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RESEARCH PAPER

Platelets increase survival of adenocarcinoma cells challenged with anticancer drugs: mechanisms and implications for chemoresistance

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BACKGROUND AND PURPOSE

Cancer cells grow without the restraints of feedback control mechanisms, leading to increased cancer cell survival. The treatment of cancer is often complicated by the lack of response to chemotherapy leading to chemoresistance and persistent survival of tumour cells. In this work we studied the role of platelets in chemotherapy-induced cancer cell death and survival.

EXPERIMENTAL APPROACH

Human adenocarcinoma cells, colonic (Caco-2) and ovarian (59 M) cells, were incubated with 5-fluorouracil (1–300 $\mu g \cdot mL^{-1}$) or paclitaxel (1–200 $\mu g \cdot mL^{-1}$) in the presence or absence of platelets (1.5×10^8 mL^{-1}) for 1, 24 or 72 h. Following incubation, cancer cells were harvested and cell survival/death was assayed using flow cytometry, Western blotting, real-time PCR, TaqMan® Gene Expression Assays and proteomics.

KEY RESULTS

Human platelets increased the survival of colonic and ovarian adenocarcinoma cells treated with two standard anticancer drugs, 5-fluorouracil and paclitaxel. In the presence of platelets, cancer cells up-regulated anti-apoptotic and down-regulated pro-apoptotic genes, increased the number of cells in the synthesis of DNA and decreased the number in the quiescent phase, increased expression of cyclins, DNA repair proteins and MAPKs. The analysis of platelet-Caco-2 secretome demonstrated the release of the chemokine RANTES, thrombospondin-1, TGF-β and clusterin. Finally, human recombinant RANTES and thrombospondin-1 improved survival of Caco-2 cells challenged with paclitaxel.

CONCLUSIONS AND IMPLICATIONS

These data demonstrate that platelets increase adenocarcinoma cells survival, proliferation and chemoresistance to standard anticancer drugs. Modulating cancer cell–platelet interactions may offer a new strategy to improve the efficacy of chemotherapy.

Abbreviations

59 M, human ovarian adenocarcinoma; 5-FU, 5-fluorouracil; Caco-2, human colonic adenocarcinoma; Chk1, checkpoint 1; CRL2014, human gingival fibroblasts; G_0/G_1, quiescent/interphase G_1 phases; G_2/M, interphase G_2/mitosis phases; PI, propidium iodide; PLT, platelets; PLTR, platelets releasate; PTX, paclitaxel; S, synthesis phase; TCIPA, tumour cell-induced platelet aggregation; TSP-1, thrombospondin-1

Introduction

Platelet cancer interactions are a highly dynamic process in which platelets undergo extensive interactions with cancer cells and cancer microenvironment facilitating the complex multi-step process of carcinogenesis including blood-borne metastasis. The first evidence for the association between vascular thrombosis and cancer was presented in 1865 when the French physician Armand Trousseau reported a high incidence of venous thrombosis in patients with gastric carcinomas. Recent clinical and experimental data confirm the relationship between cancer and thrombosis as epidemiological studies showed that 2 out of 10 cancer patients may develop thrombotic complications during the clinical course of their disease (Akl *et al.*, 2008a,b).

Moreover, thrombocytosis, which is often detected in cancer patients, is a poor prognostic factor in stomach, ovary, breast and colon cancer (Pasquini *et al.*, 1995, Santos *et al.*, 2001). Platelets contribute to different stages of cancer progression such as angiogenesis, invasion, survival in circulation and metastasis (Gupta and Massagué, 2004; Jurasz *et al.*, 2004). One of the major mechanisms involved in platelet–cancer cell interactions is tumour cell-induced platelet aggregation (TCIPA) (Radomski *et al.*, 1991). TCIPA leads to the formation of platelet–cancer aggregates that adhere to the endothelium and may cause distant embolization of the microvasculature. It has been shown that platelets involved in cancer cell–platelet aggregates generate contractile force that causes disruption of these aggregates, thus facilitating the down-stream embolization of the vasculature (Mehta, 1984; Bazou *et al.*, 2011). Moreover, the adhesion and activation of platelets by tumour cells and their platelet-fibrin-rich network-cancer emboli may shield the tumour cells from the immune system (Jurasz *et al.*, 2004; Gay and Felding-Habermann, 2011). Platelets also promote cancer cell invasion to disease-free tissues and organs (Alonso-Escolano *et al.*, 2006). In order to invade, tumour cells have the ability to degrade and remodel the extracellular matrix (ECM) via release of various proteolytic enzymes (Medina and Radomski, 2006). These include MMPs, zinc-dependent endopeptidases, which break down ECM proteins. Our research group has recently reported that platelets stimulate invasiveness of tumour cells via increased expression of MMP-9 (Alonso-Escolano *et al.*, 2006).

The objective of this research was to study if platelets have the ability to improve cancer cell survival that is decreased in response to chemotherapeutic agents 5-fluorouracil (5-FU) or paclitaxel. Both drugs have been extensively used for the treatment of metastatic colorectal and ovarian cancer. Recently, critical evaluations have been performed regarding the necessity of a bolus injection in those patients in order to achieve very high plasma concentrations of the chemotherapeutic agents (Sabharwal and Kerr, 2007). Indeed, the bolus injection of 5-FU has been shown to increase its plasma levels in combined regimens exceeding 100 $\mu g \cdot mL^{-1}$ (Tamura *et al.*, 2011) Our study shows for the first time that platelets increase survival of human ovarian and colonic adenocarcinoma cells treated with anticancer drugs paclitaxel and 5-FU at clinically relevant concentrations. We have also identified major pro-survival mechanisms involved in this effect of platelets.

Methods

The study was approved by the Trinity College Dublin Ethics Committee.

Platelet isolation and releasate

Blood was obtained from healthy volunteers who had not taken any drugs known to affect platelet function for 2 weeks prior to the study. Washed platelet suspensions (Radomski and Moncada, 1983) (1.5×10^8 platelets mL^{-1}) were prepared and re-suspended in cell culture medium free of FBS. Platelet releasate was obtained by centrifugation of collagen (10 $\mu g \cdot mL^{-1}$; Chronolog, Havertown, PA, USA)-aggregated platelets at 2000× *g* for 10 min at room temperature.

Cancer cell culture

Two human adenocarcinoma cell lines Caco-2 (colonic) and 59 M (ovarian) and one human gingival fibroblast CRL2014 were obtained from the European Cell Culture Collection (Salisbury, UK). Cell lines were cultured as previously described (Bazou *et al.*, 2011).

Cancer chemotherapeutics

Paclitaxel supplied as 6 $mg \cdot mL^{-1}$ (Medac, Hamburg, Germany) and 5-FU 25 $mg \cdot mL^{-1}$ (Medac) were obtained courtesy of the Pharmacy Department (St. James's Hospital, Dublin).

Cancer cell–platelet incubation

Platelets or platelet releasate were added to culture flasks containing subconfluent Caco-2 or 59 M cells. Control and drug-treated platelet–cancer cell cultures were incubated in the presence or absence of paclitaxel (1–200 $\mu g \cdot mL^{-1}$) or 5-FU (1–300 $\mu g \cdot mL^{-1}$) for 24 or 72 h in the absence of FBS. After incubation, conditioned media were collected and cancer cells were harvested for flow cytometry studies. For the measurement of apoptosis, platelet–cancer cell incubates were stained with Annexin-V-APC and propidium iodide (PI) for 15 min in the dark; 10 000 specific events were analysed by the FACSArray (BD Biosciences, Oxford, UK). Cell survival was expressed as a percentage of control samples. Further experiments were performed with 5-FU-treated gingival fibroblasts in the presence or absence of platelets for 72 and 144 h.

Cell cycle and cyclins

The cells were fixed in cold 70 % ethanol for 15 min. The fixed cells were divided into the appropriate number of flow cytometry tubes, containing 4×10^5 (Caco-2) and 10^4 (59 M) cells per test; 300 µL PI and 50 µL RNAse were added to the cell pellet and incubated overnight at 4°C, protected from light; 10^4 events were analysed by flow cytometry, using a low flow rate. The percentage of cells in the G_0/G_1, S and G_2/M phases were quantified using ModFit-LT™ software (Verity Software House, Topsham, ME, USA).

For cyclin determination, the cells were harvested using trypsin. The cells were washed twice in PBS and fixed in cold 70 % ethanol (diluted in PBS) and stored overnight at –20°C. The fixed cells were divided into the appropriate number of flow cytometry tubes, containing 3×10^5 (Caco-2) and 10^4 (59 M) cells per test. The cells were washed twice in PBS, and 1 mL 0.25 % Triton X-100 in PBS was added to each cell pellet. The cells were mixed and incubated for 5 min at room temperature. Each sample was filled with staining buffer [PBS

containing 1 % FCS (fetal calf serum)]. To the cell pellet, 2.5 μL of cyclins A, B1, D1 and E antibodies (cyclin A, mouse IgE, cyclin B1, mouse IgG2a, cyclin D1, mouse IgG2a, cyclin E, mouse IgG1) (Becton Dickinson, Oxford, UK) were added and incubated for 60 min at room temperature. Following incubation, FITC-conjugated secondary antibody was added and the samples were incubated for 60 min. Next, PI and RNAse were added and the samples were incubated overnight at 4°C; 10 000 events were analysed by flow cytometry.

Apoptosis gene expression

TaqMan® Gene Expression Assays (Applied Biosystems, Warrington, UK) was used for quantitative gene expression analysis of targets known to have implications for apoptosis (Meyer *et al.*, 2006).

Total cellular RNA was isolated from biological repeats of Caco-2 and 59 M cells ± platelets ± paclitaxel using miRVana kit (Ambion, New York, NY, USA). The quantity of RNA was measured by Nanodrop (Thermo Scientific, Dublin, Ireland). The quality of RNA was analysed by Agilent-2100 Bioanalyser using the RNA 6000 LabChip® kit.

For reverse transcription reaction, 1 μg of total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) was used and 100 ng of transcribed DNA (cDNA) was loaded onto the Micro Fluidic Cards and run using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Threshold cycle (C_T) results were subsequently normalized to 18S and test (cells + platelets) were calibrated against control cell level (cells only) using the comparative C_T method, $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Data are presented as fold change in gene expression relative to the control group, which was normalized to 1.

DNA damage repair pathways and MAPK pathways

Western blotting was used to measure DNA damage repair pathway proteins (Radomski *et al.*, 2002). The samples were probed with antibodies against phosphorylated BRCA1-(Ser^{1524}), Chk1-(Ser^{296}), Mre11-(Ser^{676}), p95/Nbs1-(Ser^{343}), p38-(Thr^{180}/Tyr^{182}), p42/44-(Thr^{202}/Tyr^{204}) and JNK-(Thr^{183}/Tyr^{185}) (Cell Signalling Technology, Bray, Ireland). Antibodies that detect the total levels of respective proteins were used to determine the phosphorylated fraction relative to the total fraction. β-Tubulin (Sigma, Dublin, Ireland) was used as loading control and the immunoreactive bands were quantified using densitometry.

Proteomics

The platelet–cancer cell proteome of secreted proteins was analysed using 6-plex Tandem-Mass-Tags (TMT) (Thompson *et al.*, 2003, Treumann and Thiede, 2010).

Sample. Platelet–Caco-2 incubates were collected and filtered through a 0.45 μL Millipore filter and protein concentrations were estimated using a Bradford assay (Protein Assay, Bio-Rad, Blessington, Ireland) according to the manufacturer's instructions. The equivalent of 400 μg of protein from each sample was precipitated with 2 mL of ice-cold acetone.

SDS-PAGE separation of proteins. Samples were re-dissolved with sonication in 1 X NuPage LDS sample buffer (Invitrogen, New York, NY, USA), DTT was added, samples were then heated for 5 min at 85°C and separated on a 1 mm, 12-well 10%-BIS-TRIS SDS-PAGE gel (NuPage, Invitrogen). The gel was fixed in 50% MeOH, 10% CH_3COOH, H_2O (v $v^{-1}v^{-1}$) and stained using colloidal Coomassie Blue (EZ Blue, Sigma). Each of the six lanes was cut into eight slices and the resulting 48 samples were reduced, alkylated, *in gel* digested with trypsin and derivatized with TMT reagents.

After the labelling reaction with 6-plex TMT reagents (Thermo) had been performed according to the manufacturer's instructions (lane A, 126; lane B, 127; lane C, 128; lane D, 129; lane E, 130; and lane F, 131) and the reaction had been quenched with 5% hydroxylamine, samples were pooled. The resulting eight pools were dried down in a speed vac until they were almost dry and the total volume of each sample was adjusted to 30 μL using 1% trifluoroacetic acid (TFA, Sigma).

LC-MS/MS analysis. LC-MS/MS analysis was performed on an LTQ XL orbitrap mass spectrometer (Thermo Scientific) coupled to an Ultimate 3000 nano HPLC system (further information in Item S1).

Protein identification and quantification. Using Proteome Explorer version 1.1 (Thermo), the orbitrap raw data were processed and peak lists generated from the CID spectra (for protein identification) and from the HCD spectra (for quantitation). Further information can be found in supplementary material.

Phase-contrast microscopy

Cancer cells and cancer cell–platelet incubates were viewed using Olympus CKX41 phase-contrast microscope, equipped with an Altra 20 soft imaging system (Olympus Soft Imaging Solution, Center Valley, PA, USA).

Statistics

The results are presented as mean ± SD calculated from *n* separate experiments using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). The results were compared using either Student's paired *t*-test or repeated-measures-ANOVA followed by Bonferroni's test when appropriate. $P < 0.05$ was considered statistically significant. Proteomics results were assessed using a model-based Bayesian approach as shown in Tables S1 and S2.

Results

Platelets decrease drug-induced cancer cell apoptosis and necrosis

Figures 1 and 2 show the representative traces taken from the flow cytometer demonstrating the effects of platelets on cancer cell survival. Figure 3A–D shows the measurement of apoptosis and necrosis in 59 M-platelet, Caco-2-platelet, Caco-2-platelet releasate and 59 M-platelet releasate incubates treated with increasing concentrations of paclitaxel or 5-FU. As expected, drug treatments resulted in increased number of cells undergoing apoptosis and necrosis; this effect was inhibited in the presence of platelets. These results were corroborated by phase-contrast microscopy (Figure 4). In

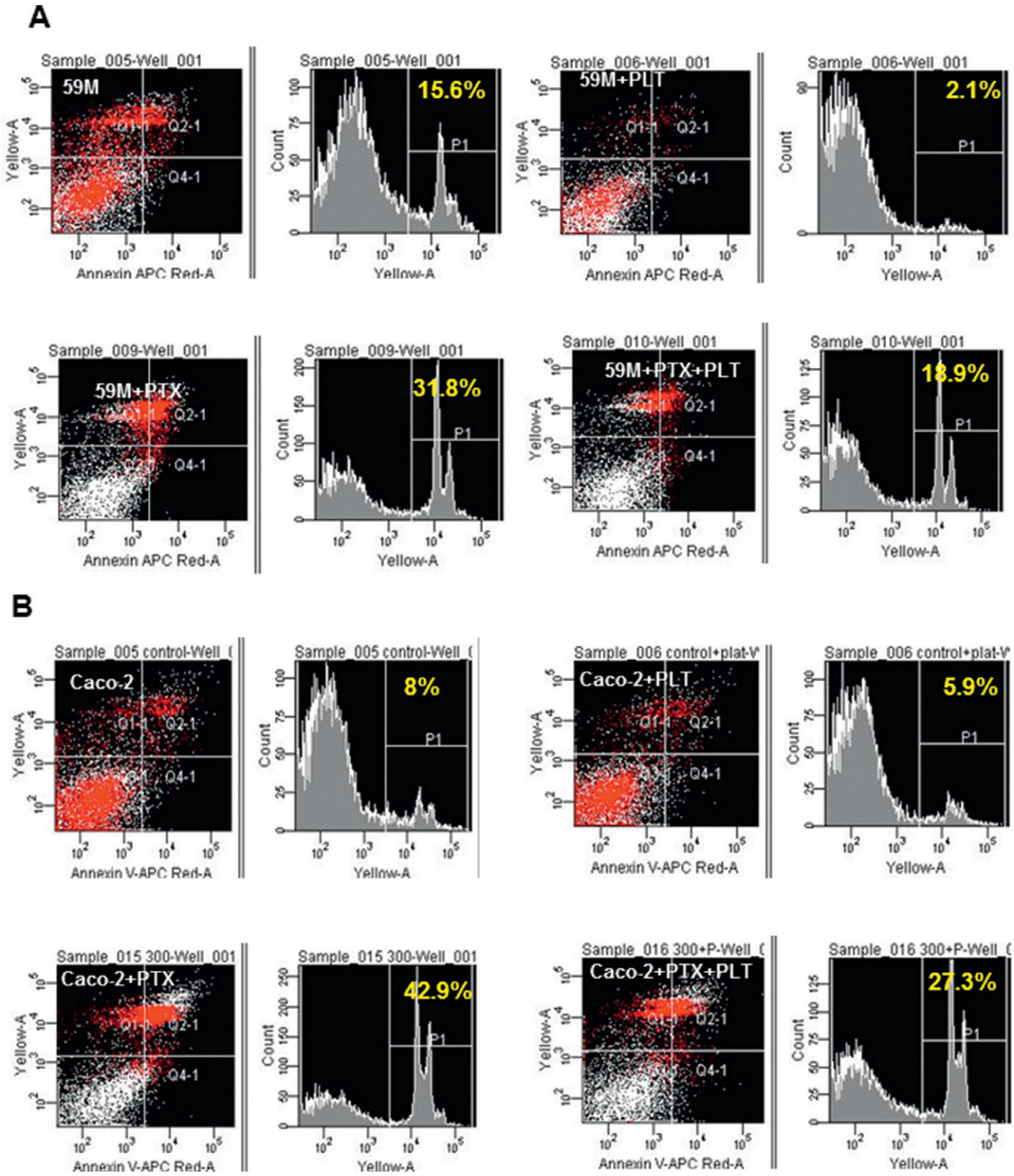

Figure 1

Platelets increase survival of adenocarcinoma ovarian 59 M and colonic Caco-2 cells in the presence of paclitaxel. (A) Representative (five experiments) dot plots of paclitaxel-treated 59 M (PTX, 200 $\mu g \cdot mL^{-1}$) cells following 24 h of incubation in the presence or absence of platelets (PLT, 1.5×10^8 mL^{-1}). (B) Representative (five experiments) dot plots of paclitaxel-treated (PTX, 200 $\mu g \cdot mL^{-1}$) Caco-2 cells following 72 h of incubation in the presence or absence of platelets (PLT, 1.5×10^8 mL^{-1}). Q1: necrosis, Q2: late apoptosis, Q3: live cells and Q4: early apoptosis. The histograms represent a gated population.

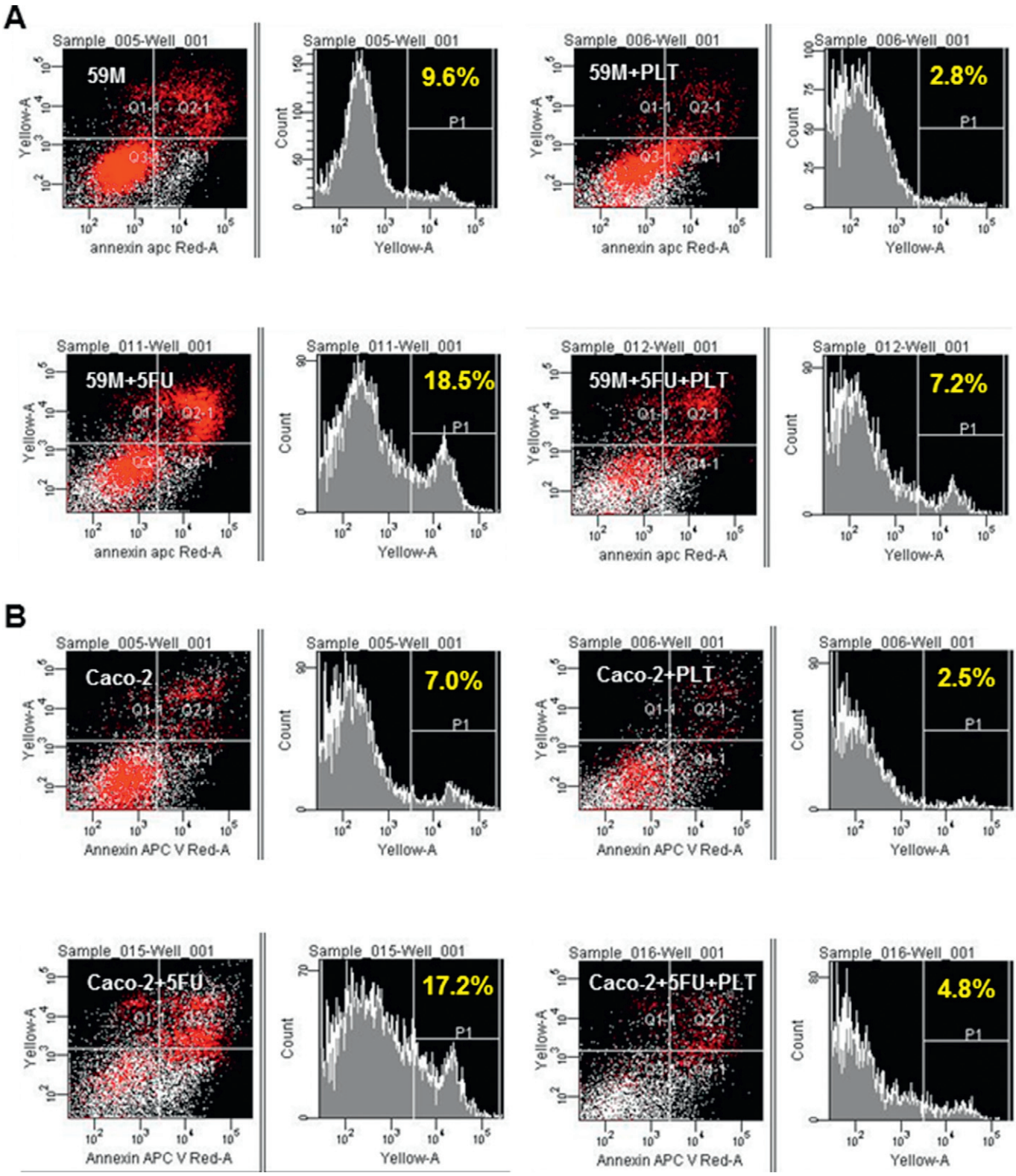

Figure 2

Platelets increase survival of adenocarcinoma ovarian 59 M and colonic Caco-2 cells in the presence of 5-fluorouracil. (A) Representative (five experiments) dot plots of 5-FU-treated (5-FU, 100 μg·mL^{-1}) 59 M cells following 72 h of incubation in the presence or absence of platelets (PLT, 1.5×10^8 mL^{-1}). (B) Representative (five experiments) dot plots of 5-FU-treated (5-FU, 100 μg·mL^{-1}) Caco-2 cells following 72 h of incubation in the presence or absence of platelets (PLT, 1.5×10^8 mL^{-1}). The histograms represent a gated population.

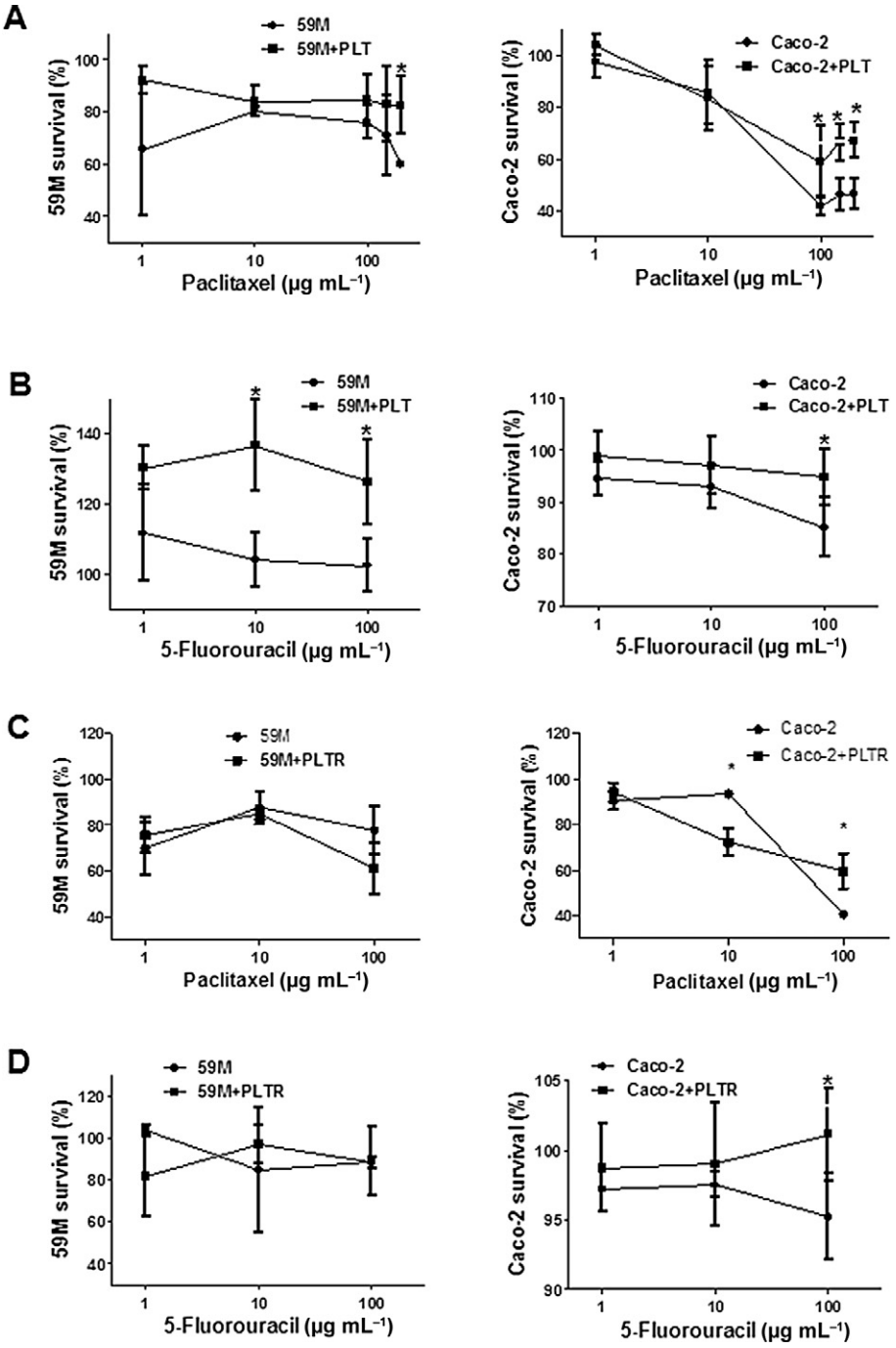

Figure 3

Concentration–response curves showing the effects of platelets on chemotherapy-challenged tumour cells. (A) Concentration–response curves showing the inhibition by platelets (PLT) of PTX-induced apoptosis in 59 M and Caco-2 cells. 59 M cells were incubated for 24 h, while Caco-2 for 72 h. Data are mean ± SD, $n = 3–5$. *$P = 0.0008$, 59 M versus 59 M + PLT and *$P < 0.0001$ Caco-2 versus Caco-2 + PLT. (B) Concentration–response curves showing the effects of PLT on 5-FU-induced apoptosis in 59 M and Caco-2 cells. Both cell types were incubated for 72 h. Data are mean ± SD, $n = 4$. *$P = 0.0082$, 59 M versus 59 M + PLT and *$P = 0.0006$ Caco-2 versus Caco-2 + PLT. (C) Concentration–response curves showing the effects of releasate (PLTR) prepared from 1.5×10^8 mL^{-1} platelets on PTX-induced apoptosis in 59 M and Caco-2 cells. 59 M cells were incubated for 24 h, while Caco-2 for 72 h. Data are mean ± SD, $n = 3–5$. 59 M versus 59 M + PLTR and *$P < 0.05$ Caco-2 versus Caco-2 + PLTR. (D) Concentration–response curves showing the effects of releasate (PLTR) prepared from 1.5×10^8 mL^{-1} platelets on 5-FU-induced apoptosis in 59 M and Caco-2 cells. Both cell types were incubated for 72 h. Data are mean ± SD, $n = 3$. 59 M versus 59 M + PLTR and *$P < 0.05$ Caco-2 versus Caco-2 + PLTR. Cell survival in the absence of drugs was set at 100%.

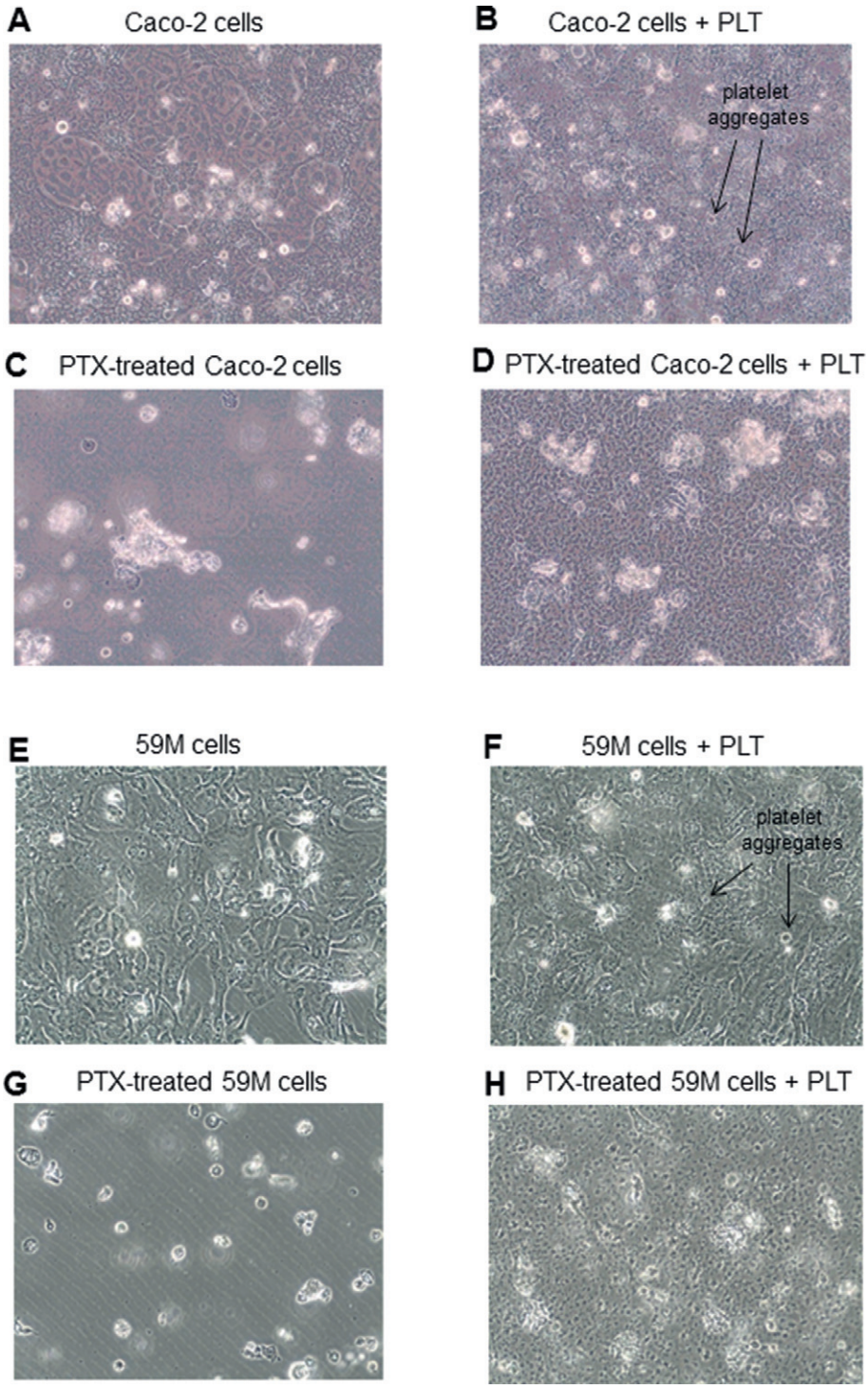

Figure 4

Representative phase-contrast micrographs showing the effects of platelets on tumour cells in the presence/absence of chemotherapeutic drugs. (A) Untreated Caco-2 cells in the absence of platelets. (B) Caco-2 cells in the presence of platelets. Small platelet aggregates can be seen. (C) PTX-treated Caco-2 cells in the absence of platelets. (D) PTX-treated Caco-2 cells in the presence of platelets. Platelets improve the morphology of PTX-treated Caco-2 cells. Caco-2 cells were incubated for 72 h in all experiments. (E) Untreated 59 M cells in the absence of platelets. (F) 59 M cells in the presence of platelets. Small platelet aggregates can be also seen. (G) PTX-treated 59 M cells in the absence of platelets. (H) PTX-treated 59 M cells in the presence of platelets. Platelets again appear to improve the morphology of PTX-treated 59 M cells. 59 M cells were incubated for 24 h in all experiments.

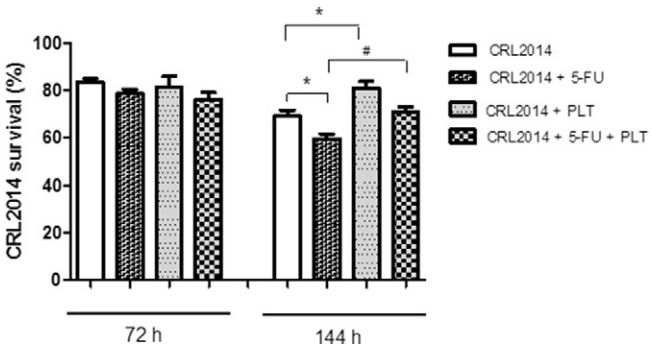

Figure 5

Time–response histogram showing the effects of platelets on chemotherapy-challenged human gingival fibroblasts. The effect of platelets (PLT, 1.5 × 10^8 mL^{-1}) on human gingival fibroblasts CRL2014 survival in the absence or presence of 5-FU (CRL2014+5-FU) after 72 h and 144 h of incubation. Data are mean ± SD, $n = 3$. *$P < 0.05$ versus control (CRL2014); # $P < 0.05$ versus 5-FU-treated CRL2014 (CRL2014 + 5-FU).

addition, knowing that 5-FU can potentially cause chemotherapy-induced oral mucositis, the effect of this drug on gingival fibroblasts was also studied. Again, 5-FU resulted in a decreased number of living cells, an effect that was abolished in the presence of platelets (Figure 5).

Platelets stimulate cancer cell cycle, DNA repair protein and MAPK levels

Next, we studied the effects of platelets on cell cycle and the expression of major cyclins such as A, B1, D1 and E, which are involved in various phases of cell cycle in the presence/absence of drugs. In the absence of anticancer drugs, after being incubated with platelets for 24 or 72 h, the number of 59 M cells residing in G_0/G_1 phase decreased and the number of cells in S, G_2/M phases increased (Figure 6A). This was associated with up-regulation of cyclin A in all cell cycle phases (Figure 6B), cyclin B1 in G_2/M phase (Figure 6C), cyclin D1 in S and G_2/M phases (Figure 6D) and cyclin E in all cell cycle phases (Figure 6E). In the presence of 5-FU, but not paclitaxel, platelets also increased the levels of cyclin A during G_0/G_1 and G_2/M phases (Figure 6B), cyclin B1 during G_0/G_1 and S phases (Figure 6C) and cyclin D1 during S phase (Figure 6D) ($n = 4$, $P > 0.05$). Platelets did not significantly modify the number of Caco-2 cells in cell cycle phases (Figure S1) ($P > 0.05$, $n = 3$).

To study the effects of platelets on DNA damage repair proteins in cancer cells challenged with paclitaxel and 5-FU, the active forms of BRCA1-(Ser^{1524}), Chk1-(Ser^{296}), Mre11-(Ser^{676}), p95/Nbs1-(Ser^{343}) proteins were measured by Western blots with antibodies directed against protein phosphorylation sites, which are responsible for activation of the proteins. Figure 7A shows that platelets resulted in significant phosphorylation of BRCA1 in 59 M and Caco-2 cells treated with paclitaxel or 5-FU. Similar results were obtained when phosphorylation of Chk1, Mre11 and p95/Nbs1 levels were measured in 59 M cells. However, platelets did not significantly modify these proteins in Caco-2 cells in the presence of treatment (Figure 7B–D). Interestingly, platelets significantly increased the phosphorylation of all the repair proteins in the absence of anticancer drugs.

The effects of platelets on MAPK pathways in cancer cells in the presence or absence of anticancer drugs was studied by measuring the active forms of p38, p42/44, JNK-p46 and JNK-p54 MAPKs by Western blots with antibodies directed against protein phosphorylation sites. The platelet treatment resulted in up-regulation of p38 and JNK-p54, in 59 M, but not in Caco-2 cells ($P > 0.05$, $n = 3$), in the presence or absence of paclitaxel/5-FU (Figure 8A and C). The activation of p42/44 and JNK-p46 was not affected by platelets in the presence of 5-FU or paclitaxel in both 59 M and Caco-2 cells (Figure 8B and D) ($P > 0.05$, $n = 3$). Interestingly, platelets significantly increased the phosphorylation of all the MAPK pathways' proteins in the absence of anticancer drugs.

Mechanisms of platelet-increased survival of cancer cells

Knowing that platelets were able to decrease both paclitaxel- and 5-FU-induced cancer cell apoptosis, the remaining experiments were carried out using paclitaxel to investigate mechanisms involved in the protective effect associated with platelets.

Firstly, we analysed the expression of genes regulating apoptosis in paclitaxel-treated 59 M and Caco-2 cells in the presence or absence of platelets. Platelets induced substantial up-regulation of anti-apoptotic genes such as BCL3, RIPK2, NF-κB1 in 59 M cells (Figure 9A) and IKBKG, BRIC5, REL and NF-κB2 in Caco-2 cells (Figure 10A). Interestingly, while some pro-apoptotic genes including PYCARD, CASP2, DAPK1, LRDD and NALP1 were down-regulated in 59 M cells (Figure 9B), others were up-regulated [i.e. NF-κBIA, NF-κBIE, CASP6 and BIK in 59 M cells (Figure 9B) and BNIP3L, CASP6 and APAF1 in Caco-2 cells (Figure 10B) ($P < 0.05$, $n = 3$)].

Secondly, the secretome of proteins released during interactions of paclitaxel-challenged Caco-2 cells with platelets was analysed in order to identify factors that may contribute to platelet-mediated cancer cell cytoprotection (Table 1). The first part of Table 1 (paclitaxel-treated Caco-2) shows proteins released from Caco-2 cells during incubation with paclitaxel, while the second part (paclitaxel-treated Caco-2 + platelets) also includes platelet proteins released during this incubation. Consequently, the paclitaxel-treated Caco-2 releasate is enriched for proteins that are (a) released from Caco-2 cells, either through secretion or through cleavage from their plasma membrane; (b) abundant Caco-2-cytosolic proteins that are derived from cellular debris generated in response to Caco-2-apoptotic or necrotic cell death. In contrast, the paclitaxel-treated Caco-2 + platelets releasate is enriched for proteins that are (c) secreted by platelets in response to interaction with the Caco-2 cells, (d) secreted by Caco-2 cells in response to interaction with platelets or (e) platelet cytosolic proteins derived from platelet debris generated following platelet activation. Many of the proteins observed in the second part of the Table 1 are proteins that are well-known to be secreted by platelets and are therefore likely to belong to category (c). The list of proteins that were identified by proteomics method can be found as Table S1. Among the proteins identified RANTES, thrombospondin-1 and clusterin are

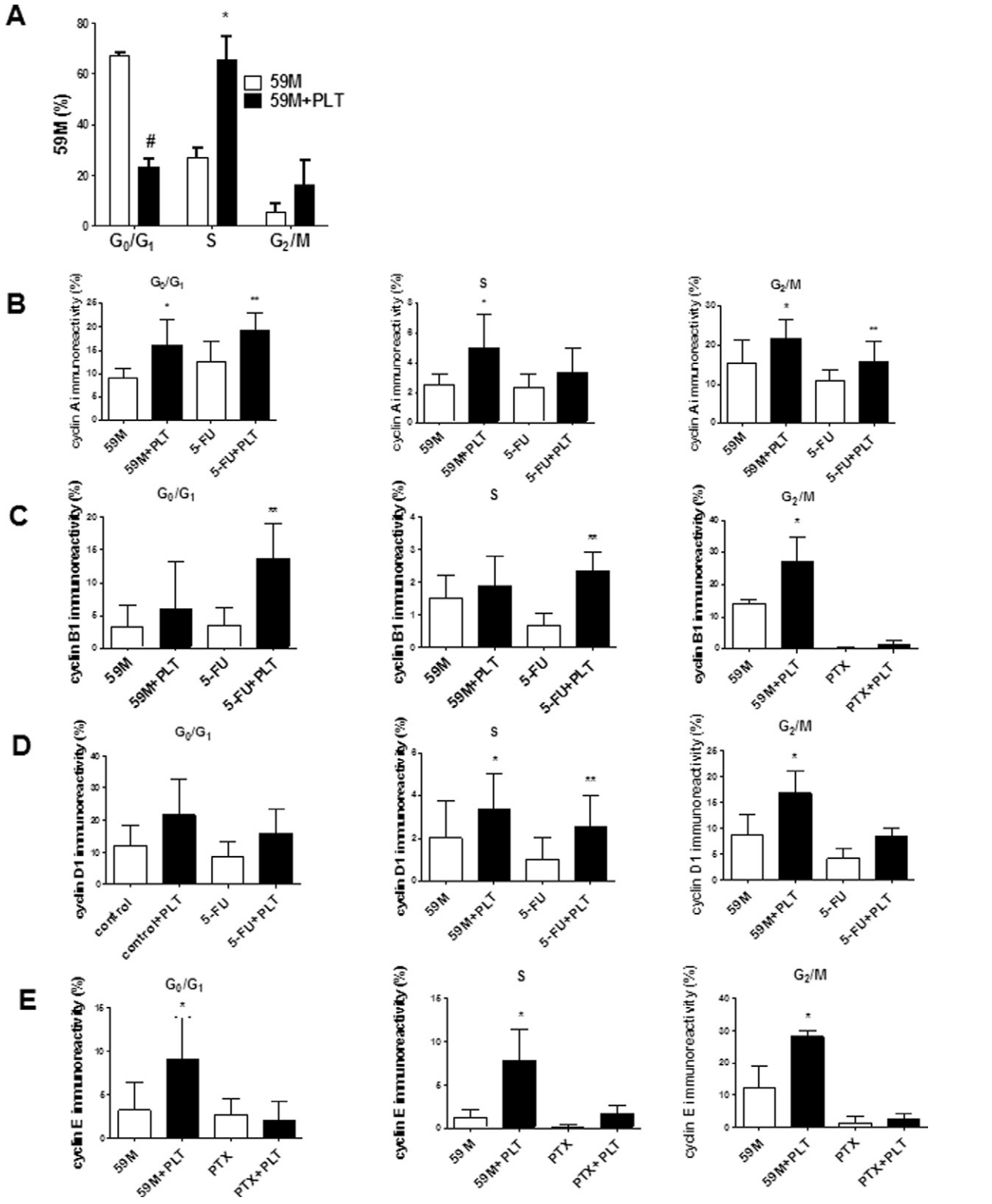

Figure 6

Effect of platelets on cell cycle and cyclin levels in 59 M cells. (A) The effects of platelets (PLT, 1.5×10^8 mL^{-1}) on G_0/G_1, S and G_2/M phases in the absence of anticancer drug. Data are mean ± SD, $n = 3$. #$P < 0.0001$, 59 M G_0/G_1 versus 59 M + PLT G_0/G_1, *$P < 0.0001$, 59 M S versus 59 M + PLT S. (B–D) Up-regulation of cyclins A, B1 and D1 in 59 M cells by PLT. 59 M cells were treated with 5-FU (200 μg·mL^{-1} for 72 h) or paclitaxel (PTX, 200 μg·mL^{-1} for 24 h) and incubated in the presence or absence of platelets (PLT, 1.5×10^8 mL^{-1}). (E) Platelets do not modulate cyclin E levels in 59 M cells challenged with paclitaxel. Data are mean ± SD, $n = 4$. *$P < 0.05$, 59 M versus 59 M + PLT, **$P < 0.05$, 5-FU versus 5-FU + PLT.

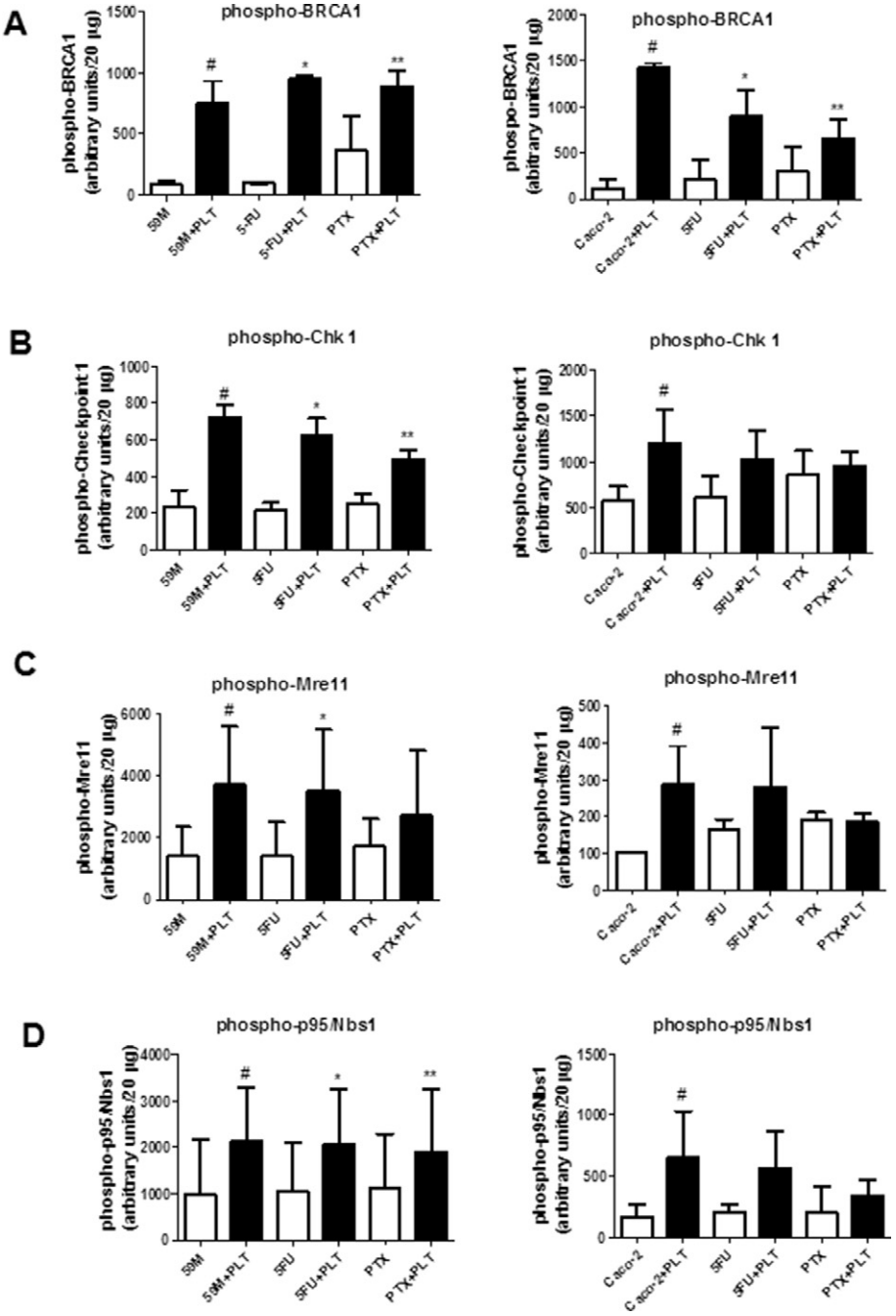

Figure 7

Platelets stimulate phosphorylation of DNA repairing proteins in 59 M and Caco-2 cells treated with 5-FU or paclitaxel. 59 M and Caco-2 cells were treated with 5-FU (300 µg·mL^{-1} for 1 h) or paclitaxel (PTX, 200 µg·mL^{-1} for 1 h) and incubated in the presence or absence of platelets (PLT, 1.5 × 10^{8} mL^{-1}). Quantitative analysis of phospho-BRCA1 (A), phospho-Chk1 (B), phospho-Mre11 (C) and phospho-p95/Nbs1 (D). Data are mean ± SD, n = 4. #P < 0.05, 59 M or Caco-2 versus 59 M + PLT or Caco-2 + PLT, *P < 0.05, 5-FU versus 5-FU + PLT, **P < 0.05, PTX versus PTX + PLT.

known to affect cell survival. Therefore, the effects of RANTES, thrombospondin-1 and clusterin were studied on the Caco-2 cell survival. The survival of these cells challenged with paclitaxel was increased by recombinant RANTES and thrombospondin-1, but not by clusterin (Figure 11A–C).

Discussion and conclusions

The key finding of our research is that platelets increase survival of adenocarcinoma cells challenged with anticancer drugs, an effect that is most likely to occur in the bloodstream

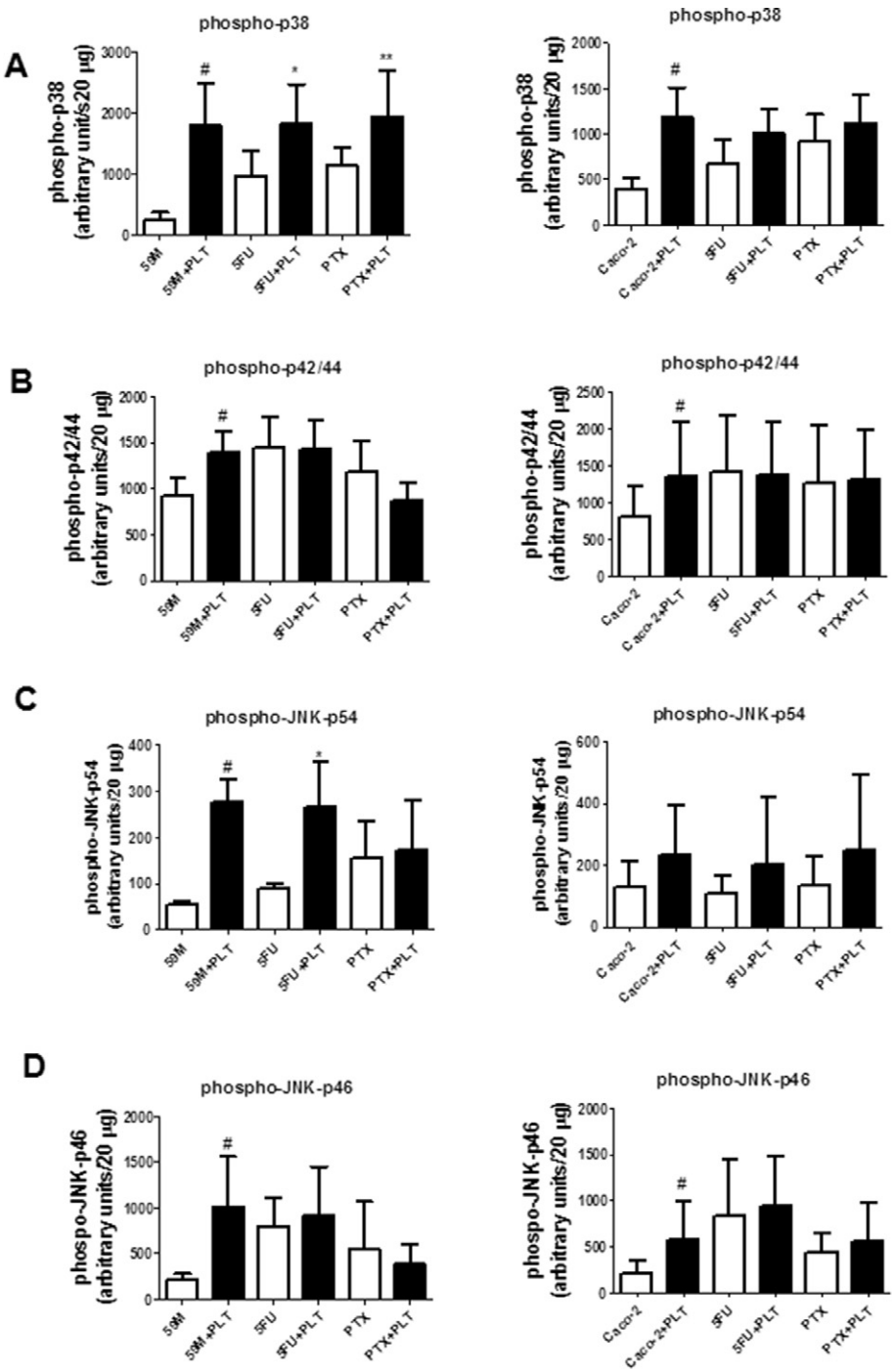

Figure 8

Platelets stimulate phosphorylation of MAPK pathways in 59 M and Caco-2 cells treated with 5-FU or paclitaxel. 59 M and Caco-2 cells were treated with 5-FU (300 µg·mL^{-1} for 1 h) or paclitaxel (PTX, 200 µg·mL^{-1} for 1 h) and incubated in the presence or absence of platelets (PLT, 1.5 × 10^{8} mL^{-1}). Quantitative analysis of phospho-p38 (A), phospho-p42/44 (B), phospho-JNK p54 (C) and phospho-JNK p46 (D). Data are mean ± SD, n = 4. #P < 0.05, 59 M or Caco-2 versus 59 M + PLT or Caco-2 + PLT, *P < 0.05, 5-FU versus 5-FU + PLT, **P < 0.05, PTX versus PTX + PLT.

as circulating cancer cells extensively interact with platelets within the vasculature. Accordingly, in the experimental model used, incubation of two human cancer cell lines (Caco-2 colon and 59 M ovarian adenocarcinomas) with two chemotherapeutic agents with different mechanisms of action [i.e. paclitaxel (mitotic spindle poison) and 5-FU (pyrimidine antagonist)] led to cell death, an effect attenuated by platelets. However, the rate of 59 M cell survival was slightly

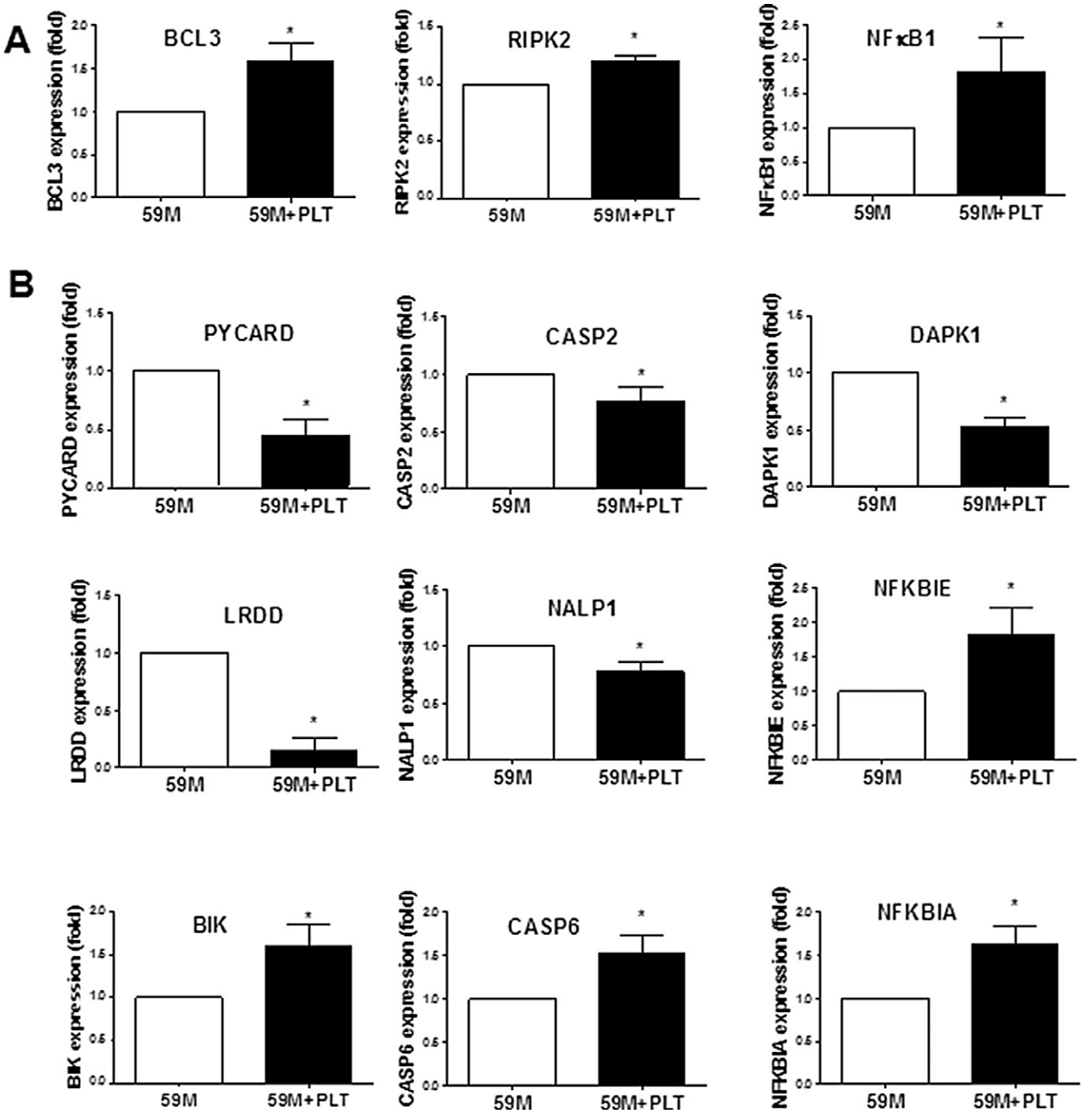

Figure 9

Platelets regulate expression of apoptosis-controlling genes in 59 M cells treated with paclitaxel. Cancer cells were incubated with paclitaxel (200 μg·mL^{-1}) for 24 h in the presence or absence of platelets (1.5×10^8 mL^{-1}, PLT). (A) Anti-apoptotic genes in 59 M cells. (B) Pro-apoptotic genes in 59 M cells. Data show relative gene expression as fold change (mean ± SD, $n = 3$. *$P < 0.05$ 59 M cells versus 59 M + PLT).

higher than that of Caco-2 cells particularly in 5-FU-treated cells. It is known that ovarian cancer cells are notorious for developing resistance to chemotherapy and various mechanisms including stimulation of DNA-repairing pathways may play a role in this process (Martinek *et al.*, 2010). The concentrations used in our study are compatible with those reported during treatment of metastatic cancers. Indeed, over the last few years, cancer has been treated in a more aggressive way than in the past requiring higher plasma concentrations. The use of bolus injections for the treatment of metastatic cancer in order to ensure efficacy of combined regimens has been recently highlighted (Tamura *et al.*, 2011).

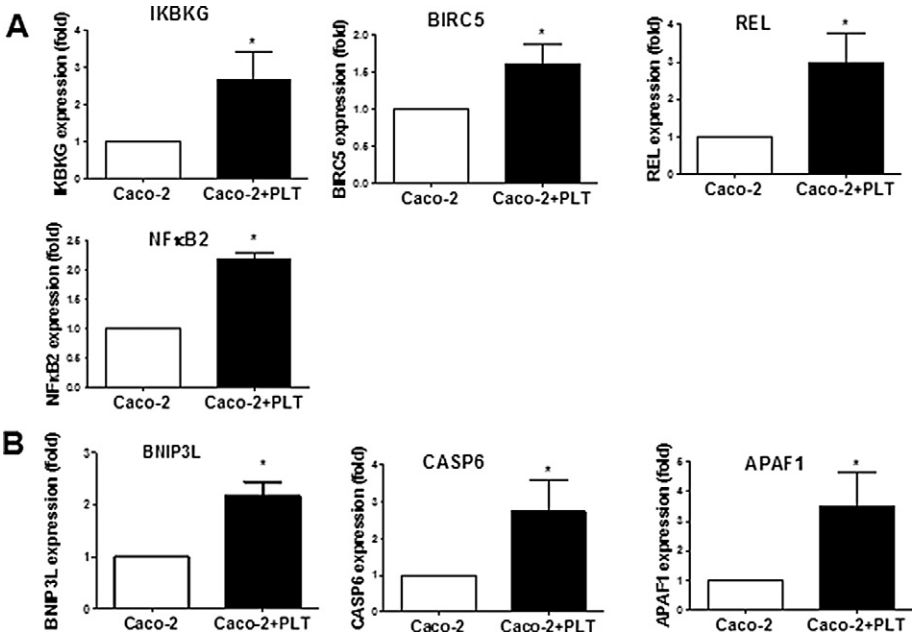

Figure 10

Platelets regulate expression of apoptosis-controlling genes in Caco-2 cells treated with paclitaxel. Cancer cells were incubated with paclitaxel (200 μg·mL^{-1}) for 24 h in the presence or absence of platelets (1.5 × 10^8 mL^{-1}, PLT). (A) Anti-apoptotic genes in Caco-2 cells. (B) Pro-apoptotic genes in Caco-2 cells. Data show relative gene expression as fold change (mean ± SD, n = 3. *P < 0.05 Caco-2 cells versus Caco-2 + PLT).

In addition, a review of preclinical reports suggests that short-term, high-dose administration of 5-FU results in growth inhibition of cancers refractory to a conventional treatment (Sobrero *et al.*, 1997). It is also worth mentioning that incubation of human gingival fibroblasts with 5-FU led to cell death but at a slower rate compared with tumour cells. This could be explained by the fact that healthy cells do not divide as fast as tumour cells. In addition, platelets were able to inhibit the effect of 5-FU on CRL2014 cells.

What mechanisms may be triggered by platelets to increase survival of adenocarcinoma cells challenged with anticancer drugs?

Firstly, platelets have the capacity to modulate the balance between pro-apoptotic and anti-apoptotic genes. The analysis of expression of apoptosis regulatory genes indicates that platelets tip the net balance towards apoptosis inhibition. For example, up-regulation of NF-κB1 and NF-κB2 indicates that platelets trigger the anti-apoptotic pathway of NF-κB (Annunziata *et al.*, 2007).

Secondly, platelets rescue cancer cells from anticancer drug-induced inhibition of cell cycle. In addition, it is also known that platelet releasate may have an important effect on cell cycle as platelet releasate from thrombin-activated platelets was found to increase the migration and proliferation of osteogenic cultures of bone marrow cells (Kark *et al.*, 2006). Indeed, 59 M ovarian cells are predominantly in the S and G_2/M phases during the cell cycle in the presence of platelets. Furthermore, in the presence of platelets cells bypassed irreversible cell cycle arrest. To study the mechanisms responsible for these effects of platelets on cell cycle, we investigated the levels of cyclin A, B1, D1 and E, the main regulators of cell cycle progression, whose overexpression has been found in a variety of cancers (Kenny *et al.*, 1999; Hwang and Clurman, 2005; Rivera *et al.*, 2006; Zhao *et al.*, 2006). Moreover, cancer migration, invasiveness, metastasis and poor patient prognosis may be linked to increased levels of cyclins (Wegiel *et al.*, 2008, Aaltonen *et al.*, 2009). Overexpression of cyclin D1 has also been linked to the development of endocrine resistance in breast cancer cells (Kenny *et al.*, 1999). We found significant up-regulation of cyclin A in the presence of platelets in untreated and 5-FU-treated 59 M cells, in all phases of the cell cycle. In contrast, platelets did not modify the regulation of cyclin A in paclitaxel-treated cells. This may be explained by direct action of paclitaxel on cyclin A (Perez-Stable, 2006). Similar to cyclin A, we found significant up-regulation of cyclin B1, D1 and E levels in the presence of platelets in 59 M cells treated with 5-FU, but not with paclitaxel. Thus, increased expression of cyclins may underpin the stimulating effect of platelets on cancer cell cycle. Interestingly, the effects of platelets on cancer cell cycle were significant in 59 M ovarian, but not colonic Caco-2 cells, presumably reflecting cell type and/or drug specificity.

Thirdly, platelets stimulate DNA repair processes. Anticancer drugs often precipitate damage of DNA and this triggers molecular mechanisms that attempt to repair DNA damage. These include factors such as BRCA1, Chk1, Mre11 and p95/Nbs1 that when activated through phosphorylation coordinate the repair of DNA lesions and the stalling of the cell cycle to allow DNA repair (Martin *et al.*, 2008). Indeed,

Table 1

The profile of proteins released during interactions of platelets with paclitaxel-treated Caco-2 cells

Paclitaxel-treated Caco-2

1. α-2-HS-glycoprotein
2. cutA divalent cation tolerance homolog (*Escherichia coli*)
3. ribosomal protein L23 pseudogene 8
4. serpin peptidase inhibitor, clade F (α-2 anti-plasmin, pigment epithelium-derived factor), member 1
5. cystatin C
6. glycine cleavage system protein H (aminomethyl carrier)
7. peroxiredoxin 4
8. group-specific component (vitamin D binding protein)
9. glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)
10. lysozyme (renal amyloidosis)
11. serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 7
12. transmembrane protein 109
13. proteasome (prosome, macropain) subunit, β type, 6
14. cadherin 17, LI cadherin (liver-intestine)
15. inter-α (globulin) inhibitor H3
16. inter-α (globulin) inhibitor H2
17. insulin-like growth factor 2 receptor
18. serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 10
19. lumican
20. gelsolin (amyloidosis, Finnish type)
21. peroxiredoxin 3
22. peroxiredoxin 5
23. α-fetoprotein
24. fibronectin 1
25. prosaposin
26. complement C4-A precursor (acidic complement C4)(C3 and PZP-like α-2-macroglobulin domain-containing protein 2)

Paclitaxel-treated Caco-2 + platelets

1. serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 1
2. haptoglobin
3. thrombospondin-1
4. fibrinogen α chain
5. Transferrin
6. platelet factor 4
7. pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)
8. apolipoprotein B (including Ag(x) antigen)
9. apolipoprotein A-I
10. serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 3
11. CD9 molecule
12. complement component 3
13. integrin, β 3 (platelet glycoprotein IIIa, antigen CD61)
14. α-2-macroglobulin
15. prolyl 4-hydroxylase, β polypeptide
16. multimerin 1
17. β-2-microglobulin
18. latent TGF-β binding protein 1
19. von Willebrand factor

Table 1

Continued

20. chemokine (C-C motif) ligand 5 RANTES
21. protein kinase C substrate 80 K-H
22. haemopexin
23. apolipoprotein A-II
24. glycoprotein V (platelet)
25. glycoprotein Ib (platelet), β polypeptide
26. peptidylprolyl isomerase F
27. heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)
28. triggering receptor expressed on myeloid cells-like 1
29. afamin
30. protein disulfide isomerase family A, member 6
31. glucosidase, α; neutral AB, inter-α (globulin) inhibitor H1
32. laminin, α 5
33. laminin, β 1
34. actin related protein 2/3 complex, subunit 4, 20 kDa
35. pregnancy zone protein
36. clusterin
37. peptidylprolyl isomerase B cyclophilin B
38. endoplasmic reticulum protein 29
39. serpin peptidase inhibitor, clade H (heat shock protein 47), member 1 (collagen binding protein 1)
40. protein disulfide isomerase family A, member 3

Caco-2 cells were treated with paclitaxel (200 $\mu g \cdot mL^{-1}$) and the protein expression determined in the presence (paclitaxel-treated Caco-2 cells + platelets, $n = 3$) or absence (paclitaxel-treated Caco-2 cells, $n = 3$) of platelets (1.5×10^8 mL^{-1}).
Proteins listed in the table were found to be present at a higher abundance in one of the two sample classes with a probability of >95%.

BRCA1 protein plays a critical role in the DNA damage recognition and in cell cycle checkpoints control that allow cell cycle progression only after DNA repair, avoiding genetic damage transmission in subsequent cell generations (Kennedy *et al.*, 2004). The activation of the checkpoint 1 (Chk1) allows repair of DNA damage, before it is replicated and passed on to daughter cells and therefore preserves the genomic integrity (Bolderson *et al.*, 2009). The Mre11 and p95/Nbs1 proteins recognize the DNA breaks and activate a variety of other proteins involved in cell cycle control and DNA repair. The Mre11 and p95/Nbs1 complex is involved in both homologous and non-homologous repair of double-strand breaks (Lavin, 2004, Lavin, 2007).

We found that the levels of active DNA-repairing agents in both ovarian and colonic adenocarcinoma are increased in the presence of platelets. Of note, this effect was significant in 5-FU but not in every repair mechanism of paclitaxel-challenged cells, again probably reflecting drug specificity.

Finally, platelets up-regulate p38 and JNK-p54 MAPKs. MAPKs, including p38, p42/44, JNK-p46 and JNK-p54 MAPKs, mediate extracellular signals and control crucial cellular processes such as proliferation, differentiation, survival, death and migration (Dhillon *et al.*, 2007). We found that platelets have the capacity to activate these proteins specifically in 59 M cells challenged with 5-FU and paclitaxel.

What factors released into cancer cell–platelet secretome could increase cancer cell survival?

Platelets may limit the access of a chemotherapeutic agent to cancer cells by drug sequestration. Platelets may also provide an anti-apoptotic mechanism to counteract pro-apoptotic effects of anticancer drugs. The first possibility appears less likely since Strieth *et al.* (2008) did not find significant interactions between platelets and paclitaxel *in vitro*. As both platelets releasate and intact platelets have the capacity to protect cancer cells from chemotherapeutic agent-induced apoptosis, drug sequestration can certainly be excluded as the sole mechanism responsible for the observed protective effect. However, the degree of protection offered by whole platelets was larger than that of releasate. This could be explained by the fact that factors associated with platelet membranes can also modulate apoptosis. Indeed, it has been previously found that platelet surface membrane receptors play an important role in mediating platelet-cancer cell interactions (Jurasz *et al.*, 2004; Janowska-Wieczorek *et al.*, 2005). Moreover, increased levels of platelet-derived microparticles transfer various surface receptors and adhesion molecules to target cells and increase survival of malignant haematopoietic cells leading to poor patient prognosis (Helley *et al.*, 2009).

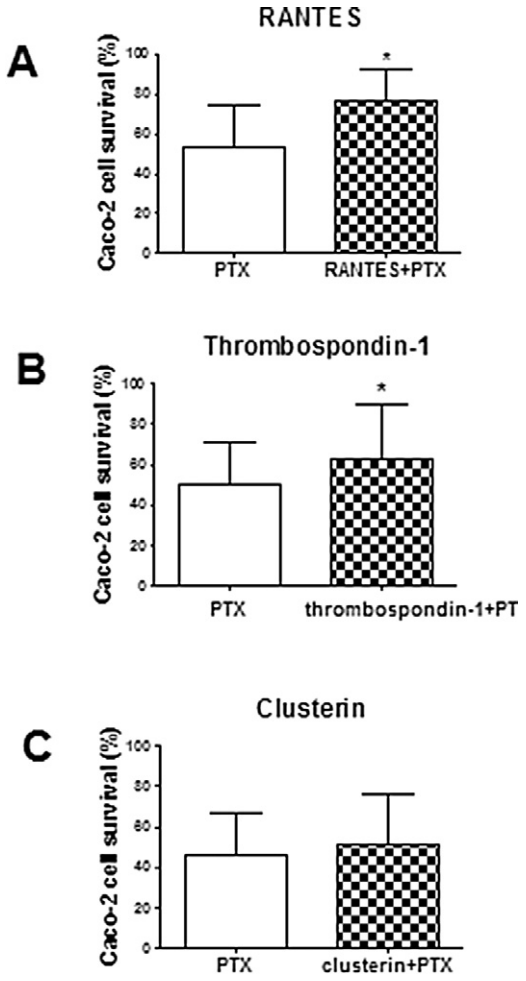

Figure 11

Effects of RANTES, thrombospondin-1 and clusterin on survival of Caco-2 cells treated with paclitaxel. Caco-2 cells were treated with paclitaxel (200 μg·mL^{-1}) for 72 h in the presence or absence of human recombinant RANTES (0.27 μg·mL^{-1}), thrombospondin-1 (1.3 μg·mL^{-1}) or clusterin (0.25 μg·mL^{-1}). Cell survival in the absence of paclitaxel was set at 100%. Data are mean ± SD, n = 3–4, *P < 0.05 paclitaxel-treated Caco-2 cells (PTX) versus cells incubated with RANTES (RANTES + PTX), thrombospondin-1 (thrombospondin-1 + PTX) or clusterin (clusterin + PTX).

In order to identify platelet factors that may protect cancer cells from damage, we used proteomics to study the secretome of proteins released during interactions of paclitaxel-treated Caco-2 cells with platelets. A number of platelet-secreted proteins are known to modulate apoptosis. These include thrombospondin-1, TGF-β, RANTES and clusterin (Borczuk *et al.*, 2007; Schniewind *et al.*, 2007; Bi *et al.*, 2010).

Clusterin is one of the glycoproteins overexpressed in, for example breast, ovarian and colon cancer; and it is a poor prognostic factor for patients (Wei *et al.*, 2009; Redondo *et al.*, 2010). A study by Park *at el.* suggested that high levels of clusterin expression by ovarian cancer cells increase paclitaxel resistance (Park *et al.*, 2008, Djeu and Wei, 2009). However, under our experimental conditions clusterin did not significantly affect the survival of Caco-2 cells.

Thrombospondin-1 (TSP-1) is stored in platelet α-granules and is released upon platelet activation (Packham and Mustard, 1984). TSP-1 may both stimulate and inhibit carcinogenesis. This glycoprotein may act as an anti-angiogenetic factor, leading to inhibition of tumour neovascularization. On the other hand, TSP-1 is involved in cell adhesion, migration and invasion of solid tumours. Qian and Tuszynski (1996) reported that TSP-1 is highly expressed in human malignant tissues and plasma of cancer patients. Higher amounts of TSP-1 receptors on cancer cells is associated with poor patient prognosis (Tuszynski and Nicosia, 1996).

TSP-1 is also known to activate TGF-β (Murphy-Ullrich and Poczatek, 2000) that inhibits host immune functions by decreasing the cytotoxicity of natural killer cells and IFN-γ secretion (Kopp *et al.*, 2009). Interestingly, in our model TSP-1 significantly increased the survival of Caco-2 cells challenged with paclitaxel.

We also found that the chemokine RANTES, which promotes cancer cell survival, proliferation and invasion (Niwa *et al.*, 2001), exerted a similar effect to that of TSP-1. However, as RANTES can also be expressed by cancer cells (Niwa *et al.*, 2001), the origin (platelet or cancer cell) of this protein in the secretome remains unclear.

It needs to be emphasized that the current experimental setup involved interactions between platelets obtained from healthy volunteers and cancer cells. Future studies will incorporate platelets obtained from patients suffering from ovarian and colonic cancers.

Our findings have implications for understanding mechanisms underlying resistance of cancer to chemotherapeutic agents

Chemoresistance in cancer still remains a major problem in anticancer drug therapy (Pennington *et al.*, 2010). Several lines of evidence support the notion that interactions between the tumour microenvironment and malignant cells may influence the apoptotic response in cancer cells and increase cell survival. For example, inhibition of NF-κB, a major pro-inflammatory transcription factor, has been shown to increase cancer cell susceptibility to paclitaxel and 5-FU when used in combination with chemotherapeutics for colon cancer (Voboril *et al.*, 2004; Kim *et al.*, 2009), prostate cancer and breast cancer (Weldon *et al.*, 2001). Furthermore, inhibition of the p42/44 MAPK enhances paclitaxel-induced apoptosis and decreases chemoresistance in colonic cancer (Xu *et al.*, 2009).

Our results underlie the importance of cancer cell–platelet interactions for the survival of adenocarcinoma challenged with high doses of anticancer drugs and provide a pharmacological rationale for designing drugs that modulate platelet–cancer interactions.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Platelets do not affect cell cycle and cyclin levels in Caco-2 cells. The effects of platelets (PLT, 1.5×10^8 mL^{-1}) on G_0/G_1, S and G_2/M phases in the presence of paclitaxel (PTX, 200 μg·mL^{-1}) (A) and 5-FU (200 μg·mL^{-1}) (B) at 72 h.
Table S1 Relative quantitation of Caco-2 releasate proteins in the presence and absence of human platelets proteins using Tandem Mass Tags. Protein data.
Table S2 Relative quantitation of Caco-2 releasate proteins in the presence and absence of human platelets proteins using Tandem Mass Tags. Peptide data.
Item S1 Further information about proteomics method.

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