Liver International ISSN 1478-3223

BASIC STUDIES

Resveratrol inhibits cell growth in a human cholangiocarcinoma cell line

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Keywords
chemotherapy – cholangiocarcinoma – cholangiocytes – multicellular tumour spheroids – resveratrol

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Received 24 December 2007
Accepted 27 February 2008

DOI:10.1111/j.1478-3231.2008.01749.x

Abstract

Background/Aims: Cholangiocarcinoma is a devastating tumour with a poor prognosis. An efficient therapy is unavailable in unoperable patients and new drugs are widely sought for and required. Resveratrol (RES) is a natural molecule with a reported anticancer effect, evaluated on different tumour cell lines. We tested the efficacy of RES on a cholangiocarcinoma cell line for the first time. *Methods:* We used the human SK-ChA-1 cell line, cultured in the classical two-dimensional model and in the three-dimensional spheroids. After RES exposure morphology, cell viability (colony-forming assay), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and cancer antigen (CA) 19-9 medium releases, cellular transglutaminase activity, karyotype and cell cycle were evaluated. *Results:* Resveratrol inhibited cell growth in both the cell culture systems used (from -15 to -80% vs untreated controls) and induced a 40-fold increase of LDH and ALP activities in the culture medium. Also, transglutaminase (TG) activity increased in the cell lysates, together with a cell cycle perturbation characterised by an accumulation in the G_1/S phase. Karyotype and CA 19-9 expression were not influenced by the treatment. *Conclusions:* The observed cytotoxic effect of RES on the human cholangiocarcinoma SK-ChA-1 cell line cultured two- and three-dimensionally suggests to further analyse its chemotherapic/chemopreventive possibilities for this kind of cancer.

Cholangiocarcinoma is a tumour originating from the epithelial cells of the biliary tree and accounts for 3% of all the gastrointestinal tumours, with an increasing incidence of the intrahepatic form in the last decades (1, 2). Cholangiocarcinoma is diagnosed with difficulty and symptoms arise late, making surgery suitable only in a limited number of patients (3). As both radiotherapy and traditional chemotherapy (5-fluorouracil and gemcitabine alone or in combination with cisplatin) showed disappointing results in terms of survival in unoperable patients, the development of new therapies is widely needed (1, 3).

Considerable interest is growing in the potential anticancer effect of the polyphenolic compound resveratrol (3,4′,5-trihydroxy-*trans*-stilbene; RES). It is a phytoalexin that naturally occurs in grapes and grape-derived foodstuffs such as red wine and nuts; in plants, RES is produced in response to injury or fungal infection (4). RES has been claimed to exert many different potential health-promoting effects, and, above all, 'anti-tumour' actions (5). In fact, an inhibiting effect on the growth of leukaemia (6), breast (7) and colorectal cancer (8) cell lines has been reported, probably linked to an arrest of the cell cycle in the S/G2 phase transition and modifications of the regulatory mechanisms as apoptosis and DNA synthesis (9). Moreover, recent studies demonstrated the cytotoxic effects and synergistic interaction with 5-fluorouracil and mytomicin C of the polyphenolic compound tannic acid, strengthening the hypothesis for the use of these natural compounds *in vivo* (10, 11).

The aim of this study was to investigate the possible effects of RES on a human cholangiocarcinoma cell

line (SK-ChA-1) (12) cultured on two- and three-dimensional cell culture systems.

Materials and methods

Cell cultures

Human cholangiocarcinoma cell line SK-ChA-1, kindly supplied by Prof. Alexander Knuth (Ludwig Institute, Frankfurt, Germany), was used. Cells were regularly maintained in exponential monolayer growth and routinely checked for mycoplasma contamination.

Two-dimensional cultures and treatments

SK-ChA-1 cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Milan, Italy), supplemented with 1% penicillin 100 U/ml, streptomycin 100 μg/ml (GIBCO), 2% L-glutamine 200 mM (GIBCO) and 10% foetal bovine serum (GIBCO). The cell line was maintained in a humidified 37 °C, 5% CO_2 incubator. For the treatments, SK-ChA-1 cells were seeded in six-well tissue culture plates (Corning, Milan, Italy) at a concentration of 2×10^5 cells/well. After 48 h, cells were exposed to increasing concentrations (8, 16, 32, 64 μM) of RES (Sigma, Milan, Italy), added directly to the culture medium, for a further 48 h.

Triplicate samples were used for each experiment. Viable cells were counted by trypan blue exclusion assay. Data are expressed as per cent of viable cells vs untreated control.

Three-dimensional cultures (multicellular tumour spheroids) and treatments

Multicellular tumour spheroids (MCTS) were prepared as described previously (13). They were initiated by seeding 2×10^5 cells/ml in 15 ml of complete Iscove modified Dulbecco's medium (GIBCO), supplemented with 1% penicillin 100 U/ml, streptomycin 100 μg/ml (GIBCO), 2% L-glutamine 200 mM (GIBCO) in polycarbonate Erlenmeyer flasks (Corning) and incubated in a gyratory rotation incubator (60 rev/min) at 37 °C in an air atmosphere (Colaver, Milan, Italy). Homotypical aggregations are visible after 4 days of culture and the MCTSs are usually complete within 7 days (mean diameter ± standard deviation, 270 ± 53.5 μm).

Multicellular tumour spheroids at the seventh day of culture were treated with RES 8, 16, 32 and 64 μM and kept for an additional 48 and 72 h. Data of five different experiments are reported.

Q-banding karyotype analysis

Metaphase cells were prepared by standard cytogenetic methods: cells were treated with colcemid 50 ng/ml (GIBCO) for 2 h. The suspension was exposed to hypotonic shock with 1% sodium citrate for 20 min at 37 °C, fixed in methanol/glacial acetic acid 3/1, air dried and mounted on slides. QFQ banding was performed on a minimum of 100 metaphases per cell line and chromosomes were karyotyped according to the International System for Human Cytogenetic Nomenclature (14).

Flow cytometric cell cycle analysis

Cells from monolayer and disaggregated spheroids were fixed in ethanol 70% and maintained at 4 °C. Fixed cells ($1–2 \times 10^6$ cells) were washed in phosphate-buffered saline (PBS; GIBCO) and stained with 1 ml of a solution containing propidium iodide (PI) 10 μg/ml in PBS, 12.5 μl RNAse (1 mg/ml in water) and maintained at 4 °C for at least 12 h. Monoparametric cell cycle analysis was performed on at least 20,000 cells for each sample by a FacsCalibur instrument (Becton Dickinson, Sunnevally, CA, USA) equipped with a 488 nm laser and the PI red fluorescence pulse was detected at 620 nm and the distribution of the different cell cycle phases was calculated (15).

Morphological evaluation

Cell culture morphology was evaluated by means of phase contrast microscopy and scanning electron microscopy (SEM) in two- and three-dimensional cell cultures, and histology in MCTSs.

To perform SEM, cells were washed twice in PBS and then fixed in 2.5% glutaraldehyde in phosphate buffer for 24 h at 4 °C. At the time of analysis, representative samples were recovered, immediately placed on a paper filter and observed in low-vacuum modality at a high voltage of 10 kV. The SEM analysis was performed using a Philips scanning electron microscope (Mod. XL20; Philips, Milan, Italy) (16).

Multicellular tumour spheroids histology was performed by fixing spheroids in 2.5% glutaraldehyde in phosphate buffer and dehydrated in increasing concentrations of ethanol (80°, 90°, 100°). Following this, an incubation of 2 h with Bio-clear (Bio-Optica, Milan, Italy) and an overnight incubation with paraffin wax were performed. The paraffin-embedded samples were cut into semithin sections (0.5 μm) and stained with haematoxylin and eosin.

Colony-forming assay

A colony-forming assay was performed by plating a single-cell suspension obtained from two- and three-dimensional cell cultures.

After treatments, cell cultures underwent trypsinic disaggregation (trypsin 0.25%, EDTA 0.02%; Sigma) and 500 cells/well were placed in six-well plates, in triplicate. Fifteen days after the seeding, colonies were fixed with methanol, stained with crystal violet and counted.

Alkaline phosphatase, lactate dehydrogenase and cancer antigen 19-9 medium release

After treatments, the medium of two- and three-dimensional cultures was collected, tested for alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities as markers of cytolysis and plasmatic membrane injury, and for cancer antigen (CA) 19-9 as a tumour marker, and compared with untreated samples (17–19).

Alkaline phosphatase and LDH activities were measured using the colorimetric technique following the manufacturer's instructions (Roche, Milan, Italy).

Cancer antigen 19-9 was measured by a sequential chemiluminescent immunometric assay following the manufacturer's instructions (DPC, Los Angeles, CA, USA).

Cellular transglutaminase activity test and immunofluorescence

Transglutaminases (TGs) are an enzymatic family present in different tissues. TG2 is the most studied and abundant in epithelium (20), and is considered to be an apoptotic marker.

Transglutaminase activity was colorimetrically analysed following the manufacturer's instructions (Covalab, Lyon, France) and the microtitre plate was measured at 450 nm with a microplate reader (Bouty Diagnostici, Milan, Italy).

In three-dimensional cell cultures, TG2 was evaluated by means of an immunofluorescence technique, as described by Salmi *et al.* (21). Unfixed sections of treated and untreated MCTSs were firstly incubated with anti-TG2 mouse antibodies (CUB 7402; NeoMarkers, Fremont, CA, USA) and then with fluorescein-conjugated antimouse immunoglobulin antibodies (Dako, Milan, Italy). Control experiments were performed using only the primary or the secondary antibody.

Statistical analysis

Each experiment has been repeated in triplicate three times. Data were expressed in percentages as mean ± standard deviation (SD) and analysed using two-tailed Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Q-banding karyotype analysis

A preliminary analysis of karyotype was performed to investigate possible genetic variations of cholangiocarcinoma cells from the originally described SK-ChA-1 cells (12). SK-ChA-1 cells showed a pseudo-triploid karyotype with a modal number of 71 chromosomes (range 65–71). The majority of analysed metaphases (70%) presented 12 marker chromosomes in which we could recognise portions of chromosomes 1, 3, 4, 11, 14, 15, 17, 18 and 19.

Effects of resveratrol treatment on SK-ChA-1 cells

Cytogenetic analysis performed after 48 h of RES exposure on two-dimensional cell cultures did not reveal structural rearrangements and/or chromosomal numerical abnormalities in comparison with the untreated cells. Q-banding karyotype analysis was not performed on MCTSs because of their partial block in the G_1 cell cycle phase.

Resveratrol contact dramatically influenced, in a dose-dependent fashion, the colony-forming assay of treated cells cultured both two- and three-dimensionally, compared with untreated cultures. In the two-dimensional cell cultures, the number of colonies, expressed as per cent of the untreated controls, after 48 h of RES 8, 16, 32 and 64 μM exposure, was 89.20 ± 12.43, 57.55 ± 11.46, 20.38 ± 2.89 and 16.30 ± 4.09 respectively (Fig. 1A). In MCTS, the number of colonies formed from a single-cell suspension, after 3 days of RES treatment (32 and 64 μM), was 67.82 ± 15.10 and 35.88 ± 4.69 respectively (Fig. 1B). Lower concentrations of RES and shorter exposure times had no effect.

Proliferation was dose-dependently inhibited in both culture systems: two-dimensional cultured cell growth, expressed as per cent of cell number at the end of the treatments, was 10% compared with untreated cells (100%) at the dose of 64 μM. In MCTS, the growth inhibition reached 50% at the highest RES dose vs untreated MCTS.

The ALP and LDH activities in the medium of RES-treated cell cultures were increased compared with untreated controls: the increased activity of both enzymes was about 40-fold in the two-dimensional cell cultures and about 20% in the MCTS (Fig. 2A and B).

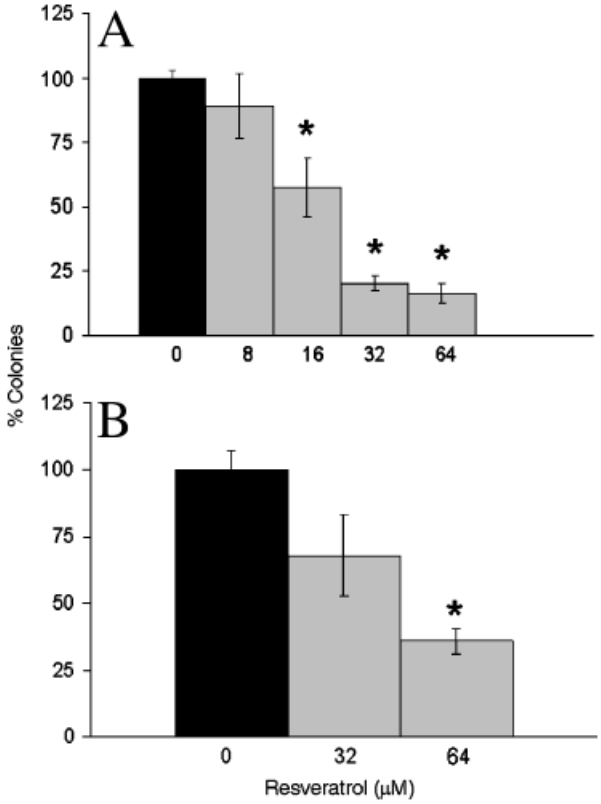

Fig. 1. Colony-forming assay of two- (A) and three-dimensional multicellular tumour spheroids (MCTS) (B) cell cultures after treatments with resveratrol at different concentrations. Two-dimensional cell cultures were treated for 48 h and MCTS for 72 h. Data are expressed as per cent of untreated controls. Standard deviations of five independent experiments are indicated. *P-value < 0.05.

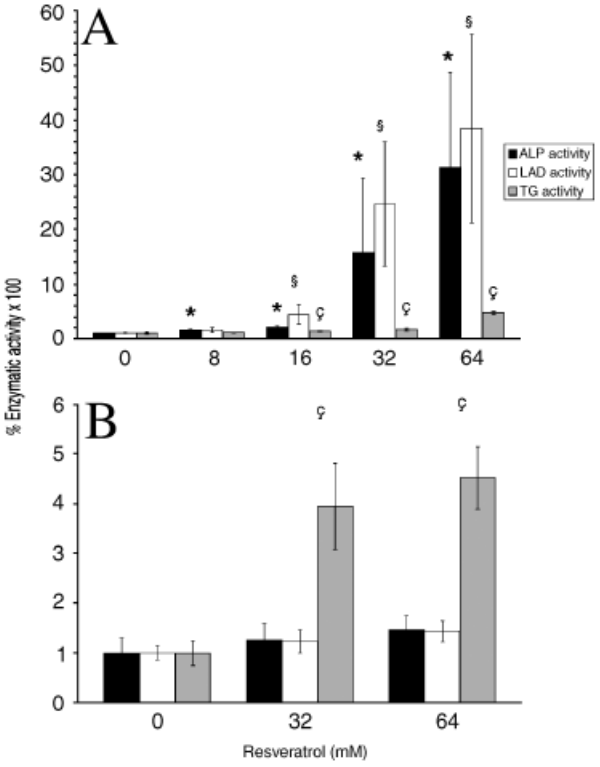

Fig. 2. Alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and transglutaminase (TG) activities in the medium of two- (A) and three-dimensional multicellular tumour spheroids (MCTS) (B) cell cultures treated with resveratrol at different concentrations. Two-dimensional cell cultures were treated for 48 h and MCTS for 72 h. Data are expressed as per cent of untreated controls. Standard deviations of five independent experiments are indicated. *, § or çP-value < 0.05.

Both untreated and treated cells did not release CA 19-9 under the experimental conditions tested.

Transglutaminase activity was increased in RES-treated cultures compared with untreated controls. In the two-dimensional cell cultures, the increase followed a dose-dependent fashion and reached 450% of the control activity at the highest RES concentration (108.33 ± 8.46, 135.37 ± 13.38, 166.83 ± 31.39 and 461.83 ± 32.49 respectively, for RES doses 8, 16, 32 and 64 μM) (Fig. 2A). In the MCTS, the TG activity increase reached 400% of untreated controls in both the RES doses tested (Fig. 2B).

In MCTS, the TG2 immunofluorescence evidenced a notable increase of the fluorescent signal in the RES-treated cell cultures, compared with the untreated MCTS. In particular, in untreated MCTS the fluorescence was localised in the peripheral ring of the spheroid, while in the RES-treated MCTS the signal was diffused throughout the spheroid structure. At greater magnification, it was possible to note that TG2 was clearly expressed in the cytoplasm of the untreated MCTS, with a picnotic pattern, while in treated cell cultures the fluorescent signal was diffused in the cytoplasm and more evident in the plasmatic membrane zone (Fig. 3).

At light microscopy, the treated two-dimensional cell cultures, compared with the untreated, showed severe cell destruction with morphological abnormalities characterised by different cell shapes, sizes and the presence of intracytoplasmatic vacuoli and inclusions; a larger number of floating cells was evident (Fig. 4).

Untreated MCTS showed a regular ovoid or a spherical shape with a smooth surface; RES-treated MCTS maintained their gross shapes and presented an irregular surface.

At histology, the untreated three-dimensional cell cultures were characterized by a solid cellular growth with the formation of focal ductal-like structures, whereas the treated MCTS had an irregular surface with clusters of cells detaching from the body of the

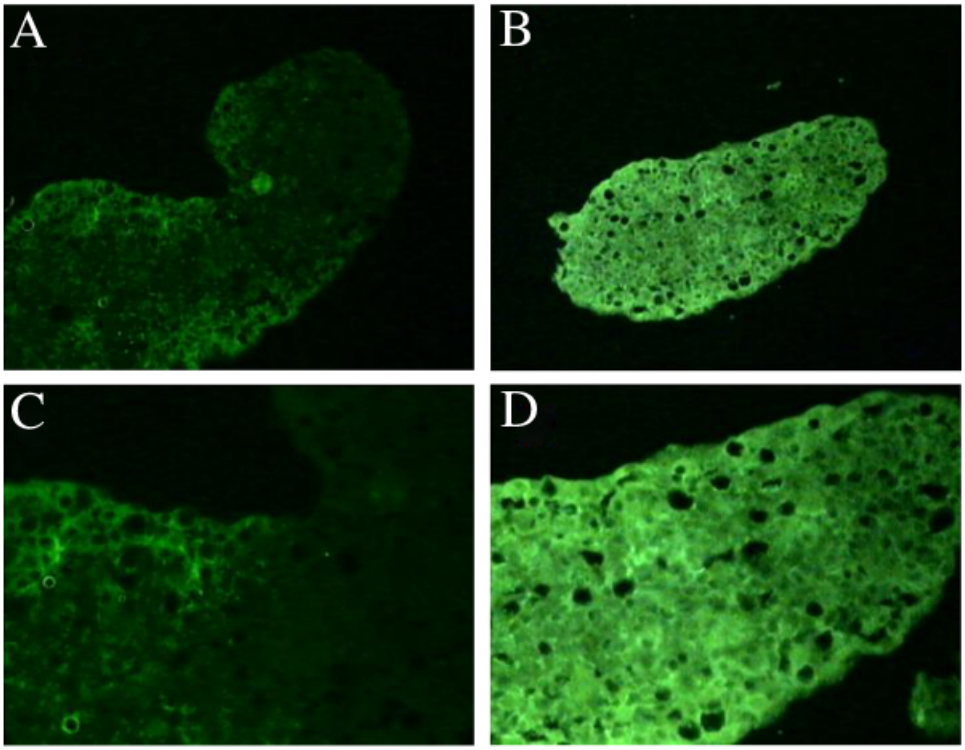

Fig. 3. Transglutaminase 2 (TG2) immunofluorescence of SK-ChA-1 three-dimensional cell cultures treated with resveratrol 64 μM for 72 h (B, D) and untreated controls (A, C). The major fluorescence signal present in the treated cell cultures is notable. Magnification shows the cytoplasmatic localisation of TG2 in the untreated cells and the diffuse pattern in the treated cells, with a major signal in the submembraneous zone of the cell.

main spheroid structure, and signs of necrosis in the core (Fig. 5).

At SEM, untreated MCTS appeared densely organised and tightly packed with duct-like structures formed by the interaction between large and flat, and small and round cells (Fig. 6). Treated MCTS lost the three-dimensional structure and partially collapsed; at $\times$ 3000 magnification, an altered plasmatic membrane surface and blebs were observed (Fig. 6F).

Cell cycle analysis

Figure 7A shows the DNA histograms of the SK-ChA-1 cells obtained from two-dimensional cell cultures and evaluated by fluorescent-activated cell sorting. The untreated cultures showed a pattern typical of cells in the exponential growth phase. At 24 h, RES caused an accumulation in the G_1/S boundary phases, and this was in agreement with the reduced growth of the treated cells compared with the control ones. At 48 h, the majority of treated cells were in the S-early phase: between 24 and 48 h, the number of cells slightly decreased, also suggesting a cytotoxic effect of RES. At 72 h, the DNA histograms of the treated cells were similar to those of controls and the number of cells started to increase, suggesting a repair of the damage caused by RES. In MCTS, different cell cycle perturbations were found. Figure 7B shows the cell cycle perturbations evaluated after a 72-h exposure with 32 or 64 μM RES, and those after 7 days of culture. The majority of the treated cells were in the G_0/G_1 phases; however, the cell cycle phase distribution was similar in the control ones.

Discussion

Our data demonstrate for the first time that RES has *in vitro* cytotoxic effects on the cholangiocarcinoma cells. However, conducting studies on cholangiocarcinoma and its therapeutic approaches is difficult because of the lack of suitable *in vitro* models, the low percentage (5%) of cholangiocytes inside the hepatic mass and their peculiar anatomical topography (1, 22). In this study, we used the SK-ChA-1 cell line, derived from a human cholangiocarcinoma (12), and cultured by two different models: the two- and the three-dimensional cell culture systems. The three-dimensional cell culture system MCTS is nowadays

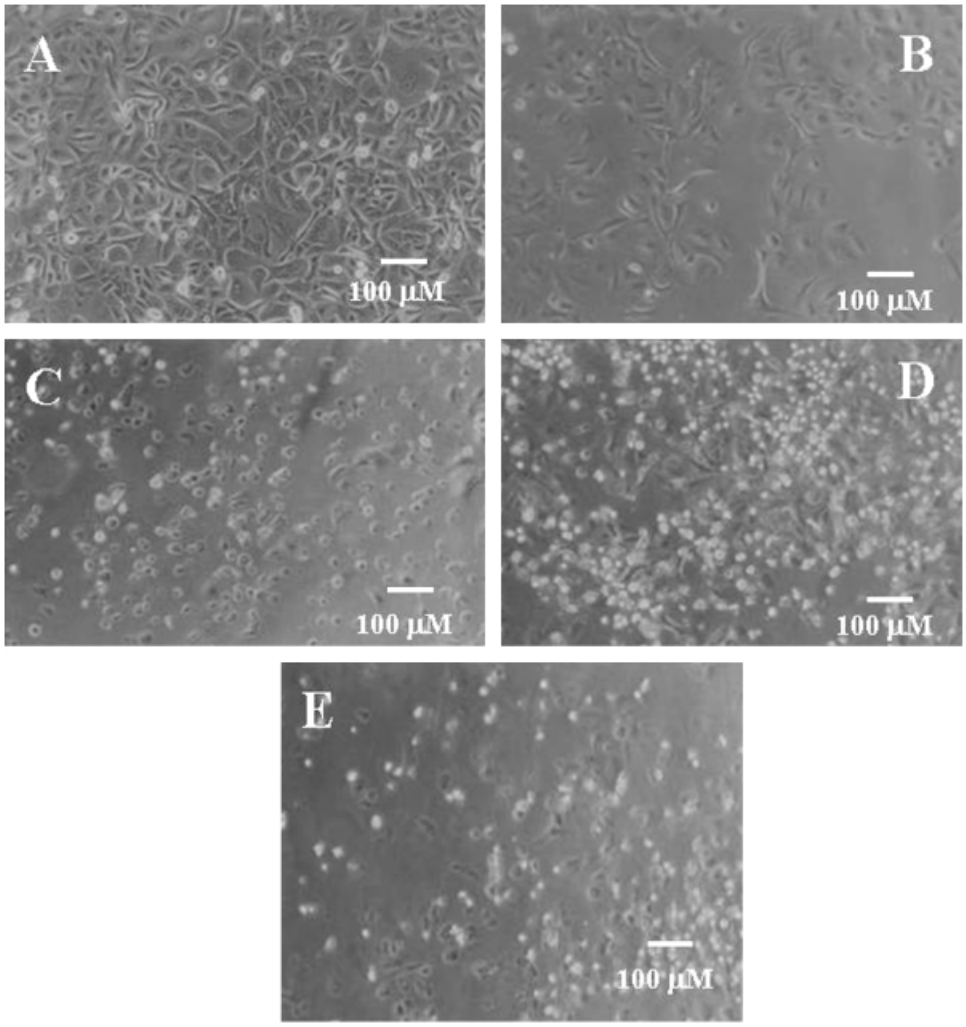

Fig. 4. Phase contrast microscopy of two-dimensional SK-ChA-1 cell cultures treated with resveratrol at increasing doses: untreated (A), 8 μM (B), 16 μM (C), 32 μM (D) and 64 μM (E) for 48 h. The increasing presence of floating cells dependent on the resveratrol dose is notable (clusters of floating cells are indicated by white arrows). Magnification × 10.

considered to be pivotal in '*in vitro*' oncological research (23). In fact, in the MCTS cultures, the drug uptake can be different from that in the traditional monolayer system as only superficial cells are directly in contact with the medium, and there are different cell-to-cell contacts and intercellular signalling (23).

Among the molecules under investigation for their anticancer potential both as chemopreventive and chemotherapeutic agents, RES is one of the most promising (5). Researchers are joining the challenge testing RES on different human cancer cell lines and animals (6–8). Our study is the first to present the biological effects of RES on a cholangiocarcinoma cell line. This type of cancer has a poor prognosis and an increasing prevalence in young people; actually, unoperable patients, who are the majority, are encouraged to follow chemotherapy and/or radiotherapy clinical trials. In fact, traditional therapies do not change the survival rate because they could have difficulties in arriving at cholangiocytes through the haematic flow (1–3). From this point of view, RES could be effective in the cholangiocarcinoma treatment because of its metabolism (5). In fact, orally taken RES is rapidly absorbed by the intestinal mucosa and reaches hepatocytes, where it actively accumulates and from where it is secreted into the bile (24). Nevertheless, RES bioavailability is crucial, considering its practical application; although different studies (25, 26) have shown a low plasmatic peak after RES oral administration and a short half-life, nothing is known about RES portal and bile concentrations in humans, its cholehepatic shunting and the maximum

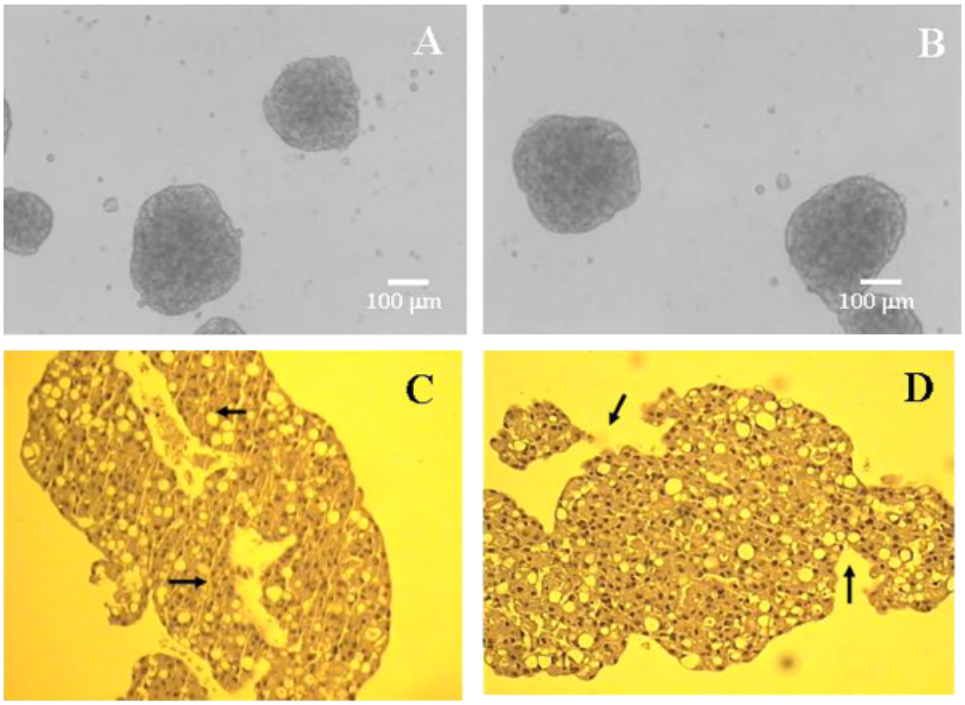

Fig. 5. Light microscopy and histology of three-dimensional multicellular tumour spheroids SK-ChA-1 cell cultures untreated (A and C) or treated with resveratrol 64 μM for 72 h (B and D). In (C), the presence of large ductal-like structures (black arrows) and in (D) the presence of cellular clusters partially or totally detached from the main spheroid body (black arrows) are notable.

tolerated dose (27). Animal studies showed that RES actively accumulates and penetrates into the liver and has a relevant enterohepatic circulation (28, 29). Thus, it is possible that hepatic and portal concentrations could be different from the plasmatic one as reported for other drugs and molecules (adriamycin, methylergometrine and bile acids) (30–32).

The dose-dependent cytotoxicity of RES is present in both the experimental models we used and MCTS confirm the major 'chemoresistence' of this model, which is sensitive only to the higher doses of RES and after a longer contact-time (23). The LDH and ALP release in the medium suggests an alteration in the integrity of the cholangiocyte's plasma membrane with leakage of large molecules and cytolysis (17, 18), in a dose-dependent fashion.

This toxic effect appears to be the cause of an apoptotic mechanism, as shown by the morphological findings and by the increased intracellular TG activity. TGs are a family of intracellular Ca^{2+}-dependent enzymes that catalyse post-translational modifications of proteins by establishing glutamyl–lysine cross-links and/or covalent incorporation of di- and polyamines (20). TG2 is the most abundant in the epithelial cells and is related to apoptosis, where it acts during the final phases of the processes, in the protein packaging (20). While we for the first time demonstrated the implication of TG in the RES cytotoxic mechanism, other authors showed RES involvement in other apoptotic pathways (7, 8). Scarlatti *et al*. (7) demonstrated an increase of apoptosis in MDA-MB-231 breast cancer cell line induced by RES through an increase of ceramide cellular content.

A perturbation of the cell cycle was observed in the two-dimensional cell cultures: while the untreated cells were in the exponential growth phase, after 24 h of treatment, cells accumulated in the G_1/S phase and after 48 h in the S-early phase. These findings, reported also in other cell lines treated with RES and associated with an upregulation of cyclins (9), suggest that the RES ability to block these sensible cell cycle phases has a chemotherapeutic potential, possibly in association with other molecules (33). The effect of RES on the cell cycle in MCTS was less evident, but similarly present: MCTS did not disaggregate but showed morphological alterations, characterised by the presence of apoptotic blebs. In addition, the culture medium was enriched in suspended single cells, presumably discharged from the MCTS themselves. The significant increase in TG activity and the major expression of TG2 demonstrated by immunofluorescence are representative of the occurrence of an active apoptosis (20).

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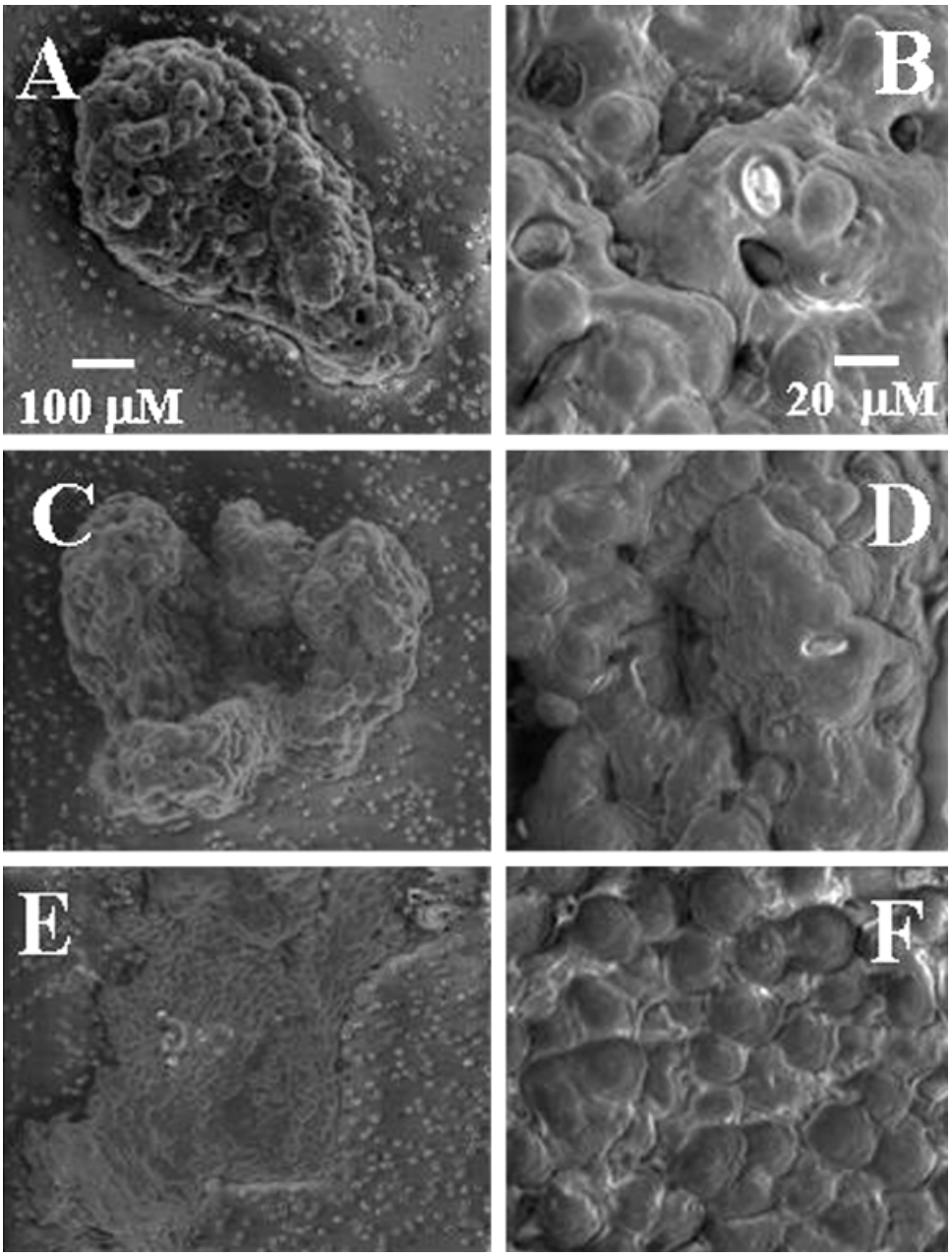

Fig. 6. Scanning electron microscopy of three-dimensional multicellular tumour spheroids (MCTS) SK-ChA-1 cell cultures treated with resveratrol for 72 h. (A and B) control MCTS, (C and D) 32 μM resveratrol-treated MCTS, (E and F) 64 μM resveratrol-treated MCTS. In (F), the partial loss of the intercellular adhesion is notable. × 600 (A, C, E) and × 3000 (B, D, F) magnification.

In the literature, it is still debated whether the effect of RES on the cell cycle is reversible or directly induces apoptosis (5): in our two-dimensional model, the apoptotic damage was partially repaired after 72 h, suggesting the coexistence of different pathways of damage and a partial reversibility of the process.

SK-ChA-1 cellular monolayers do not express CA 19-9 (12). In our experiments, neither the different culture system (MCTS) nor the RES treatment induced the secretion of this marker, making this antigen unsuitable for further studies.

Compared with the previous studies by Marienfeld *et al*. (10) and Naus *et al*. (11) conducted on different cholangiocarcinoma cell lines, cultured two-dimensionally and treated with the polyphenolic compound tannic acid, RES demonstrated a higher cytotoxic and pro-apoptotic potential. It is possible to speculate that the activity of this class of molecules on the cell cycle

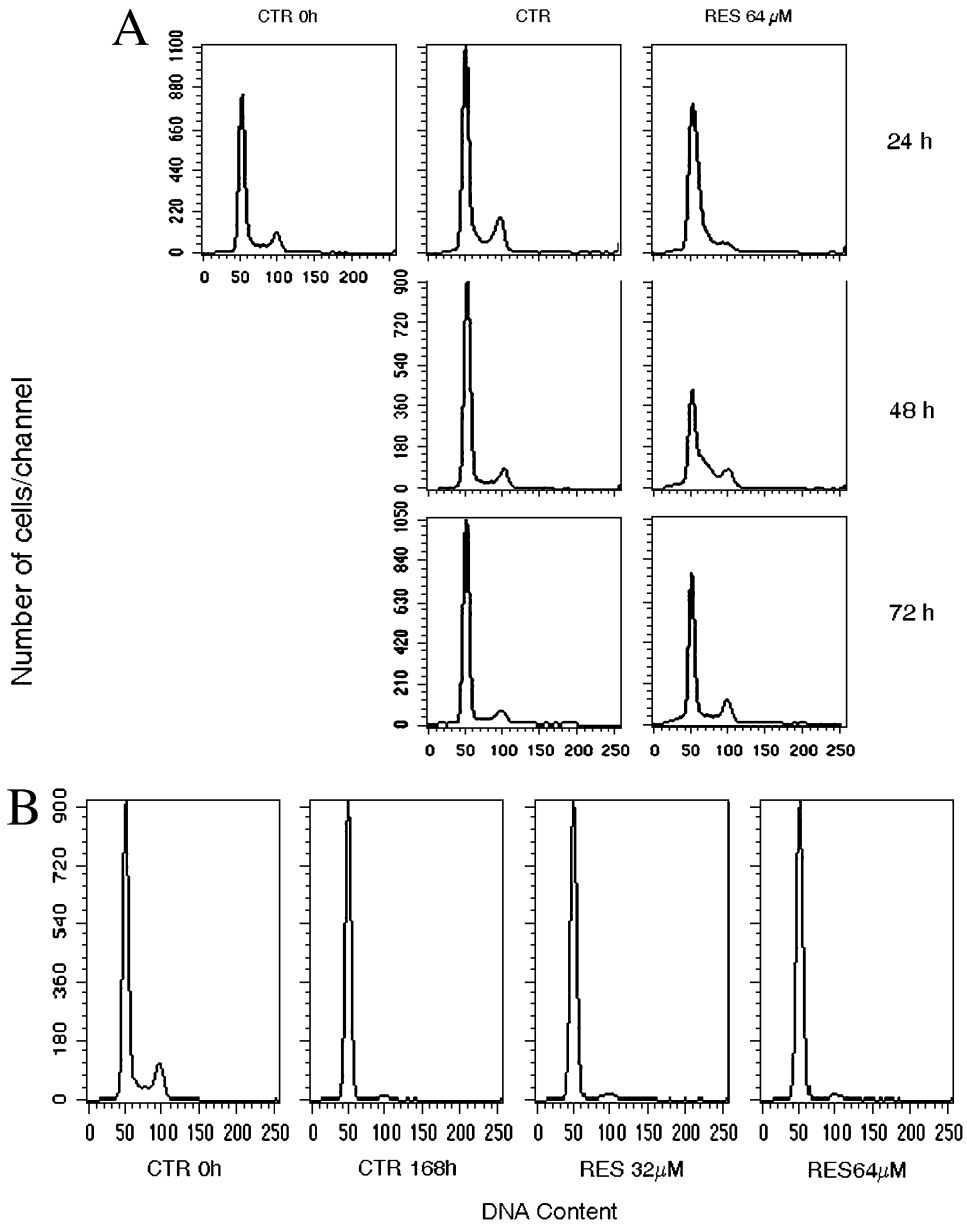

Fig. 7. Flow cytometric cell cycle analysis of two- (A) and three-dimensional (B) SK-ChA-1 cell growth evaluated at different times during resveratrol (RES) exposure. In (B), the RES exposure time was 72 h. CTR, untreated control cells; RES, resveratrol.

could have a synergistic effect with other chemotherapy agents, as suggested by the cell cycle block in the G_1/S phase.

With all the caution called for in drawing conclusions from *in vitro* cell line experiments, our data obtained in two different *in vitro* models, together with the recent knowledge concerning RES anticancer properties, suggest further investigations for a possible chemotherapic role of RES in the treatment of cholangiocarcinoma. Theoretically, should our findings be confirmed on other cell lines and animal models, and the pharmacokinetics of RES in humans be clarified, it could be suggested for trials as a chemotherapeutic drug in unoperable patients. It could be administered in combination with other chemotherapic agents (34), orally or directly injected into the biliary tract through a retrograde catheterism, as palliative care applied on biliary stents for a long local release or as a chemopreventive drug in high-risk patients like those affected by primary sclerosing cholangitis or hepatic cystic diseases (1).

Acknowledgements

We thank Prof. Riccardo Ghidoni for his help in the preparation of the manuscript and Kevin Smart for the English revision.

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