Toxicology in Vitro 24 (2010) 830–834

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Acrylamide catalytically inhibits topoisomerase II in V79 cells

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ARTICLE INFO

Article history:
Received 3 July 2009
Accepted 8 December 2009
Available online 16 December 2009

Keywords:
Acrylamide
Topoisomerase II
V79 cells

ABSTRACT

The vinyl monomer acrylamide is characterized by the presence of an α,β-unsaturated carbonyl group that makes it reactive towards thiol, hydroxyl or amino groups and towards the nucleophilic centers in DNA. The ability of acrylamide to chemically modify protein thiols has prompted us to consider topoisomerase II as one possible target of acrylamide, since agents targeting protein sulfhydryl groups act as either catalytic inhibitors or poisons of topoisomerase II. Nuclear extracts from V79 Chinese hamster cells incubated with acrylamide reduced topoisomerase II activity as inferred by an inability to convert kinetoplast DNA to the decatenated form. Nuclear extracts incubated with acrylamide pre-incubated with DTT converted kinetoplast DNA to the decatenated form, suggesting that acrylamide influences topoisomerase II activity through reaction with sulfhydryl groups on the enzyme. Furthermore, acrylamide did not induce the pBR322 DNA cleavage, as assessed by cleavage assay; thus, it cannot be regarded as a poison of topoisomerase II. As a catalytic inhibitor, acrylamide antagonizes the effect of etoposide, a topoisomerase II poison, as determined by clonogenic assay in V79 cells. This antagonism is confirmed by band depletion assay, from which it can be inferred that acrylamide reduces the level of catalytically active cellular topoisomerase II available for the action of etoposide.

1. Introduction

The vinyl monomer acrylamide (CH_2=CH–$CONH_2$), generally used in numerous industrial applications, has been classified by the International Agency for Research on Cancer as "probably carcinogenic to humans" (group 2A) (IARC, 1994); the discovery that acrylamide can be formed in heated foodstuffs (Tareke et al., 2002) has prompted research in various areas including epidemiological studies of cancers in humans. It has been reported that the genotoxic effects of acrylamide are really exerted by its metabolite glycidamide, which represents the ultimate carcinogen (Rice, 2005). Recently, it has been proposed that DNA damage by acrylamide in metabolically competent cells is mediated by oxidative stress (Jiang et al., 2007; Zhang et al., 2009). However, several studies have suggested that acrylamide itself can be cytotoxic and genotoxic; in fact, acrylamide has been demonstrated both to interact directly with DNA via a Michael-type reaction forming various adducts (Besaratinia and Pfeifer, 2005) and to induce transformational (Park et al., 2002)) and clastogenic effects in cells lacking CYP2E1 (Tsuda et al., 1993; Puppel et al., 2005; Martins et al., 2007), the isozyme involved in the epoxidation of acrylamide to glycidamide (Ghanayem et al., 2005). It is still unsolved by which mechanism pure acrylamide can produce toxicological effects. Among the different proposals, an interesting hypothesis is that acrylamide triggers mutagenesis by non-genotoxic or epigenetic mechanisms (Besaratinia and Pfeifer, 2005). One possible epigenetic mode of action may be related to the affinity of acrylamide for macromolecular sulfhydryl, hydroxyl or amino groups (Friedman, 2003; Besaratinia and Pfeifer, 2007). The ability of acrylamide to chemically modify protein thiols has prompted us to consider topoisomerase II as one possible target of acrylamide, since it has been demonstrated that agents targeting protein sulfhydryl groups act as either catalytic inhibitors or poisons of topoisomerase II (Hasinoff et al., 2005).

Topoisomerase II acts by passing an intact double helix through a transient double-stranded break that it creates in a separate segment of DNA. To maintain genomic integrity during the DNA strand passage event, the enzyme forms covalent bonds with DNA. These covalent topoisomerase II-cleaved DNA intermediates are normally tolerated by cells, but when significantly increased, they induce many DNA strand breaks that result in chromosomal aberrations, sister chromatid exchanges and cell death (Fortune and Osheroff, 2000). Most topoisomerase II inhibitors, such as etoposide (VP16), increase topoisomerase II-cleaved DNA intermediates by inhibiting the ability of topoisomerase II to religate DNA breaks (Yang et al., 1985); other poisons, such as thiol-reactive quinones, appear to act primarily by enhancing the levels of DNA cleavage mediated by topoisomerase II (Wang et al., 2001). Another major class of topoisomerase II-directed drugs is represented by the catalytic inhibitors which act by a variety of mechanisms and induce significantly different cellular effects. Aclarubicin,

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doi:10.1016/j.tiv.2009.12.010

intercalated into DNA, prevents the binding of topoisomerase II to DNA (Jensen et al., 1990); merbarone prevents cleavage of the gate-DNA strand acting at the same stage in the catalytic cycle as the topoisomerase II poisons (Fortune and Osheroff, 1998). Other catalytic inhibitors are sulfhydryl-reactive agents, such as maleimide (Jensen et al., 2002), cisplatin (Hasinoff et al., 2005) and purine analogs (Jensen et al., 2005).

In the present study, we utilized V79 Chinese hamster cells, a cell line devoid of CYP activity (Doehmer et al., 1988; Glatt et al., 2005), to investigate the effect of pure acrylamide on topoisomerase II activity in nuclear extracts, by performing kinetoplast DNA (kDNA) decatenation assays and pBR322 cleavage assays; moreover, to determine whether acrylamide antagonizes topoisomerase II poison, we carried out clonogenic assays and band depletion assays in cells submitted to single and combined treatments with acrylamide and VP16.

2. Materials and methods

2.1. Cell culture and reagents

V79 Chinese hamster cell lines were routinely cultured in D-MEM (Gibco-Invitrogen, Paisley, UK), supplemented with 5% fetal calf serum (Gibco, Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37 °C in a 5% CO_2 humidified incubator. Acrylamide (USB Corporation, Staufen, Germany) was freshly prepared by dissolving it in double-distilled sterile water; VP16 (Sigma–Aldrich, St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich) was diluted in double-distilled sterile water.

2.2. Clonogenic assay

Colony-forming assays were performed according to the following procedure. Cells (250) were plated in P-6 dishes, left for 18 h, and then exposed to acrylamide (0.1–10 mM) for periods of time variable from 1 h to 24 h or VP16 (0, 1.5, 2.5, 5, 7.5 μM) for 60 min, in complete medium. At the end of each treatment, the culture medium was removed and the cells were washed twice in PBS before being incubated in fresh medium for 10 days; the colonies were then stained with 0.1% Methylene Blue.

The same procedure was performed to evaluate the effects of the combined treatments in which the cells were pre-treated with acrylamide (0, 1, 5, 7 mM) for 1 h before exposure to 5.0 μM VP16 for 1 h.

Each set of single and combined treatments was carried out at least three times. For each experiment, cell survival was expressed as the percentage of untreated cells. When DMSO was used due to solubility problems, the final concentration of DMSO did not exceed 0.5% (v/v). Through the use of appropriate controls, this amount of DMSO was shown to have no significant effect on cell growth.

2.3. Preparation of nuclear extracts

Exponentially growing V79 cells were collected, washed once with PBS, suspended in 3 ml of cold TEMP buffer (10 mM Tris–HCl, pH 7.5; 1 mM EDTA; 4 mM $MgCl_2$; 0.5 mM PMSF) and incubated on ice for 15 min. Then, the cells were lysed by 70 strokes in a Dounce homogenizer. The lysed cells were centrifuged at 1500*g* for 10 min. The pelleted nuclei were washed two times with TEMP buffer, centrifuged again as described above, resuspended in 2 volumes of cold TEP buffer (same as TEMP but lacking $MgCl_2$) plus an equal volume of 1 M NaCl and incubated on ice for 60 min. The samples were then centrifuged at 15,000*g* for 20 min. Total protein amount was measured by the Bradford assay (Bradford, 1976).

2.4. Kinetoplast DNA decatenation assay

Kinetoplast DNA (kDNA; TopoGEN, Port Orange, FL, USA) decatenation assays were performed by utilizing nuclear extracts (200 ng) from untreated cells incubated either with acrylamide (0, 1, 2, 3, 4, 5 and 7 mM) for 45 min or with acrylamide (0, 1, 5, and 7 mM) pre-incubated for 10 min at 37 °C with 0.5 mM dithiothreitol (DTT) in 10 mM Tris–HCl, pH 8. According to the manufacturer's instructions, kDNA (250 ng) was incubated in 20 μl of reaction buffer [50 mM Tris–HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, and 0.5 mM DTT] at 37 °C for 30 min. Decatenation products were electrophoresed on a 1% Tris/borate/EDTA (TBE) ethidium bromide (0.5 μg/ml) agarose gel.

2.5. pBR322 DNA cleavage assay

DNA cleavage assays using nuclear extracts (200 ng) from untreated cells were performed as described by Hasinoff et al. (2005), with minor modifications. Briefly, 20 μl of reaction mixture contained 125 ng of pBR322 plasmid DNA, 0.5 mM ATP in assay buffer [10 mM Tris–HCl, 50 mM KCl, 50 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5% (v/v) glycerol, pH 8.0], acrylamide (0, 1, 5, 7 mM) or VP16 (100 μM). The order of addition was assay buffer, DNA, acrylamide or VP16, and then nuclear extracts. The reaction mixture was incubated at 37 °C for 15 min, quenched with 1% (v/v) SDS/25 mM Na_2EDTA and then treated with 0.25 mg/ml proteinase K (Invitrogen) at 55 °C for 60 min. The samples were separated by electrophoresis (2 h at 10 V/cm) on a 1% TBE ethidium bromide agarose gel and the linear pBR322 DNA was identified by comparison with linear pBR322 DNA produced by the action of the restriction enzyme BamHI (New England BioLabs, Beverly, MA, USA) acting on a single site on pBR322 DNA.

2.6. Band depletion assay

Band depletion assays, according to Sehested et al. (1998), were carried out by Western blotting of topoisomerase II on nuclear extracts obtained from cells exposed to single and combined treatments with acrylamide (1, 5 and 7 mM) and VP16 (5 μM) for 1 h. For the detection of topoisomerase II, the polyclonal primary antibody anti-p170 form of human topoisomerase IIα (TopoGEN) was used. Goat anti-mouse immunoglobulin secondary antibody 1:6000 (Sigma–Aldrich) application and visualization were performed using the Roche Chemiluminescence Kit (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions. Total protein amount was measured by the Bradford assay (Bradford, 1976). Control of protein content in each lane was evaluated by staining gels with Coomassie Blue and the membranes with Ponceau (Sigma–Aldrich).

3. Results

3.1. Clonogenic assay

Results from clonogenic assays at different times and different concentrations of acrylamide are shown in Fig. 1A. Cell survival was about 75% of untreated V79 cells after exposure to the lower doses (0.1–1 mM) for all the treatment times and was reduced by exposure to the higher concentrations (7–10 mM) for 6 h. Further experiments were performed by utilizing doses of acrylamide (1, 5 and 7 mM, for 1 h) which had only a mild effect on cell survival (Fig. 2B).