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Transglutaminase 2 Mediates the Cytotoxicity of Resveratrol in a Human Cholangiocarcinoma and Gallbladder Cancer Cell Lines

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ABSTRACT

Resveratrol is a polyphenolic compound extracted from plants and is also a constituent of red wine. Our aim was to evaluate if the cytotoxic effect of resveratrol (RES) on cholangiocarcinoma (CC) and gallbladder cancer (GBC) cell lines could be abolished by TG2 inhibition. Human CC and GBC cell lines (SK-ChA-1 and MZ-ChA-1), grown in a three-dimensional cell culture system (MCTS, multicellular tumor spheroids), were treated for 72 h with RES (32, 64 μM) alone or combined with different TG2 inhibitors (Cystamine, B003, T101). We investigated: cells viability; cell morphology with light microscopy (LM) and transmission electron microscopy (TEM); immunoreactivity with immunohistochemistry; Q-Banding karyotype analysis; TG2 activity; Western blotting. RES treatment induced a significant inhibition of cell growth, ranging from 24% to 76% in both cell lines. The inhibitors successfully reduced TG2 activity without any variation of protein quantity as demonstrated by immunohistochemistry and Western blot. TG2 inhibition resulted in cell growth normalization. In addition, morphologic analysis by light and transmission electron microscopy confirmed the cytotoxic effect of RES and its reduction consequent to TG2 inhibition. Our data demonstrated a connection between the cytotoxic effect of RES in SK-ChA-1 and MZ-ChA-1 and TG2 activity.

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Introduction

Tumors originating from the epithelial cells of the biliary tract include cholangiocarcinoma (CC) and gallbladder cancer (GBC).

CC accounts for 3% of all gastrointestinal tumors, with an increasing incidence of the intrahepatic form over the last decades, especially in high-risk group as in patients with sclerosing cholangitis (1–3). Nowadays the curative treatment for patients with CC is only complete surgical resection. However, difficulties in the early diagnosis of CC and its high metastatic potential mean that most CC patients will have regional or distant metastases at the time of clinical diagnosis. Few specific diagnostic or therapeutic tools are available because of the limited information available on the molecular pathogenesis of CC, and new therapies are widely needed (4). GBC is linked to several important risk factors among which gallstone disease (5). It is associated with the female gender and has a typical geographic expansion (Indo-Gangetic plains of India, Mapuche Indians in Chile and South America). GBC grows through a number of passages before turning into aggressive malignancy. The exposure to any carcinogenic agents possibly converts the normal epithelium of the gall bladder, evolving in an invasive carcinoma over the years. The incidence of GBC alludes to the role of genetic and environmental factors associated with the progression and generation of the disease. Studies establish no genetic markers and future research will be useful to understand them (6).

Resveratrol (3,5,4′-trihydroxy-trans-stilbene, RES) is a natural phytoalexin produced by a great variety of plants, such as grape vine (*Vitis vinifera*), peanuts, mulberries, and cranberries. RES is produced in response to injury or fungal infection (7) and as a

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wide spectrum of anticancer activities both in vitro and in vivo (8,9). Resveratrol supposedly mediates, at least in part, the purported disease-preventing actions of red wine and is also considered to prevent cardiovascular diseases and morbidity associated with obesity and old age (10–12).

Recent research on RES has been met with great interest; it has demonstrated that resveratrol inhibits cell growth in a human CC cell line; data has showed for the first time that RES has in vitro cytotoxic effects on the CC cell line SK-ChA-1 (13). Studies were conducted using two different models, the two- and three-dimensional cell culture systems: MCTS, multicellular tumor spheroids. Spheroids are a cellular model considered pivotal in in vitro oncological research (14,15).

Another study demonstrates the role of RES as a naturally chemopreventive agent. It regulates and suppresses some mechanisms associated with tumor events: that is, origination and promotion. RES reduces the resistance of cholangiocarcinoma, making their cells more susceptible to the chemotherapeutic agents in vitro and in vivo (16). Also in GBC, some studies have confirmed the capacity of RES to suppress the proliferation of cells, considering it as a therapy (17).

The cytotoxic effect mediated by RES could be the cause of an apoptotic mechanism, showed by increased intracellular TG2 activity. TGs are a family of intracellular Ca^{++}-dependent enzymes, which catalyze the post-translational modifications of proteins by establishing glutamyl-lysine crosslinks and/or covalent incorporation of di- and polyamines (18). Recent studies have demonstrated that cancer cells express elevated levels of TG2 and how these protein families have been found altered in different studies of cell survival and apoptosis (19,20).

The aim of the present study was to evaluate if the cytotoxic effect of RES on a cholangiocarcinoma and a gallbladder cancer cell lines would be abolished by TG2 inhibition.

Material and Methods

Cell Cultures

SK-ChA-1 (human cholangiocarcinoma) and MZ-ChA-1 (gallbladder cancer) cell lines were used. The cells were commonly conserved in exponential monolayer growth and regularly tested for mycoplasma contamination, as previously described (21). The successive cultures used for analysis were assessed to be at passage 60.

SK-ChA-1 and MZ-ChA-1 cells were grown in DMEM medium (GIBCO, Italy), added with 1% penicillin—100 U/mL-streptomycin 100 μg/mL (GIBCO, Italy)—2% L-glutamine 200 mM (GIBCO, Italy) and 10% fetal bovine serum (GIBCO, Italy). The cell lines were preserved in a humidified incubator at 37°C and 5% CO_2.

Three-Dimensional Cultures (Multicellular Tumor Spheroids) and Treatments

MCTSs were arranged as formerly described (Roncoroni et al., 2008). They were initiated by seeding 2×10^5 cells/mL in 15 mL of complete Iscove Modified Dulbecco's Medium (IMDM–GIBCO, Italy), added with 1% penicillin 100 U/mL streptomycin 100 μg/mL (GIBCO, Italy), 2% L-glutamine 200 mM (GIBCO, Italy) in polycarbonate Erlenmeyer flasks (Corning, Italy) and incubated in a gyratory rotation incubator (60 rpm) at 37°C in air atmosphere (Colaver, Italy). Homotypical aggregations were observable after 4 days of culture and the MCTSs were commonly complete within 7 days (mean diameter $\pm$ standard deviation, 270 $\pm$ 53.5 μm).

MCTSs at the 7th day of culture were treated with RES 32 and 64 μM and retained for supplementary 72 hours.

The data from the five experiments carried out are presented.

Spheroids Resveratrol Treatments With or Without Transglutaminase 2 Inhibitors

Resveratrol (Chemical Company, Ann Arbor, MI, USA): starting concentration 128 mM.

Inhibitor: Cystamine (Aldrich Chemica Company) 0.5 μM.

Inhibitor: B003 (Boc-DON-Gln-Ile-Val-OMe) and T101 (1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]) imidazolium chloride (Zedira GmbH) 5 μM.

Spheroids treatments for both SK-ChA-1 and MZ-ChA-1 were performed after 7 days from seeding and for additional 72 h as follows: negative control (ethanol absolute vehicle), RES 32 μM, RES 64 μM, RES 32 μM + cystamine, RES 64 μM + cystamine, RES 32 μM + B003, RES 64 μM + B003, RES 32 μM + T101, RES 64 μM + T101.

The data of the five different experiments are reported.

Q-Banding Karyotype Analysis

Metaphase cells were arranged by standard cytogenetic techniques: cells were treated with Colcemid 50 ng/mL (GIBCO, Italy) for 2 h. The suspension was exposed to hypotonic shock for 20 min at 37°C with 1% sodium citrate, fixed in methanol/glacial acetic

acid 3/1, air dried, and assembled on slides. QFQ banding was executed on a min. 100 metaphases per cell line and chromosomes were karyotyped according to the International System for Human Cytogenetic Nomenclature (ISCN) (22).

Histology, Light (LM), and Transmission Electron Microscopy (TEM)

In order to avoid any loss of three-dimensional cell cultures they were colored with toluidine blue and filtered through tissue bags, fixed in 4% buffered formalin, dehydrated and paraffin-embedded, while for ultrastructural examination the cells were washed twice in PBS (GIBCO, Italy), fixed in 2.5% glutaraldehyde in phosphate buffer, postfixed in osmium tetroxide, dehydrated and embedded in epoxy resin. After the first fixation, in order to avoid spheroids dispersion during processing, they were entrapped in a warm liquid 2% agar solution, becoming solid at room temperature, and then cut in small cubes ($2 \times 2 \times 1$ mm). Semithin sections (0.5 μm) were stained with toluidine blue for light microscopy observation and ultrathin sections (50–60 nm) were counterstained with uranyl acetate, lead citrate and observed in a Jeol JEM 1010 (Tokyo, Japan) transmission electron microscope.

Colony-Forming Assay

Colony-forming assay was executed by plating a single-cell suspension achieved from three-dimensional cell cultures.

After RES treatment alone or in combination with inhibitors, MCTSs underwent trypsinic disaggregation (trypsin 0.25%-EDTA 0.02%—Sigma, Italy) to achieve single-cell suspensions: 500 cells per well were positioned in six-well plates, in triplicate. After 15 days from seeding, the colonies became observable and were fixed with methanol, colored with crystal violet and counted. The surviving fraction was calculated and compared with the plating efficiency of the untreated samples. The control group is a negative control; spheroids are treated only with the vehicle of resveratrol (ethanol absolute).

Cytotoxicity Assay (MTT)

The tetrazolium bromide (MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to determine cell proliferation according to the manufacturer's instructions. The MTT assay is based on the ability of viable cells to metabolize the tetrazolium salt to blue formazan colorless in the mitochondria.

After RES treatment alone or in combination with inhibitors, MCTSs underwent trypsinic disaggregation (trypsin 0.25%-EDTA 0.02%—Sigma, Italy) to achieve single-cell suspensions. SK-ChA-1 and MZ-ChA-1 cells were seeded in 96-well plates by adding 200 μl/well of a suspension of 3×104 cells/well. After cells reached 80% confluence, the medium was removed and 50 μl MTT (5 mg MTT with 1 ml PBS) was added diluted in the medium 1:4. After incubating for 4 h, the medium was removed and 150 μl DMSO was added to each well. The spectrophotometric absorbance of each well was analyzed by means of a microplate reader at 550 nm (Wallac Victor Multilabel Counter 1420). Cell viability was expressed as the percentage of untreated control cells.

Immunohistochemistry (IHC)

IHC was performed on histologic sections from paraffin-embedded samples, pretreated for antigen retrieval in citrate buffer, incubated with a mouse monoclonal antibody against tissue transglutaminase (TG2) (clone TG 100, Thermo Fisher Scientific, Waltham, MA, USA) eluted 1:5000 or with an anticleaved Caspase-3 (Asp1275) monoclonal antibody (cl-CASP3; cat #9661; Cell Signaling Technology) eluted 1:1000. Endogenous peroxidase activity was blocked by hydrogen peroxide (0.3%) for 10 min. All the slides were counterstained with hematoxylin. The negative controls were incubated in the absence of primary antibodies. Reactions were detected by the Novolink Max polymer detection system (Novocastra Laboratories L.T.D., Leica Microsystem), following the manufacturer's instructions, using Diaminobenzidine (DAB) as chromogen.

Immunohistochemistry was performed using a Biogenex i6000 Automated Staining System (Biogenex).

Cellular Transglutaminase Activity Test

TG2 activity was colorimetrically examined, following the manufacturer's guidelines (Covalab, France). For a short time, the cells were suspended in 1 mL distilled water in addition to a 20 μL protease inhibitor cocktail without EDTA (Pierce, USA); 50 μL of this suspension was distributed in each well on a 96-well microtiter plate with covalently coupled CBZ-Gln-Gly and incubated with calcium, dithiothereitol, and biotinylated cadaverine and then strepavidin-labelled peroxidase was added and peroxidase activity revealed with H_2O_2 and tetramethyl benzidine. Optical density was evaluated at 450 nm with a microplate reader (Bouty Diagnostici, Italy). The results were normalized referring to the cell number and expressed as percent, compared to untreated samples.

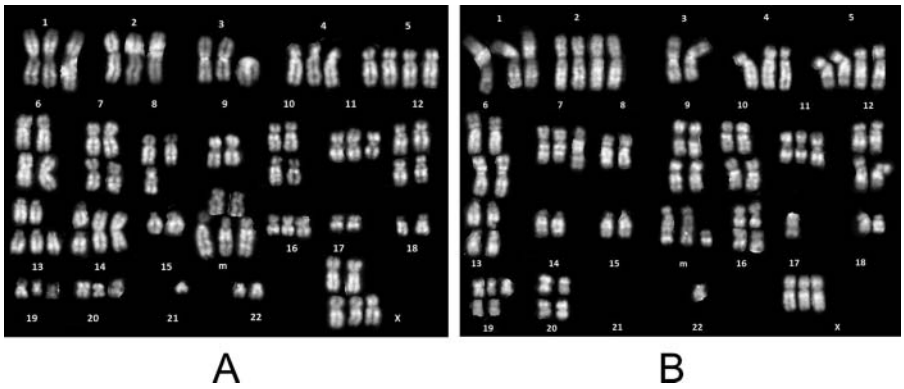

Figure 1. A: Karyotype of MZ-ChA-1 cell line showing 77, XX XXX, der (14;14) (q10;q10)der (14;14)(q10;q10), +5 mar. B: Karyotype of SK-ChA-1 cell line showing 71, XXX, + 3 mar. Karyotype Q-banding analysis was nominated according to the International System for Human Cytogenetic Nomenclature 2016. Schematic illustrations of the karyotype findings were produced by the cytogenetic data analysis system (Chromowin Plus, Tesi Imaging).

Western Blotting

To detect tissue transglutaminase (TG2), the cells (5×10^6) were homogenized and lysed for 30 min in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% TRITON, 0.1% SDS, 1% sodium deoxycholate, 4 mM sodium pyrophosphate, 50 mM NaF, 25 mM Na3VO4, 2 mM PMSF, and a protease inhibitor cocktail (Sigma). The lysate was centrifuged in an Eppendorf centrifuge (13,500 rpm) for 30 min at 4°C, and the supernatant was saved for analysis. The total cell lysates were used for Western blotting. Samples for each analysis (10 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Gels were transferred to a nitrocellulose membrane and then placed in a blocking solution consisting of TBST (10 mM Tris [pH 8.0], 150 mM NaCl, and 0.05% Tween 20) and 5% skim milk for 1 h. The blotted membranes were incubated with the monoclonal TG2 antibody (Biodesign International, Inc.). After incubation with appropriate secondary antibodies and extensive washing, the antigens were detected by means of chemiluminescence using an ECL Plus immunodetection kit (Amersham Co.). The proteins were densitometrically quantified, making sure that the signals were in the linear range. All the data were calculated by comparing the intensity of the bands. The values were calculated after normalization to the amount of β-actin.

Statistical Analysis

Each experiment was performed in triplicate for three times. Data were stated in percentages as mean ± standard deviation (SD) and examined using two-tailed Student's *t*-test. $P < 0.05$ was considered as statistically significant. The statistical analysis for the study was carried out by a biomedical statistician.

Results

Cellular Q-Banding Karyotype, Transglutaminase Activity, Clonogenic Test, and MTT Cytotoxicity Assay

An analysis was carried out to examine any possible genetic differences of cells from the initially described SK-ChA-1 (22) and MZ-ChA-1. SK-ChA-1 cells presented a pseudo-triploid karyotype with a modal number of 71 chromosomes (range 65–71). The majority of the analyzed metaphases (70%) presented 12 marker-chromosomes in which we could identify portions of

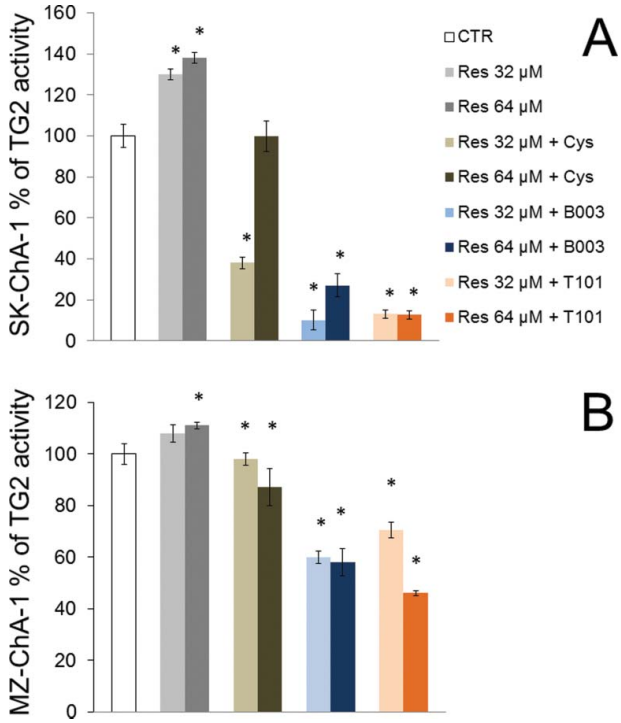

Figure 2. Cellular transglutaminase activity respectively in SK-ChA-1 (A) and MZ-ChA-1 cells (B) at different concentrations of RES alone or combined with the different inhibitors.

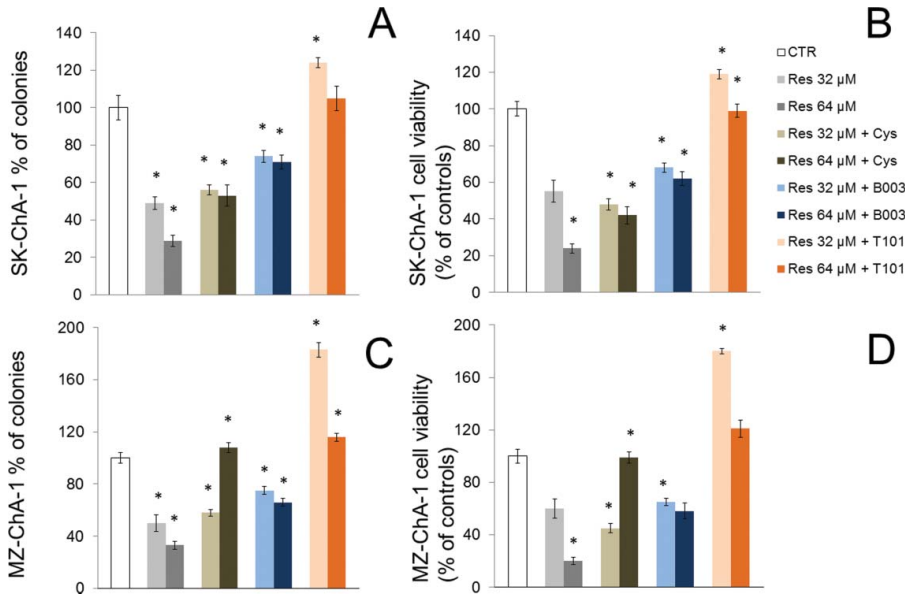

Figure 3. Colony-forming and MTT cytotoxicity assay of three-dimensional multicellular tumor spheroids (MCTS), respectively, of SK-ChA-1 (A, B) and MZ-ChA-1 cells (C, D) at different concentrations of RES alone or combined with the different inhibitors. Data are expressed as percentage of untreated controls. The standard deviations of the five independent experiments are indicated. $^{*}P$ value $<$ 0.05.

chromosomes 1, 3, 4, 11, 14, 15, 17, 18, 19, as previously described (13). MZ-ChA-1 cells showed a pseudo-triploid karyotype with a modal number of 77 chromosomes: (range 68–71) 66% of the analyzed metaphases presented from two to nine marker-chromosomes (not identifiable with Q-banding) in which we could recognize portions of chromosomes 3, 4, 5. In the figures the karyotypes shown represent the modal number of the two cell lines in monolayer (Fig. 1A–1B). It was not possible to evaluate the spheroids karyotype, because MCTS are three-dimensional culture with a partial block of mitosis.

Transglutaminase 2 activity was increased in RES-treated cultures compared with untreated controls: at the highest concentration of 64 μM in SK-ChA-1 the activity increased 34%, in MZ-ChA-1 10% *vs.* 100% controls.

The normalization of cell growth was associated to the inhibition of TG2 activity both in SK-ChA-1 and MZ-ChA-1 (Fig. 2A–2B). The clonogenicity of cells derived from MCTS treated with RES for 72 h at the concentrations of 32 and 64 μM showed a significant inhibition of cell growth: for SK-ChA-1 49% and 29%, for MZ-ChA-1 52% and 33% *vs.* 100% controls. The number of colonies changed, decreasing with high doses of RES. The clonogenicity of cells derived from MCTS treated for 72 h with RES combined with the three TG2 inhibitors (cystamine, B003 and T101) prevented growth inhibition in both cell lines. Cell growth for SK-ChA-1 with RES 32 μM and the three different inhibitors (cystamine, B003 and T101)

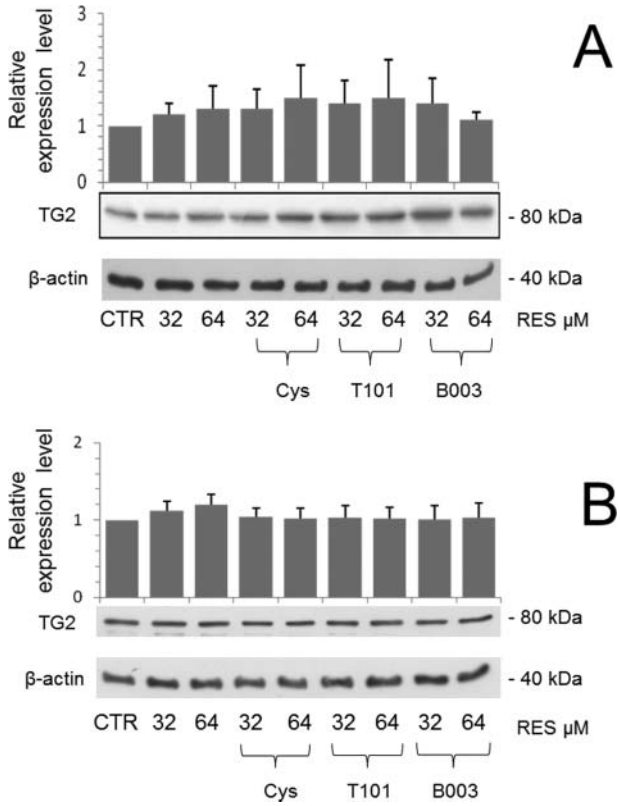

Figure 4. Western blot analysis of transglutaminase (TG2) in SK-ChA-1 (A) and MZ-ChA-1 (B) cells subjected to different treatments. A representative blot from three independent experiments was shown. The blot was reprobed using the antibody against β-actin as a loading control. The histogram showed the densitometric quantification relative to *C*-values.

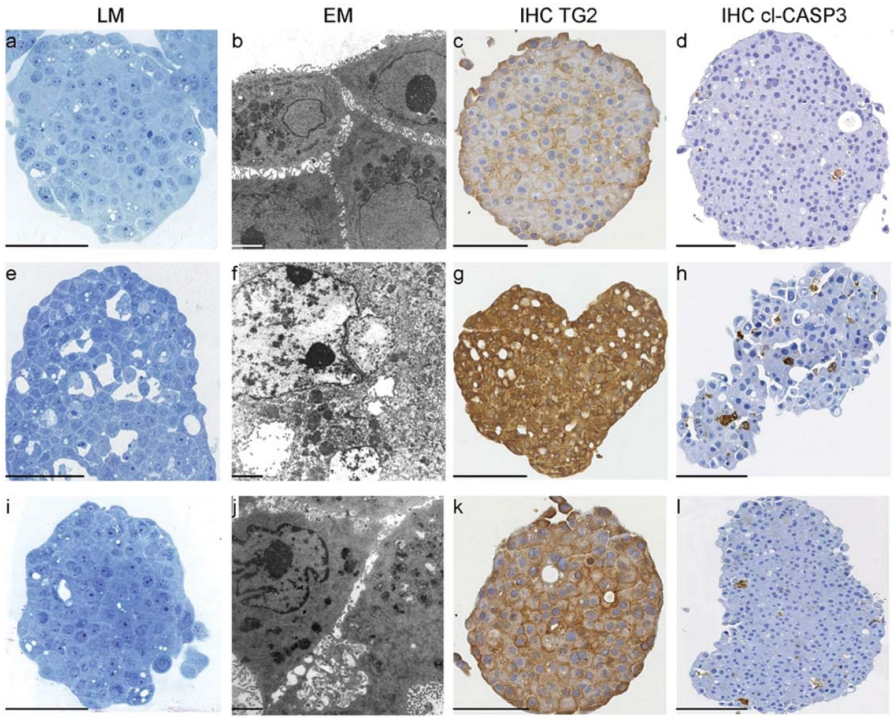

Figure 5. Representative images from the cholangiocarcinoma MZ-ChA-1 cell line, grown in a three-dimensional cell culture system, untreated control (A–D); or treated for 72 h with RES 64 μM either alone (E–H) or in combination with the TG2 inhibitor T101 (I–L) are shown. LM-Light Microscopy (A, E, I; toluidine blue staining on semithin sections from resin-embedded samples; scale bar, 100 μM); EM, electron Microscopy on ultrathin section from the same samples of LM (B, F, J; scale bar 2 μM); IHC, immunohistochemistry for transglutaminase 2 (TG2; C, G, K) or cleaved Caspase 3 (D, H, L; cl-CASP3) (scale bar, 100 μM).

increased 7%, 25%, and 75%, respectively (Fig. 3A); for MZ-ChA-1 8%, 25%, and 133% *vs.* the percentage of colony growth from cells treated only with RES (Fig. 3C). Both lines in co-treatment RES 64 μM /TG2 inhibitors (respectively cystamine, B003 and T101) showed increases of 24%, 42%, and 76% (SK-ChA-1) and 75%, 33%, 83% (MZ-ChA-1) *vs.* the percentage of colony growth from cells treated only with RES. MTT cytotoxicity assay confirmed the clonigenic test (Fig. 3B–3D).

Transglutaminase 2 Western Blot

Western blot analysis was carried out to evaluate the changes of TG2 expression in the MZ-ChA-1 and SK-ChA-1 cells being studied. We have demonstrated that the TG2 protein content remains at control level with all the treatments, despite the marked increases in TG2 enzyme activity with RES and decreases with the different inhibitors (Fig. 4A–4B).

Light Microscopy, Transmission Electron Microscopy (TEM), and Immunohistochemistry

The control spheroids of both cell lines (Fig. 5) showed a regular shape with a normal ultrastructure of cells encircled by short microvilla and joined by desmosome-like junctions, with weak, scattered immunopositivity for anti TG2. A few cells were apoptotic, as determined by cleaved caspase-3 staining (cl-CASPASE3) (Fig. 5A–D). The cells forming spheroids, treated with resveratrol, appeared severely damaged with numerous vacuoles, loss of organelles integrity, diffuse strong immunoreactivity for anti-TG2 antibody and presence of cl-CASPASE3-positive cells (Fig. 5E–H). The best results were obtained by T101 TG2 inhibitor treatment: the spheroids showed less vacuoles, almost normal ultrastructure, and weak immunoreactivity with both anti-TG2 and cleaved caspase-3 antibodies (Fig. 5I–L). The MZ-ChA-1 cell line as shown in Fig. 5 was the more representative of the two cultures.

Conclusions

The present study demonstrates a relationship between TG2 inhibition and prevention of the cytotoxic effect of RES in tumors of the biliary tract.

Cancer is a social problem in both the developing and developed countries. Nowadays, the main treatments for cancer are chemotherapy, radiotherapy, surgery and when possible, chemoprevention. The most important drugs used in chemotherapy cause several unwanted

effects (hair loss, bone marrow suppression, drug resistance, gastrointestinal lesions, neurological dysfunction, and cardiac toxicity) (23–25). Great interest is paid to the research of new anti-cancer agents with better efficacy and fewer side effects. Natural compounds are great sources to develop new remedies for different diseases. A natural compound as resveratrol is known to be a potent antioxidant; it has anti-inflammatory effects and inhibits the proliferation of a variety of cancer cells (26,27). The understanding of the mechanisms involved in RES cytotoxicity is worthwhile in order to gain a greater appreciation of RES anti-cancer properties and to progress the investigation on the possible chemotherapic/chemopreventive role of RES in the treatment of cholangiocarcinoma and gallbladder cancer (28). Recent studies (29) have evaluated the concentrations of RES and its metabolites in colorectal tissue of humans who ingested resveratrol: there was indication that resveratrol exerted a small reduction of cell proliferation in colorectal tissue after ingestion, so as to elicit pharmacological effects. Recent pilot studies of SRT501, micronized RES, demonstrated that the polyphenol administered to patients with colorectal cancer and hepatic metastases can reach potentially active concentrations in human tissues that are distant from the GI tract, such as the liver. Moreover, cleaved caspase-3, which is a marker of apoptosis, significantly increased in malignant hepatic tissue following SRT501 treatment (30). Other studies have demonstrated a role for low-dosage resveratrol in the sensitivity of cholangiocarcinoma to chemotherapy-induced cell death. In addition, it has been demonstrated that the suppression of cytochrome P450 1b1 (Cyp1b1) expression may be one mechanism by which resveratrol makes these cells more susceptible to chemotherapy (31).

Our recent data (13) have demonstrated for the first time that RES has in vitro cytotoxic effects on cholangiocarcinoma cells. We used a SK-ChA-1 cell line derived from human cholangiocarcinoma, using two different models: two- and three-dimensional cell culture systems (MCTS, Multi-cellular Tumor Spheroids). Unlike conventional monolayer cell culture systems, MCTS maintains the specific morphological and biochemical properties of the corresponding in vivo tissue and remains in a differentiated and functionally active state for many weeks, thus making it possible to study the long-term effects of various xenobiotics (13). In the present study, we have confirmed the cytotoxic effect of resveratrol both in a human cholangiocarcinoma cell line (SK-ChA-1) and a gallbladder cancer cell line (MZ-ChA-1) spheroids. Q-banding analysis only showed the karyotype of the monolayers culture (Fig. 1), because MCTS are partially blocked in mitosis; no Q-banding analysis could be performed.

The toxic effect appears to be the cause of an apoptotic mechanism; cleaved caspase-3 immunohistochemistry is positive for spheroids treated with RES. The apoptotic effect is simultaneously demonstrated by the increase of intracellular Transglutaminase type 2 (Fig. 5). TG2 is present in the epithelial cells and is involved in apoptosis, acting during the final phases of the process, in the protein packaging (18,32). We considered choosing TG2, because our previous studies (13) evaluated the implications of TG2 in the RES cytotoxic mechanism. Moreover, TG2 is widely involved in cancerous disease. We cotreated RES with three different TG2 inhibitors: cystamine and the two selective inhibitors B003 and T101. Cystamine inhibits TG2 in two different ways: the TG2 active site formed a stable complex with cystamine by oxidation of the disulfide bond in TG2. Moreover, a cystamine-derived metabolite (MEA, betamercaptoethanolamine) is a TG2 inhibitor able to compete for transamidation, limiting TG2 expression by reducing the binding of transcription factors to promoters (33). Previous studies (34) demonstrated that cystamine may be an effective sensitizer of TRAIL-induced apoptosis in cancer cells expressing high levels of TG2.

T101 inhibits TG2 by factor XIIIa inactivation and B003 is a TG2 catalytic site-specific inhibitor (35–37).

The cotreatment RES/TG2 inhibitors prevented the growth inhibition in both cell lines; in the clonogenic and in MTT cytotoxicity assay at the dose of 32 and 64 μM RES cytotoxicity reverted, caused by the direct involvement of the TG2 inhibitions. Moreover, the T101 inhibitor permits a better reversion of RES cytotoxicity both in SK-ChA-1 and MZ-ChA-1 cell lines. As an additional confirmation, TG2 activity with the three different inhibitors decreases in the cotreatment with the inhibitors, drastically in the SK-ChA-1 cell line. The MZ-ChA-1 cell line has a great ability to grow new colonies after combination with the three inhibitors, even more at the dose of 64 μM combined with cystamine, thus demonstrating the capability to overcome the cytotoxic effect caused by resveratrol. All morphological, optical, and ultrastructural results, together with immunohistochemical data, support our hypothesis that TG2 activity mediates the cytotoxic effect of resveratrol on three-dimensional cell cultures. In Western blot analysis, the expression of TG2 does not change with RES and the three different inhibitors employed.

Our data on these two different cell lines, corroborated by some recent studies concerning RES anti-cancer properties (38), can open the possibility to candidate resveratrol in the treatment of biliary and gallbladder cancer; RES could have a possible chemotherapeutic/chemopreventive role. Clinical treatments should be improved, especially with regard to clarifying the

pharmacokinetics of RES in humans: inoperable patients could be treated with RES in combination with other chemotherapeutic agents. The polyphenol could be also administrated as a chemopreventive drug to patients with a high risk to develop cancer, like patients affected by hepatic cystic disease or sclerosing cholangitis.

For all these reasons the study of the mechanism, which RES supposedly works with, is of great interest to support further studies of dietary supplementation with RES as a potential gastrointestinal cancer-preventing agent.

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