Letter

Release of heat shock proteins from human neuroblastoma cells exposed to acrylamide

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ABSTRACT — When human neuroblastoma cells (SH-SY5Y) were exposed to 0.5 - 5 mM acrylamide for 18 hr, the levels of heat shock proteins (HSPs) of 90, 70 and 27 kDa (Hsp90, Hsp70, and Hsp27, respectively) were elevated in the incubation media depending on the dose of acrylamide whereas only the Hsp70 level increased within cells. U0126, a specific inhibitor of extracellular signal-regulated protein kinase kinase and a potent suppressor of the cytotoxicity of acrylamide, suppressed the increase in the levels of all HSPs in the incubation media but not their expression within cells. Total protein concentrations in the incubation media increased depending on the dose of acrylamide, and this increase was associated with the increasing number of bands detected by silver staining after SDS-polyacrylamide gel electrophoresis. One of the clearest bands was identified as Hsp90 by peptide mass fingerprinting. Thus, acrylamide causes release of proteins, including that of HSPs, from SH-SY5Y cells. HSP in extracellular fluid may be a good indicator of cytotoxicity of acrylamide.

Key words: Acrylamide; Neurotoxicity; SH-SY5Y cells; Heat shock protein; ERK; Extracellular fluid

INTRODUCTION

Acrylamide has long been known to be a potent neurotoxin in humans and animals (Igisu *et al.*, 1975), although the mechanisms of its action have not been clarified. One of the characteristic morphological alterations caused by acrylamide is distal axonal swelling associated with the accumulation of neurofilaments (Gold and Schaumburg, 2000). Acrylamide also causes aggregation of intermediate filaments in cultured cells (Blain *et al.*, 2006).

Heat shock proteins (HSPs) are molecular chaperones for proteins; they are present in all cells, and serve to prevent protein misfolding and aggregation (Hartl and Hayer-Hartl, 2002). Judging from the morphological changes induced by acrylamide, it possibly has an effect on levels of HSPs. Indeed, Gold *et al.* (2004) reported low immunostaining of 70 kDa HSP (Hsp70) in the brain of rats intoxicated with acrylamide. Saito *et al.* (2005) reported that a high concentration of acrylamide (10 mM) enhanced the expression and release of HSPs in nonneuronal cells (HeLa cells and normal rat kidney cells). In our previous studies, we found that acrylamide causes apoptosis in

human neuronal cells (SH-SY5Y) and that signal transduction mediated by extracellular signal-regulated protein kinase (ERK) plays an important role (Okuno *et al.*, 2006; Sumizawa and Igisu, 2007), while HSPs have been shown to influence signal transduction leading to cell death (Xanthoudakis and Nicholson, 2000). We therefore examined HSPs inside and outside SH-SY5Y cells exposed to acrylamide.

MATERIALS AND METHODS

Materials

Bio-Safe CBB G-250 Stain and Aurum Serum Protein Mini Kit were purchased from Bio-Rad (Hercules, CA, USA). Silver Stain Kit was obtained from Wako Pure Chemical Industries (Osaka, Japan). Polyclonal antibodies against Hsp90, Hsp70 and Hsp27 from Stressgen (Victoria, BC, Canada). Polyclonal anti-actin and HRP-conjugated donkey anti-goat IgG from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immobilon-P Transfer Membrane and Immobilon Western Detection Reagents (chemiluminescent HRP substrate) from Milli-

pore Corporation (Billerica, MA, USA). Aprotinin and Igepal CA-630 from Sigma (St. Louis, MO, USA). SH-SY5Y human neuroblastoma cells from American Type Culture Collection. The sources of all other chemicals and kits were the same as described previously (Okuno *et al.*, 2006; Sumizawa and Igisu, 2007).

Cell culture and treatments

SH-SY5Y cells were grown as described previously (Sumizawa and Igisu, 2007), and the exponentially growing cells were plated at $1.3\text{--}2.4 \times 10^6$ cells/well in 6-well culture plates and cultured for 2 days prior to the experiments. The medium was changed to 1 ml of serum-free DMEM/F12 containing acrylamide or without it and cells were incubated for 18 hr. The serum-free medium was used to avoid possible binding of acrylamide to the serum proteins. Incubation in serum-free DMEM/F12 for 24 hr did not lower the viability of SH-SY5Y cells. To examine the effect of U0126, cells were pretreated for 1 hr with U0126 in serum-free medium and then exposed to acrylamide for 18 hr. Heat shock treatment was performed as described by Quigney *et al.* (2003), i.e., cells were placed at 41.5°C in a humidified 5% CO₂ atmosphere for 1 hr and then allowed to recover at 37°C for 6 hr before they were exposed to acrylamide. The cytotoxicity detection (lactate dehydrogenase (LDH) assay) and cell viability tests (water soluble tetrazolium (WST)-8 assay) were carried out as described previously (Sumizawa and Igisu, 2007).

Immunoblotting

After removing the medium, culture plates were rinsed with ice-cold phosphate buffered saline (PBS) containing 1% aprotinin, cells were scraped with 100 µl of RIPA buffer (PBS containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/ml phenylmethylsulphonyl fluoride, 30 ml/ml of aprotinin and 1 mM sodium orthovanadate). The cell lysate was passed through a 21-gauge needle 10 times. After left on ice for 30 min, the cell lysate was centrifuged at $15,000 \times g$ for 20 min at 4°C. The supernatant was used as the total cell lysate. One-hundredth volume of the culture medium or 15 or 30 µg of protein from the total cell lysate was loaded onto 10% SDS-polyacrylamide gels. The proteins were transferred onto an Immobilon-P membrane, and the membrane was treated with polyclonal primary antibodies (against Hsp90, Hsp70, Hsp27, or actin), horse radish peroxidase (HRP)-conjugated secondary antibodies, followed by chemiluminescent HRP substrate. The intensities of the bands were quantified by a CoolSaver AE-6955 using CS Analyzer Ver2.0 (ATTO Corporation, Tokyo, Japan).

Protein analysis

The concentrations of the proteins were measured by Protein Assay based on the Bradford method. Ten µl of the culture medium was resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE), and proteins were stained using Silver Stain Kit (Wako) or Coomassie Brilliant Blue (CBB).

For peptide mass fingerprinting, culture media corresponding to 40 µg of proteins were half-saturated with ammonium sulfate and left overnight at 4°C. After centrifugation at $15,000 \times g$ for 20 min at 4°C, the pellet was resuspended in 80 µl of PBS. After electrophoresis on a 10% SDS-polyacrylamide gel, the proteins were stained with CBB. Clear bands of approximately 90 kDa were excised from the gel and placed in distilled water (1 ml). Peptide mass fingerprinting was carried out at Hitachi High-Tech Science Systems Corporation (Hitachinaka, Japan) using oMALDI-Qq-TOF MS/MS QSTAR Pulsar i (Applied Biosystems), MASCOT (Matrix Science) and NCBI nr (Taxonomy: human).

RESULTS AND DISCUSSION

Among the HSPs we examined, only the level of Hsp70 was elevated within the cells exposed to acrylamide (Fig. 1a). This elevation in the Hsp70 level was not suppressed by U0126 (Fig. 2a), a specific inhibitor of extracellular signal-regulated protein kinase kinase. In our previous studies, U0126 suppressed all indices of acrylamide cytotoxicity, including lowered trypan blue exclusion and LDH leakage, increased p53 phosphorylation, and activation of caspase 3 associated with increase in cell population in the subG1 phase (Okuno *et al.*, 2006; Sumizawa and Igisu, 2007). This indicates that the elevation of the intracellular Hsp70 level was not related to the toxicity of acrylamide. In addition, while heat shock caused a nearly 3-fold increase in the level of Hsp70 within cells (but no increase in the levels of Hsp90 or Hsp27), the cells subjected to heat shock did not differ in response (evaluated by WST-8 and LDH assay) to acrylamide (1 - 5 mM) from the untreated ones (data not shown).

In contrast, the levels of Hsp90, Hsp70 and Hsp27 in the incubation media increased depending on the dose of acrylamide (Fig. 1b). Moreover, 5 µM of U0126 suppressed the levels of HSP by more than 75% (Fig. 2b). Hence, extracellular HSPs appear to be closely related to the cytotoxicity of acrylamide.

Conventionally, HSPs have been regarded as an important part of the defense mechanisms of the cell because they are often expressed in response to various noxious stimuli such as oxygen deprivation, radiation and chemi-

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cals (Hartl and Hayer-Hartl, 2002). Recent studies have revealed extracellular functions of HSPs. For instance, Hsp90- α when expressed extracellularly activates proteinase and increases the invasiveness of tumor cells (Eustace

et al., 2004). Exogenous Hsp70 binds with high affinity to the plasma membrane and activates NF- κ B (Asea *et al.*, 2000). Hsp70 is released from cells involving lysosomal endosomes, independent of de novo synthesis or cell death

Fig. 1. Effects of acrylamide on intracellular (a) and extracellular (b) levels of heat shock proteins. SH-SY5Y cells were incubated with or without acrylamide for 18 hr. Representative immunoblots from 6 independent experiments are shown. In a densitometric analysis, the intracellular Hsp70 level was elevated significantly by 4 and 5 mM acrylamide ($p < 0.001$; ANOVA followed by the Bonferroni test) but no elevation in the levels of Hsp90 and Hsp27 was observed.

(Mambula and Calderwood, 2006). Thus HSPs can be secreted from cells via an active secretion pathway and can affect other cells. The results of the present experiment do not clarify whether the extracellular release of HSPs is caused by active processes or simply due to the damage to the cell membrane.

Apparently, when a high dose of acrylamide was administered, the increase in the extracellular protein level was not limited to that of HSPs because the concentrations

($\mu\text{g/ml}$, mean $\pm$ SD, and $n=3$) of the total proteins were 49.2 ± 4.6 (when not exposed to acrylamide), 68.8 ± 11.5 (when exposed to 1 mM of acrylamide), 79.1 ± 15.6 (2 mM acrylamide), 103.3 ± 20 (3 mM acrylamide), 115.6 ± 17.5 (4 mM acrylamide) and 121.7 ± 17.5 (5 mM acrylamide). Furthermore, numerous bands were observed with silver staining after the SDS-PAGE of the proteins in the incubation media (Fig. 3). Peptide mass fingerprinting revealed that the 90 kDa band obtained following SDS-

Fig. 2. Effects of extracellular signal-regulated protein kinase kinase inhibitor (U0126) on intracellular (a) and extracellular (b) levels of heat shock proteins. After SH-SY5Y cells were treated with U0126 for 1 hr, acrylamide (final concentration of 3 mM) was added and incubated further for 18 hr. Representative immunoblots from 3 independent experiments are shown.

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Fig. 3. Proteins in incubation media detected by silver staining following SDS-polyacrylamide gel electrophoresis. After SH-SY5Y cells were incubated with or without acrylamide for 18 hr, 10 μ l of the incubation media was loaded. For peptide mass fingerprinting, the bands (after staining with Coomassie Brilliant Blue) indicated by the arrow were excised, and found to contain both Hsp90- α and Hsp90- β .

PAGE contained both Hsp90- α and Hsp90- β (Homo sapiens) (This was also confirmed by immunoblotting). Thus, although the involvement of active secretion of HSPs cannot be ruled out, it appears that acrylamide impairs plasma membrane, at least at high doses, resulting in nonspecific leakage of proteins.

Although the pathogenetic significance of the changes in the levels of HSPs inside and outside SH-SY5Y cells is not yet clear, in view of the fact that HSPs are predominantly an intracellular component, extracellular levels of HSP may be a sensitive indicator of acrylamide toxicity. HSPs appear to be especially useful because their determination does not depend on enzymatic activities that may be inhibited by chemicals. Furthermore, the measurement is unlikely to be affected by the reducing substances such as glutathione whereas many methods to evaluate cytotoxicity including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), WST-8 and LDH assay are dependent on the changes of reduced form of nicotinamide adenine dinucleotide.

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