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Toxicity Assessment of Wastewater by Proteomics Analysis

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The effects of effluent from a wastewater treatment plant (EWWTP) on intestinal epithelial Caco-2 cells, a human intestinal epithelial cell line derived from a human colon carcinoma, were investigated. Previous studies have shown that the wastewater constituents nonylphenol and lipopolysaccharide (LPS) induce the overexpression of specific proteins (galectin-3, glutathione S-transferase A2 subunit, peroxiredoxin-1, and heat shock protein 90, beta (HSP90b)). In this study, the first screening of EWWTP was carried out using the HSP47-transformed cell assay, which is a highly sensitive toxicity assay. From the results of proteomics analysis of human intestinal Caco-2 cells treated with EWWTP, we found the overexpression of specific proteins, namely, elongation factor 1β and enolase 1. These results suggest that specific proteins can be used as biomarkers for the risk assessment of water and wastewater.

1. Introduction

Because cells lining the alimentary tract serve as the first line of defense against xenobiotics that orally enter the body, the impact of these compounds on these cells should be determined. The human intestinal epithelial cell line Caco-2 derived from a human colon carcinoma is considered a model intestinal epithelium.(1) Because Caco-2 cells can be cultured on microporous filters and can form monolayers, the measurement of changes in the paracellular ion flux linked to tight-junction disruption can be carried out.(2) As a target cell type, the intestinal epithelium is a good subject for determining protein expression profiles by proteomics analysis. In this study, the effects of wastewater constituents on Caco-2 cells were determined.

In previous studies, results of proteomics analysis showed that galectin-3, a galactose-specific lectin, the glutathione S-transferase A2 subunit, and peroxiredoxin-1 are overexpressed by nonylphenol-treated cells,(3) whereas heat shock protein 90, beta (HSP90b) is overexpressed by lipopolysaccharide (LPS)-treated cells.(4) Nonylphenol was used because it is a typical component of detergent, whereas LPS, which can be the main toxic substance in the effluent, was used because it is a component of activated sludge bacteria.(5)

These overexpressed proteins are associated with protection from toxic compounds. Galectin-3 is associated with the suppression of apoptosis and cell death.(6,7) The glutathione S-transferase A2 subunit is related to protection against oxidative dam-

age.[8] Peroxiredoxin-1 is associated with cellular defense against oxidative stress.[9] HSP90b is related to endotoxin activities.[10]

In this study, we investigated the toxicity of effluent from a wastewater treatment plant (EWWTP) using HSP47-transformed cells and proteomics analysis. The purpose of this study is to identify biomarkers that can be used for the risk assessment of water and wastewater.

2. Materials and Methods

Effluent samples obtained from a wastewater treatment plant in June, July, September, and October were used for the assays. These samples were filter-sterilized using a 0.45-µm membrane filter (Millipore, MA, USA). The chemical oxygen demand (COD) of the samples was determined by the standard dichromate reflux method.[11]

Two different cell lines were used for these experiments: the human intestinal cancer cell line Caco-2 and Chinese hamster ovary (CHO) cells with (HSP47 (+)) or without (HSP47 (–)) an HSP promoter.

CHO cells with (+) or without (–) an HSP47 promoter were provided by Dr. Yokota of Kaneka Corp. and were grown as an adherent monolayer in 75-cm^2 tissue culture flasks using Nutrient Mixture F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 200 µg/ml G418 (Gibco BRL 13075-015) and 0.1 g/L kanamycin (Sigma). The cultures were maintained in a 5% CO_2 incubator at 37°C. The doubling time of HSP47 (+) cells was 20 h, whereas that of HSP47 (–) cells was 18 h.

The human colon adenocarcinoma cell line Caco-2 was provided by Dr. Shimizu of the University of Tokyo, Japan. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids (NEAAs), 4 mM l-glutamine in a 95% air/5% CO_2 atmosphere at 37°C. The cells were subcultured at a split ratio of 3 to 8 every three days. Only reagent-grade or cell-culture-grade reagents were used in this study.

HSP47-transformed cells were trypsinized and plated onto 96-well plates at an initial concentration of 1×10^4 cells per well in 100 µl of Nutrient Mixture F-12 medium supplemented with 10% FBS, 0.1% G418, and 0.2% kanamycin solution. The cells were allowed to attach for 48 h before adding 100 µl of samples diluted 10-fold or 20-fold with the medium followed by incubation for 3 h in a 5% CO_2 incubator at 37°C. The medium was then carefully removed and the cells were washed twice with phosphate-buffered saline (PBS) (–). Fifty microliters of lysis buffer (Promega) was then added and the plates were incubated for 30 min at room temperature (RT).

Twenty microliters of cell lysate was transferred to a new plate, to which 100 µl of the substrate solution (10 mM $NaH_2PO_4\bullet2H_2O$, 100 mM NaCl, 1% bovine serum albumin (BSA), 0.005% NaN_3, 1 mM $MgCl_2\bullet6H_2O$, and 1% 4-methylumbelliferyl-β galacticose (MUG), pH 7) was added to induce the conversion of MUG into galactose and methylumbelliferyl.[12] After allowing the reaction to occur in the dark for 30 min at RT, 60 µl of reaction stop buffer (1 M glycine-NaOH (pH 10.3)) was added and the fluorescence at 365 nm excitation/450 nm emission was then determined using a multidetection microplate reader (Powerscan HT, Biotek Instruments, USA).

For proteomics analysis, after incubation (3 h, 10% EWWTP), the proteins were extracted by cell lysis in a solution of 4% (v/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 7 M urea, 2 M thiourea, 25 mM spermine base (Sigma), 1 M ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 5 mM 4-(2-aminoethyl) benzene-sulfonylflouride (AEBSF), followed by centrifugation at 100,000 *g* for 1 h at 15°C. Protein samples from the treated or nontreated cells were quantified using the two-dimensional (2D) Quant kit (Amersham Biosciences, Uppsala, Sweden) and resuspended at 1 µg/µl in a 350-µl

solution of 8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) immobilized pH gradient (IPG) buffer (pHs 3–10) (Amersham Biosciences, Uppsala, Sweden), and 10 mM DTT. Solubilized proteins were electrophoresed in the first dimension following the Amersham protocol using a flatbed electrophoresis system (Ettan IPGphor II, Amersham Biosciences, Uppsala, Sweden) and 18-cm IPG dry strips (Amersham Biosciences, Uppsala, Sweden) with a pH linear range of 3–10. After isoelectric focusing, the IPG strips were re-equilibrated for 20 min in a solution of 2% (w/v) sodium dodecyl sulfate (SDS), 6 M urea, 30% (v/v) glycerol, 0.05 M Tris-HCl (pH 8.8), and 2% (w/v) DTT, and for 20 min in a solution of 2% (w/v) SDS, 6 M urea, 30% (v/v) glycerol, 0.05 M Tris-HCl (pH 8.8), and 5% (w/v) iodoacetamide. The strip was placed on a gradient SDS-PAGE gel (12–14% (w/v) polyacrylamide) and run at 600 V, and 400 mA for 30 min and at 600 V, and 40 mA for 5 h using the Ettan DALTsix electrophoresis system (Amersham Biosciences, Uppsala, Sweden). The proteins were visualized by Coomassie Brilliant Blue (CBB) staining (using PhastGel Blue R-350, Amersham Biosciences, Uppsala, Sweden) for spot analysis, and the gel was analyzed using ImageMaster 2D Elite (Amersham Biosciences, Uppsala, Sweden). The protein spot of interest was excised from the gel, washed and digested in-gel with trypsin (sequencing grade, Boehringer Mannheim, Germany). All matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired using a Voyager-DE STR mass spectrometer (Applied Biosystems). The matrix solution was prepared by dissolving 10 mg of α-cyano-4-hydroxycinnamic acid (Sigma, USA) in 1 ml of 50% acetonitrile and 0.1% trifluoroacetic acid in deionized water. The obtained peptide sequence tags were used to identify the protein of interest by searching databases using BLAST (http://www.ncbi.nlm.nih.gov:80/blast) or the Prospector software mass spectrometry (MS)-Tag (http://prospector.ucsf.edu/).

Statistical analyses such as Student's t-test were carried out. Differences in means were considered significant at $p<0.05$. All experiments were conducted at least three times.

3. Results and Discussion

The toxicity of EWWTP can be determined using HSP47-transformed CHO cells by incubating the latter with the test compound and measuring the activity of β-galactosidase. When introduced into a chromosome, the HSP47 plasmid can express β-galactosidase efficiently during stress induction. Experimental CHO cells were transformed by inserting the β-galactosidase gene downstream of the HSP47 promoter, whereas control CHO cells had the β-galactosidase gene under the control of the SV40pA promoter.[13] Isoda *et al.*[12] developed this highly sensitive system for detecting trace amounts of environmental pollutants and natural toxins. It has already been revealed that the production of stress proteins is induced as a result of the reaction of cells with a stressor such as heat, a chemical substance or a heavy metal. Therefore, upon stress induction, the effects of the hazardous chemicals can be quantitatively evaluated by measuring the activity of the reporter gene. It has already been revealed that the production of stress proteins is induced as a result of the reaction of a cell with hazardous chemicals.[12] The HSP47-transformed CHO cells responded to low concentrations (0.1–1 nM) of various hazardous chemical substances.

These results indicate that the defense mechanisms in cells exposed to low concentrations of hazardous chemical substances or nature toxic substances can be detected with high sensitivity.

The results of the HSP47 assay are shown in Fig. 1. The cells were treated for 3 h with EWWTP from four sampling periods (June, July, September, and October). A significant toxic effect was observed in the cells treated with the June, July, and September samples, with the latter showing the strongest effect.

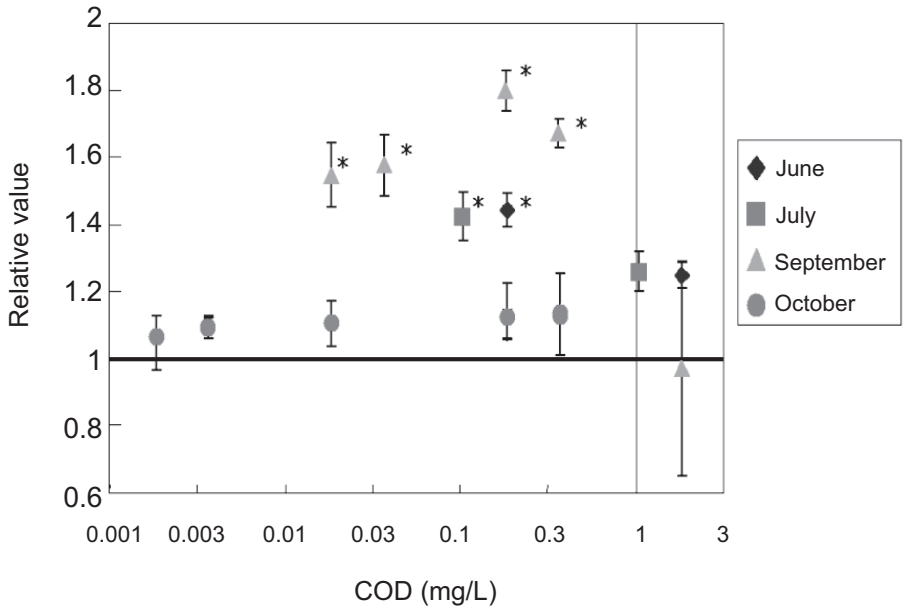

Fig. 1. Stress response of HSP cells incubated with EWWTP for 3 h. The stress response of treated cells was quantified by measuring their β-galactosidase activity versus that of the control. *Statistically significant versus negative control (t-test, $p<0.05$). Data represent results of three independent experiments (±SD).

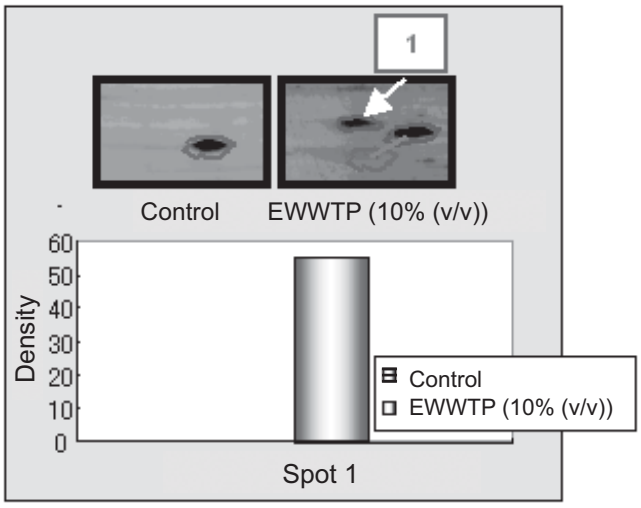

Fig. 2. Comparison between 2D protein profiles of Caco-2 cells incubated with or without 10% EWWTP for 3 h and intensity of spot 1.

August is the rainy season in Japan. During this time, abundant wastewater containing dust, pollutants, and hazardous compounds enters into wastewater treatment plants. Therefore, we consider that the abundant rain makes the environmental toxicity of samples in September worse.

To evaluate the effects of the effluent from EWWTP on the intestinal epithelial Caco-2 cells, a human intestinal epithelial cell line derived from a human colon carcinoma, we used 2D electrophoresis (2DE) and MS. The extracted proteins were first subjected to 2DE. The two spots indicated by arrows in Figs. 2 and 3 show the highest protein expression levels in EWWTP (September sample)-treated cells. To

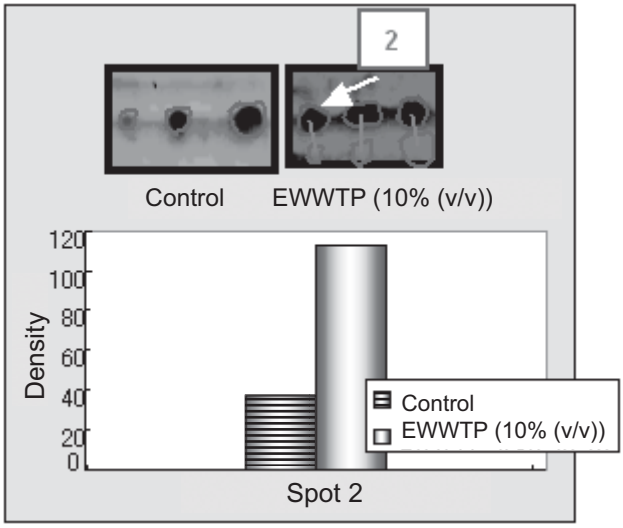

Fig. 3. Comparison between 2D protein profiles of Caco-2 cells incubated with or without 10% EWWTP for 3 h and intensity of spot 2.

Table 1
Overexpressed proteins as determined by proteomics analysis.

Spot number	Treatment	Protein	Molecular weight (kDa)	Isoelectric point (pI)	Spot density (untreated)	Spot density (treated)
1	EWWTP (10 % (v/v))	elongation factor 1β	30	4.0	0	55
2	EWWTP (10 % (v/v))	enolase 1	50	7.3	37	112

identify these proteins, the spot was subjected to tryptic digestion and MALDI-TOF mass analysis. Spots 1 and 2 were identified by MALDI-TOF MS as elongation factor 1β and enolase 1, respectively (Table 1).

Elongation factor 1β is known to be involved in the induction of allergic reactions, such as urticaria and itching. Studies have shown that elongation factor 1β contributes to allergic manifestation.[14–16] The overexpression of enolase 1 has been observed in various tumors.[17,18] The overexpression of enolase 1 is required in both energy and protein synthesis/degradation pathways in rapidly growing tumors. On the other hand, it has been reported that enolase 1 is overexpressed during oxidative stress.[19] Thus, the enhanced expressions of elongation factor 1β and enolase 1 by EWWTP-treated Caco-2 cells possibly represent a consortium of protective mechanisms against environmental toxicity.

In conclusion, this study has shown that incubating Caco-2 cells for 3 h with EWWTP is sufficient for enhancing the expressions of elongation factor 1β and enolase 1. These enhancements may possibly be responses against environmental toxicity. These results suggest that specific proteins, such as elongation factor 1β and enolase 1, can be used as biomarkers for the risk assessment of water and wastewater.

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