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Effects of the rare elements lanthanum and cerium on the growth of colorectal and hepatic cancer cell lines

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ABSTRACT

Human HT-29 and HepG2 cell lines were employed to test the effects of increasing concentrations of two rare earth elements (REEs), namely cerium (Ce) and lanthanum (La), alone or in combination. Effects on cell proliferation were measured using MTT assay, luciferase-based assays and proliferating cell nuclear antigen expression, while cell mortality and type of cell death was determined by Annexin V-FTC test using flow cytometry. Modulation of 84 genes involved in oxidative stress pathways was also studied using RT-PCR based arrays. Major alterations in selected genes compared to basal expression levels of respective control groups were found in the cells exposed to 600 μM Ce for 48 h. In HepG2 cells, 51 out of 84 genes were significantly up- or down-regulated, while in HT-29 cells only 16 genes were significantly up- or down-regulated. Dosage of REEs seems to be the pivotal factor for switching the biological effects from down- to up-regulation of cell growth; thus, low concentrations promoted cell survival and proliferation, but when concentrations increased, REEs exerted anti-proliferative and cytostatic/cytotoxic effects.

The molecular mechanisms underlying these effects are still not well-defined and further analysis of the mechanisms that result in inhibition or induction of cell proliferation are crucially important.

1. Introduction

Lanthanum (La) and cerium (Ce) are rare earth elements (REEs) that have many uses in industries throughout the world. As a group, REEs constitute the fifteenth most abundant component of the Earth's crust; Ce is present in the Earth's crust in higher quantities than lead, molybdenum or arsenic, and La occurs in similar quantities to lead, rendering the label “rare” misleading (Redling, 2006). In the last decade, REEs have become some of the most common xenobiotics in our surroundings (Zhao et al., 2004), and they are widely used in industry, stockbreeding and medicine, especially as trace fertilizers in agriculture, they can therefore be concentrated in the food chain.

China contains the largest mineral deposits of REEs in the world (Drew et al., 1990; Yang and A., 2006), and the application of REEs as feed additives for livestock has been practiced in China for decades; La, Ce and other REEs are used as feed additives in animal production (Redling, 2006). Numerous reports in the Chinese literature have described that a small amount of these REE mixtures in the diet can increase the body weight gain of pigs, cattle, sheep and chicken, and it has been reported that they also increase milk and egg production (He and Xia, 1998; Rambeck and Wehr, 2000; Zhu et al., 1994).

REEs have several properties that make them attractive alternatives to antibiotics for promoting growth. Generally, absorption of orally-applied REEs is low, with > 95% being recovered in the feces of animals (Redling, 2006). As a result, the chances of residues being present in the meat are low, with studies reporting no higher levels of REEs in the muscle tissue of supplemented animals than those fed commercial diets (Redling, 2006).

Although very small amounts of REEs are absorbed into the body when they are supplemented orally (He and Rambeck, 2000), even such small amounts of REEs in the animal body may have an effect on the metabolism through influencing hormones such as triiodothyronine (He et al., 2003) or growth hormone (Wang and Xu, 2003). Results have indicated that supplementing growth media with REEs can affect adipogenesis and lipogenesis rates of 3T3-L1 mouse cells, and that these effects may depend upon the dose or type of REE applied.

Around a dozen studies, especially in pigs, have been performed using western animal production conditions (Rambeck et al., 2004). Most of these studies in piglets and in fattening pigs have provided significant data, indicating that REEs imported from China can improve

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weight gain and feed conversion (Rambeck et al., 2004). In addition, there have been no reports of the development of bacterial resistance in treated animals (Redling, 2006).

However, from October 2004, only feed additives that have passed a renewed European Food Safety Authority (EFSA) procedure can be marketed in the European Union. Under these new rules, feed additives will be categorized as technological additives, zootechnical additives, or coccidiostats and histomostats (Khan, 2004). If REEs are characterized as trace elements, they are considered nutritional additives, if they enhance the digestibility or stabilize the gut flora, they are zootechnical additives. In Switzerland, REEs obtained a temporary registration under the trade name “Lancer” to be supplemented as other essential trace elements in the feed of piglets and pigs at a concentration of 150–300 mg REE per kg feed.

The EFSA recently evaluated the safety and efficacy of Lancer as a feed additive for weaned piglets (EFSA, 2013). Lancer is a feed additive mainly consisting of two REEs, namely La and Ce, and although one study (Von Rosenberg et al., 2013) suggests that La and Ce are not deposited in tissues in piglets, this is apparently not consistent with data found in other species, e.g. cattle (Schwabe et al., 2011). Consequently, the EFSA could not conclude that there is no potential for consumer exposure; in addition, in the absence of an established NOAEL (no observed adverse effect level) for the target species, the EFSA was unable to relate any possible exposure with the evidence of a safe dose.

In view of the increasing requests of application of lanthanides for improving animal growth, which may increase the possibility of human exposure, it is becoming necessary to obtain in-depth information on their environmental toxicity in humans (Dai et al., 2002; Xue et al., 2009). It is well known that the biological effect spectrum of REEs is wide and the dose-response relationship is complicated. Studies in rats have shown that REEs can induce chromosome damage of blood lymphocytes (Xu et al., 2000) and liver damage (Nakamura et al., 1997), depress learning and memory (Li et al., 2000), increase or suppress cell-mediated immunity of the spleen (Liu et al., 2000), and change the expression levels of certain genes (Zhao et al., 2004).

The safety evaluation of the effects of REEs in animals and humans is difficult to assess, and it is necessary to find sensitive biomarkers, utilizing different techniques to acquire a deeper understanding of their mechanism.

In this perspective, we explored the effects of different dosages of La and Ce on cell viability and proliferation in two human cancer cells lines, human colorectal (HT-29) and hepatocellular (HepG2), and we studied the modulation of 84 genes that are involved in pathways of oxidative stress in humans. To our knowledge, there are no previous studies considering all these aspects to elucidate the biological and molecular mechanism of REE toxicity in humans.

2. Material and methods

2.1. Chemicals

Cerium (III) chloride heptahydrate, 99.9% purity grade and lanthanum chloride heptahydrate, 99.999% purity grade were purchased from Sigma–Aldrich (Milan, Italy). All other chemicals and reagents used were of analytical grade.

2.2. Cell culture

Human hepatoma HepG2 and colon adenocarcinoma HT-29 cell lines were cultured, respectively, in DMEM and McCoy's 5A media containing 10% fetal bovine serum (Gibco BRL, Grand Island, USA), penicillin, 100 µg/ml streptomycin and amphotericin B (Sigma Chemical Co., St Luis, MO) at 37 °C in 5% CO_2. Different batches of described media were prepared by supplementing with La and Ce, alone or in combination, in concentrations ranging from 0.1 µM to 10 mM, followed by filtration with filtropur 0.2 µm (SARSTEDT). Different biological replicates for each treatment condition, defined by exposure time and compound concentration, were then prepared for the following analysis steps: quantification of effective exposure doses by ICP-MS, cell mortality and cell cycle progression by flow-cytometry, cell proliferation by MTT and luciferase-based viability assays, and finally gene expression profiling by quantitative RT-PCR. Negative control cell cultures for each time point and for both cell types were included in the experiments.

2.3. ICP-MS

The concentrations of the different lanthanide solutions were checked by ICP-MS, in order to take into consideration the possible effects of filtration and precipitation of the studied compounds in the media used for cultured cells. Determination of Ce and La was performed after wet digestion with acids and oxidants (HNO_3 and H_2O_2) of the highest quality grade (Suprapure). Approximately 0.5 g of material was subjected to microwave digestion (microwave oven ETHOS 1 from Milestone S.r.l., Sorisole (BG), Italy) with 7 ml of HNO_3 (70% v/v) and 1.5 ml of H_2O_2 (30% v/v). Samples were then taken to a final weight of 50 g with ultrapure water (Arium611VF system from Sartorius Stedim Italy S.p.A., Antella - Bagno a Ripoli, (FI), Italy). Ce and La determination was performed with ICP-MS (XseriesII, Thermo Scientific, Bremen, Germany) after daily optimization of instrumental parameters and external standard calibration curve; terbium was used as an internal standard.

2.4. Cell proliferation assays

MTT assays were performed to determine cell proliferation (Mosmann, 1983). HepG2 cells and HT-29 cells were treated in 96-well plates with fixed concentrations of Ce and La (Table 1). After incubating cells for 24 h, 48 h and 72 h with La and Ce alone or as a mixture, cell proliferation was estimated using the MTT assay as follows: 5 mg ml^{-1} of MTT reagent (Sigma–Aldrich) was added and incubated for 3 h at 37 °C in a humidified atmosphere (5% CO_2). After incubation, the media was removed and 100 µl of DMSO was added to each well to dissolve the formazan (the metabolic product from MTT). Absorbance at 590 nm was then measured in a microplate reader (Spectramax, Gemini, EM). Results are expressed as the percentage of means ± SD (standard deviation) of three experiments, each conducted with six replicates, calculated with respect to the controls, which are considered as 100%. After preliminary analysis by MTT on a broad range of La and Ce concentrations, cell viability was also evaluated on both cell lines by using RealTime-Glo™ MT Cell Viability Assay (Promega Italia, MI). This confirmation analysis was limited to the most relevant concentrations previously selected by MTT. Briefly, HepG2 cells and HT-29 cells were plated into 96-well opaque-walled assay plates (10×10^4 cells/well), treated with La and Ce alone or as a mixture and incubated for 24 and 72 h. After treatment, cells were incubated for 10 min in the cell culture incubator with a RealTime-Glo™ reagent according to the manufacturer's protocol. Luminescence was then measured on a Glomax Multi Detection System Promega with an integration time of 0.5 s per well. The luminescent signal correlates with the number of metabolically-active cells. Results are provided as means of luminescence values (RLU) ± SD of two experiments conducted with six replicates.

2.5. Western blotting

HepG2 and HT-29 cells were plated in 6-well plates (10^5 cells/well) and treated for 24 and 72 h, respectively, with La and Ce alone or as a mixture. Total cell lysates, obtained as previously described (Cannito et al., 2008), were subjected to sodium dodecyl sulfate-polyacrylamide gel-electrophoresis on 12% acrylamide gels, incubated with the required primary antibodies (PCNA sc-25,280; primary antibody dilution was in agreement with the manufacturer's instructions), followed by