown to contain direct-acting compounds that induce DNA damage in
Detection of gene-specific DNA damage in EBV

For further analysis, therefore, the EBNA-1 region was chosen, due to the recent descriptions of multiple mutations in the EBNA-1 gene and protein in biopsies from nasopharyngeal carcinoma and in different viral strains (29,30), and the oncogenic potential of EBNA-1 (31). We have proceeded to cleaving the DNA, whether biotin-labelled or not, into fragments by a combination of restriction enzymes (Bam HI and Hind III), which should result in a 2932-bp-fragment of EBV containing the EBNA-1 gene (29–31, and references therein), and some similarly-sized fragments from cellular DNA. Fragments of around 3 kb were separated on an agarose gel. Biotin-labelled and unlabelled DNA were recovered by a gel extraction kit, brought into a high-salt binding buffer (34), and bound to streptavidin-coated beads. Bound DNA was subjected to PCR using EBNA-1-specific primers (35).

The PCR assay demonstrated that EBNA-1, as verified by DNA sequencing (not shown), is present in streptavidin-bound DNA from treated cells, but is absent in streptavidin-bound DNA from control cells (Figure 3). Since it can be expected that only a fraction of all EBV episomes would be damaged by CS, the EBNA-1 sequence was also detected in DNA not bound to streptavidin-beads, but not in fragments of other sizes (not shown).

CS at all three concentrations induced bulk DNA damage as shown by terminal filling-in under our present conditions (Figure 2). When damage in the EBNA-1 region of EBV episomes was investigated at CS dilution 1:30 (Figure 3), the band at 387 bp representing this region appeared weaker at a CS dilution of 1:100 and stronger at CS 1 (not shown), but proper quantification by PCR has not yet been done. It is interesting to note that damage in control cells is present at ~40% of the level observed in CS 0.03-treated cells; the absence of DNA damage in the EBV gene may, however, indicate that this damage is preferentially located in the cellular DNA. In future, analysis at higher resolution may be possible by ligation-mediated PCR provided damage occurs in such a way that at least 100 DNA sequences with damage at the same nucleotide become available for the PCR reaction (47).

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References


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