Pancreatic cancer cells retain the epithelial-related phenotype and modify mitotic spindle microtubules after the administration of ukrain *in vitro*

Nicoletta Gagliano[a], Tatiana Volpari[a], Marco Clerici[a,c], Letizia Pettinari[a], Isabella Barajon[a], Nicola Portinaro[b], Graziano Colombo[c], Aldo Milzani[c], Isabella Dalle-Donne[c] and Carla Martinelli[a]

The aim of this study is to characterize the phenotype of pancreatic ductal adenocarcinoma (PDAC) cells in relation to the expression of epithelial-to-mesenchymal transition (EMT) markers and determine whether ukrain, an anticancer drug based on the alkaloids extracted from greater celandine, modulates *in vitro* the malignant behavior of PDAC cells in order to extend our understanding of its therapeutic potential. Three cell lines (HPAF-II, HPAC, and PL45) were treated with ukrain (5, 10, and 20 μmol/l) for 48 h or left untreated (control). Cell proliferation was assessed by growth curves. Apoptosis was determined by Hoechst nuclear staining and by cytochrome *c* and caspase-8 expressions. The EMT markers E-cadherin, β-catenin, and vimentin, as well as actin and tubulin cytoskeletons, were analyzed by immunofluorescence. Interphase and mitotic microtubules as well as abnormal mitotic figures were studied by fluorescence microscopy after tubulin immunolabeling. Ukrain strongly suppressed cell proliferation and induced apoptosis possibly through an extrinsic pathway as cytochrome *c* immunoreactivity suggested that the integrity of the mitochondria was not affected. Tubulin expression indicated an antiproliferative effect of ukrain on the basis of alterations in mitotic spindle microtubule dynamics, leading to abnormal mitosis. Membranous E-cadherin/β-catenin immunoreactivity was similarly expressed in control-treated and ukrain-treated cells, although the drug upregulated E-cadherin in cell lysates. Our results suggest that ukrain exerts its chemotherapeutic action on PDAC cells targeting mitotic spindle microtubules, leading to abnormal mitosis and apoptosis, and favoring cell cohesiveness. The differentiated epithelial phenotype of HPAF-II, HPAC, and PL45 cell lines concomitant with a highly invasive potential suggests that further experiments will be necessary to definitively clarify the role of EMT in PDAC progression. *Anti-Cancer Drugs* 00:000–000 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2012, **00**:000–000

Keywords: apoptosis, epithelial-to-mesenchymal transition, mitotic spindle, pancreatic ductal adenocarcinoma, ukrain

Departments of [a]Biomedical Sciences for Health, EML-Extracellular Matrix Laboratory, [b]Medical Biotechnologies and Translational Medicine, School of Medicine and [c]Life Sciences, Università degli Studi di Milano, Milan, Italy

Correspondence to Nicoletta Gagliano, PhD, Department of Biomedical Sciences for Health, EML-Extracellular Matrix Laboratory, University of Milan, Via Fratelli Cervi 93, 20090 Segrate, Milan, Italy
Tel: +39 02 50 330 450; fax: +39 02 50 330 452;
e-mail: nicoletta.gagliano@unimi.it

Received 10 January 2012 **Revised form accepted** 20 April 2012

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the USA and results in an estimated 227 000 deaths per year worldwide [1]. PDAC is one of the most aggressive tumors and, because of its metastasis-prone and locally resistant nature, is characterized by a very poor prognosis and a 5-year survival of less than 5% for all stages of the disease [2–6]. The high mortality of PDAC could be partly because of the ability of cancer cells to acquire invasive characteristics. Thus, the tumors in most patients diagnosed with pancreatic cancer are unresectable and the patients usually die from metastatic cancer dissemination even after treatment using existing therapeutic protocols [2].

As for most carcinomas, the phenotypic switch of pancreatic epithelial cells to mesenchymal cells, the so-called epithelial-to-mesenchymal transition (EMT), plays a pivotal role in progression toward malignancy [7,8]. During EMT, the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype render tumor cells invasive and with the ability to disseminate, forming distant metastases [7,9]. The loss of E-cadherin is considered a key marker of EMT. E-cadherin is a transmembrane glycoprotein whose intracellular domain is linked to β-catenin to form the E-cadherin/β-catenin complex, mediating cell–cell adhesion and playing a major role in the control of epithelial cell architecture and differentiation [10–12]. In addition to the dissolution of junction proteins and the loss of epithelial proteins, EMT is accompanied by increased expression of mesenchymal proteins, such as the intermediate filament protein vimentin, and the actin cytoskeleton reorganization [7].

Current therapy for PDAC is surgery followed by adjuvant radiotherapy and chemotherapy for early-stage disease and palliative chemotherapy for advanced disease [3]. Gemcitabine is the standard drug in both adjuvant and

0959-4973 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32835507bc

palliative treatments [13,14]. To improve therapeutic outcome, it is necessary to identify novel agents or combination therapy that might be helpful in treating pancreatic cancer patients with fewer side effects.

Ukrain (UK, NSC 631570) is an anticancer drug based on the alkaloids extracted from greater celandine (*Chelidonium majus*, a member of the *Papaveraceae* family) [15], made water soluble by derivatization with thiotepa. Some preclinical and clinical investigations suggest that UK, given as a single drug or in combination with conventional chemotherapy, is clinically effective in the treatment of a range of solid tumors including those in the colon, rectum, breast, pancreas, bladder, and ovary [16–20]. It has been shown that UK is a safe and effective drug in the treatment of patients with unresectable [19,21] and resected [22] pancreatic cancer. When used as adjuvant chemotherapy in combination with gemcitabine, UK seems to lead to prolonged survival [22].

Considering the key role of EMT in the progression of carcinoma, we aimed to characterize *in vitro* the phenotype of three PDAC cell lines (HPAF-II, HPAC, and PL45), with particular focus on the analysis of some key markers of EMT, and we examined the possible modulation of PDAC cells by UK, to assess its potential therapeutic effect on pancreatic cancer. We also analyzed the effect of UK on cell proliferation and apoptosis pathways to extend our understanding of the mechanisms of antitumor and chemopreventive potentials of UK. We show that HPAF-II, HPAC, and PL45 cells retain *in vitro* a differentiated epithelial phenotype, and that UK seems to exert its chemotherapeutic action by suppressing the dynamic instability of mitotic spindle microtubules, leading to abnormal mitosis and apoptosis, and possibly favoring cell cohesiveness to counteract tumor invasion.

Methods

Cell cultures

For this study, three human pancreatic cancer cell lines from ductal adenocarcinoma were used: HPAF-II, HPAC, and PL45 (American Type Culture Collection). Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/l glutamine, antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin), and 0.25 μg/ml amphotericin B.

Drug preparation

UK (kindly provided by Nowicky Pharma, Vienna, Austria) was obtained as a 5 mg water-soluble *C. majus* alkaloid thiophosphoric acid derivative in 5 ml of water for injection. According to the manufacturer's instructions, the stock solution was stored at room temperature.

Ukrain treatment

Pancreas cancer cells were treated at 80% of confluence with three final concentrations of UK (5, 10, and 20 μmol/l). Untreated cells served as controls (CTs). The doses and timing of UK were chosen on the basis of our previous results [23,24]. CTs and UK-treated cells were incubated for 48 h in a serum-free medium and then harvested by trypsinization (trypsin 0.25%; EDTA 0.1% in PBS). Each cell line was cultured in duplicate.

Cell morphology and viability

Cell morphology of CTs and UK-treated cells was analyzed by phase-contrast microscopy using a Leica DM IL microscope (Leica Microsystems, Wetzlar, Germany). Cell viability was determined by trypan blue exclusion. Cells were plated onto six-well plates (300 000 cells/well) and allowed to attach. Fresh medium containing 5, 10, and 20 μmol/l of UK was added, and cells were counted after 0, 24, 48, and 72 h.

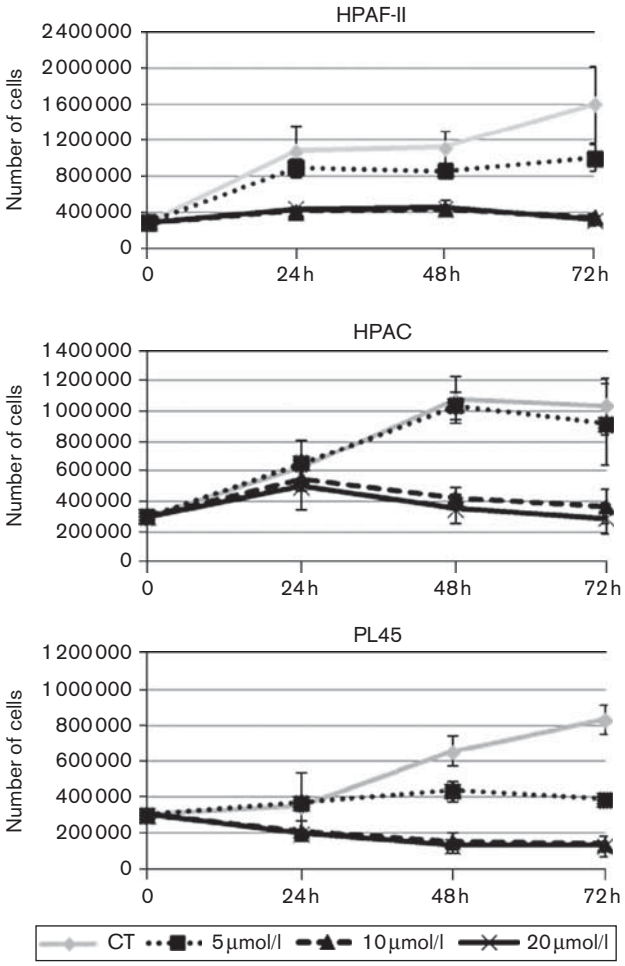
Apoptosis

The apoptosis ratio in CTs and UK-treated cells was analyzed at the 24, 48, and 72 h time points using Hoechst 33258 and propidium iodide (PI). The assay allows the fluorescence detection of the compacted state of chromatin in apoptotic cells. The blue-fluorescent Hoechst 33258 dye stains the condensed chromatin of apoptotic cells more brightly than the chromatin of normal cells, and the red-fluorescent PI dye is permeant only to dead cells. The staining pattern resulting from the simultaneous use of these dyes allows the differentiation of normal, apoptotic, and dead cell populations. Briefly, cells were harvested and washed in cold PBS. Cell density was adjusted to $\sim 1 \times 10^6$ cells/ml in PBS. A volume of 1 μl of Hoechst 33258 stock solution (1 mg/ml stock in water) was added for 1 ml of cell suspension and cells were incubated for 20 min. Then, 1 μl of PI (30 μg/ml stock) was added and cells were observed under the fluorescence microscope. The apoptotic/necrotic rate was calculated as a ratio of apoptotic/necrotic cells to the total number of cells per field. For each sample, six fields were analyzed.

Immunofluorescence microscopy

PDAC cells were cultured on round coverslips, 12 mm in diameter, placed in 24-well culture plates. After 48 h, CTs and UK-treated cells were washed in PBS, fixed in 4% paraformaldehyde in PBS containing 2% sucrose for 5 min at room temperature, postfixed in 70% ethanol, and stored at −20°C until use. The cells were then washed in PBS three times and incubated for 1 h at room temperature with the monoclonal primary antibodies: anti-E-cadherin (1 : 500; Becton Dickinson, Buccinasco, Milan, Italy), anti-tubulin (1 : 2000; Sigma-Aldrich, Milan, Italy), and anti-vimentin (1 : 100; Novocastra, Leica Microsystems, Newcastle, UK). Secondary antibody conjugated with Alexa 488 (1 : 500; Molecular Probes, Invitrogen, Life Technologies, Monza, Italy) was applied for 1 h at room temperature, followed by rinsing with PBS. Negative CTs were incubated excluding the primary antibody.

Fig. 1



Cell viability. Time-dependent effect of ukrain on cultured pancreatic ductal adenocarcinoma (PDAC) cell proliferation. Cells were plated in six-well plates (300 000 cells/well) and allowed to attach. Fresh medium containing 5, 10, and 20 μmol/l ukrain was added, and cells were counted at the indicated times. Untreated PDAC cells were used as controls (CTs). Data are means ± SD for duplicate experiments.

For actin cytoskeleton detection, rhodamine-phalloidin (Sigma-Aldrich) was used. Cells were incubated with 50 μmol/l rhodamine–phalloidin in PBS containing 0.2% triton X-100 for 45 min in the dark and then washed extensively in PBS.

After the labeling procedure was completed, the coverslips were incubated for 15 min with 4′6-diamidino-2-phenylindole (DAPI) and mounted onto glass slides using Mowiol (Sigma-Aldrich) mounting medium. The cells were photographed using a digital camera connected to a Nikon Eclipse microscope (Nikon Instruments, Calenzano, Florence, Italy).

Mitotic index

In each field, the number of cells showing normal and abnormal mitoses was counted under the fluorescence microscope at × 20 magnification. For each sample, six fields were analyzed. The number of cells showing normal and abnormal mitoses was calculated as a ratio to the total number of mitoses and expressed as percentage.

Western blot

Cells lysates were prepared in 50 mmol/l Tris-HCl, pH 7.6, 150 mmol/l NaCl, 1% Triton X-100, 5 mmol/l EDTA, 150 mmol/l $MgCl_2$, 1% SDS, proteases inhibitors, and 1 mmol/l sodium orthovanadate. The lysates were incubated on ice for 30 min and centrifuged at 14 000*g*, for 10 min, at 4°C, to remove cell debris. Cell lysates (35 μg of total proteins) were diluted in SDS–sample buffer, loaded on a 12% SDS–polyacrylamide gel, separated under reducing and denaturing conditions at 80 V according to Laemmli, and transferred at 90 V to a nitrocellulose membrane in 0.025 mol/l Tris, 192 mmol/l glycine, and 20% methanol, pH 8.3, for 90 min. After electroblotting, the membranes were air dried and blocked for 1 h in 5% (w/v) nonfat dry milk in Tris-buffered saline Tween-20 (TBST; 150 mmol/l NaCl, 10 mmol/l Tris pH8.0/Tween-20 0.05%). For E-cadherin evaluation, membranes were incubated for 1 h at room temperature in monoclonal antibody to E-cadherin (1 : 2500 in TBST; Becton Dickinson) and, after washing, in horseradish peroxidase-conjugated rabbit anti-mouse serum (1 : 6000 dilution in TBST; Sigma). Immunoreactive bands were observed using the Opti-4CN substrate (Bio-Rad, Segrate, Milan, Italy). Caspase-8 expression was analyzed in cell lysates using a monoclonal antibody to caspase-8 (1 : 400 dilution in TBST, Santa Cruz Biotechnology, Santa Cruz, California, USA), shown by the horseradish peroxidase-conjugated rabbit anti-mouse serum (1 : 40000 dilution in TBST; Sigma) and the amplified Opti-4CN substrate (Bio-Rad). To confirm equal loading, membranes were reprobed with monoclonal antibody to α-tubulin (1 : 2000 dilution in TBST; Sigma).

Results

Cell morphology and viability

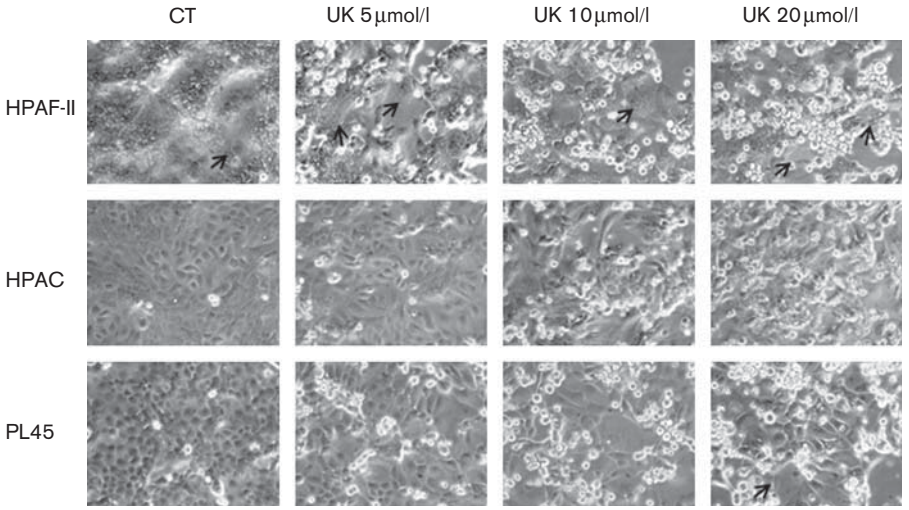
Growth curves showed that the administration of UK strongly affected cell proliferation in all three cell lines (Fig. 1). Inhibition of cell growth was evident starting from 24 h at all doses of UK in HPAF-II, 48 and 72 h after the administration of 10 and 20 μmol/l of UK in HPAC, and 48 and 72 h at all doses in PL45 cells.

Phase-contrast microscopy analysis indicated that untreated HPAF-II, HPAC, and PL45 cells showed a morphology consistent with an epithelial-like phenotype: they showed a rounded–polygonal shape and they grew as tight aggregates. Cell morphology was not modified by the administration of UK after 48 h (Fig. 2).

Microtubule organization and mitosis

In our previous study [23], we found that UK caused marked cell cycle arrest in the G2/M phase of HPAF-II,

Fig. 2



Cell morphology. Phase-contrast microphotographs showing the cell morphology of controls (CTs) and ukrain (UK)-treated PDAC cells after 48 h. CT cells were rounded–polygonal and grew adherent, and UK did not modify their morphology. HPAF-II cells show a heterogeneous size and frequent, large cells were evident (arrows). Large cells were also detectable in HPAC and PL45, but they were less frequent. Original magnification: × 20.

HPAC, and PL45 cells; therefore, we determined whether UK affects microtubule organization by indirect immunofluorescence using a monoclonal anti-tubulin antibody. The interphase microtubule network showed a normal arrangement and organization, and was not affected by the administration of UK (data not shown). Mitotic spindle microtubules were normally arranged in CT cells of the three cell lines considered. In particular, we observed some anaphase, telophase, or cytokinesis figures in the CT PDAC cell lines, indicating that untreated cells were able to complete cell division (Fig. 3a and b). By contrast, mitotic spindle microtubules were strongly modified in all UK-treated cells. Starting from 5 μmol/l of UK, the number of mitoses per field increased, showing evident abnormal mitotic spindles and the concomitant abnormal alignment of chromosomes during mitosis (Fig. 3c–f). UK-treated cells frequently showed more than one microtubule-organizing center and the mitotic spindles were tripolar or tetrapolar and sometimes multipolar. In some cases, the mitotic spindle was monopolar, containing microtubules radiating toward the periphery of the cell and the chromosomes were arranged as disorganized balls or as a ring (Fig. 3e). Very frequently, we observed an asymmetric arrangement of the spindle microtubules and also an asymmetric separation of the chromosomes in the two dividing cells. Anaphase or telophase figures were very rare or completely absent in UK-treated cells, suggesting that UK blocked the progression through mitosis. Some cells showed a 'grape-like' nuclear morphology (Fig. 3f) typical of cells undergoing mitotic slippage, and some large cells containing macronuclei were observed (Fig. 3g) as a result of abnormal mitosis.

To quantify this observation, we measured the ratio of abnormal mitoses in CTs and UK-treated cells. The abnormal mitosis ratio (Fig. 4) was higher after the administration of UK. In particular, the percentage of abnormal mitoses increased dose dependently in HPAF-II and HPAC cells, whereas in PL45, all mitoses were abnormal, starting from administration of 5 μmol/l UK.

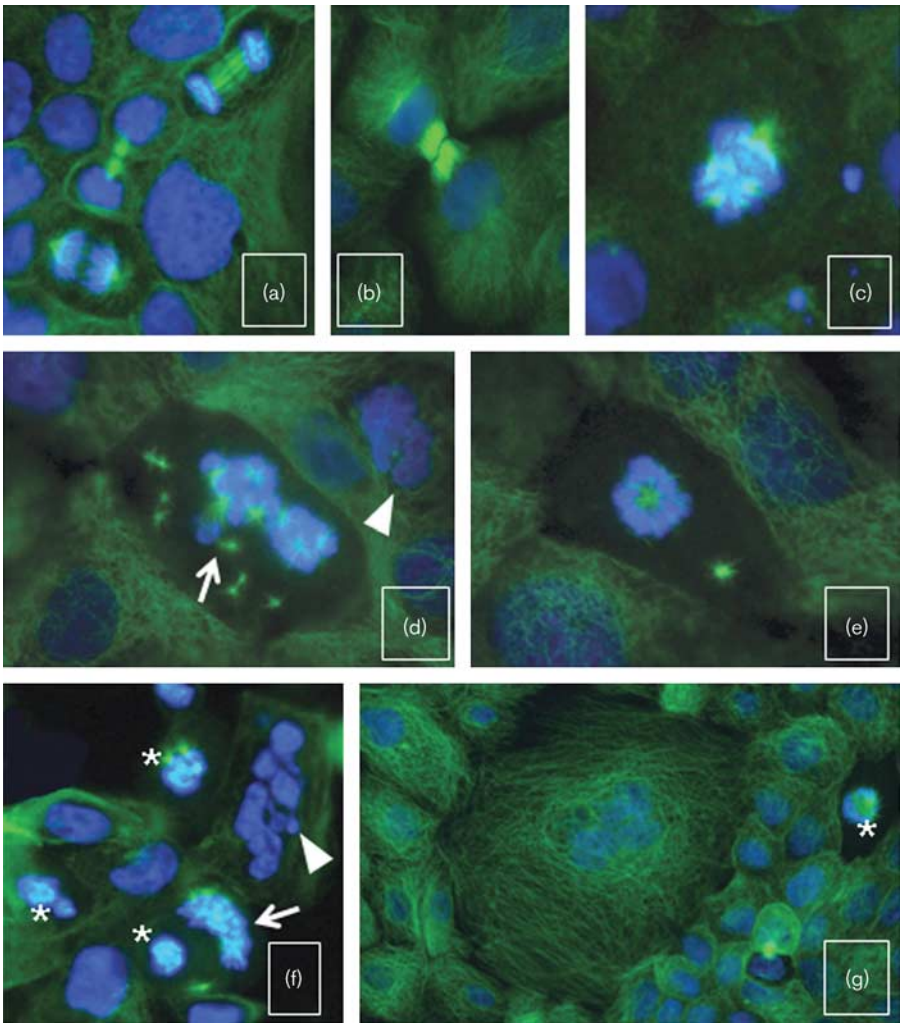
Apoptosis

We examined whether the drug was effective in inducing apoptosis using the blue-fluorescent Hoechst 33258 dye. Our results indicated an overall dose-dependent increase in the number of apoptotic cells after the three time points considered (Fig. 5a). Some necrotic cells were evident 24 and 48 h after the administration of 20 μmol/l UK and 72 h after treatment with 10 μmol/l UK in HPAF-II cells. In HPAC and PL45 cells, necrotic cells were evident starting after the administration of 10 μmol/l UK after all the time points considered (Fig. 5a).

To determine whether UK-induced apoptosis involves intrinsic or extrinsic apoptosis mechanisms, we analyzed cytochrome *c* and caspase-8 expressions.

Cytochrome *c* was similarly expressed in both CTs and UK-treated cells: a punctuate immunoreactivity in the cytoplasm in both CTs and treated cells (Fig. 5b), suggesting that mitochondrial integrity is not affected by the drug. In contrast, we observed increased procaspase-8 and caspase-8 levels in UK-treated cells (Fig. 6a and b).

Fig. 3



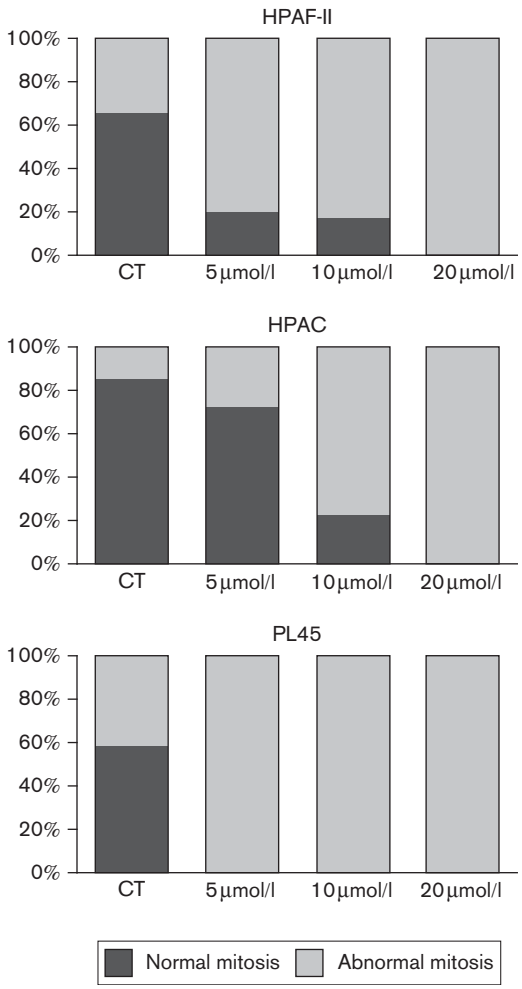
Tubulin cytoskeleton and nuclear morphology. Microphotographs showing representative immunofluorescence analysis showing the tubulin cytoskeleton during interphase and mitosis. In control (CT) cells, many normal mitoses were observed, and some anaphases, telophases, and cytokinesis were evident (a, b). In ukrain-treated cells, frequent abnormal mitoses were detected, characterized by multipolar mitotic spindles (asterisks) and asymmetric chromosome separation in daughter cells (arrows; c, d; asterisks in f and g). Many cells had monopolar mitotic spindles and chromosomes arranged as a ring (e). Some giant cells showing a well-preserved tubulin cytoskeleton and macronuclei were observed (g). Arrowheads: grape-like nuclei. Green: tubulin; blue: 4′6-diamidino-2-phenylindole. Original magnification: × 60.

Epithelial-to-mesenchymal transition markers analysis

As the phenotypic switch between the epithelial type and mesenchymal type plays a key role in the progression of pancreatic cancer, we analyzed the effect of UK on the EMT-related cell phenotype. Immunofluorescence analysis indicated that all of the PDAC cells considered express E-cadherin and β-catenin, and that this expression was present at the cell boundary. This pattern, consistent with the expression of E-cadherin in differentiated epithelial cells, was observed by immunofluorescence analysis in CTs as well as in UK-treated cells (Figs 7a and 8a). Although E-cadherin expression at the cell–cell boundary was similar in CTs and UK-treated cells, interestingly, western blot analysis indicated an upregulation of E-cadherin in UK-treated cells compared with CTs (Fig. 7b and c).

Phalloidin staining of F-actin showed a different arrangement of microfilaments in the three PDAC cell lines. In HPAF-II, no evident stress fibers were observed and F-actin staining had the same membranous distribution as that for E-cadherin and β-catenin immunoreactivities. In HPAC, F-actin was similarly expressed as in HPAF-II cells, but membranous staining was less evident and frequent F-actin bundles were observed in the cytoplasm. By contrast, in PL45 cells, F-actin staining was diffused in the cytoplasm of CTs and UK-treated cells (Fig. 8b). However, a slight reactivity was observed at the cell–cell

Fig. 4



Normal and abnormal mitoses. Bar graphs showing the percentage of normal and abnormal mitoses. In each field, normal and abnormal mitoses were counted and expressed as a ratio to the total number of mitosis.

contacts in a few PL45 cells only after the administration of 20 μmol/l UK, suggesting the formation of cortical bundles consistent with the acquisition of a more epithelial-like phenotype.

Immunoreactivity for the intermediate filament vimentin was not detectable in both CTs and UK-treated PDAC cells (data not shown).

Discussion

We determined *in vitro* whether UK affects PDAC cell proliferation, apoptosis, the expression of EMT markers, and cytoskeleton arrangement. UK reduced PDAC cell proliferation 24, 48, and 72 h after treatment. This was evident after the 10 μmol/l dose and suggests that the drug exerts an antiproliferative effect, possibly elicited, according to previous findings, by the blockage of cell cycle and induction of apoptosis [23,24–28].

Immunofluorescence analysis of tubulin indicated a high number of mitoses in UK-treated compared with CT cells with very scarce or absent anaphase, telophase, and cytokinesis figures, pointing to a blockage of the cell cycle at the G2/M phase, as suggested previously by cell cycle analysis in the same cells [23]. The concomitant increase in abnormal tripolar or quadripolar mitotic spindles in UK-treated cells suggests that the G2/M arrest induced by UK treatment is caused by the generation of nonfunctional mitotic spindles, accounting for both the subsequent mitotic arrest and the noncorrect distribution of chromosomes in the daughter cells.

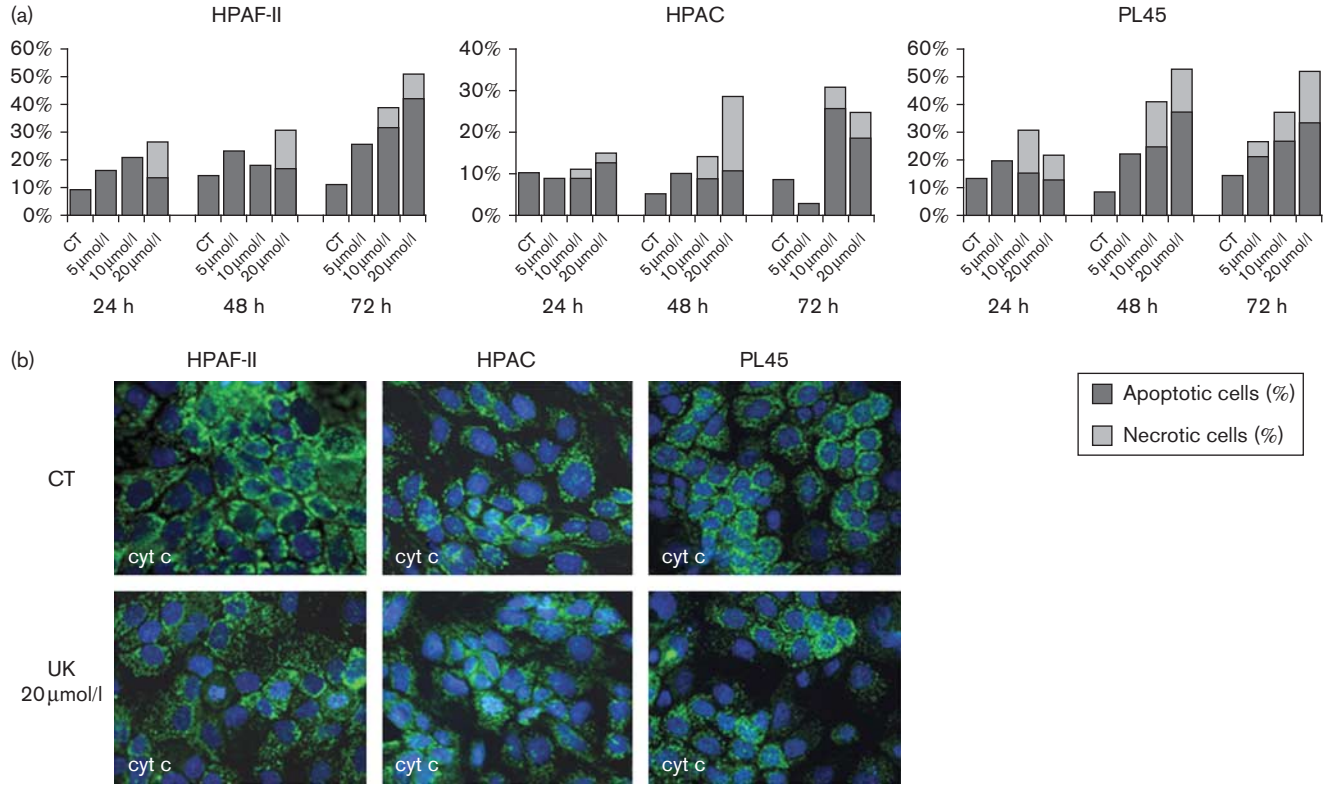
The generation of aberrant spindles may occur as a consequence of the suppression of microtubule dynamics. Considering that microtubules from spindles are highly dynamic and show a turnover rate 20-fold faster than interphasic microtubules [29], interference with their dynamic behavior could result in morphologically abnormal spindles and, therefore, abnormal mitosis. Therefore, the antiproliferative effect of UK could be primarily based on the interference with the dynamics of spindle microtubules rather than their depolymerization.

This effect of UK on the microtubules of the mitotic spindle seems very similar to that elicited by paclitaxel on cancer cells [30]. Interestingly, our results point to a different effect of paclitaxel and UK on the interphase microtubule, as the former acts as a stabilizing agent, inducing the density of interphase microtubules and the formation of long thick microtubule bundles surrounding the nucleus. By contrast, the interphase microtubule network remained unmodified after the administration of UK.

The effect of UK as a microtubule-targeting agent was also confirmed by the observation of cells consisting of multiple macronuclei after the administration of 20 μmol/l UK, as observed in cells escaping mitotic arrest [31,32]. Cells arrested at mitosis can escape checkpoint arrest and enter interphase without undergoing division. This response, termed 'mitotic slippage', is characterized by the unique multinucleated 'grape-like' nuclear morphology [31]. Cells that undergo mitotic slippage will likely fail to proliferate and will die. Our results suggest that the antiproliferative effect of UK may be based on the stabilization of microtubule dynamics, preventing the completion of mitosis and, in part, ultimately reverting to an abnormal multinucleate interphase condition.

Inhibition of progress through mitosis ultimately results in apoptotic cell death and may be important in the antitumor actions of the drug. By fluorescence microscopy, we observed several cells characterized by a 'grape-like' nuclear morphology typical of cells undergoing mitotic slippage, and some large cells containing macronuclei as a result of abnormal mitosis. These conditions suggest that UK-treated cells may undergo apoptosis;

Fig. 5



Apoptosis analysis. (a) Percentages of apoptotic and necrotic cells after the administration of ukrain (UK) measured using Hoechst 33258 and propidium iodide (PI) after 24, 48, and 72 h. Hoechst 33258 and PI double stain were used to evaluate the percentages of apoptosis. Hoechst 33258-positive and PI-negative cells were designated as apoptotic, and Hoechst-positive and PI-positive cells showed necrosis. (b) Representative microphotographs showing immunofluorescence detection of cytochrome *c* in controls (CTs) and 20 µmol/l UK-treated pancreatic ductal adenocarcinoma cells. Untreated CTs and UK-treated cells showed a similar pattern characterized by a punctate cytoplasmic staining pattern typical for the localization of cytochrome *c* into intact mitochondria. Original magnification: × 40.

therefore, we analyzed whether apoptosis mechanisms are triggered in PDAC cells after the administration of UK.

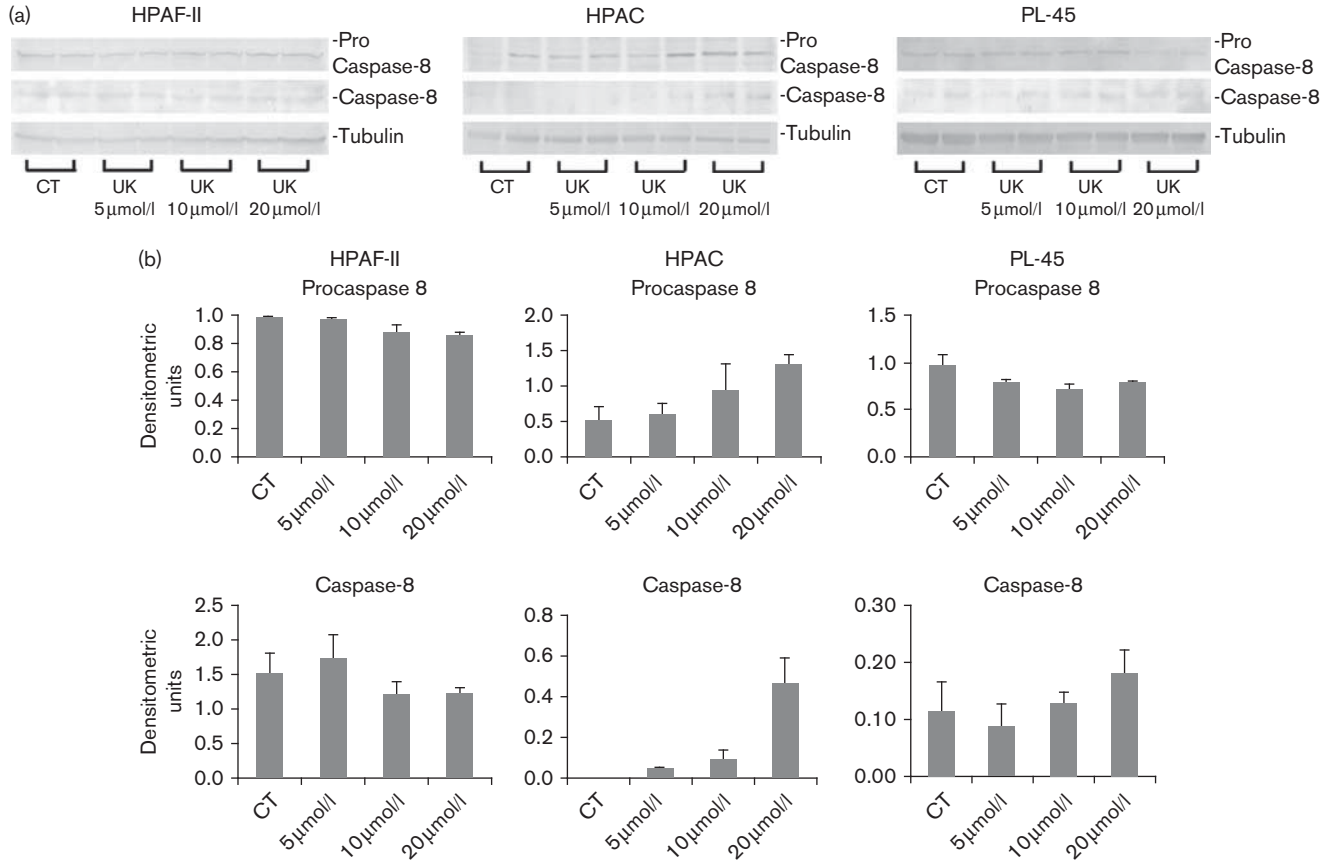
Apoptosis is a highly regulated form of cell death involving complex intracellular proteolysis in which the main executors are caspases. The apoptotic mechanism may be triggered either by the extrinsic death receptor-dependent pathway or by the intrinsic mitochondrial pathway. The latter is characterized by the disruption of the mitochondrial integrity with the release of proapototic proteins, including cytochrome *c*, from the mitochondrial intermembrane space into the cytosol, resulting in the activation of caspase-9 that finally induces cell death [33]. Our data show that UK significantly induced apoptosis in cultured PDAC cells. The immunofluorescence analysis of cytochrome *c* distribution, however, showed that UK did not trigger mitochondrial damage as cytochrome *c* is not released by the mitochondria into the cytosol, suggesting that the intrinsic mitochondrial pathway is not the target of the drug, as reported previously for UK-treated glioblastoma cells [27]. Therefore, we analyzed caspase-8 expression to determine whether the extrinsic apoptosis pathway was responsible for the dose-dependent apoptosis evidenced using Hoechst staining.

Procaspase-8 was upregulated after the administration of UK, and active caspase-8 levels were strongly induced after the administration of UK, suggesting that UK may trigger the apoptosis extrinsic pathway in PDAC cells.

Loss of intercellular adhesion and increased motility promote tumor cell invasion. Acquisition of the metastatic phenotype of cancer cells consists of multiple steps, including EMT, a complex stepwise process characterized by the loss of epithelial adhesion properties following changes in E-cadherin expression patterns and disruption of the adherens junction, induction of matrix metalloproteinases (MMPs), leading to the disruption of basement membranes, and enhanced migration and invasion [7]. Therefore, during tumor progression, EMT allows tumor cells to acquire the capacity to infiltrate surrounding tissue and metastasize at distant sites [34].

An early essential event of EMT is the loss of epithelial phenotype and cell–cell adhesion driven by the down-regulation of E-cadherin, a well-characterized adhesive

Fig. 6



Caspase expression. (a) Representative western blots showing procaspase-8 and caspase-8 protein expression in controls and ukrain-treated pancreatic ductal adenocarcinoma cells. (b) Bar graphs showing procaspase-8 and activated caspase-8 after densitometric scanning of immunoreactive bands obtained by a western blot. Procaspase-8 and caspase-8 protein levels were normalized on tubulin expression. Data are expressed as densitometric units and are means±SD for duplicate experiments.

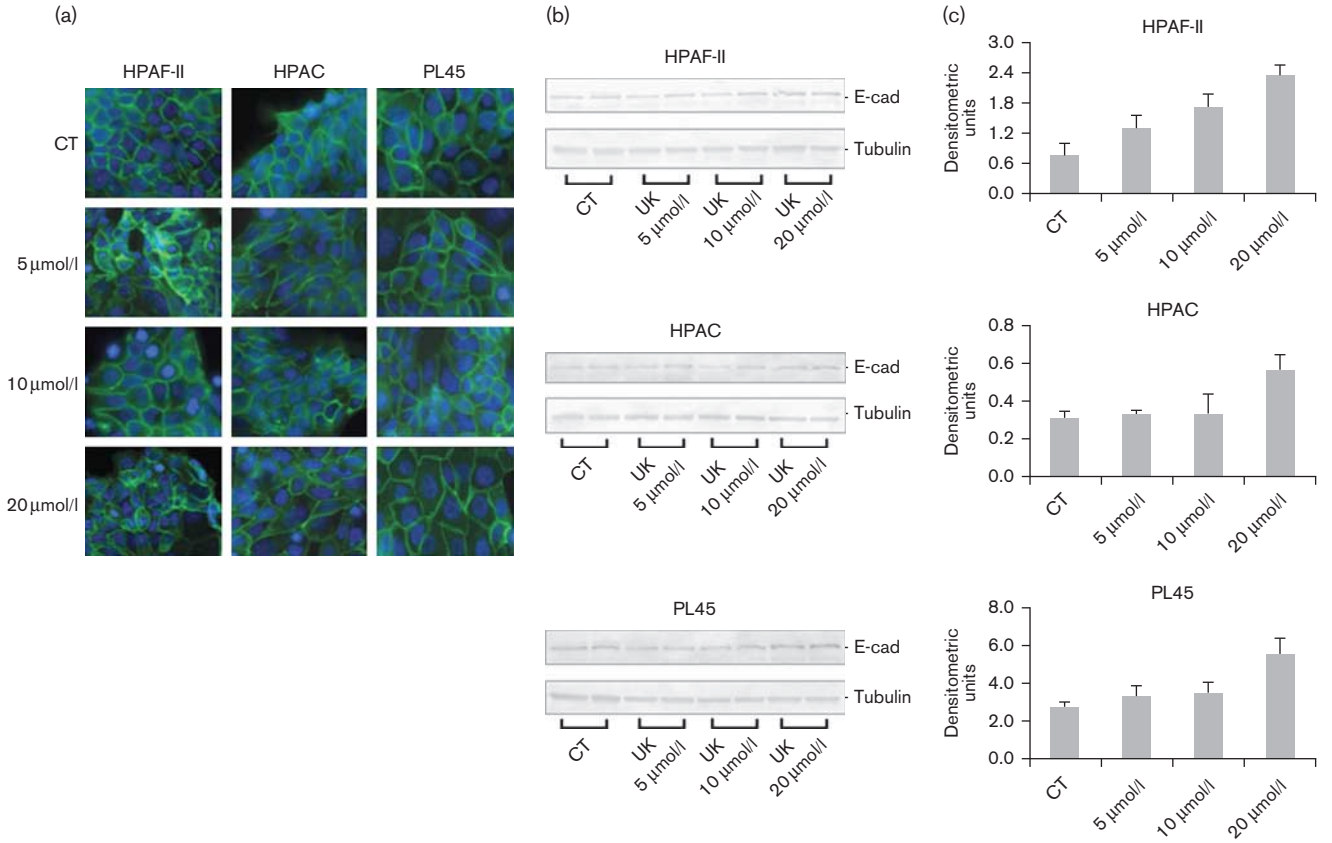
junction protein expressed in differentiated and polarized epithelial cells. The graded loss of E-cadherin, the core transmembrane protein of the adherens junction of epithelial cells, correlates with the aggressiveness of numerous carcinomas and the worsening of prognosis, whereas the forced expression of E-cadherin suppresses tumor development in various in-vitro and in-vivo tumor experimental models [35]. E-cadherin downregulation leads to the release of the E-cadherin/β-catenin complex from the membrane, the elimination of cell–cell junctions [36], and β-catenin nuclear translocation, during which it may function as a transcriptional coactivator [37].

It was also shown that pancreatic cancers show a reduced expression of E-cadherin and an increased expression of N-cadherin that, in primary tumors, are significantly related to the histological grade [38] and to the invasive and undifferentiated phenotypes [39].

Our data on the immunofluorescence analysis show that untreated cells express E-cadherin and β-catenin at the cell–cell boundary, showing a strong immunoreactive signal at the plasma membrane level. This suggests that the adherent junctions are retained by PDAC cells that have an epithelial differentiated phenotype, although they are highly malignant and invasive, as reported previously [23]. UK did not apparently modify E-cadherin expression at the morphological level, but the protein levels tended to be upregulated after the administration of UK, suggesting that the drug may favor cell-to-cell adhesion through adherent junctions.

We examined the actin cytoskeleton as changes in the arrangement of actin filaments are common features in cellular transformation [40]. In particular, during EMT, a marked reorganization of the cytoskeleton occurs, and the actin subcortical mesh in epithelial cells is dissociated [41]. In transformed cells, a crucial alteration in the actin cytoskeleton leads to the disappearance of the marginal actin filament bundles, consistent with a decrease in cell–cell adhesion and alteration in the motile behavior of epithelial cells [42]. We did not observe evident actin stress fibers in CT PDAC cells. In HPAF-II, actin staining was diffuse in the cytoplasm and was

Fig. 7



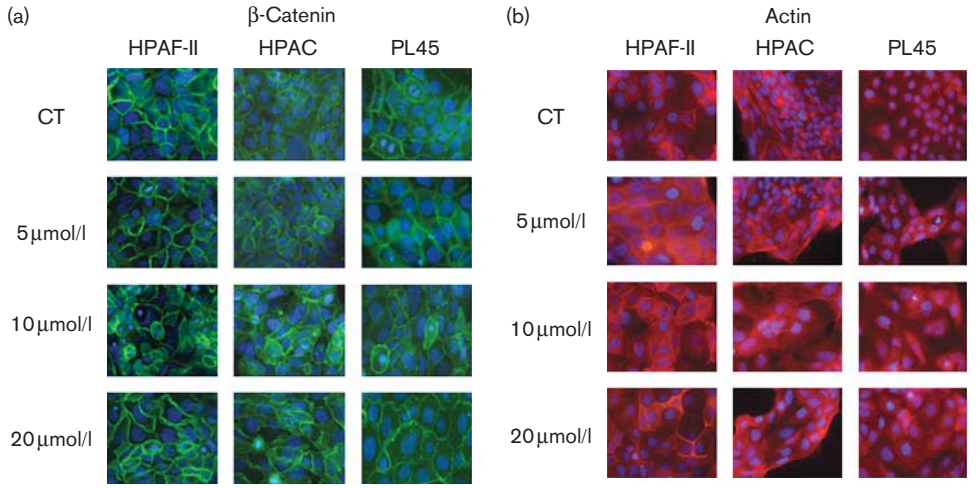
E-cadherin expression. (a) Representative immunofluorescence analysis showing E-cadherin expression in pancreatic ductal adenocarcinoma (PDAC) cells. E-cadherin immunoreactivity is high in both controls (CTs) and ukrain (UK)-treated cells and located at the cell–cell contacts. Green: E-cadherin; blue: 4′6-diamidino-2-phenylindole. Original magnification: × 60. (b) Representative western blots showing E-cadherin protein expression in CTs and UK-treated PDAC cells. Immunoreactive bands were obtained using a monoclonal antibody to E-cadherin. (c) Bar graphs showing E-cadherin expression after normalization on tubulin. Data are expressed as densitometric units and are means±SD for duplicate experiments.

evident at the cell–cell contact, consistent with the epithelial-like phenotype of these cells. In HPAC, actin immunoreactivity was slight at the cell boundary and some actin filaments were evident in the cytoplasm in both CTs and UK-treated cells. In contrast, CT PL45 cells had diffuse actin immunoreactivity only in the cytoplasm, as well as after the administration of 5 and 10 µmol/l UK. In 20 µmol/l UK-treated cells, a slight immunoreactivity was detectable at the cell–cell contact in a few cells, suggesting that the drug may favor actin cytoskeleton reorganization and the formation of cortical filament bundles, typical of the epithelial phenotype. Our data, therefore, point to a different organization of microfilaments in PDAC cell lines, and no effects of UK on actin cytoskeleton arrangement were evident.

Vimentin is a widely used marker of EMT. Silencing vimentin causes mesenchymal cells to adopt epithelial shapes, and following transfection with vimentin, cDNA epithelial cells adopt mesenchymal shapes coincident with the assembly of vimentin intermediate filaments. Vimentin was not detected in our experimental model.

The overall pattern of EMT-related markers in PDAC cells that we observed, in particular with regard to the E-cadherin/β-catenin complex and vimentin expression pattern, is apparently in contrast to that in classic EMT pathways. In fact, untreated HPAF-II, HPAC, and PL45 PDAC cells retain characteristics of the well-differentiated epithelial phenotype, although they express high levels and activity of MMP-2 and MMP-9, and a high motile/invasive potential under the same experimental conditions, as shown in our previous study [23]. However, UK was reported to significantly reduce MMPs activity and the migration/invasion in these cells [23]. In particular, MMP-9 activity was decreased by UK in HPAF-II cells and in all the cell lines considered, respectively. This was an interesting finding, as MMP-9 is expressed to the same extent in both carcinoma and host stromal cells. In the same cell lines, the gene expression for MMP-9 was correlated with the expression

Fig. 8



β-catenin expression and actin organization. (a) Representative immunofluorescence analysis of β-catenin showing immunoreactivity located at the plasma membrane level, as observed for E-cadherin. (b) Actin cytoskeleton arrangement in pancreatic ductal adenocarcinoma cells. Actin localization at the cell–cell contacts in HPAF-II cells is consistent with the presence of cortical bundles typical of epithelial cells. HPAC cytoplasm contains evident actin bundles and fluorescence is observed in some cells at the cell–cell contacts in both controls (CTs) and ukrain (UK)-treated cells. A different pattern was found in PL45 cells, in which the actin–phalloidin signal is diffuse in the cytoplasm in CTs and after the administration of 5 and 10 μmol/l UK. A slight reactivity was observed at the cell–cell contacts in a few cells only after the administration of 20 μmol/l UK, suggesting the formation of cortical bundles consistent with the acquisition of a more epithelial-like phenotype. Green: β-catenin; red: actin-phalloidin; blue: 4′6-diamidino-2-phenylindole. Original magnification: × 60.

of miR-21, which was reported to exert proinvasion and proangiogenic effects, and to induce resistance to anticancer agents [43,44]. Further studies analyzing the effect of UK on miR-21 expression could be useful to understand the possible effect of the drug on this oncogenic regulator playing a key role in PDAC.

Considering that the loss of E-cadherin has been reported as a pivotal event during EMT in carcinogenesis, a considerable number of cell lines are expected to show a decrease in or loss of E-cadherin expression. However, experimental evidence supports our findings showing that six of seven PDAC cell lines retain the typical E-cadherin expression at the plasma membrane, and that the expressions of EMT markers in PDAC are similar to those in benign pancreatic ducts [45]. To support the consistency of our data and hypotheses, Funel *et al.* [46] showed that microdissected PDAC cells and primary cell cultures obtained from the same PDAC tissues had the same phenotype and behavior, and that the results obtained on using PDAC primary cell lines and commercial cell lines were overlapping. In this complex scenario, we can provide some hypotheses to explain this apparent inconsistency between the retained epithelial phenotype and the malignancy of pancreatic cancer carcinoma cells.

Several pieces of experimental evidence suggest that many invasive and metastatic carcinomas have not undergone a complete EMT or even lack signs of EMT, and possess morphological and molecular characteristics consistent with well-differentiated epithelia, including high levels of E-cadherin, as observed in our study, and the presence of epithelial junctions and cell polarity [47]. Carcinoma cells showing an epithelial morphology were observed in prostate [48] and breast cancers [49,50]. Moreover, E-cadherin continued to be complexed with catenins even within metastatic carcinomas [51]. Therefore, the general assumption of an inverse correlation between E-cadherin expression and the invasive potential of carcinoma cells is not absolute.

Another point to be considered is that an incomplete EMT may occur, thus suggesting that the transition to an aggressive malignant phenotype is not an 'all or nothing' event, but rather a multistep process that manifests in a broad range of phenotypic changes. Moreover, the steps characterizing the EMT multistep process do not necessarily occur consecutively and are not all necessarily present in a given sample of EMT [47]. These two situations are supported by the evidence that full EMT is rarely observed in biopsies from patients with pancreatic cancer [52]. In fact, in PDAC biopsies, loss in membrane E-cadherin expression was reported in ~30–50% of cases, and a similar pattern was observed for β-catenin [53–55].

A particular factor to be considered at this point is related to the two possible modalities adopted by carcinoma cells for invasiveness. Single carcinoma cells showing a complete EMT-related phenotype are highly motile and invade surrounding tissues as individual mesenchymal-like cells. However, invasive carcinoma cells may adopt a collective migration pattern characterized by invasive multicellular aggregates containing well-differentiated

cells that retain an epithelial morphology and, above all, cohesiveness, that collectively migrate within the adjacent tissues. Collectively migrating cells maintain their cell–cell junctions, and migrate in sheets, tubes, and clusters, forming protrusions such as pseudopodia or invadopodia, in which the release of MMPs leads to the proteolytic breakdown of ECM in the tumor environment [56]. A significant number of cancers, including certain breast, epithelial prostate, large cell lung, and ovarian cancers, are, by pathological criteria, characterized as invasive and malignant, and yet, they do not lose E-cadherin expression and show collective migration and invasion [52,56]. Therefore, both collective migration and incomplete EMT allow invasiveness as would a complete EMT-related mesenchymal phenotype. In particular, collectively migrating cells express specific MMPs such as MMP-2 for remodeling ECM [57], and this is also observed in the highly invasive HPAF-II, HPAC, and PL45 cell lines [23].

Our findings of the concomitant expression of E-cadherin/β-catenin complex at the cell–cell boundary and the high levels of MMPs are consistent with the phenotype of cells adopting a collective migration mechanism, but suggest that further experiments will be necessary to definitively clarify the role of EMT in the progression of PDAC.

Conclusion

Considered as a whole, our results suggest that UK may exert some effects on pancreatic cancer cells. In particular, UK seems to exert its chemotherapeutic and antiproliferative action by suppressing the dynamic instability of mitotic spindle microtubules, leading to abnormal mitosis and apoptosis.

Conversely, our results on the EMT-related phenotype suggest that PDAC cell lines retain an epithelial-like phenotype that is not significantly modified by the administration of UK, although upregulation of E-cadherin induced in UK-treated cells is consistent with a higher cohesiveness that may counteract tumor invasion.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

1 Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. *Lancet* 2011; **378**:607–620.
2 Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics. 2009. *CA Cancer J Clin* 2009; **59**:225–249.
3 Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet* 2004; **363**:1049–1057.
4 Sohn TA, Yeo CJ, Cameron JL, Koniaris L, Kaushal S, Abrams RA, *et al.* Resected adenocarcinoma of the pancreas-616 patients: results, outcomes, and prognostic indicators. *J Gastrointest Surg* 2000; **4**:567–579.
5 Liu Y, Brand RE, Turzhitsky V, Kim YL, Roy HK, Hasabou N, *et al.* Optical markers in duodenal mucosa predict the presence of pancreatic cancer. *Clin Cancer Res* 2007; **13**:4392–4399.
6 Ghaneh P, Costello E, Neoptolemos JP. Biology and management of pancreatic cancer. *Gut* 2007; **56**:1134–1152.
7 Thiery JP. Epithelial–mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002; **2**:442–454.
8 Cano CE, Motoo Y, Iovanna JL. Epithelial-to-mesenchymal transition in pancreatic adenocarcinoma. *Scientific World J* 2010; **10**:1947–1957.
9 Kang Y, Massagué J. Epithelial–mesenchymal transitions: twist in development and metastasis. *Cell* 2004; **118**:277–279.
10 Semb H, Christofori G. The tumor-suppressor function of E-cadherin. *Am J Hum Genet* 1998; **63**:1588–1593.
11 Hirohashi S. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol* 1998; **153**:333–339.
12 El-Bahrawy MA, Pignatelli M. E-cadherin and catenins: molecules with versatile roles in normal and neoplastic epithelial cell biology. *Microsc Res Tech* 1998; **43**:224–232.
13 Burris HA, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, *et al.* Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**:2403–2413.
14 Mössner J. What's new in therapy of pancreatic cancer? *Dig Dis* 2010; **28**:679–683.
15 Kretmair H. Hypericum perforatum, St John's wort. *Pharmazie* 1950; **5**: 85–88.
16 Susak YM, Zemskov VS, Yaremchuk OY, Kravchenco OB, Yatsyk IM, Korsh OB. Comparison of chemotherapy and X-ray therapy with Ukrain monotherapy for colorectal cancer. *Drugs Exp Clin Res* 1996; **22**:115–122.
17 Uglianitsa KN, Nefyodov LI, Brzosko W. Evaluation of the efficacy of ukrain in the treatment of breast cancer: clinical and laboratory studies. *Drugs Exp Clin Res* 1998; **24**:231–239.
18 Uglianitsa KN, Nechiporenko NA, Nefyodov LI, Brzosko WJ. Ukrain therapy of stage T1N0M0 bladder cancer patients. *Drugs Exp Clin Res* 1998; **24**:227–230.
19 Zemskov O, Prokopchuk Y, Susak S, Zemskov O, Tkachenko Y, Hodysh W, *et al.* Efficacy of ukrain in the treatment of pancreatic cancer. *Langenbecks Arch* 2002; **387**:84–89.
20 Ernst E, Schmidt K. Ukrain – a new cancer cure? A systematic review of randomised clinical trials. *BMC Cancer* 2005; **5**:69.
21 Gansauge F, Ramadani M, Pressmar J, Gansauge S, Muehling B, Stecker K, *et al.* NSC-631570 (ukrain) in the palliative treatment of pancreatic cancer. Results of a phase II trial. *Langenbecks Arch Surg* 2002; **386**:570–574.
22 Gansauge F, Ramadani M, Schwarz M, Beger HG, Lotspeich E, Poch B. The clinical efficacy of adjuvant systemic chemotherapy with gemcitabine and NSC-631570 in advanced pancreatic cancer. *Hepatogastroenterology* 2007; **54**:917–920.
23 Funel N, Costa F, Pettinari L, Taddeo A, Sala A, Chiriva-Internati M, *et al.* Ukrain affects pancreas cancer cell phenotype in vitro by targeting MMP-9 and intra-/extracellular SPARC expression. *Pancreatology* 2010; **10**: 545–552.
24 Gagliano N, Pettinari L, Aureli M, Colombo E, Costa F, Carminati R, *et al.* Malignant phenotype of renal cell carcinoma cells is switched by ukrain administration in vitro. *Anticancer Drugs* 2011; **22**:749–762.
25 Liepins A, Nowicky JW, Bustamante JO, Lam E. Induction of bimodal programmed cell death in malignant cells by the derivative ukrain NSC-631570. *Drugs Exp Clin Res* 1996; **22**:73–79.
26 Roublevskaia IN, Polevoda BV, Ludlow JW, Haake AR. Induced G2/M arrest and apoptosis in human epidermoid carcinoma cell lines by semisynthetic drug ukrain. *Anticancer Res* 2000; **20**:3163–3167.
27 Gagliano N, Moscheni C, Torri C, Donetti E, Magnani I, Costa F, *et al.* Ukrain modulates glial fibrillary acidic protein, but not connexin 43 expression, and induces apoptosis in human cultured glioblastoma cells. *Anti-cancer Drugs* 2007; **18**:669–676.
28 Nowicky JW, Hiesmayr W, Nowicky W, Liepins A. Influence of ukrain on DNA, RNA and protein synthesis in malignant cells. *Drugs Exp Clin Res* 1996; **22**:81–91.
29 Saxton WM, Stemple DL, Leslie RJ, Salmon ED, Zavortink M, McIntosh JR. Tubulin dynamics in cultured mammalian cells. *J Cell Biol* 1984; **99**: 2175–2186.
30 Yvon AMC, Wadsworth P, Jordan MA. Taxol suppresses dynamics of individual microtubules in living human tumor cells. *Mol Biol Cell* 1999; **10**:947–959.
31 Elhajouji A, Cunha M, Kirsch-Volders M. Spindle poisons can induce polyploidy by mitotic slippage and micronucleate mononucleates in the cytokinesis-block assay. *Mutagenesis* 1998; **13**:193–198.
32 Meek DW. The role of p53 in the response to mitotic spindle damage. *Pathol Biol* 2000; **48**:246–254.

33 Scholz C, Wieder T, Starck L, Essmann F, Schulze-Osthoff K, Dorken B, *et al*. Arsenic trioxide triggers a regulated form of caspase-independent necrotic cell death via the mitochondrial death pathway. *Oncogene* 2005; **24**:1904–1913.

34 Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial–mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* 2006; **172**:973–981.

35 Nelson WJ, Nusse R. Convergence of Wnt, β-catenin, and cadherin pathways. *Science* 2004; **303**:1483–1487.

36 Marambaud P, Shioi J, Serban G, Georgakopoulos A, Sarner S, Nagy V, *et al*. A presenilin-1/γ-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J* 2002; **21**:1948–1956.

37 Van Es JH, Barker N, Clevers H. You WNT some, you lose some: oncogenes in the WNT signaling pathway. *Curr Opin Genet Dev* 2003; **13**:28–33.

38 Nakajiama S, Doi R, Toyoda E, Tsuji S, Wada M, Koizumi M, *et al*. N-cadherin expression and epithelial–mesenchymal transition in pancreatic carcinoma. *Clin Cancer Res* 2004; **10**:4125–4133.

39 Joo YE, Rew JS, Park CS, Kim SJ. Expression of E-cadherin, alpha and β-catenins in patients with pancreatic adenocarcinoma. *Pancreatology* 2002; **2**:129–137.

40 Pawlak G, Helfman DM. Cytoskeletal changes in cell transformation and tumorigenesis. *Curr Opin Genet Dev* 2001; **11**:41–47.

41 Savagner P. Leaving the neighborhood: molecular mechanisms involved during epithelial–mesenchymal transition. *Bioessays* 2001; **23**:912–923.

42 Ayollo DV, Zhitnyak IY, Vasiliev JM, Gloushankova NA. Rearrangements of the actin cytoskeleton and E-cadherin-based adherens junctions caused by neoplasic transformation change cell–cell interactions. *PLoS One* 2009; **4**:e8027.

43 Giovannetti E, Funel N, Peters GJ, Del Chiaro M, Erozenci LA, Vasile E, *et al*. MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Res* 2010; **70**:4528–4538.

44 Hwang JH, Voortman J, Giovannetti E, Steinberg SM, Leon LG, Kim YT, *et al*. Identification of microRNA-21 as a biomarker for chemoresistance and clinical outcome following adjuvant therapy in resectable pancreatic cancer. *PLoS One* 2010; **5**:e10630.

45 Cates JM, Byrd RH, Fohn LE, Tatsas AD, Washington MK, Black CC. Epithelial–mesenchymal transition markers in pancreatic ductal adenocarcinoma. *Pancreas* 2009; **38**:e1–e6.

46 Funel N, Giovannetti E, Del Chiaro M, Mey V, Pollina LE, Nannizzi S, *et al*. Laser microdissection and primary cell cultures improve pharma-cogenetic analysis in pancreatic adenocarcinoma. *Lab Invest* 2008; **88**:773–784.

47 Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res* 2006; **66**:8319–8326.

48 Christiansen JJ, Rajasekaran SA, Inge L, Cheng L, Anilkumar G, Bander NH, *et al*. N-glycosylation and microtubule integrity are involved in apical targeting of prostate-specific membrane antigen: implications for immunotherapy. *Mol Cancer Ther* 2005; **4**:704–714.

49 Ng WK. Fine-needle aspiration cytology findings of an uncommon micropapillary variant of pure mucinous carcinoma of the breast: review of patients over an 8-year period. *Cancer* 2002; **96**:280–288.

50 Tan DS, Potts HW, Leong AC, Gillett CE, Skilton D, Harris WH, *et al*. The biological and prognostic significance of cell polarity and E-cadherin in grade I infiltrating ductal carcinoma of the breast. *J Pathol* 1999; **189**: 20–27.

51 Kartenbeck J, Haselmann U, Gassler N. Synthesis of junctional proteins in metastasizing colon cancer cells. *Eur J Cell Biol* 2005; **84**:417–430.

52 Wicki A, Lehembre F, Wick N, Hantusch B, Kerjaschki D, Christofori G. Tumor invasion in the absence of epithelial–mesenchymal transition: podoplanin-mediated remodeling of the actin cytoskeleton. *Cancer Cell* 2006; **9**:261–272.

53 Pryczynicz A, Guzinska-Ustymowicz K, Dymicka-Piekarska V, Czyzewska J, Kemona A. Expression of matrix metalloproteinase 9 in pancreatic ductal carcinoma is associated with tumor metastasis formation. *Folia Histochem Cytobiol* 2007; **45**:37–40.

54 Li YJ, Ji XR. Relationship between expression of E-cadherin–catenin complex and clinicopathologic characteristics of pancreatic cancer. *World J Gastroenterol* 2003; **9**:368–372.

55 Pignatelli M, Ansari TW, Gunter P, Liu D, Hirano S, Takeichi M, *et al*. Loss of membranous E-cadherin expression in pancreatic cancer: correlation with lymph node metastasis, high grade, and advanced stage. *J Pathol* 1994; **174**:243–248.

56 Yilmaz M, Christofori G. Mechanisms of motility in metastasizing cells. *Mol Cancer Res* 2010; **8**:629–642.

57 Yilmaz M, Christofori G, Lehembre F. Distinct mechanisms of tumor invasion and metastasis. *Trends Mol Med* 2007; **13**:535–541.