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Acrylamide-induced apoptosis in rat primary astrocytes and human astrocytoma cell lines

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ABSTRACT

This study aimed to evaluate the acrylamide (ACR)-induced apoptotic effects on rat primary astrocytes and three human astrocytoma-derived cell lines (U-1240 MG, U-87 MG, and U-251 MG). As determined through the MTT assay, treatment with 1 and 2 mM ACR for 24–72 h resulted in decreased cell viability in all cells. Decreases in cell viability could be blocked in all cells with the exception of U-251 MG cells by Z-DEVD FMK. ACR-induced dose-dependent apoptotic effects were also demonstrated by increases in the sub-G_1 phase cell population in all cells. The decreased expressions of pro-caspase 3, 8, and 9 and the interruption of the mitochondrial membrane potential were observed in all cells. Exposure to 2 mM ACR for 48 h resulted in increased Bax/Bcl-2 ratios in primary astrocytes and U-87 MG cells, whereas the overexpression of Bcl-2 was observed in U-1240 MG and U-251 MG cells. The ACR-induced increases in the levels of p53 and pp53 in primary astrocytes could be attenuated by caffeine. These results suggest the existence of a common apoptotic pathway among all cell types and that U-87 MG cells may be a suitable substitute *in vitro* model for primary astrocytes in future studies on ACR-induced neurotoxicity.

1. Introduction

Acrylamide (ACR) is a commonly encountered water-soluble vinyl monomer that is utilized in a wide variety of industrial applications and scientific studies (Besaratinia and Pfeifer, 2007). ACR can also be formed through food processing at high temperatures via the Maillard reaction (Mottram et al., 2002). The existence of ACR in the daily diet and in living and working environments has potentially imposed major threats to the health of the general public and generates concerns regarding its pathogenic mechanisms (Mottram et al., 2002; Stadler et al., 2002). ACR can easily diffuse through various biological membranes due to its relatively simple structure and low molecular weight (Besaratinia and Pfeifer, 2007). After ingestion, ACR can be further metabolized by cytochrome P-450 CYP2E1 to glycidamide (GLY). Both ACR and GLY have been suggested to be accountable for glutathione depletion, which results in a reduced regeneration of the sulfhydryl group in proteins and thus hinders their functions (Sumner et al., 1992). ACR and GLY have been proposed to be genotoxic and carcinogenic agents. Doerge et al. (2005) reported that GLY can lead to the formation of protein and DNA adducts and the induction of DNA strand breaks. Exposure to ACR at high concentrations has also been reported to induce the mutation of TP3, a gene encoding p53, which is confirmed to be responsible for tumor suppression (Besaratinia and Pfeifer, 2004). In addition, the reproductive toxicity and neurotoxicity induced by ACR and GLY have also been well documented, and both physiological systems may share some common characteristics related to their pathogenesis (Exon, 2006). Several possible molecular mechanisms related to ACR- and GLY-induced neurotoxicity have been proposed (LoPachin, 2005). First, ACR affects the fast anterograde transportation of nerve growth factors between the nerve cell body and distal axons by interacting with kinesin-related motor proteins in neurofilaments, which results in neuron death. Second, nerve terminal degeneration results from reduced neurotransmitter release due to the interruption of the fusion processes of synaptic vesicles with their target membrane through the binding of ACR to the sulfhydryl groups on membrane fusing protein (Sickles et al., 1996).

A growing body of evidence shows that astrocytes are essential for the maintenance of proper neuron function in the brain. It has been reported that neuronal survival may be sustained through the distribution of astrocytes in the midbrain via the epidermal growth factor receptor signaling pathway (Wagner et al., 2006). In an *in vitro* model, the inflammatory states of glia cells can trigger neuronal cell death via mitogen-activated protein kinase, c-Jun N-terminal kinases, and p38 (Xie et al., 2004). Additionally, through the release of glutamate, a neuro-active substance, astrocytes are suggested to play an important role in synaptic regulation (Ricci et al., 2009). Recently, interactions between astrocytes and neurons involved in neurological dysfunctions, such as stroke, migraine, epilepsy, multiple sclerosis in central nervous system, and Alzheimer's

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disease, were also intensively reviewed (Brambilla et al., 2013). The participation of astrocytes in the regulation of cerebral blood flow, the defensive mechanisms against oxidative stress, and microglial activation and differentiation during brain inflammation are evidently related to the above-mentioned disorders. Based on these studies, the investigation of ACR-induced toxicity in astrocytes is warranted.

ACR was previously considered to be a neurotoxicant rather than an astrotoxin (Cookson and Pentreath, 1994). This hypothesis was confirmed by Holden and Coleman (2007), who found that the exposure of human astrocytoma cell lines (U-251 MG, U-373 MG and CCF-STTG1) to ACR for 24 h imposed no significant influences on their viability and the expression of glial fibrillary acidic protein (GFAP). However, our laboratory provided the first demonstration that a longer exposure (up to 72 h) to ACR at a concentration of 2 mM or higher is necessary for astrocytic cells (U-1240 MG) to show apoptotic and astrogliotic responses in a time- and dose-dependent manner (Chen et al., 2009). In addition, we also reported that treatment with 2 mM ACR for 48 h induced significant increases in p53, pp53, Cdk2, p27, p21, cyclin D_1, G_0/G_1-arrested cell population, and DNA damage in a U-1240 MG culture. The blockage of cell cycle arrest through treatment with caffeine indicated that ATM/ATR kinase is also involved in ACR-induced astrocytic toxicities. In contrast, U-87 MG and U-251 MG cells appeared to have different protein expression profiles than those observed in U-1240 MG cells after ACR exposure (Chen et al., 2010). Furthermore, ACR-induced mitochondrial collapse followed by apoptosis was evident by the findings reported by Chen et al. (2013), who observed an increase in the release of cytochrome c and decreases in the expressions of poly(ADP-ribose) polymerase and pro-caspase 3 and in the Bax/Bcl-2 ratio in U-1240 MG cells.

Although many astrocytic cell lines had been employed in ACR-induced cytotoxic studies, the response of primary astrocytes to ACR remains to be identified due to the limited evidence available. Despite the fact that Mead and Pentreath (1998) compared the EC_{50} values between rat primary astrocytes and C6 glioma cells by examining the neutral red uptake and the expression of GFAP induced by 40 chemicals and concluded that primary astrocytes are more vulnerable to ACR than C6 glioma cells, these researchers also reported that astrocytes have a relatively higher resistance against the toxicity induced by amyloid β-peptide, which could have both neurotrophic or neurotoxic effects on neurons in the pathogenesis of Alzheimer's disease, than rat C-6 glioma and human 1321NI astrocytoma cells (Pentreath and Mead, 2004).

Together with our previous findings, it is necessary to further assess the differences in ACR-induced toxic responses between primary astrocytes and astrocytoma cell lines. The aims of this study were to investigate the responses of rat primary astrocytes and to evaluate whether astrocytoma cells are suitable substitute *in vitro* models for primary astrocytes for the elucidation of the mechanisms of ACR-induced cytotoxicity. Three different astrocytoma cell lines, namely U-1240 MG, U-251 MG, and U-87 MG (LaRocca et al., 1989), were used in this comparative study against primary astrocytes for the examination of cell viability, cell population, apoptotic proteins, and mitochondrial membrane potential.

2. Materials and methods

2.1. Chemicals and reagents

ACR, ammonium per-sulfate, ethanol, glycine, methanol, NaCl, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), and lysis buffer were purchased from Sigma–Aldrich (St Louis, MO, USA). Dimethyl sulphoxide (DMSO), triton X-100, KCl, and tetramethylethylenediamine were purchased from J.T. Baker (Philipsburg, NJ, USA). Protein markers, loading buffer, and protein dye were purchased from Fermentas (Vilnius, Lithuania). Polyvinylidene fluoride and Na_2HPO_4 were purchased from Merck KGaA (Darmstadt, Germany). The antibody against GFAP was purchased from Ebioscience (San Diego, CA, USA). The antibody against pro-caspase 3 was obtained from Abcam (Cambridgeshire, UK). Antibodies against pro-caspase 8, Bcl-2, Bax, and tubulin, and an antibody conjugated to horseradish peroxidase against rabbit IgG were purchased from GeneTex (Irvine, CA, USA). The antibody against pro-caspase 9 and the caspase-3/CPP32 inhibitor Z-DEVD-FMK were purchased from Biovision (San Francisco, CA, USA). Skim milk powder was purchased from Anchor (Auckland, NZ). Dulbecco's modified Eagle's medium (DMEM), culture reagents, and buffers were purchased from Gibco (Rockville, MD, USA).

2.2. Cell cultures

The human astrocytoma cell lines U-1240 MG, U-87 MG, and U-251 MG were obtained from Ohio State University (Columbus, OH, USA). The cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in a 5% CO_2 100% humidified atmosphere. Rat primary astrocytes were collected from the brain cortices of one- to two-day-old Sprague–Dawley (SD) rats using a modified procedure from a previously published protocol (McCarthy and de Vellis, 1980). The collected primary cells and all of the cell lines were cultured for at least 5 days in the same media under the same culture conditions. When the primary cells reached confluence, the culture was shaken for 18 h at 280 rpm to remove the microglia and oligodendrocytes. The remaining attached astrocyte monolayer was then dissociated by 0.5% (v/v) trypsin/EDTA solution, centrifuged at 2000 rpm for 5 min, re-suspended with 10 ml of DMEM, and cultured at a density of 4×10^5 cells for 5 days. The identification of these cells was first obtained by staining with anti-GFAP antibody and further confirmed through flow cytometry. Ninety-five percent of the collected cells with positive GFAP antibody staining were proven to be of astrocyte origin. All of the animal handling procedures followed the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the National Taiwan University.

2.3. ACR and caffeine treatments

The ACR stock solution (1 M) was prepared with double-distilled water (ddH_2O). The designated ACR concentrations (0, 0.1, 0.5, 1, and 2 mM) were added to the cell cultures, and the cells were cultured for different durations (0, 24, 48, and 72 h) as reported previously (Chen et al., 2009). To determine the role of ATM/ATR kinase in the DNA damage response to ACR treatment, the cell were pre-treated with caffeine at various concentrations (0.25, 0.5, 1, and 2 mM) prior to ACR exposure, as in our previously published protocol (Chen et al., 2010).

2.4. Analysis of cell viabilities

The cell viabilities of the primary astrocytes and astrocytoma cells were analyzed through an MTT assay (Chen et al., 2013). In brief, the cells were cultured in a 96-well microtiter plate at a density of 1000–5000 cells per well in a final volume of 100 μl of culture medium for 1 day prior to the experiment. After the treatment was completed, the cells were immediately co-incubated with 10 μl of MTT solution (0.5 mg/ml) for 3 h at 37 °C. The medium