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Cholesterol homeostasis and autophagic flux in perifosine-treated human hepatoblastoma HepG2 and glioblastoma U-87 MG cell lines

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ABSTRACT

Perifosine exerts an antiproliferative effect on HepG2 and U-87 MG cells and also interferes with the transport of cholesterol from the plasma membrane to the endoplasmic reticulum (ER). Recently we demonstrated that exposure of U-87 MG cells to perifosine causes an accumulation of autophagosomes. We have now expanded the study to establish the molecular mechanism by which perifosine interferes with the autophagic process. Using transmission electron microscopy, we report that the treatment of HepG2 and U-87 MG cells with perifosine causes an intense cytoplasmic vacuolization identified as autophagic vesicles. The accumulation of autophagosomes induced by perifosine is due to a blockage of the autophagic flux, thereby affecting cell proliferation. Perifosine also provokes a differential ER stress response in the HepG2 and U-87 MG cell lines. We have also demonstrated a relationship between the deregulation of cholesterol transport and the inhibition of the autophagic flux prompted by perifosine. Thus our findings clearly demonstrate that perifosine impairs the autophagic flux in HepG2 and U-87 MG cells, which is related to defects in intracellular cholesterol transport. Our study is relevant for anticancer therapy because tumour cells exhibit autophagy as a pro-survival mechanism. Further research to identify the precise mechanisms of autophagy maturation and the role of cholesterol may provide new insights into the antiproliferative action of perifosine.

1. Introduction

Brain and liver tumours are two of the most malignant types of cancers, posing major health problems and presenting especially difficult challenges to therapy. Evidence increasingly points to a connection between lipid metabolism and cancer, characterized mainly by an alteration in the mechanisms that regulate cholesterol homeostasis [1]. In this context, some researchers have proposed a potential strategy to treat glioblastoma by blocking the uptake of cholesterol into brain-cancer cells [2]. Other authors have reported that the active protrusion formed by invasive tumour cells is regulated by the cholesterol balance at the plasma membrane [3], and Kuzu et al. [4] have recently shown that some compounds mediate cancer-cell death by inhibiting intracellular cholesterol transport, leading to a homeostatic imbalance in the lysosomal-endosomal cell compartments.

Antitumour alkylphospholipids (APLs), such as perifosine, hexadecylphosphocholine (miltefosine), erucylphosphocholine, and edelfosine, are lipid analogues which exert antitumour activity against a broad spectrum of established tumour-cell lines [5]. There is growing interest in the biological activity of these lipid analogues as they do not interact with DNA but selectively inhibit the growth of transformed cells at concentrations that do not affect normal cells [6], and thus could well complement existing DNA-directed anticancer chemotherapies.

In previous studies, we have investigated the effects that these APLs exert upon cholesterol metabolism and have found that all of

Abbreviations: APLs, alkylphospholipids; CQ, chloroquine; ER, endoplasmic reticulum; LDLR, low-density lipoprotein receptor; MEM, minimal essential medium; FBS, foetal bovine serum; TEM, transmission electron microscopy; TLC, thin-layer chromatography; TN, tunicamycin.

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them share a common mechanism of action to impede cholesterol from reaching the endoplasmic reticulum (ER), thus markedly reducing intracellular cholesterol esterification in HepG2 (human hepatoblastoma) and U-87 MG (human glioblastoma) cells. We have also demonstrated that APLs not only block cholesterol esterification in both cell lines but also increase its synthesis and internalization, with 3-hydroxy-3-methylglutaryl-CoA reductase and low-density lipoprotein receptor (LDLR) being up-regulated [7–9]. Consequently, exposure of these cells to APLs leads to a deregulation of cholesterol homeostasis. In relation to this, we have recently reported that exposure of U-87 MG cells to APLs substantially alters the intracellular lipid metabolism and induces autophagosome accumulation [9]. Autophagy is a process of bulk degradation that in mammals is important for the turnover of long-lived proteins and acts as a pro-survival mechanism during starvation. However, undue activation of autophagy can lead to a type of cell death that is distinct from apoptosis [10]. It is now recognized that the level of autophagy needs to be finely regulated within a certain range and that excessive or deficient autophagy may trigger various diseases. The interplay between autophagy and lipid metabolism is complex; autophagy regulates lipid metabolism, and alterations in intracellular lipid content are likely to be important in the autophagy pathway [11,12], but the underlying mechanisms remain unclear.

These observations prompted us to explore how perifosine, as a representative APL, could affect the autophagic process in human cancer-cell lines. Here, we report that perifosine impairs the autophagy flux related to a defect in cholesterol transport. Our study emphasizes that autophagy is a survival mechanism for HepG2 and U-87 MG cells, this being consistent with the fact that, by inhibiting the basal autophagic flux, perifosine interferes with the proliferation of these tumour cells.

2. Materials and methods

2.1. Materials

Foetal bovine serum (FBS) was obtained from the Cell Culture Company (Pasching, Austria). Minimal essential medium (MEM), thin-layer chromatography (TLC) plates, Fluoromount, chloroquine (CQ) and the protease-inhibitor cocktail were from Sigma–Aldrich (Madrid, Spain) and [1,2-^{3}H(N)]cholesterol was from America Radiolabeled Chemicals, Inc (Saint Louis, MO, USA). Perifosine was obtained from Selleck Chemicals (Ontario, Canada). Cell Proliferation Reagent WST-1 kit was from Roche (Madrid, Spain). Polyclonal anti-human primary antibodies (β-actin, Beclin-1, CHOP/GADD153, GRP78/BiP, LC3) and Alexa Fluor 568 or horseradish peroxidase (HRP)-linked secondary IgGs were from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell culture

Human hepatoma HepG2 and human glioblastoma U-87 MG cell lines were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). The cells were cultured in MEM containing 10% heat-inactivated FBS supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, in a humid atmosphere with 5% CO_2 at 37 °C, and subcultured at a ratio of 1:10 once a week.

2.3. Assays for cell proliferation

Cells were seeded onto 96-well plates (10,000 cells/well) and maintained in MEM/10% FBS for 24 h. The culture medium was then replaced with fresh MEM/10% FBS and the cells incubated for 24 and 48 h without or with different concentrations of perifosine in the absence or presence of CQ. After incubation, 10 μl of Cell Proliferation Reagent WST-1 was added and the cells were incubated for 2 h at 37 °C and 5% CO_2. The absorbance of the formazan product in each well was measured directly in the plates at a wavelength of 450 nm using an ELx800 microplate reader (Biotek Instruments, Inc., Potton, UK). The reference wavelength was 600 nm.

2.4. Transmission electron microscopy

HepG2 and U-87 MG cells were seeded in 6-well plates and allowed to grow for 24 h. Subsequently, either 20 μM perifosine or a vehicle as control was added and left for 24 h in MEM/10% FBS. The cells were collected using trypsin and centrifuged at 1500 rpm for 5 min in MEM/10% FBS. The cell pellets were fixed in 2.5% glutaraldehyde plus 2% paraformaldehyde in 0.05 M cacodylate buffer for 4 h at 4 °C. The samples were washed three times with cacodylate buffer and postfixed in an aqueous solution of 1% OsO_4 containing 1% potassium ferrocyanide for 1 h at 4 °C in darkness. The following washes were carried out: 0.15% tannic acid in cacodylate buffer, cacodylate buffer, and water, all at room temperature. The samples were left in 2% uranyl acetate for 2 h and washed several times in water before being dehydrated at 4 °C in ethanol solutions rising from 50% to 100%. Next, the samples were placed into resin [EMbed 812/100% ethanol (1/1)] for 60 min at room temperature, the same resin at a 2:1 ratio for 60 min, and then resin without ethanol overnight. For polymerization, the samples were incubated in pure resin for 48 h at 60 °C. Ultrafine sections (50–70 nm) were cut using a Leica Ultramicrotome R (Leica Microsystems, Barcelona, Spain) and contrasted using 1% aqueous uranyl acetate for 5 min and lead citrate in a CO_2-depleted atmosphere for 4 min [13]. A Libra 120 Plus electron microscope (Carl Zeiss microscopy, Jena, Germany) was used to study the sections.

2.5. Transport of cholesterol from the plasma membrane to the endoplasmic reticulum

The cells were seeded in 12-well plates at 60–70% confluency and cultured in MEM/10% FBS in the absence or presence of 20 μM perifosine and/or 20 μM CQ for 6 and 24 h. After treatment, the medium was removed and replaced with fresh medium containing 1 μCi of [1,2-^{3}H(N)]cholesterol for 60 min at room temperature to label the plasma membrane [14]. The cells were washed twice with PBS containing 0.5 mg/ml of BSA prewarmed to 37 °C to remove any unincorporated label. The cells were then incubated at 37 °C in MEM for 60 min, and afterwards the medium was removed and the cellular lipids were extracted. Cholesterol and cholesteryl esters were separated by TLC using a solvent of n-hexane/ethyl ether/acetic acid (80:20:2, v/v/v). The spots were rendered visible by exposure to iodine vapour and radiometric measurements of scraped lipid spots were made by liquid scintillation using a Beckman 6000-TA counter (Madrid, Spain).

2.6. Immunoblotting analysis

Cells growing in log-phase were incubated for 6 and 24 h with MEM/10% FBS in the absence (PBS as vehicle) or presence of the assayed compounds. The cells were washed twice, scraped into ice-cold PBS (pH 7.4) and centrifuged at 100 × *g* for 10 min at 4 °C. The cell pellets were suspended in 0.3 ml ice-cold lysis buffer consisting of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 and a protease inhibitor cocktail, and incubated on ice for 30 min with occasional shaking. Cell lysates were centrifuged at 10,000 × *g* for 15 min at 4 °C and supernatants were stored at −80 °C until used; an aliquot was taken to determine protein