Effect of linear alkylbenzene sulfonate (LAS) on human intestinal Caco-2 cells at non cytotoxic concentrations

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Abstract Linear alkylbenzene sulfonate (LAS) is a cytotoxic synthetic anionic surfactant widely present in the environment due to its large-scale production and intensive use in the detergency field. In this study, we investigated the effect of LAS (CAS No. 25155-30-0) at non cytotoxic concentrations on human intestinal Caco-2 cells using different in vitro bioassays. As results, LAS increased Caco-2 cell proliferation at concentrations ranging from 1 to 15 ppm, more significantly for shorter exposure time (24 h), confirmed using flow cytometry and trypan blue exclusion methods. Moreover, proteomics analysis revealed that this effect was associated with an over-expression of elongation factor 2 and

dipeptidyl peptidase 3, and a down-regulation of 14-3-3 protein theta, confirmed at mRNA level using real-time PCR. These findings suggest that LAS at non cytotoxic concentrations, similar to those observed at wastewater treatment plants outlets, increases the growth rate of colon cancer cells, raising thereby its tumor promotion effect potential.

Keywords Linear alkylbenzene sulfonate · Caco-2 cells · Cell proliferation · Proteomics · Elongation factor-2 · Dipeptidyl peptidase 3 · 14-3-3 Protein theta

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Introduction

Toxicants released into the environment are generally found at concentrations much lower than those at

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which they may exert cytotoxicity. This is mainly due to the developments made in the treatment technologies used in the wastewater treatment plants, and the environmental regulations imposed by national and international legislations. In contrast, many of these toxicants were shown to have carcinogenic or tumor promotion effects at low concentrations. As examples, glyphosate, a cytotoxic organophosphate herbicide, showed a tumor promoting potential in skin HaCaT cells by increasing cell proliferation and preventing apoptosis at low concentrations (George and Shukla 2013). Also, it has been reported that exposure to low-concentration arsenic promotes cell proliferation and carcinogenesis both in vitro and in vivo (Wu et al. 2013). Such proliferative effects at low sub-lethal concentrations were observed for many other environmental toxicants such as phthalates, okadaic acid, etc. (Chen and Chien 2013; Del Campo et al. 2013).

Linear alkylbenzene sulfonates (LASs) are among the major anionic surfactants used in detergents, such as laundry powders and dishwashing products. The total annual consumption of LASs in Europe only is approximately 430,000 tons estimated for the period of 2005, of which nearly 350,000 tons are derived from household use as reported in HERA (Human & Environmental Risk Assessment on ingredients of European household cleaning products: Linear Alkylbenzene Sulphonate) (2013). After use, such detergent compounds are discharged into the environment, and contribute to the contamination of different environmental compartments (e.g. rivers, ground water, soils and seashore). The concentration of LASs in raw sewage ranges from 1 to 21 mg/l, but after treatment it decreases to a range of 0.008–5.91 mg/ml in treated effluent, depending on the treatment process used (Mungray and Kumar 2009). Furthermore, LASs are considered as non-carcinogenic (Yam et al. 1984) toxic compounds to different terrestrial and aquatic living organisms (Debelius et al. 2008; Krogh et al. 2007; Marin et al. 1991). However, their effect at low non cytotoxic concentrations on human cell line has not been well investigated.

Moreover, toxicologists all over the world are calling to reduce the animal use in toxicology tests and substitute them by animals and human cell lines and tissue as in vitro models (MacGregor et al. 2001). In this respect, and considering the relevance of intestine as a target organ for orally administrated contaminants, the human intestinal Caco-2 cell line,

widely used as in vitro cell model for cancer and toxicological studies (Natoli et al. 2009; Vicente et al. 2014), was selected to reproduce colon cancer cells.

The current study was designed to investigate LAS ($C_{12}H_{25}C_6H_4SO_3Na$) effect on human intestinal Caco-2 cells at low non-cytotoxic concentrations, using different bioassays. We additionally performed proteomics in order to identify some possible molecular actors associated with the observed enhanced proliferation in LAS-exposed Caco-2 cells.

Materials and methods

Cell culture

Human intestinal Caco-2 cells, provided by Dr. Makoto Shimizu of the University of Tokyo, Japan, were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (Biowest, Nuaillé, France), 1 % non-essential amino acids (Cosmo Bio, Tokyo, Japan) and 1 % Penicillin/Streptomycin (Lonza Walkersville, Inc., Walkersville, MD, USA) and incubated in a 95 % air and 5 % CO₂ atmosphere at 37 °C. Cell passages were carried out at a split ratio of 1:3, using 0.25 % trypsin (1 mM EDTA), when cell confluence reaches 80 %. All experiments were performed with cultures between passages 5 and 12.

Linear alkylbenzene sulfonate

Linear alkylbenzene sulfonate (LAS) used in this study was purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan (Cat. No. 195-07682) as crystalline powder, having the following CAS NUMBER: 25155-30-0, and the formula " $C_{12}H_{25}C_6H_4SO_3Na$ ". LAS solutions used for all experiments were prepared in the culture media already set for caco-2 cell culture.

Cell proliferation

Cell proliferation was assessed using the conventional MTT reduction assay. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was purchased from DOJINDO (Tokyo, Japan). The cultured Caco-2 cells in 96-well plates at 10⁵ cell/ml density, were treated with different concentrations of LAS: 1, 5,

10 and 15 ppm, for 24 and 48 h, then 10 μ l of MTT stock solution (5 mg/ml) was added to the culture medium and incubated for 6 h at 37 °C. The formazan was dissolved in 100 μ l 10 % SDS (W/V). Cell proliferation was determined as the mean of absorbance at 570 nm using a microliter plate reader, and expressed as the percentage of the control.

Cell number and cell viability

The cell number and viability were assessed using flow cytometry according to the manufacturer instructions. Briefly, Caco-2 cells were seeded at a cell density of 2×10^5 cell/ml in Petri dishes. After 24 h, cells were treated with 5 and 15 ppm LAS for 24 h, while control cells were not treated. Then, cells were harvested, suspended in Guava ViaCount reagent and allowed to be stained for at least 5 min in darkness. The cell number and viability were measured by Guava PCA flow cytometry (Guava Technologies, Hayward, CA, USA).

Intracellular ROS measurement

The determination of intracellular ROS was performed using OxiSelect™ Intracellular ROS Assay Kit, from CELL BIOLABS, INC. (San Diego, CA, USA), according to the manufacturer protocol. Caco-2 cells were cultured in clear bottom black 96-well plate for 24 h, then pre-incubated for 60 min with DCFH-DA (2', 7'-Dichlorodihydrofluorescein diacetate). For test plates, LAS solution of 5 ppm was then added to the cells. Cells in control plates were not treated. H₂O₂ at 100 μ M was used as positive control. After different incubation times of 3, 12 and 24 h for LAS treatments and 1 h for H₂O₂ treatment, the DCF fluorescence was measured using plate reader at 480/530 nm excitation/emission wavelengths and results were presented as percentage of control of DCF fluorescence intensity.

Proteomics

Protein extraction and quantification

Caco-2 cells were seeded at a cell density of 2×10^5 cell/ml density in Petri dishes. After 24 h, cells in assay dishes were treated with 5 ppm LAS for 24 h, while control cells were not treated. Then total proteins were extracted in lysis buffer as described

by Tsolmon et al. (2011). After centrifugation, supernatant was assayed for protein content by Plus One 2D Quant kit (GE Healthcare, Piscataway, NJ, USA), following the manufacturer's instructions, and then stored at −80 °C.

Two-dimensional gel electrophoresis (2-DE)

First dimension was carried out using the EttanIPGphor II (Amersham Biosciences) as previously described by Tsolmon et al. (2011). Briefly, samples containing equal quantities of protein (400 mg) were diluted to 350 μ l with rehydration buffer, then subjected to Isoelectric focusing (IEF) after overnight rehydration on ImmobilineDryStrips (24 cm; pH 3–10 linear). Focusing was performed as follows: 1 h at 500 V, 1 h at 1000 V, 3 h at 10,000 V and 2 h 45 min at 10 000 V for a total of 56 kVh. After equilibration with DTT (Dithiothreitol) and iodoacetamide-containing buffer, strips were transferred onto 10 % vertical slab gels and SDS-PAGE was run at 280 Wh on EttanDaltSix electrophoresis system from Amersham Biosciences.

2-DE gels image analysis

Separated proteins were stained with Coomassie R-350 stain (GE Healthcare) in 30 % methanol and 10 % acetic acid. Gels were scanned at 600 dpi resolution and differential spot expression was performed using ImageMaster™ 2D Platinum 5.0 software (Amersham Biosciences). After automatic spot detection and matching, the authenticity and outline of each spot was validated by eyes and edited manually where necessary. The pairs were labeled with annotations that define tie points for gel matching. Then, after normalization to the volume of all spots in the gel, the normalized volume for each spot in the control gel was compared to the normalized volume of matched spot in the treated samples gels.

In-gel digestion and mass spectrometry

As previously described by Tsolmon et al. (2011), spots were excised and treated with the destaining solution (25 mM ammonium bicarbonate NH₄HCO₃ in 50 % Acetonitrile; Wako, Tokyo, Japan). Gel pieces were washed in 100 ml acetonitrile for 5 min and briefly dried at room temperature followed by

reduction with 10 mM DTT in 25 mM NH_4HCO_3 and alkylation with 55 mM iodoacetamide in 25 mM NH_4HCO_3 . Spots were digested overnight by 10 mg/ml trypsin in 50 mM NH_4HCO_3 at 37 °C. Ten microliters of peptide mixtures were analyzed by on-line capillary UltiMate 3000 proteomics MDLC system (Dionex, Sunnyvale, CA, USA) coupled to a nanospray 3200QTrap MS/MS system (Applied Biosystems). The obtained peaks were searched using MASCOT search engine against Swiss-Prot database consisting of *Homo sapiens* sequences. The proteins with ion scores greater than 34 were significant for the Swiss-Prot database ($p < 0.05$).

Gene expression: real-time PCR

The expression of elongation factor 2 (EF2), dipeptidyl peptidase 3 (DPP3) and 14-3-3 protein theta (14-3-3T), in treated and non-treated Caco-2 cells, were determined by real-time PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal positive control. Primers used for these experiments were purchased from Applied Biosystems. Primers for EF2 (Hs01555212_g1), DPP3 (Hs00189032_m1), 14-3-3T (Hs03045200_g1) and GAPDH (Hs02758991_g1) were inventoried TaqMan® Gene Expression Assays. DNA-free total RNA of cultured cells was isolated with Isogen kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Briefly, cells were seeded at 2×10^5 cell/ml in Petri dishes, cultured for 24 h and then exposed to 0 (non-treated: control), 5 and 15 ppm LAS for 24 h. Isolated RNA was ethanol precipitated, quantified and quality assessed by Nanodrop ND-1000 spec-trophotometer. About 1 mg of total RNA was submitted to reverse transcription with Superscript III (Invitrogen) using oligo(dT) primers according to the manufacturer's protocol and cDNA was synthesized using thermal cycler (Applied Biosystems, Waltham, MA, USA). cDNA amplification reactions were run on Applied Biosystems 7500/7500 fast RT-PCR system.

Statistical analysis

Data are presented as mean $\pm$ SD. Statistical analyses of changes, for each time and concentration point compared to control, were performed using a paired two-tailed Student's *t* test. A p -value < 0.05 was considered statistically significant.

Fig. 1 Reduction of MTT by Caco-2 cells after exposure to different LAS concentrations for 24 and 48 h; cells were seeded in 96-well plates at 10^5 cell/ml; cell proliferation was determined as the mean of absorbance at 570 nm and expressed as the percentage of control; results represent the mean $\pm$ SD of at three independent experiments; *significant increases compared to the control: * $p < 0.05$; ** $p < 0.01$

Results and discussion

The effect of LAS on the cell proliferation in human intestinal Caco-2 cells was assessed for a range of low concentrations (1–15 ppm LAS) and two different exposure times (24 and 48 h) using MTT assay. As shown in Fig. 1, LAS significantly increased Caco-2 cell proliferation, more importantly for 24 h, to reach a maximum level of 147 ± 13 % of control at 15 ppm, which is interestingly the opposite of LAS cytotoxic

Fig. 2 Cell number and viability of Caco-2 cells exposed to 5 and 15 ppm LAS for 24 h; cells were seeded at 2×10^5 cell/ml in Petri dishes; control represents non-treated Caco-2 cells; cell number and cell viability were measured using flow cytometry; results represent the mean $\pm$ SD of three independent experiments; **significant difference from the control ($p < 0.01$)

effect, previously reported for concentrations higher than 50 ppm and exposure times longer than 24 h (Bradai et al. 2014).

Additionally, cell number and viability of LAS-treated Caco-2 cells at 5 and 15 ppm were investigated using flow cytometry. As shown in Fig. 2, total cell number increased significantly in LAS-treated Caco-2 cells compared to the control ($8.22 \times 10^6 \pm 0.59 \times 10^6$ cell/ml and $1.08 \times 10^7 \pm 0.66 \times 10^6$ cell/ml for 5 and 15 ppm LAS, respectively, compared to $5.23 \times 10^6 \pm 0.29 \times 10^6$ cell/ml for control). Furthermore, a slight decrease in the cell viability was noticed when LAS concentration raised up to 15 ppm (Fig. 2). This effect was also observed at similar LAS concentrations using Trypan blue exclusion method (Fig. S1).

It is well known that reactive oxygen species (ROS) signaling is involved in cell proliferation and survival as highlighted in different studies proving that ROS act as positive regulator of cell proliferation (Ray et al. 2012; Sauer et al. 2001). Moreover, intestinal epithelial cells proliferative activity was demonstrated to be associated with the oxidative challenge which stimulates proliferative responses (Aw 2003). Therefore, the intracellular ROS level changes were investigated in non-exposed and LAS-exposed Caco-2 cells to 5 ppm LAS for different treatment times (Fig. 3). Results showed a slight but significant ROS level increase in LAS-exposed Caco-2 cells after 12 and 24 h exposure. These results suggest that the observed increase in

Fig. 3 Intracellular ROS level in Caco-2 cells exposed to 5 ppm LAS for 3, 12 and 24 h, and 100 μ M H₂O₂ for 1 h; Cells were seeded in 96-well plates at 10^5 cell/ml; results are expressed as fold of control, where control represent non-exposed Caco-2 cells; data represent the mean $\pm$ SD of at least three independent experiments; *significant difference from the control: * $p < 0.05$; ** $p < 0.01$

LAS-exposed Caco-2 cells proliferation is associated with increased ROS production. In accordance with our findings, it was demonstrated that pro-oxidant state plays an important role in the proliferative capacity of cancer mouse keratinocyte cell line, providing evidence for a functional role of elevated ROS levels in tumor promotion (Gupta et al. 1999).

LAS is known to be a non-cancer inducer compound (Yam et al. 1984) and even though Caco-2 cells express estrogen receptors (Campbell-Thompson et al. 2001), LAS and its degradation products have been reported to be not estrogenic (Navas et al. 1999). Interestingly, our findings suggest that LAS at non cytotoxic concentrations and for short exposure time (24 h) increases colon cancer cells proliferation. This effect is also known for some chemicals which are not carcinogenic, but have a selective effect of tumor promotion on initiated cells, such as cholic acid, which is a colon cancer promoter that has effect only on aberrant colon cells (initiated cancer cells) via increasing their proliferation (Corpet et al. 1997).

In order to investigate some possible molecular actors associated with LAS-induced proliferation in Caco-2 cells, proteomics approach was performed. In this respect, protein expression changes occurring in 5 ppm LAS treated Caco-2 cells for 24 h. This concentration was chosen because, in contrast with 15 ppm, it showed increased cell proliferation only with no cytotoxicity or reduced viability (Fig. 2), thus avoiding the apparition of molecular changes related to cytotoxicity. The expression patterns of protein spots appearing on CBB-stained 2-D gels were analyzed using ImageMaster™ 2D platinum 5.0 software, and spots whose expressions were significantly changed in protein profiles of LAS-exposed cells versus non-exposed cells, with a percentage of volume modification exceeding 30 %, were selected for identification (Fig. 4).

The selected protein spots were identified by LC/MS/MS on the basis of peptide mass matching with theoretical peptide mass in tryptic digests of all known proteins of human species. The identified proteins with their theoretical and observed pI and M_w values, accession numbers and scores are presented in Table 1. The analysis of the identified proteins was conducted regarding their background information, functions and implication in cell proliferation, cell cycle and cancer, and accordingly, three proteins: Elongation factor 2 (EF2), Dipeptidyl peptidase 3

Fig. 4 Representation of 2-Dimensional CBB-stained gel illustrating exemplary protein expression in Caco-2 cells with enlarged images of differentially expressed proteins after exposure to 5 ppm LAS for 24 h (a); Relative volumes (fold change of control) of protein spots (b); Spots volumes were obtained using ImageMaster™ 2D platinum software; results represent the mean $\pm$ SD of two independent experiments; *significant difference from the control ($p < 0.05$)

Table 1 Summary of differentially expressed proteins in Caco-2 cells exposed to 5 ppm LAS for 24 h

Spot no.	Accession no.	Score	Calculated <i>pI</i> /observed <i>pI</i>	Calculated Mw/observed Mw (kDa)	Protein name	Function category
1	P13639	404	6.41/7.00	96/102	Elongation factor 2	Translation elongation factor activity
2	Q9NY33	171	5.02/5.20	82/76	Dipeptidyl peptidase 3	Dipeptidyl-peptidase activity
3	P27348	155	4.68/4.75	28/26	14-3-3 protein theta	Regulation of signaling pathways
4	P11142	318	5.37/5.50	71/65	Heat shock cognate 71 kDa protein	Chaperone/stress response
5	Q14697	101	5.74/6.00	107/110	Neutral alpha-glucosidase AB	glucan 1,3-alpha-glucosidase activity
6	Q9P157	36	5.92/6.00	71/65	Serum albumin	Bending protein/transport

(DPP3) and 14-3-3 protein theta (14-3-3T), were selected for further investigation.

It is well known, that in protein synthesis, the elongation step is critically governed by EF2 which is solely responsible for translocation of codons from A to P ribosome sites. The overexpression of this protein

is observed in many cancers (ovarian, gastric and colon cancers) (Alaiya et al. 1997; White-Gilbertson et al. 2009). In fact EF2 influences cell cycle progression and enhances the cell growth through the promotion of G₂/M phase progression as reported by Nakamura et al. (2009) for gastrointestinal cancer

cells. Proteomics results showed that the LAS-induced increase in Caco-2 cells proliferation was associated with the overexpression of EF2 (spot 1 in Fig. 4) and this was confirmed at mRNA level using real-time PCR for treatments with 5 and 15 ppm LAS for 24 h (Fig. 5), where the EF2 mRNA was significantly overexpressed in both treatments (3.79 ± 0.83 and 3.37 ± 0.28 fold of control for 5 and 15 ppm LAS treatments, respectively).

Furthermore, the importance of aminopeptidases in cell proliferation has been revealed by several studies which demonstrated the growth modulatory effects of aminopeptidase inhibitors (Ino et al. 1992, 1994; Sekine et al. 2001). The aminopeptidase DPP3 has been reported to be associated with few cancers. In fact it was found to be overexpressed with increased activity in ovarian and endometrial cancers (Simaga et al. 1998; Prajapati and Chauhan 2011), more markedly with the aggressiveness of the cancer (Simaga et al. 2003). In this study, Proteomics results revealed that LAS at concentration of 5 ppm induced the overexpression of DPP3 in Caco-2 cells after 24 h of exposure (spot 2 in Fig. 4). These results were confirmed at gene level in Caco-2 cells exposed to 5 and 15 ppm LAS for 24 h (Fig. 5), and similar pattern for both treatments were observed (4.88 ± 1.02 and

3.28 ± 0.23 fold of control for 5 and 15 ppm LAS treatments, respectively) which suggest the involvement of DPP3 in the LAS-induced proliferation effect on Caco-2 cells at those concentrations.

14-3-3 proteins play key roles in the regulation of central physiological pathways such as mitogenesis, cell survival signaling, cell cycle and apoptosis (van Hemert et al. 2001). They are also involved in the regulation of various oncogenes and tumor suppressor genes (Tzivion et al. 2006). Some 14-3-3 isoforms are negative regulators of cell cycle and are down-regulated in different cancers (ovarian, prostate and endometrial carcinomas) (Mhawech et al. 2005; Urano et al. 2004). 14-3-3T negatively regulates the kinase activity of PDPk1 (3-phosphoinositide-dependent protein kinase 1) (Sato et al. 2002), which is crucial for cell proliferation and cell cycle progression (Nakamura et al. 2008), and an activator of NF-kappa B pathway (Lee et al. 2005) which in its turn is well recognized as central activator of the anti-apoptotic cascades (Escárcega et al. 2007). Consequently, the down-regulation of 14-3-3T has a pro-survival impact on cells and promotes cell proliferation. In our study, LAS treatment at 5 ppm in Caco-2 cells significantly down-regulated 14-3-3T at translational level (0.44 ± 0.12 fold of control) (spot 3 in Fig. 4), confirmed as well at mRNA level (Fig. 5) for both 5 and 15 ppm LAS after 24 h of exposure, where gene expression decreased to 0.78 ± 0.27 and 0.71 ± 0.08 fold of control, respectively.

Fig. 5 Relative gene expression of Elongation factor 2 (EF2), Dipeptidyl peptidase 3 (DPP3) and 14-3-3 protein theta (14-3-3T) in Caco-2 cells exposed to 5 and 15 ppm LAS for 24 h. Cells were seeded at 2×10^5 cell/ml in Petri dishes; data are expressed as fold-change of control; EF2, DPP3 and 14-3-3T mRNA levels are normalized to GAPDH mRNA levels; results represent the mean $\pm$ SD of three independent experiments; *significant differences from the control: $*p < 0.05$; ** $p < 0.01$

Conclusions

Taken together, our data suggest that LAS, at concentrations similar to those observed in wastewater treatment plants outlets, may have a tumor promotion effect on colon cancer cells through increasing cell proliferation, in association with the overexpression of EF2 and DPP3 and the down-regulation of 14-3-3T. Therefore, more attention should be paid to the adverse effects of toxicants even at low non-cytotoxic concentrations. The use of human in vitro cell models can provide important additional data on toxicological effects. Thus it can help in the improvement of the scientific basis of risk assessment through targeting different endpoints thanks to the increasing list of available normal and cancer cell lines obtained from different tissue origins.

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Conflict of interest The authors have no conflict of interest to disclose.

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