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The effects of fluoride on cell migration, cell proliferation, and cell metabolism in GH_4C_1 pituitary tumour cells

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ABSTRACT

The consumption of drinking water rich in fluoride has toxic effects on the central nervous system. In cell biology research, fluoride is currently used as a phosphatase inhibitor. The aim of the present study was to evaluate the effect of fluoride on different physiological processes in GH_4C_1 pituitary tumour cells. We used a range of different fluoride concentrations, from levels below normal human serum concentrations (0.23 and 1.2 μmol/L) to those observed in chronically exposed persons (10.7 μmol/L) and above (107 and 1072 μmol/L). Treatment of 10.7 μmol/L fluoride resulted in a discrete induction of DNA synthesis, without a change in cell number. Cell migration, a behaviour stimulated by growth factors, was increased in cells treated with 2.4 μmol/L. At this fluoride concentration, changes in phosphorylation status of both cytoskeletal and cytosolic protein fractions, as well as in actin cytoskeletal arrangements were observed. The GH_4C_1 fluoride treated cells had significantly less cellular protein than control cells, suggesting an effect of fluoride on hormone secretion and protein synthesis in this endocrine cell. The bioreduction of MTT was significantly increased with a wide range of fluoride concentrations. With the highest fluoride concentration, 1072 μmol/L, all of the analysed parameters were significantly reduced, suggesting that this dose is highly toxic in GH_4C_1 cells. Our results show that biologically relevant concentrations of fluoride are capable of increasing cell migration in tumour cells, suggesting that exposure to fluoride could stimulate tumour invasion.

1. Introduction

Fluorine is the most electronegative and reactive element, and it commonly forms salts as fluoride, such as sodium fluoride (NaF). In this state, fluoride presents an oxidative state of −1 (F^-). Fluoride occurs naturally in the earth's crust and is the 13th most abundant element (Riedel, 2004). It occurs naturally in many bodies of water due to the weathering of volcanic rock and elution of soil. The concentration of fluoride depends on diverse physical, chemical and geological factors, such as the composition of the soil, the firmness and porosity of rocks, the pH of the surroundings, the deepness of the water level in wells and volcanic activity (ATSDR, 2003), and it also depends on anthropogenic activity. The major sources of fluoride in humans are through diet (water, food, and beverages), and fluoride-containing dental products (ATSDR, 2003). Therefore, the majority of fluoride enters the body via the intestinal epithelium (Monsour and Kruger, 1985; Martinez-Mier et al., 2003). In Mexico, the primary source of fluoride is water, which is taken from naturally contaminated aqueous mantles situated in Sonora, San Luis Potosí and the "Comarca Lagunera" (Del Razo et al., 1993; Hurtado-Jiménez and Gardea-Torresdey, 2004). Several million people are exposed worldwide to high concentrations of fluoride by consuming drinking water (NRC, 2006). Aside from skeletal and dental fluorosis, a diminished intellectual capacity, diminished cardiac contractility, hepatic and renal degeneration and necrosis, and a decrease in fertility are often observed in endemic fluoride areas (Grimaldo et al., 1995; Rocha-Amador et al., 2007; Xiong et al., 2007).

Among the systemic toxic manifestations in humans, the main toxic effect of fluoride on cellular metabolism is its interaction with enzymes. In most cases, fluoride inhibits enzymatic activity, but in other cases, fluoride ions actually stimulate enzymatic activity. The mechanism depends on the type of enzyme that is affected. Fluoride ions or hydrogen fluoride can bind to functional amino acid groups surrounding the active centre of an enzyme. Fluoride ions can also induce significant conformational changes by binding to positively

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charged residues far away from the active centre. In general fluoride ions are capable of binding to metalloenzymes containing Mg^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Mo^{4+} or Ni^{2+} (in order of affinity) in their active centre. Phosphatases and kinases that require Mg^{2+} as cofactor are inhibited by fluoride due to the formation of a quaternary enzyme–substrate/phosphate–Mg^{2+}–F^- complex or the effect of fluoride on the phosphorylation process. This inhibitory capability of fluoride is used in research when phosphatases, especially acid phosphatases, need to be inactivated (Adamek et al., 2005). Moreover, it is well known that inhibition of phosphatases can activate intracellular pathways such as growth factors and can induce proliferation (Schlessinger, 2000). With respect to cellular function, one of the most important cellular activities, cell motility, is a cycle of membrane protrusion, formation of new cell attachment processes, and detachment of the oldest processes. Phosphorylation and de-phosphorylation of the protein machinery are key events that allow this cycle, and growth factors are important activators (Horwitz and Parsons, 1999; Vicente-Manzanares et al., 2005). As fluoride affects different molecular targets, it is possible that fluoride induces important changes in cell migration behaviour. However, few studies have been performed to investigate the role of fluoride as an element that is altered in the extracellular milieu and can affect the migration behaviour of cells. It is important to know how tumour cells are affected by the presence of fluoride, as these cells have a more active migration capacity. To this end, we decided to study a tumour cell line, the rat pituitary GH_4C_1 cell line, in order to analyse the effect of fluoride on cell migration and proliferation. It is important to note that these cells secrete growth hormone and, to a lesser extent, prolactin. In previous studies, we have observed that these cells exhibit significant migration activity (Azorín et al., 2008).

2. Materials and methods

2.1. Materials

F-10 culture medium, horse serum, foetal bovine serum, transferrin, Dulbecco's phosphate buffered saline (PBS), trypsin, and soybean trypsin inhibitor were purchased from GIBCO, USA. TO-PRO 3 iodide was purchase from Molecular Probes, Invitrogen (USA). All other substances were purchase from Sigma Chem. Co. (USA).

2.2. Cell culture

GH_4C_1 pituitary tumour cells (Tashjian et al., 1968) were purchase from American Type Culture Collection (USA). All of the assays were performed with the same batch of cells that were from the second passage of the cell aliquot obtained directly from ATCC. Cells were cultured with F-10 medium supplemented with 292 μg/mL L-glutamine, 15% horse serum, and 2.5% foetal bovine serum. After the cells were harvested, they were challenged with different concentrations of NaF in a culture medium enriched with serum for 24 h. The NaF concentrations used in the present study were equivalent to 0.23, 1.2, 2.4, 10.7, 107, and 1072 μmol/L of fluoride. We evaluated effects of low and high concentrations of fluoride because of a reported hormesis effect of fluoride exposure (NRC, 2006), which could explain some of the paradoxical effects associated with this contaminant.

2.3. MTT viability and proliferation assay

The MTT assay is based on the transformation of the yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenases (primarily succinate dehydrogenase; Slater et al., 1963). Aliquots of 4500 GH_4C_1 cells were seeded into each well of a 96-well plate culture with serum-enriched medium. After 5 days, the culture medium was replaced with fresh serum-enriched medium and challenged with different concentrations of fluoride. The MTT assay was performed after 20 h. Because we needed to inhibit cell proliferation in the migration assay, proliferation curves were performed to obtain an effective inhibitory mitomycin-C using the MTT proliferation assay. The optimal inhibitory concentration of mitomycin-C for GH_4C_1 cell proliferation was 5 ng/mL.

2.4. Cell proliferation capacity assay

The ^{3}H-thymidine incorporation assay was used to investigate the effect of fluoride on GH_4C_1 cell proliferation. Aliquots of 400,000 cells/well were seeded into a 24-well cell culture cluster plate and incubated with serum-enriched medium for 6 h, followed by a 24-h culture period with defined medium (without foetal bovine serum) in order to arrest the cell cycle. At the end of this period the medium was replaced with a serum-enriched medium containing different concentrations of fluoride. The cells were incubated for 20 h, followed by a 4-h incubation period in the presence of 10 μCi ^{3}H-thymidine. The non-incorporated ^{3}H-thymidine was washed away, and the cell proteins were precipitated with 5% (w/v) trichloroacetic acid solution followed by an ethanol wash. The proteins were then dissolved into 0.1 M NaOH, and neutralised with 0.1 M HCl. The tritium content was measure using a liquid scintillation counter.

2.5. Migration assay

To analyse the migration behaviour of GH_4C_1 cells exposed to different concentrations of fluoride, we used the Boyden chamber system (Mertsch et al., 2008). The Boyden chamber assay is based on the capacity of cells to migrate through a porous membrane placed between two compartments. Cells that have passed through the membrane are collected on a poly-lysine covered cover slip placed in the lower compartment. For these experiments, cells were inhibited with 5 ng/mL of mitomycin-C in order to avoid changes in cell number due to cell proliferation. Aliquots of 300,000 cells in serum-enriched medium were placed in the Boyden chamber (Millicell, Millipore, USA), and different concentrations of fluoride were added. After 24 h, migrated cells were fixed with 3% (v/v) paraformaldehyde in PBS for 20 min and stained with Toluidine Blue. The number of migrated cells was obtained by counting the cells in 10 fields observed with a direct microscope and a 20× objective.

2.6. Cell number and adhesion assay

To evaluate cell number, the cresyl violet assay was used (Kueng et al., 1989; Quesney et al., 2001; Carrington et al., 2006; Zeng et al., 2008). Aliquots of 20,000 cells/mL were cultured for 24 h, and then the medium was replaced with fresh medium, and different concentrations of fluoride were added. After a 24-h culture period, a 2× fixation solution was added (2% paraformaldehyde, 0.2% picric acid and 2.5% sucrose in PBS), and cells were fixed for 48 h at 4 °C. The cells were then washed, dried, and dyed with 0.1% cresyl violet acetate for 30 min. Cells were washed and dried again, and a solubilisation solution of 10% acetic acid was added to the cells. The coloured solution was quantified using a spectrophotometer (Labsystems Multiscan, Labsystems, Finland) at 590 nm. In order to evaluate cell adhesion capacity, cells were initially processed in the same way as above for the cell number evaluation. These cells were then incubated for 24 h in medium containing fluoride. The cells suspended in the culture medium were collected, and the cells adhered to the bottom of the culture well were washed twice with PBS supplemented with Ca^{2+}. The PBS of the washes was taken together with the incubation medium. The cells suspended in the medium and washing PBS were centrifuged at 800 rpm for 10 min. The precipitated cells and the cells attached to the bottom of the well were processed with the cresyl violet technique. For protein quantification, the same protocol was used. However, after the 24 h incubation with fluoride, the cells were washed with PBS, disrupted with deionised water and scrapped. Protein quantification of the cell samples was performed using the micro BCA Protein Assay kit (Pierce, USA) following the provider's instructions and using bovine albumin (reagent pure, Research Organics, USA) as a protein reference.

2.7. Actin cytoskeleton arrangement assay

To visualise the actin cytoskeleton arrangement of GH_4C_1 cells, a rhodamine–phalloidin conjugate was used. Cells were incubated with serum-enriched medium supplemented with different concentrations of fluoride for 24 h. At the end of the fluoride exposure period, cells were fixed with 3.5% paraformaldehyde, 5% sucrose, and 3 mM $MgCl_2$, in PBS without Ca^{2+} for 30 min. The cells were then permeabilised with 0.1% Triton-X100 in PBS for 3 min, and F-actin was stained with phalloidin conjugated with rhodamine. Cover slips were mounted with Vectashield (Vector Lab., USA) and observed with a confocal microscope (Leica TCS SP2, Leica, Germany) using a 63× optical objective.

2.8. Western blot

The cytoskeletal or triton-insoluble fraction and the cytosol or triton-soluble fraction of cells exposed to different concentrations of fluoride for 24 h period were obtained with PHEM buffer [20 mM PIPES, 25 mM HEPES, 2 mM EDTA, 4 mM $MgCl_2$, 1 mM NaF, 10 mM Na_3VO_4, protease inhibitor cocktail (Complete, Roche, DE), 1 mM PMSF with or without 0.1% (v/v) Triton X-100, pH 7.4] described by Toral et al. (2007). The fractions were lysed with RIPA buffer [150 mM NaCl, 50 mM Tris, 10% (v/v) glycerol, 0.1% (v/v) SDS, 1% (v/v) Triton X-100, 23 mM deoxycholic acid, 5 mM NaF, 2 mM Na_3VO_4, protease inhibitor cocktail, 4 mM PMSF, pH 7.4]. After sample protein quantification, 40 μg/protein/sample of soluble or insoluble fractions were resolved by 10% SDS-PAGE. Phosphorylated proteins in tyrosine and serine protein kinase C (PKC)-substrates in both fractions were determined by Western blotting using 1:1000 primary antibodies for both phospho-residues and secondary antibodies conjugated with peroxidase. Actin was used as a loading control.

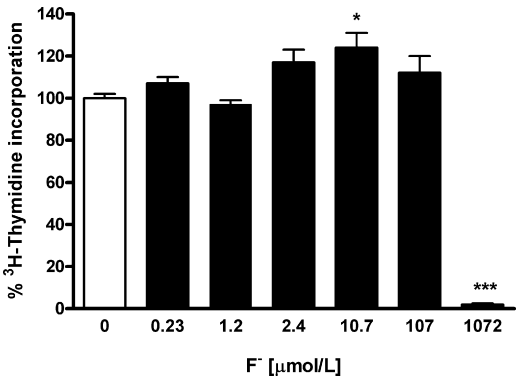

Fig. 1. Percent of ^{3}H-thymidine incorporation of GH_4C_1 cells incubated for 24 h with different fluoride concentrations. Values were normalized to percent with respect to control group. Open bar corresponded to control cells and filled bars corresponded to fluoride treated cells. Values are expressed as mean ± SE of four experiments. $^*p < 0.05$ and $^{***}p < 0.001$ control compared to fluoride treated groups.

2.9. Data analysis

All values are expressed as the mean ± SEM of at least three independent experiments. Statistical analysis of the cell migration data was performed using Kruskal–Wallis non-parametric ANOVA followed by Dunn's multiple comparison test. Results of the other assays were statistically analysed by one-way ANOVA followed by the Tukey–Kramer multiple comparison test. These statistical tests were performed using the statistical software GraphPad InStat 1.14 v. 3 (San Diego, CA, USA). Differences between treatments were considered significant when the p-value was less than 0.05.

3. Results

3.1. Effect of fluoride on GH_4C_1 cell DNA synthesis

The fluoride concentration range used in this study was from 0.23 (a concentration below normal serum levels) to 1072 μmol/L. We studied the capacity of GH_4C_1 cells challenged with fluoride for 20 h to enter into the S-phase of the cell cycle by measuring thymidine incorporation. GH_4C_1 cells showed a rate of thymidine incorporation of 93,866 ± 5106 dpm. As shown in Fig. 1, GH_4C_1 cells cultured with serum and with different concentrations of fluoride displayed an increased cell proliferation rate only with 10.7 μmol/L fluoride. It was clear that the highest concentration of fluoride, 1072 μmol/L, inhibits DNA synthesis, as almost no incorporation was observed (Fig. 1).

3.2. Effect of fluoride on GH_4C_1 cell number

In order to evaluate the effect of different concentrations of fluoride on GH_4C_1 cell number we used the cresyl violet acetate method. This method has been extensively used to measure changes in cell number (Kueng et al., 1989; Quesney et al., 2001; Carrington et al., 2006; Zhang et al., 2008). Fig. 2 shows the percent of cresyl violet in GH_4C_1 cells for the different experimental conditions used. No net change in cell number could be observed in the cells incubated with 0.23–107 μmol/L fluoride (Fig. 2). However, a significant reduction in cell number, 65%, was observed with a fluoride concentration of 1072 μmol/L (Fig. 2).

3.3. Effect of fluoride on MTT metabolism in GH_4C_1 cells

In Fig. 3, the effects of different fluoride concentrations on MTT reduction activity in GH_4C_1 cells are shown. Cells showed an increase in MTT metabolism when fluoride was present at concentrations of 2.4, 10.7 and 107 μmol/L. The highest fluoride concentration, 1072 μmol/L, induced a significant decrease (42%) in MTT metabolism.

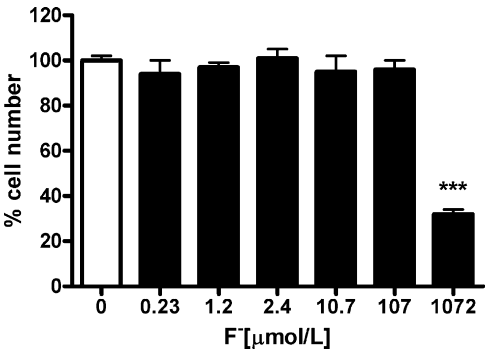

Fig. 2. Percent of cell number measurement of GH_4C_1 cells incubated for 24 h with different fluoride concentrations. Changes in cell number were determined by cresyl violet cell staining method. Values were normalized to percent with respect to control group. Open bar corresponded to control cells and filled bars corresponded to fluoride treated cells. Values are expressed as mean ± SE of three independent experiments. $^{***}p < 0.001$ control compared to fluoride treated groups.

3.4. Effect of fluoride on proteins in GH_4C_1 cells

Cellular protein quantifications of GH_4C_1 cell lysates incubated for 24 h with different concentrations of fluoride are presented in Fig. 4. In the presence of fluoride, a decrease in cellular protein

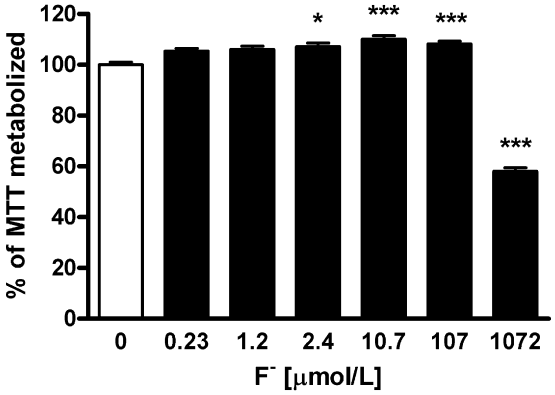

Fig. 3. Percent of MTT metabolised by GH_4C_1 cells incubated with different fluoride concentrations during a 24-h period. Values were normalized to percent with respect to control group. Open bar corresponded to control cells and filled bars corresponded to fluoride treated cells. Values are expressed as mean ± SE of three independent experiments. $^*p < 0.05$ and $^{***}p < 0.001$ control compared to fluoride treated groups.

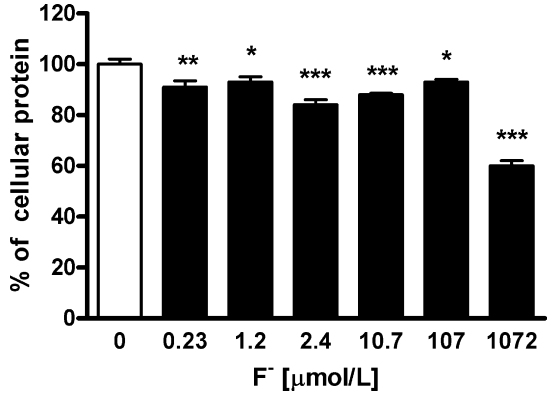

Fig. 4. Percent of total protein content of GH_4C_1 cells incubated for 24 h with different fluoride concentrations. Values were normalized to percent with respect to control group. Open bar corresponded to control cells and filled bars corresponded to fluoride treated cells. Values are expressed as mean ± SE of four independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ control compared to fluoride treated groups.

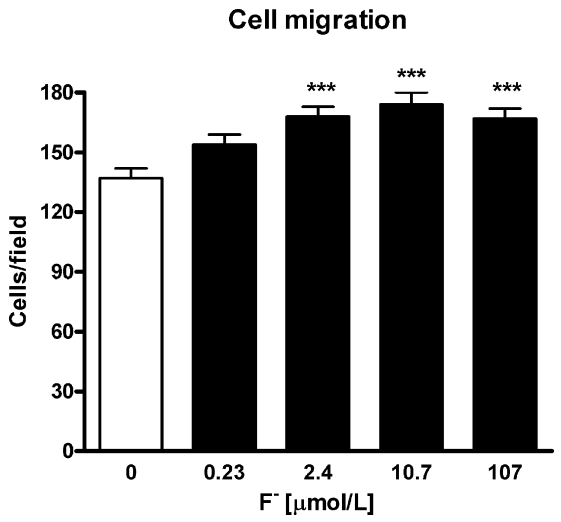

Fig. 5. Cell migration in a Boyden chamber system of GH_4C_1 cells treated with different fluoride concentrations. Cells were allowed to migrate during a 24-h period, fixed and stained. After were counted from ten random fields using a light microscope and a 20× objective. Open bar corresponded to control cells and filled bars corresponded to fluoride treated cells. Values are expressed as mean ± SE of three independent experiments. ***$p < 0.001$ control compared to fluoride treated groups.

concentration was observed, and the magnitude of the decrease changed according to the fluoride concentration used. However, no correlation was observed between the magnitude of protein reduction and fluoride concentration.

3.5. Effect of fluoride on cell migration and adhesion

GH_4C_1 cells were assayed for cell migration in a Boyden chamber. Before the cells were assayed, they were treated with 5 ng/mL mitomycin-C. Cells were allowed to migrate for a 24-h period with or without fluoride (Fig. 5). The rate of cell migration was 137 ± 5 cells/field/24 h for cells without fluoride. Treatment with 2.4 μmol/L fluoride induced a 22% increase in the rate of migration. A similar increase (27.5% and 21.5%) was observed with 10.7 and 107 μmol/L fluoride respectively (Fig. 5). Cell adhesion decreased (without statistical significance) 17 ± 2%, 23 ± 2.5%, 26 ± 2% with 2.4, 10.7 and 107 μmol/L fluoride respectively. However, a significant change in cell detachment was observed with the fluoride concentration of 1072 μmol/L, with more cells in suspension than attached. Only 13.3 ± 1.7% of cells were maintained attached.

3.6. Actin cytoskeleton arrangement

Fig. 6 shows the filamentous actin arrangements of GH_4C_1 cells cultured in enriched medium and increasing concentrations of fluoride. The actin cytoskeleton arrangement of control GH_4C_1 cells showed polyhedral cells with thin actin fibres in cell borders, extensive actin blebs and small punctuate actin condensations at the cell substrate adhesion level (Fig. 6A). Treatment with 1.2 μmol/L fluoride induced small lamellipodia, and the punctuated actin structures were more abundant than in control cells (Fig. 6C). An increase in fluoride treatment to 2.4 μmol/L fluoride induced thick cortical rings and more blebs (Fig. 6D). A further increase in bleb formation was observed with 10.7 μmol/L fluoride (Fig. 6E). However, a tenfold higher fluoride concentration was able to induce an arrangement of actin around the whole cell membrane as a lamellipodia, resembling belt of multiple intercrossed fibres (Fig. 6F). With the highest fluoride concentration, 1072 μmol/L, few fibres with low definition could be seen, and round zones resembling holes were present (Fig. 6G).

3.7. Cytoskeletal protein phosphorylation

Proteins associated with actin cytoskeleton were obtained taking the advantage that they are insoluble in Triton X-100. The cytosolic fraction, corresponded to those proteins soluble in Triton. Both protein fractions were resolved by SDS-PAGE (10%) followed by Western blotting analysis for immunodetection of proteins phosphorylated at serine and tyrosine residues. The antibody against phospho-serine residues is specific for PKC serine substrates. Fig. 7 shows the protein phosphorylation pattern of the soluble and insoluble fractions of GH_4C_1 cells incubated with different concentrations of fluoride in a serum-enriched medium. It is interesting to note that with the different fluoride concentrations, the phosphorylation pattern changed from a more phosphorylated state to a less phosphorylated state or *vice versa*.

4. Discussion

Our results show that biologically relevant concentrations of fluoride affect different physiological behaviours of GH_4C_1 anterior pituitary tumour cells. The fluoride concentrations used in the present study were chosen based on serum and urine concentrations observed in human populations: from zones with low/normal fluoride concentrations in water, in which fluoride concentrations of 2.4 μmol/L have been observed, and from areas with high concentrations of fluoride in water, in which serum fluoride concentrations of 10.7–80 μmol/L have been observed (Gupta et al., 2001; Schüttle, 2003; Xiong et al., 2007). Moreover, we have assayed other fluoride concentrations: low concentrations of 0.23 and 1.22 μmol/L, and two higher concentrations of 107 and 1072 μmol/L, which are similar to urine concentrations observed in areas with fluorosis (Gupta et al., 2001; Rocha-Amador et al., 2007). Unfortunately, information about comparative analyses between fluoride in soft tissues, plasma, and urine is scare. Additional measurements of fluoride in soft tissue after chronic exposure are needed. Inkielewicz and Krechniak (2003) showed that fluoride concentrations in soft rat tissues (kidney, liver, brain, and testis) were similar to those in the urine of rats exposed to fluoride via drinking water, where levels ranged from 32 to 272 μM. The limited information of fluoride concentration in human soft tissue shows that the pineal gland contains the highest concentration of fluoride in the body (15 mM) (Luke, 2001) compared with concentrations of 100–350 mM found in bone ash of people with preclinical skeletal fluorosis and osteosclerosis of the pelvis and vertebral column (Franke et al., 1975).

It is important to analyse the role of fluoride in tumour cell behaviour. Recently, a significant increase in pituitary adenoma incidence and aggressiveness has been observed in Mexico (Lopez-Gonzalez and Sotelo, 2000; Velásquez-Pérez et al., 2004). The cells used in the present study are a clone from a cell line obtained from a pituitary tumour induced by X-rays in an adult female rat (Tashjian et al., 1968). The GH_4C_1 cells express the somatolactotrope phenotype and secrete growth hormone. These cells proliferate actively in the serum-enriched medium used in the present study; they increase 100% in 48 h (data not shown). In order to evaluate the effect of fluoride in these cells, and considering that phosphatase inhibition by fluoride mimics activation of growth factor receptors, we analysed changes in proliferation behaviour. We have evaluated the ability of fluoride to modify the rate of DNA synthesis at the initiation of the cell cycle when cells were previously arrested. We observed that fluoride increased thymidine incorporation modestly at a concentration above normal serum levels, and that higher concentrations did not modify the cell cycle. This result suggests a weak effect of fluoride on the initiation machinery of the cell cycle. Nevertheless, a net increase in cell number in response to fluoride was not observed, confirming a modest activity of fluoride on cell pro-

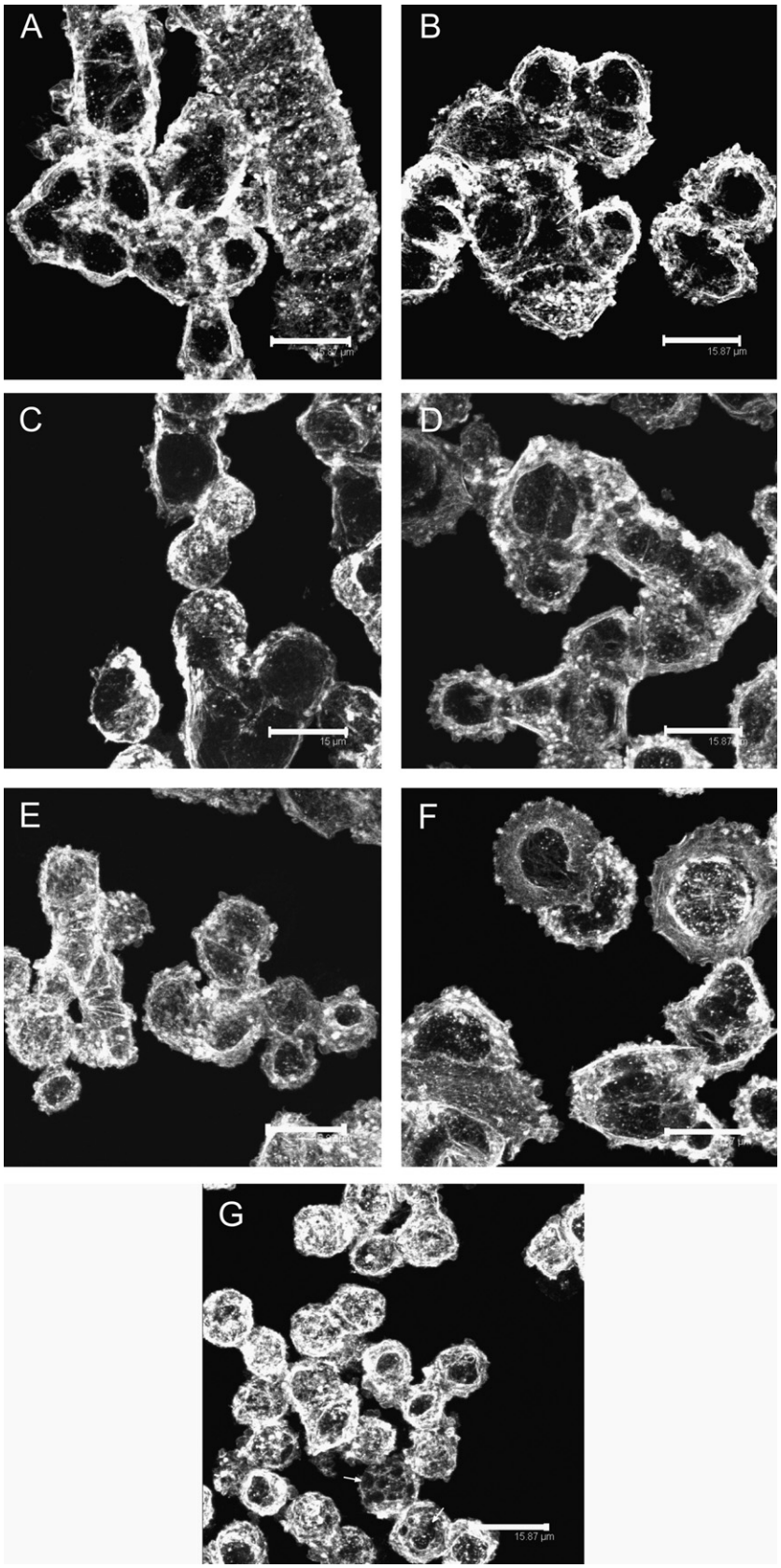

Fig. 6. Actin cytoskeletal arrangement of GH_4C_1 cells treated with different fluoride concentrations. Cells were incubated without or with fluoride and after 24 h were fixed and F-actin was stained with TRITC labeled phalloidin. Cells were observed at a confocal microscope. Panel A corresponds to control cells (no fluoride treatment), and the rest of the panels corresponded to cells treated with different fluoride concentrations: 0.23 μmol/L (panel B), 1.2 μmol/L (panel C), 2.4 μmol/L (panel D), 10.7 μmol/L (panel E), 107 μmol/L (panel F), and 1072 μmol/L (panel G). The arrow indicates images resembling holes. Bar = 15.87 μm.

liferation. With respect to the MTT assay, which is classically used to evaluate cell proliferation and viability, our results showed that fluoride treatment induced a significant increase in formazan salt metabolism. Therefore, this technique cannot be used to analyse either viability or proliferation in GH_4C_1 cells under these experimental conditions. The analysis of GH_4C_1 cell migration behaviour revealed that migration was clearly stimulated by fluoride, while there were only minor changes in cell adhesion with fluoride treatment. When we examined actin cytoskeleton arrangement, we observed an increase in bleb formation at the same fluoride concentration in which cell migration was significantly increased. Blebs are membrane protrusions with high actin polymerisation activity and are associated with cell migration (Fackler and Grosse, 2008). However, a more striking change in actin cytoskeleton was observed with cells treated with 107 μmol/L fluoride, with cells exhibiting a more flattened shape with lamellipodia and blebs, suggestive of an active migration behaviour. Nevertheless, there were also actin filaments arrayed in arcs, suggesting an increase in cell adhesion (Small et al., 1999; Chhabra and Higgs, 2007). The migration data of these cells showed no further increment in this behaviour, which is in agreement with the type of actin structures observed. These results suggest that the concentrations of fluoride observed in people exposed to low levels of fluoride are capable of stimulating the intracellular signals involved in actin cytoskeletal arrays typical of those involved in cell motility in GH_4C_1 cells. At higher concentrations of fluoride, the cells looked as if they had activated a different pathway for actin cytoskeletal regulation and had less motility. When we looked for changes in cytoskele-

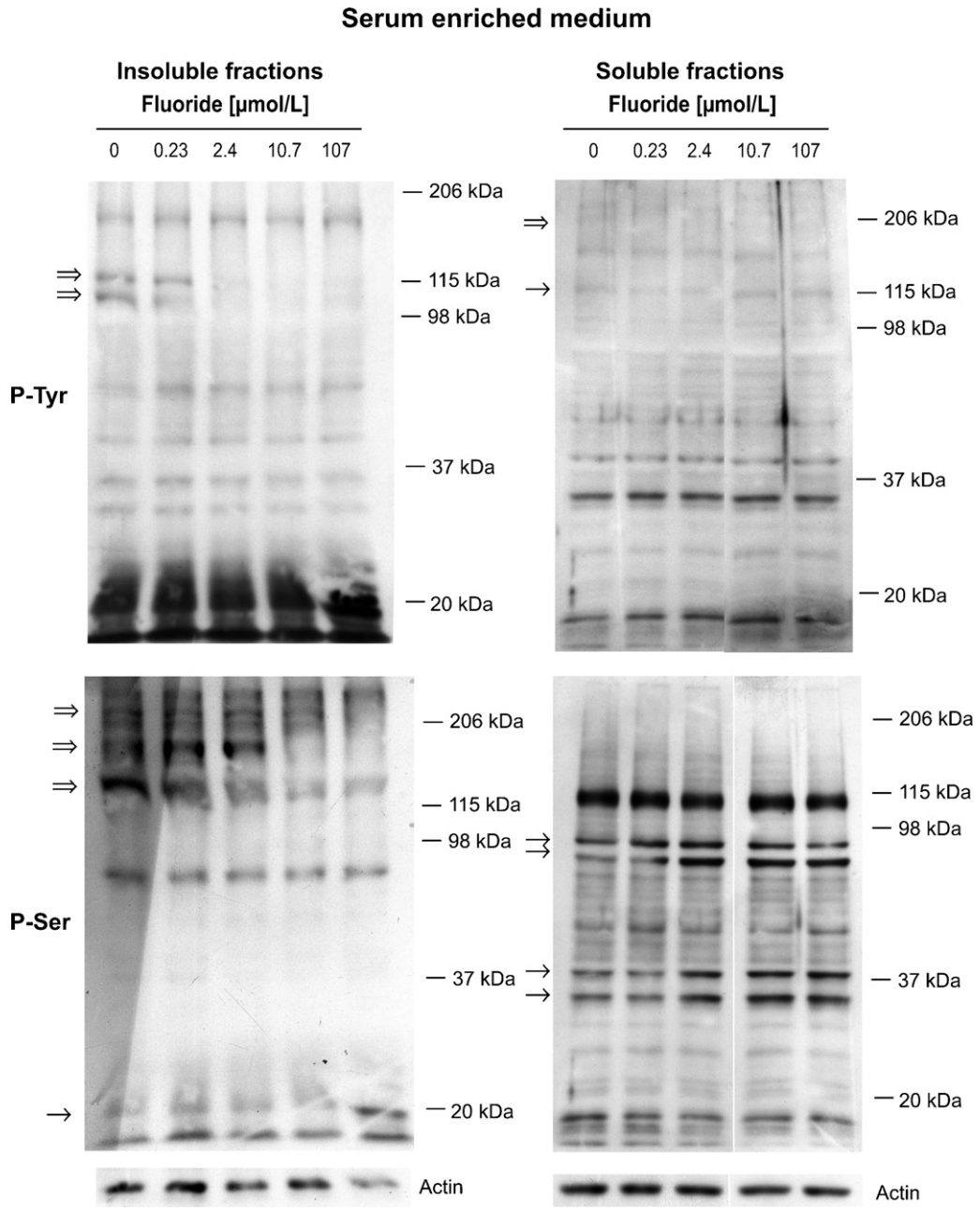

Fig. 7. Phosphorylation of cytoskeletal and cytosolic proteins of GH_4C_1 cells incubated with different fluoride concentrations. After a 24-h treatment period with fluoride the proteins associated with the actin cytoskeleton (insoluble fraction) or in the cytosol (soluble fraction) were obtained and resolved by SDS-PAGE followed by a Western Blot for phosphor-tyrosines and phospho-serines. Arrows indicate changes in phosphorylation of a protein band. Thin arrow represents phosphorylation increase and empty arrow phosphorylation decrease.

tal protein phosphorylation, a pattern of increasing or decreasing amount of phosphorylated tyrosine and PKC-phosphorylated serine proteins with different sizes was observed. It is interesting to note that the effect of fluoride on the phosphorylation of the protein bands was in one direction with the broad range of fluoride concentrations used. Interestingly, most of the changes in protein phosphorylation were observed since 2.4 μmol/L fluoride, the concentration at which cells exhibited an increase in cell motility behaviour. However, we would like to identify actin cytoskeletal associated proteins and analyse their phosphorylation state in response to fluoride exposure. These results support the hypothesis that the intracellular pathways involved in the cell migration of these tumour cells are stimulated by fluoride concentrations similar to those observed in the serum of people exposed to low fluoride concentrations in drinking water, suggesting an increase of tumour invasion risk.

The other metabolic processes analysed in this study (dehydrogenase metabolism and protein cellular concentration) showed that fluoride affects different processes in different ways. Our results showed a decrease in cellular protein concentration in response to fluoride treatment. These results agree with the studies of Kubota et al. (2005) and Sharma et al. (2008) in ameloblast cells, of Wang et al. (2005) in hepatocytes, and of Zhang et al. (2007) in neurons. In ameloblast cells, 1 mM fluoride induces a significant decrease in protein secretion by inducing endoplasmic reticulum stress (Sharma et al., 2008). In GH_4C_1 cells, was observed a decrease in total cellular protein concentration with all the range of fluoride concentrations evaluated. With the higher concentrations of fluoride, this effect could be associated to an induced endoplasmic reticulum stress that further decreases protein concentration by inhibiting protein synthesis, which has been reported in fluoride exposure. On the other hand, with the lower concentrations of fluoride, this effect could be explained as a significant increment of the basal secretion of growth hormone and prolactin in these endocrine cells. Our data of tetrazolium salt metabolism showed a stimulatory effect of fluoride treatment. The MTT method is largely used to evaluate cell culture growth and cell viability. It has been documented that mitochondrial succinate dehydrogenases are affected by fluoride (Stachowska et al., 2000; Adamek et al., 2005). According to these data, we expected to see a decrease in MTT metabolism in the presence of fluoride in GH_4C_1 cells. However, MTT is also metabolised by enzymes of the glycolytic pathway (Berridge et al., 1996; Liu et al., 1997), and in other membrane compartments of the cell (Bernas and Dobrucki, 2002). As fluoride inhibits a number of enzymes in the glycolytic pathway, resulting in a decrease of lactate and pyruvate (Adamek et al., 2005), MTT could be more likely metabolised by lactate dehydrogenase, resulting in a modest increase in MTT reduction in response to fluoride.

With the highest fluoride concentration used, 1072 μmol/L, was obtained a very significant decrease in all of the parameters that we have analysed. The abrupt fall in cell number suggests that this fluoride concentration is highly toxic for GH_4C_1 cells and that the cell have detached from the substrate. The cell adhesion assay performed in this study verified that GH_4C_1 cells have detached, and one of the primary causes of this is cell death. This cell death could be due to an increase in GH_4C_1 cellular apoptosis and/or necrosis. Fluoride induction of cellular apoptosis has been proposed to be one of its mayor effects (Elliot et al., 2001; Wang et al., 2004; Zhong et al., 2005; He and Chen, 2006; Zhang et al., 2007). In summary, fluoride induces changes in cellular behaviour in GH_4C_1 cells, as low concentrations of fluoride stimulate cell migration and cellular bioreduction, and decrease cellular protein concentration. We can conclude that biological relevant concentrations of fluoride can modify important aspects of the cellular behaviour of pituitary tumour cells and can contribute to a more pathological behaviour, favouring cell invasion.

Conflict of interest

None.

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