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APOPTOSIS AND CELL PROLIFERATION IN PROXIMAL TUBULAR CELLS EXPOSED TO APOPTOTIC BODIES. NOVEL PATHOPHYSIOLOGICAL IMPLICATIONS IN CISPLATIN-INDUCED RENAL INJURY

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SUMMARY

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The therapeutic efficacy of the antineoplastic drug cisplatin is limited by its nephrotoxicity, which affects particularly to proximal tubular cells (PTC). Cisplatin-induced cytotoxicity appears to be multifactorial and involves inflammation, oxidative stress as well as apoptosis. We have recently shown that the cyclo-oxygenase-2 (COX-2)/intracellular prostaglandin E_2 (iPGE$_2$)/EP receptor pathway mediates the apoptotic effect of cisplatin on human proximal tubular HK-2 cells. Here, we studied the effects on HK-2 cells of apoptotic bodies (ABs) generated after treatment of HK-2 cells with cisplatin. We found that ABs inhibited cell growth, induced apoptosis and increased COX-2 expression and iPGE$_2$ in ABs-recipient HK-2 cells. Inhibition of the COX-2/iPGE$_2$/EP receptor pathway in these cells prevented the effects of ABs without interfering with their internalization. Interestingly, 2nd generation ABs (i.e. ABs released by cells undergoing apoptosis upon treatment with ABs) did not trigger apoptosis in naïve HK-2 cells, and stimulated cell proliferation through the COX-2/iPGE$_2$/EP receptor pathway. These results suggest that ABs, through iPGE$_2$-dependent mechanisms, might have a relevant role in the natural history of cisplatin-induced acute kidney failure because they contribute first to the propagation of the noxious effects of cisplatin to non-injured PTC and then to the promotion of the proliferative tubular response required for proximal tubule repair. Since iPGE$_2$ also mediates both cisplatin-induced HK-2 cell apoptosis, intervention in the COX-2/iPGE$_2$/EP receptor pathway might provide us with new therapeutic avenues in patients with cisplatin-induced acute kidney injury.

KEYWORDS: Apoptotic bodies, cisplatin, intracellular prostaglandin E2, proximal tubular cells.

ABBREVIATIONS:

ABs, Apoptotic bodies; BCA, Bicinchoninic acid assay; BG, bromocresol green; BrdU, 5′-Br-2′-deoxyuridine; BSA, Bovine serum albumin; COX-2 , Cyclooxygenase 2; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal bovine serum; FITC, Fluorescein isothiocyanate; ITS, Insulin-transferrine-selenium; iPGE2, intracellular prostaglandin E2; PBS, Phosphate-buffered saline; PGT, Prostaglandin Transporter PI, Propidium Iodie; PTC, Proximal tubular cells; RLU/s, Relative Luminescence Units; RT-PCR, Reverse transcription polymerase chain reaction; SDS, Sodium dodecyl sulfate; SEM, Scanning electron microscope; UV, Ultraviolet.

INTRODUCTION

Cisplatin (cis-diamminedichloroplatinum II) is widely used for the treatment of solid tumours, including non-small cell lung carcinoma, testicular, head and neck, ovarian, and uterine cervical. However, renal toxicity, which affects to 25–40% of cisplatin-treated patients, restricts the clinical use of this drug in the treatment of cancer therapy [1]. The uptake of cisplatin by renal tubular cells –particularly proximal tubular cells (PTC)- leads to tubular injury and cell death. In PTC, cisplatin-induced cytotoxicity appears to be multifactorial and involves inflammation, oxidative stress, and apoptosis via caspases [2].

Prostaglandin E_2 (PGE_2) has been shown to play a role in cisplatin nephrotoxicity in mice [3] and in cultured human PTC [4]. PGE_2 biosynthesis involves three sequential steps [5]: the release by phospholipase A_2 of arachidonic acid from membrane glycerophospholipids, the conversion of arachidonic acid to prostaglandin H_2 by cyclooxygenase (COX) isoenzymes (COX-1 and COX-2) and finally isomerization of prostaglandin H_2 to PGE_2 by prostaglandin E synthases such as microsomal PGE synthase-1 (mPGES-1). The renal content in PGE_2 increases in cisplatin-treated mice in parallel to an increase in the expression of COX-2 and mPGES-1 whereas mPGES-1 null mice, are resistant to cisplatin-induced both renal dysfunction and structural damage [3]. Furthermore, the renal PGE_2 content in cisplatin-treated mPGES-1 null mice underwent a very mild increase while at the same time renal damage promoted by cytokine expression and renal oxidative stress were suppressed. This phenotype was recapitulated in cisplatin-treated wild-type mice which were previously treated with celecoxib, a COX-2 inhibitor [3]

It is widely assumed that PGE_2 exerts its actions via membrane spanning G-protein coupled EP receptors, which implies that PGE_2 must reach the extracellular compartment to activate EP receptors [5]. However, we have previously found in cultured human proximal tubular HK-2 cells that *intracellular* prostaglandin E_2 ($iPGE_2$), most likely acting through *intracellular* PGE_2 EP receptors, mediates cisplatin-induced apoptotic cell death [4]. Since PGE_2, once synthesized, is quickly released to the extracellular medium, any increase in $iPGE_2$ involves its return (recycling) from the extracellular compartment via the prostaglandin uptake transporter (PGT). [6]. This implied that inhibition of PGT in human proximal tubular HK-2 cells prevents cisplatin-induced apoptosis. Thus cell death is mediated by $iPGE_2$ at least in this cell type. [4]. In summary, our previous results indicated that the COX-2/$iPGE_2$/EP receptor pathway mediates the apoptotic effect of cisplatin on proximal tubular HK2 cells.

Extracellular vesicles play an important role in intercellular communication. They typically include exosomes, microparticles and apoptotic bodies (ABs), which are distinguishable according to the size, mechanism of formation, surface markers and content [7, 8]. ABs (> 1 μm) are the largest membrane-derived extracellular vesicles and they are only produced during the disassembly of apoptotic cells [9]. ABs express phosphatidylserine on the outer face of the

membrane, which allows for their rapid recognition and engulfment by professional phagocytes or neighboring ‘amateur’ phagocytic cells (such as epithelial cells, mesangial cells, vascular smooth muscle cells, hepatic cells, and endothelial cells) and contributes thereby to the clearance of apoptotic cells [10]. The importance of ABs formation in disease is emerging because they may contribute to the development of various pathological states through the transport of the biomolecules (DNA, microRNA, lipids and protein) they harbour [11, 12]. However, to date, the focus of research on cisplatin-induced apoptosis has been primarily directed towards the signaling pathways involved in the death of tubular cells and no attention has been paid to the role of ABs generated by the apoptosis of renal parenchymal cells in the pathogenesis of cisplatin-induced acute kidney injury. In the present work, we have addressed this issue through studying the role of ABs generated by cisplatin-treated human renal proximal tubular HK-2 cells in the propagation of the effects of cisplatin on cell viability and cell proliferation to untreated PTC. Our results suggest that ABs from apoptotic PTC may act as vectors of intercellular communication that propagate the effects of cisplatin along neighboring tubular cells.

METHODS

Reagents

Cisplatin, bromocresol green (BG), AH6809, GW627368X, diclofenac, propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) were purchased from Sigma (St. Louis, MO, USA.). Etoricoxib and Z-VAD-FMK were from Sequoia research products (Berkshire, UK) and Calbiochem (Darmstadt, Germany), respectively. Pierce BCA-200 Protein Assay Kit was from ThermoFisher (Grand Island, NY, USA.) and Nocodazole, PVDF membranes and Western blotting luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA. USA.). 5′-Br-2′-deoxyuridine (BrdU), Cell Tracker™ CM-DiI and ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) were purchased from BD Biosciences (Palo Alto, CA. USA) and Invitrogen (Carlsbad, CA, USA) respectively. Annexin-V–FITC (fluorescein isothiocyanate)/propidium iodide (PI) apoptosis detection kit and Pierce BCA-200 Protein Assay Kit were from Canvax (Córdoba, Spain) and (ThermoFisher. Grand Island, NY, USA.), respectively. TriReagent was purchased from Vitro (Madrid, Spain). Antibodies were obtained from the following sources: anti-BrdU was from BD Biosciences (Palo Alto, CA. USA.) and anti-caspase 3 active was from Cell Signaling Technology (Leiden, The Netherlands) , anti-COX-2 was from Abcam (Cambridge, UK), and phalloidin CruzFluor™ 488 Conjugate were from Santacruz (Santa Cruz, CA. USA.), Anti-β-actin and rabbit anti-mouse IgG peroxidasa conjugate from Sigma (St. Louis, MO, USA.), α-mouse-Alexa-Fluor® 488 and α-rabbit-Alexa-Fluor®488 were from Invitrogen (Carlsbad, CA, USA). The human COX-2 luciferase reporter construct phPES2 containing the promoter fragment -327 to +59 was a gift from Dr. Hiroyasu Inoue (Nara Women's University, Nara, Japan).

Cell culture

Human renal proximal tubular epithelial cells (HK-2) were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA.). HK-2 cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/amphoterycin B and 1% glutamine (Invitrogen. Carlsbad, CA, USA) and 1% insulin-transferrine-selenium (ThermoFisher, Grand Island, NY, USA.). The culture was performed in a humidified 5 % CO_2 environment at 37 °C. In all experiments, cells were plated at 70–90 % confluence.

Isolation of ABs from apoptotic HK-2 cells and assessment of their internalization in recipient, intact HK-2 cells

HK-2 cells were committed to apoptosis by incubation with 50 μM cisplatin for 24 hours. Induction of apoptosis and formation of ABs were checked by inverted phase contrast microscopy and confirmed by annexin V-FITC staining followed by flow cytometry, as previously described [4]. ABs were further characterized by scanning electron microscopy and fluorescence microscopy analysis. ABs were isolated as follows: medium from apoptotic HK-2 cells was collected without detachment of intact cells and clarified from cell debris and dead cells by centrifugation (500 x g, 5 minutes). This ABs-containg medium was further centrifuged (5,000 x g, 10 minutes) to isolate the ABs. Pelleted ABs were washed with PBS twice and resuspended in complete culture medium. ABs were immediately used for experiments. When required, apoptosis was induced in HK-2 cells by UV exposure (4000μW/cm^2) for 120 seg or nutrient deprivation for 7 days and ABs were isolated following the same procedure indicated for ABs from cisplatin-treated HK-2 cell. ABs were quantified by measuring their protein content using the Pierce BCA-200 Protein Assay Kit (to this end, ABs were previously resuspended in PBS)

For studies on scanning electron microscopy HK-2 cells were grown on 12 mm^2 cover glass and treated with ABs for 2 h. ABs were isolated and then attached on poly-L-lysine coated coverslips. ABs and cells were fixed in 1.5% glutaraldehyde in PBS for 30 min. After washing twice with PBS, the samples were dehydrated in graded ethanol solutions for 5 min each (50%, 70%, 80%, 95%, and 100%) then completely dehydrated by immersing them in a solution 100% for 15 min followed by air–drying. Dried samples were further processed with gold coating, viewed with Hitachi S-3000N scanning electron microscope from SiDi service (Universidad Autónoma de Madrid).

For studies on the internalization of ABs, ABs-recipient HK-2 cells (i.e. naïve HK-2 cells) were grown on cover slips. Parallely, to obtain fluorescently labelled ABs (red), ABs-producer cells were incubated with Cell Tracker™, a lipophilic dye that conjugates to thiol-containing peptides and proteins, prior to induction of apoptosis with cisplatin (24 h treatment). Subsequently, ABs-

recipient HK-2 cells were incubated with Cell Tracker™-labelled ABs for up to 4 h. Alternatively, recipient cells were pre-incubated for 1 h with 10 µM nocodazole, an inhibitor of microtubule formation, before being incubated with labelled ABs. Cells were then washed, fixed and stained with phalloidin CruzFluor™ (green) in order to visualize filamentous actin. Afterwards cover slips were transferred onto slides, mounted with ProLong Gold antifade-DAPI and the uptake of ABs was detected by confocal microscopy.

MTT assay

Cell viability was determined using a colorimetric assay with MTT. The MTT assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. Cells were plated in 24-well plates (4 x 10^4 cells/ well). After being treated with ABs, 0.1 mg/ml MTT was added to the plates for 2 h. Formazine was dissolved in 250 µl DMSO and the absorbance was detected at 570 nm using an ELISA plate reader. Absorbances were transformed into number of cells through a cell titration curve using a range between 1 x 10^4 and 10 x 10^4 cells/well. The cell number was measured manually with a hemocytometer (this method permits effective discrimination of live from dead cells using trypan blue exclusion).

Labelling of proliferating cells with 5'-Br-2'-deoxyuridine (BrdU)

DNA synthesis was assessed by BrdU uptake. 4 × 10^4 cells/well were cultured in 24-well plates and incubated with ABs. Cells were then washed and pulsed with 10 µM BrdU during the last 1 h of incubation and afterwards, they were fixed with 2 % paraformaldehyde, for 15 min. The incorporation of BrdU was determined by immunological detection by confocal laser scan microscopy as previously described [13].

Annexin-V–FITC apoptosis assay

Apoptotic and necrotic HK-2 cells were detected using an annexin-V–FITC/Propidium iodide (PI) apoptosis detection kit on a flow cytometer as previously described [4]. Live HK-2 cells showed no staining, early apoptotic cells were positive to annexin V staining, late apoptotic cells showed both PI and annexin V staining and necrotic cells were positive to PI.

Immunofluorescence analysis of iPGE$_2$ and cleaved caspase-3

HK-2 cells (4 x 10^4 cells/glass coverslip) were fixed with 2% paraformaldehyde and permeabilized with 0.1% (v/v) Triton X-100. After being washed, cells were blocked with 4% bovine serum albumin and incubated overnight at 4 °C with anti-PGE$_2$ (1:100 dilution) or anti-cleaved caspase 3 (1:50 dilution) antibodies. Cells were then incubated at 37 °C with α-rabbit-Alexa-Fluor® 488 (1:1000) for 1 h in the darkness. Coverslips were mounted with ProLong Gold antifade reagent with DAPI. Immunofluorescence detection was performed using a Leica SP5

confocal microscope (Leica Microsystems, Wetzlar, Germany), through the Confocal Microscopy Service (ICTS ‘NANBIOSIS’ U17) of the Biomedical Research Networking Centre on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN at the University of Alcalá, Madrid, Spain) (www.uah.es/enlaces/investigacion.shtm).

For detection of PGE_2 in ABs, ABs-producer cells were incubated with Cell Tracker™ prior to induction of apoptosis with cisplatin to obtain fluorescently labelled ABs. ABs were attached on poly-L-lysine coated slides, and then, were fixed with 2% paraformaldehyde in PBS for 10 min, permeabilized with 0.1 % Triton X-100 for 10 min, blocked with 4 % bovine serum albumin and incubated for 2 h at 37 ºC with anti-PGE2 (1:50 dilution). Then, ABs were washed with PBS and incubated with α-rabbit-Alexa-Fluor® 488 (1:400 dilution) for 1 h in the darkness. Finally, slides were washed, mounted with ProLong Gold antifade reagent and immnunofluorecence detection was performed as above.

Western blot analysis of COX-2

Cells were split into six-well plates at a density of 15×10^4 cells/well and incubated for 24 h before incubation with ABs. Then, immunoblotting was performed essentially as described previously [4]. Antibodies working dilutions were: 1:1000 for COX-2 and 1:5000 for β-actin.

Single-step real-time quantitative RT-PCR

Total cell RNA from HK-2 cells was isolated with TriReagent (Sigma, St. Louis, MO) and real-time quantitative RT-PCR analysis was performed in 2 ng samples using SYBR Green PCR master mix (Applied Biosystems), in one-step RT-PCR protocol as previously described [14]. Primer sequences for genes were as follows (sequences 5’–3’): COX-2 sense: GATACTCAGGCAGAGATGATCTACCC, antisense: AGACCAGGCACCAGACCAAAGA; β-actin sense: AGAAGGATTCCTATGTGGGCG and antisense: CATGTC CCAGTTGGTGAC.

Luciferase assay of COX-2 promoter activity

Cells were split into p35 plates at a density of 15×10^4 cells/plate 24 h before transfection. Cells were then transfected with both human COX-2-luciferase and renilla luciferase reporter plasmids, as previously described [14]. After being incubated with ABs, COX-2 firefly luciferase activity was determined as indicated elsewhere [14].

Statistical analysis

Each experiment was repeated at least three times. The results are expressed as the mean ± SD. They were subjected to one-way analysis of variance (ANOVA) following by the Bonferroni's test for multiple comparisons. The level of significance was set at $P \leq 0.05$.

RESULTS

Characterization of ABs released from cisplatin-treated apoptotic HK-2 cells. Internalization of ABs in control HK-2 cells

ABs represent a population of membrane vesicles, budding directly from the plasma membrane. ABs can mediate intercellular communication through the transport of biomolecules that they harbour and may subsequently contribute to the development of various pathological states [11, 12]. Here, we aimed to evaluate the role of ABs in mediating and propagating the tubular injury induced by cisplatin. To this end, we first generated ABs by incubating human proximal tubular HK-2 cell line with 50 μM cisplatin for 24 h (Fig. 1 a upper panel, right).

After the initial generation, we then identified ABs through several approaches: a) specific staining with annexin V (Fig. 1 a) which demonstrated ABs origin from apoptotic cells; b) by scanning electron microscopy we demonstrated that apoptotic cells and ABs display the characteristic morphology (Fig. 1 b). ABs size ranged between 0.57 and 2.27 μm (Figs 1 a lower panel) and their protein content was 166.26±28 μg/mL. The microscopical aspect of ABs derived from HK-2 cells can be seen in Fig. 1 b lower panel.

Previous studies have shown that PTC are capable of ingesting apoptotic bodies by phagocytosis [15]. Indeed, the addition of Cell Tracker™-labelled ABs to HK-2 cells resulted in their internalization, as assessed by the increase in cell fluorescence (Fig. 1 c). Furthermore, pre-incubation of cells with microtubule inhibitor nocodazole, an inhibitor of phagocytosis, fully prevented the increase in cell fluorescence in HK-2 cells incubated with labelled ABs (Fig. 1 d).

Finally, because PGE_2 increases in HK2 cells undergoing apoptosis after being exposed to cisplatin [4], we asked whether ABs from cisplatin-treated HK-2 cells contain PGE_2. As shown in Fig. 1 e most, but not all ABs, contained PGE_2.

ABs generated by cisplatin-treated HK-2 cells induce apoptotic cell death in control HK-2 cells

Cisplatin reduces the viability of HK-2 cells in a dose-dependent manner, as assessed by MTT assay [4]. We hypothesized that ABs from cisplatin-treated HK-2 cells might propagate the tubular injury induced by cisplatin. In order to test this hypothesis, we studied the effect of increasing amounts of freshly isolated ABs from cisplatin-treated HK-2 cells on the viability of control HK-2 cells, as assessed by MTT assay. To this end, ABs-recipient HK-2 cells (≈ 40,000 cells) were incubated for 24 h with the ABs produced by cisplatin-treated HK-2 cells. The number of ABs-producer cells ranged from 10,000 cells (i.e. ratio ABs-producer cells to ABs-recipient cells = 0.25:1) to 120.000 (i.e. ratio ABs-producer cells to ABs-recipient cells = 3:1). As

shown in Fig. 2 a, ABs reduced viability of HK-2 cells when the ratio ABs-producer cells to ABs-recipient cells was 1:1 (i.e.: 166 ± 28 µg ABs protein) or higher. Worth mentioning, no obvious dose-dependent effect was observed for ratios higher than 1:1. Further confocal microscopy studies with the following types of staining: DAPI (Fig. 2 b upper panel), active caspase-3 (Fig. 2 b lower panel), as well as flow cytometry studies with annexin V-FITC/PI (Fig. 2 c), indicated that ABs-treated HK-2 cells underwent apoptotic cell death.

We next studied the effect on HK-2 cell death of transient exposure to ABs from cisplatin-treated HK-2 cells. HK-2 cells were exposed to ABs (ratio ABs-producer cells to ABs-recipient cells = 2:1) for incubations times ranging 2 h to 24 h. Afterwards, ABs were removed through extensive washing, cells were incubated in a complete medium for up to 24 h (total incubation time with ABs and the complete medium was 24 h), and cell death was assessed by MTT assay. As shown in Fig 2 d, 4 h incubation with ABs was enough to diminish cell viability in recipient HK-2 cells. Furthermore, in these conditions the level of cell death after incubation with ABs for 8 h was similar to that achieved when HK-2 cells were incubated for 24 h with ABs.

In summary, the results shown in Fig. 2 are in concurrence with the view that ABs might propagate the tubular cell injury induced by cisplatin.

Role of the COX-2/iPGE2/EP receptor pathway in ABs-induced HK-2 cell death.

1. Inhibition of the COX-2/iPGE$_2$/EP receptor pathway prevents ABs-induced HK-2 cell death

We previously found in cultured human PTC that cisplatin increases COX-2 expression and iPGE$_2$ [4]. These changes could be pathologically relevant due to the fact that inhibition of COX-2, EP receptors or PGT resulted in prevention of cisplatin-induced HK-2 cell death [4]. If ABs contributed to the propagation of tubular cell injury triggered by cisplatin, one might expect that they will activate pathways involved in cisplatin-induced cell death such as the COX-2/iPGE2/EP receptor pathway. To test this hypothesis, HK-2 cells were preincubated for 1 h with the following compounds: etoricoxib (COX-2 inhibitor), BG (PGT inhibitor), AH6809 or GW627368 (antagonists of EP1-3 receptors or EP4 receptors, respectively). Since ABs released by cisplatin-treated HK-2 cells induced apoptotic cell death (Fig. 2), we also pre-incubated cells with pan-caspase inhibitor Z-VAD-FMK in order to achieve full prevention of ABs-induced cell death. Next, HK-2 cells were exposed to ABs for 8 h and cell death was assessed 24 h later by annexin V/PI staining subsequently followed by flow cytometry. Our results indicated that all the compounds tested prevented, at least partially, ABs-induced cell death. Worth noting is that COX-2 inhibitor etoricoxib fully abrogated it (Fig. 3 a) and that, besides etoricoxib, nimesulide (another COX-2 inhibitor which is non-structurally related to etoricoxib) also inhibit completely ABs-induced cell death (results are not shown). Therefore, COX-2 plays a critical role in the apoptotic cell death induced by ABs in HK-2 cells through a mechanism partially dependent on iPGE$_2$.

2. ABs increase intracellular PGE_2 and COX-2 expression in HK-2 cells.

We next asked whether ABs released by cisplatin-treated HK-2 cells increased the intracellular levels of PGE_2 and the expression of COX-2 in recipient HK-2 cells. As displayed in Fig. 3 b, exposure of HK-2 cells to ABs resulted in higher levels of $iPGE_2$ and increased expression of COX-2 Even though our primary interest was not in the mechanism through which ABs increased the expression of COX-2, as a preliminary approach we studied the contribution of transcriptional mechanisms. We studied by real-time RT-PCR analysis the effect of ABs released by cisplatin-treated HK-2 cells on COX-2 mRNA expression in recipient HK-2 cells and found that it increased in a time-dependent manner (Fig. 3 c). Transient transfection assay with pHPES2 (− 327/+59), a plasmid that expresses firefly Luc under the control of the human COX-2 gene promoter showed that ABs also increased the activity of the human COX-2 gene promoter (Fig. 3 c, inset). Taken together, these results suggest that transcriptional mechanisms are involved in ABs-induced COX-2 up-regulation. In summary, ABs released by cisplatin-treated HK-2 cells determine transcriptional up-regulation of COX-2 expression in recipient HK-2 cells, which might explain the increase of $iPGE_2$ in these cells.

3. Internalization of ABs in HK-2 cells is only slightly dependent on the COX-2/iPGE2/EP receptor pathway

The phagocytic removal of ABs, which ensures the natural transfer of macromolecules between ABs-producer and ABs-recipient cells, is a regulated process. This process involves their recognition by engulfment receptors followed by the activation of signalling cascades, which in turn leads to reorganization of actin filament networks (a critical event for the successful engulfment of ABs and apoptotic cells) [16]. We then analysed the possible role of the COX-2/$iPGE_2$/EP receptor pathway in the regulation of the phagocytosis process. We addressed this issue by studying the effect of inhibitors/antagonists of the COX-2/$iPGE_2$/EP receptor pathway on the internalization of Cell-Tracker^{TM}-labelled ABs (as assessed by the increase in cell fluorescence intensity in ABs-recipient cells after 4 h incubation with fluorescence-labelled ABs). As shown in Fig.3 d, all the compounds tested had a very mild effect on the internalization of ABs, which suggested that this was not the main mechanism through which the intervention in the COX-2/$iPGE_2$/EP receptor pathway prevented ABs-induced HK-2 cell death.

4. Inhibition of the COX-2/$iPGE_2$/EP receptor pathway *after* the internalization of ABs by HK-2 cells abrogates ABs-induced cell death.

Since a diminished internalization of ABs did not fully explain the prevention of ABs-induced HK-2 cell death by inhibitors of the COX-2/iPGE2/EP receptor pathway, we tested the hypothesis that inhibitors of the COX-2/iPGE2/EP receptor pathway may act on the mechanism that triggers apoptosis following ABs internalization.

In order to substantiate this hypothesis, naïve HK-2 cells (i.e. ABs-recipient cells) were first incubated for 4 h with ABs produced by HK-2 cells treated with cisplatin. ABs-recipient cells

were then washed and incubated for 24 h in the presence of the inhibitors/antagonists of the COX-2/iPGE2/EP receptor pathway. In these conditions, cell death (as assessed by flow cytometry after annexin V/PI staining) was fully prevented by all the inhibitors tested (Fig. 3 e). Interestingly, in these conditions, the effect of the antagonists of EP receptors and the inhibitor of PGT, when added after incubation with ABs, were even stronger than when they were added before incubation (compare Fig. 3 a with Fig 3 e). In summary, the results shown in Fig. 3 e confirm that the inhibition of the COX-2/iPGE$_2$/EP receptor pathway is capable of preventing ABs-induced HK-2 cell death by interfering with the mechanism by which ABs triggers apoptosis.

Further studies on the ABs released by cisplatin-treated HK-2 cells

1. Characterization of the macromolecules harboured in ABs.

We have hypothesized that ABs generated by apoptotic PTC act as a cell-to-cell communication system that might propagate the tubular injury induced by cisplatin. The hypothesis implies that apoptosis of PTC cells upon treatment with cisplatin may even occur in cells that have not been directly exposed to cisplatin. Thus, apoptosis on these cells might occur as a consequence of transferring signaling macromolecules -including but not limited to proteins, DNA and RNA- harboured in ABs produced by cisplatin-exposed PTC.

To further characterize the nature of the macromolecules responsible for ABs-induced cell death, HK-2 cells were incubated for 24 h with ABs that were previously subjected to one of the following treatments: i) incubation in boiling water to denature proteins ii) RNase or DNase to eliminate RNAs or DNAs, respectively and iii) trypsin to digest adhesive proteins on the ABs surface. In these studies, we took advantage of the fact that incubation for 24 h of HK-2 cells with ABs does not affect cell proliferation (Fig. 4 d). Therefore, we used MTT assay to assess the effect of ABs on cell survival. While DNase did not affect the lethal effect of ABs, the remaining treatments resulted in partial (incubation with RNase) or total (incubation with trypsin or boiling water) prevention of ABs-induced cell death (Fig. 4 a). These results suggest that ABs are able to propagate apoptosis through the transport of proteins and RNAs from cisplatin-exposed apoptotic HK-2 cells to recipient, naïve PTC which may be distant from affected cells.

2. ABs generated by HK-2 cells subjected to ultraviolet exposure or nutrient deprivation also induce apoptosis in naïve HK-2 cells. Role of the COX-2/iPGE$_2$/EP receptor pathway.

Since cisplatin accumulates inside cultured PTC [17], it is theoretically possible that it is also present in ABs from cisplatin-treated HK-2 cells. This would explain the fact that the COX-2-iPGE$_2$-EP receptor pathway mediates HK-2 apoptosis induced by both cisplatin or by ABs from cisplatin-treated cells. As a corollary, induction of HK-2 cell apoptosis by stimuli such ultraviolet (UV) exposure or nutrient deprivation will not necessarily result in the release of ABs able to trigger apoptosis (in ABs-recipient HK-2 cells). This is due to the absence of cisplatin in these ABs. Alternatively, even if these ABs were able to induce apoptosis, it will not necessarily be

mediated by the COX-2/iPGE$_2$/EP receptor pathway because this pathway might be exclusive to ABs from cisplatin-treated cells. In order to address these relevant issues, recipient HK-2 cells were incubated with ABs previously isolated from HK-2 cells. They were then subsequently either exposed to UV irradiation with an UV lamp for 120 sec or subjected to nutrient deprivation for 7 days. Our results (Fig. 4 b) indicated that these ABs were also able to induce apoptotic cell death. In fact, the extent of apoptosis, as assessed by annexin V/PI staining, was similar to that induced by ABs from cisplatin-treated cells. These results rule out cisplatin as a key component of ABs apoptotic capabilities, at least in this cell type.

We next studied the role of the COX-2/iPGE$_2$/EP receptor pathway in the cell death induced by ABs released by HK-2 cells exposed to UV irradiation or subjected to nutrient deprivation. Prior to being exposed to ABs for 24h, recipient HK-2 cells were pre-incubated with diclofenac, etoricoxib, bromocresol green or GW627368. Then, cell death was assessed by MTT assay. As shown in Fig. 4 c, in the case of ABs generated by UV exposure –left panel-, all the inhibitors/antagonists of the COX-2/iPGE$_2$/EP receptor pathway partially prevented their lethal effect. In the case of ABs generated by nutrient deprivation -right panel-, EP4 antagonist GW627368X and PGT inhibitor bromocresol green fully prevented ABs-induced cell death, whereas the NSAIDs tested did not prevent it. In summary, these results indicate that, besides cisplatin-induced ABs, ABs generated by HK-2 cells subjected to UV exposure or nutrient deprivation also induce apoptosis in ABs-recipient HK-2 cells. However, the role of the COX-2/iPGE$_2$/EP receptor pathway is not as clear as in the case of apoptosis induced by ABs from cisplatin-treated cells

3. ABs generated by cisplatin-treated HK-2 cells inhibit cell proliferation and induce the release of 2nd generation ABs that stimulate cell proliferation without triggering apoptosis in ABs-recipient HK-2 cells.

Because cisplatin reduces PTC proliferation as well as it induces apoptosis [4,18,19], we studied in the naïve HK-2 cells the effects on BrdU incorporation of ABs from cisplatin-treated HK-2 cells. As shown in Fig. 4 d (left), ABs inhibited HK-2 cell proliferation. This effect was delayed in comparison with the onset of apoptosis, because it was only evident after incubation for 48 h. These results are in agreement with the view that ABs are able to propagate the effects of cisplatin to intact PTC.

In the same way, it has been shown that dying cells promote wound repair through tubular cell proliferation in cisplatin-induced acute kidney injury in rats [20]. Thus, we hypothesized that cisplatin-induced PTC apoptosis might have two sequential effects. First, ABs released by PTC (i.e. 1st generation ABs) propagate to naïve PTC noxious effects of cisplatin such as apoptotic cell death and inhibition of cell proliferation and in the second stage, wherein ABs-exposed PTC undergo apoptosis, they shed ABs (2nd generation ABs) that do not trigger apoptosis but instead stimulate cell proliferation. In order to test this hypothesis, 2nd generation ABs were generated as follows. First, control HK-2 cells were incubated for 8 h with ABs from cisplatin-treated HK-2

cells (ratio ABs-producer cells to ABs recipient cells = 2:1). Cells were then washed to remove ABs and incubated for 16 h in a complete medium. In these conditions cells underwent apoptosis (as previously shown in Fig. 2 d), thereby releasing 2nd generation ABs. Naïve HK-2 cells were then incubated for 24 h with 2nd generation ABs (ratio ABs-producer cells to ABs-recipient cells = 2:1). Afterwards the percentage of proliferating cells and apoptotic cells was assessed by BrdU incorporation assay and flow cytometry, respectively. Our results indicated that in contrast to 1st generation ABs, 2nd generation ABs did not trigger apoptosis (results are not shown) but enhance cell proliferation (Fig. 4 d, right)

Finally, in order to assess the role of the COX-2/iPGE$_2$/EP receptor pathway in the effects of ABs on cell proliferation, the experiments shown in Fig. 4 d were repeated in HK-2 cells which were previously incubated with inhibitors/antagonists of this pathway. All the substances tested prevented both the inhibition of HK-2 cell proliferation by 1st generation ABs (Fig. 4 e, upper panel) and the stimulation of cell proliferation by 2nd generation ABs (Fig. 4 e, lower panel).

In summary, these results indicate that i) cisplatin-treated apoptotic HK-2 cells shed 1st generation ABs that inhibit cell proliferation and trigger apoptosis (as shown before) in naïve HK-2 cells, which gives raise to 2nd generation ABs that induce cell proliferation and ii) the COX-2/iPGE$_2$/EP receptor pathway plays a relevant role in the effects of ABs. As we shall discuss, these results suggest that ABs from PTC exposed to cisplatin may have a relevant role in the natural history of cisplatin-induced acute kidney failure and that intervention in the COX-2/iPGE$_2$/EP receptor pathway may be a useful therapeutic approach in this pathological condition.

DISCUSSION

Apoptosis of PTC plays a significant role in cisplatin nephrotoxicity. The cellular pathways of cisplatin-induced PTC apoptosis have been extensively examined and involve activation of intrinsic and extrinsic apoptotic cascades [21]. On the contrary, the role of ABs in the propagation of the noxious effects of cisplatin to tubular cells has not been previously studied. This is probably due to the fact that ABs have long been considered to be only a part of the downstream process resulting in apoptotic cell morphological changes. Consequently, a very limited number of studies have addressed the role of ABs in the development of pathological states. Herein, we studied the role of ABs generated by PTC in propagating the effects of cisplatin to tubular cells by mediating signalling communication along tubular cells. We found that ABs from cisplatin-treated HK-2 cells induced apoptosis and inhibited cell growth in naïve HK-2 cells, but also stimulated cell proliferation through the release of 2nd generation ABs. These effects, which were critically dependent on activation of the COX-2/iPGE$_2$/EP receptor pathway, indicate that ABs are capable of dual effect. They can reproduce noxious effects of cisplatin on PTC and promote the wound repair response on these cells. Therefore, ABs may

play a relevant role in the propagation of the renal tubular changes during cisplatin-induced acute kidney failure and its recovery.

ABs were initially involved in facilitating apoptotic cell removal by phagocytic cells through helping them to engulf smaller 'bite-size' fragments of apoptotic cells [16]. Ironically, even this unique recognized biological effect of ABs was subsequently challenged because it was shown that phagocytes also engulf the apoptotic cells 'in whole' [10]. In the last couple of years the concepts about the biological role of ABs have evolved rapidly and nowadays ABs have been implicated in the pathogenesis of various diseases including tumorigenesis [22], autoimmunity [12, 23,24], inflammation [11] and viral infection [11] .Our data, in which ABs generated by HK-2 cells under different apoptotic stimuli such as cisplatin, UV exposure or nutrient deprivation induce apoptosis in naïve HK-2 cells, are in agreement with the importance of ABs formation in pathological settings. The current view in the field is that the transfer of macromolecules (i.e. proteins, microRNA and DNA) between ABs-producer and ABs-recipient cells contribute to the development of several pathological states [11, 12]. Our present results fit well this view because manoeuvres which degrade proteins and RNA hamper the ability of ABs to propagate the noxious effects of cisplatin in healthy cultured PTC.

Whether ABs from apoptotic PTC propagate cisplatin's nephrotoxicity to healthy tubular cells in an *in vivo* scenario, should be addressed in specific experiments, although previous work supports this possibility: it has already shown in vivo and ex vivo experiments that cisplatin-induced PTC apoptosis results in Fas ligand-mediated apoptotic death of cells of adjacent tubules [25]. In addition, since most (but not all) ABs from cisplatin-treated HK-2 cells contain PGE_2 (Fig. 1 e), it can be speculated that the capture of these ABs by healthy tubular cells might contribute to propagate the effects of cisplatin.

ABs are recognized by "Eat-Me" signals such as phosphatidylserine. While in healthy cells phosphatidylserine is mainly present on the inner leaflet of the plasma membrane, it is translocated to outer leaflet on ABs and membranes from apoptotic cells [10]. In the injured tubule, phagocytosis and clearance of ABs and dead cells are mainly performed by surviving PTCs expressing the phosphatidylserine receptor KIM-1 [15, 26]. KIM-1 is an apoptotic cell phagocytic receptor expressed in PTCs that confers them the ability of phagocytosis [15] and that it is specifically upregulated in PTCs following treatment with cisplatin [27]. It is likely that KIM-1 is involved in the uptake of ABs by HK-2 cells because they express this phagocytic receptor [28]. However, other KIM-1-independent pathways might contribute to the engulfment of ABs by PTC since KIM-1–negative tubules are also able to engulf apoptotic cells (although less efficiently than KIM-1 positive ones [15].

Previous studies have already shown that activation of EP receptors by PGE_2 or microinjection of PGE_2 induce apoptosis in several cell types [29-36]. Regarding cisplatin´s nephropathy, we have previously found that the $iPGE_2$ mediates cisplatin-induced apoptosis in PTC and in the present work we show that the COX-2/$iPGE_2$/EP receptor pathway also plays a relevant role in

the mechanism through which ABs generated by cisplatin-treated PTC propagate apoptosis to intact PTC. The fact that intervention in the COX-2/iPGE$_2$/EP receptor pathway also reduced the loss of cell viability of healthy PTC exposed to ABs generated by other stimuli (i.e. UV exposure or nutrient deprivation), highlights the relevance of this pathway in the apoptosis induced by ABs in PTC. Nevertheless, it is unlikely that an ABs-dependent increase in iPGE$_2$ itself is responsible for PTC apoptosis in cisplatin´s nephrotoxicity because cell viability was unaffected when iPGE$_2$ was enhanced by treatment with PGE$_2$ (unpublished observations). Therefore, additional mechanisms besides the increase in iPGE$_2$ might be required for the induction of PTC apoptosis from ABs generated by cisplatin-treated PTC. If Fas ligand where expressed on ABs, it would be a potential candidate, given that its expression on apoptotic PTC is responsible for the propagation of the noxious effect of cisplatin to intact tubular cells [25]. However, specific experiments should be conducted to identify the mediators that cooperate with iPGE$_2$ in the induction of PTC apoptosis by ABs generated by cisplatin-treated PTC.

The central hypothesis of this work is that ABs generated by PTC propagate the effects of cisplatin along the proximal tubule. We have shown first that ABs from cisplatin-treated cells are capable of reproducing two early noxious effects of the drug on PTC, namely apoptosis and inhibition of cell proliferation. However, in a later stage, dying cells promote wound repair through tubular cell proliferation as previously observed in rats with cisplatin-induced acute kidney injury [20].

Therefore, we hypothesized that the ABs responsible for the propagation of cell proliferation should be generated in a second stage by ABs-exposed apoptotic cells. Accordingly, ABs generated by cisplatin-treated HK-2 cells induced the release of 2nd generation ABs that stimulated PTC proliferation but not apoptosis. Besides confirming our hypothesis, these results indicate that ABs do not have always have the same effects on recipient cells. This suggests that the content of ABs in molecules that mediate intercellular communication is dependent on the stimulus that triggers apoptosis. Clearly, understanding the differential mechanism through which ABs induce apoptosis or cell proliferation is a very relevant issue and a great challenge because ABs transport many molecules (such are cytokines, bioactive lipids, metabolites, proteins, mRNA and miRNAs) that play a critical role in intercellular [37]. For instance, our results in Fig. 4 indicate that RNAs (presumably mRNAs or miRNAs) and peptides/proteins play a role in the differential mechanism through which ABs induce apoptosis or cell proliferation. Therefore, arrays of cytokines, mRNAs and miRNAs as well as proteomics might shed light on the differential mechanism through which ABs induce apoptosis or cell proliferation. In fact, shotgun proteomics have identified six different proteins unique to ABs of human PTC, as compared to their healthy counterparts [38] and other studies have also reported a drastic difference in 1028 proteins between intact cells and ABs [39].

In summary, our results suggest that ABs may have a relevant role in the natural history of cisplatin-induced acute kidney failure because they contribute first to the propagation of the noxious effects of cisplatin to non-injured PTC and promote later the proliferative tubular

response required for proximal tubule repair. On the other hand, the fact that the effects of ABs on PTC are mediated by $iPGE_2$, provide us with new therapeutic avenues based in intervention in the COX-2/iPGE2/EP receptor pathway in patients with cisplatin-induced acute kidney injury.

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LEGENDS TO FIGURES

Fig.1. Characterization of ABs released from cisplatin-treated apoptotic HK-2 cells. Internalization of ABs in control HK-2 cells a) Flow cytometry analysis of ABs using annexin V-FITC/forward scatter dot plot b) *Scanning electron microscopy images of apoptotic human renal proximal tubular HK-2 cells and ABs isolated from them.* HK-2 cells were treated with 50 μM

cisplatin for 24 h. Upper panel left: control; upper panel right: extensive blebbing in apoptotic HK-2 cells upon treatment with cisplatin; lower panel left: ABs isolated from the incubation medium of cisplatin-treated cells; lower panel right: ABs settled in intact cells. Control HK-2 cells were incubated for 4 h with ABs isolated from cisplatin-treated HK-2 cells c) *Internalization of ABs in HK-2 cells*. Cells were first incubated with Cell Tracker™, then they were treated with cisplatin as in b) and shed ABs were isolated from the incubation medium. Next, ABs-recipient HK-2 cells were incubated ABs (red) for up to 4 h and cells were then stained with phalloidin (green) and DAPI in order to visualize filamentous actin and cell nuclei, respectively (original magnification, 40X). d) *Inhibitor of phagocytosis nocodazole prevents the internalization of ABs in HK-2 cells.* ABs-recipient HK-2 cells were pre-incubated for 1 h with 10 µM nocodazole, an inhibitor of microtubule formation. Then, cells were incubated with labelled ABs as in c. e) *Expression of PGE_2 in ABs*. ABs-producer cells were incubated with Cell Tracker™ prior to induction of apoptosis with cisplatin to obtain fluorescently labelled ABs. Then, ABs were attached on poly-L-lysine coated slides, incubated with anti-PGE_2 and further processed as indicated in Methods (original magnification 60X).

Fig.2. ABs generated by cisplatin-treated HK-2 cells induce apoptotic cell death and inhibit cell proliferation in control HK-2 cells a) *Reduction of cell viability (MTT assay).* b) *Evidences of apoptosis: apoptotic-like nuclear morphology (DAPI staining) and cleaved caspase-3 staining*. Cells were grown on coverslips (original magnification 40X). c) *Quantitative analysis of apoptotic cells.* The bars show the sum, normalized to control values, of the percent of annexin V+/PI−cells (i.e. early apoptotic cells with preserved plasma membrane integrity) and the percent of annexin V+/PI + cells (i.e. late apoptotic cells), as determined by flow cytometry. d) *Reduction of cell viability (MTT assay) upon transient exposure to ABs.* HK-2 cells were exposed to ABs for incubations times ranging 2 h to 24 h. Then, ABs were removed and cells were incubated in complete medium for up to 24 h (total incubation time with ABs and complete medium was 24 h).

General information: Unless otherwise indicated, the ratio ABs-producer cells to ABs-recipient cells was always 2:1. For MTT and immunofluorescence studies ABs, which were isolated from ≅ 80,000 cells treated with 50 µM cisplatin for 24 h, were added to ≅ 40,000 intact HK-2 cells. For flow cytometry studies, ABs were isolated from ≅ 400,000 cisplatin-treated cells and added to ≅ 200,000 intact HK-2 cells. Microphotographs are representative examples of at least three independent experiments. Bars and error bars in graphs: Each bar represents the mean ± SD of 3 different experiments

Fig.3: Role of the COX-2-iPGE2-EP receptor pathway in ABs-induced HK-2 cell death. a) *Inhibition of the COX-2-iPGE2-EP receptor pathway prevents ABs-induced HK-2 cell death.* Cells were pre-incubated for 1 h with antagonists/inhibitors of the pathway and then they were incubated with ABs for 8h, washed and incubated for 24 h in medium which was replenished again with the inhibitors/antagonists. The bars show the sum, normalized to control values, of

the percent of apoptotic and necrotic cells, as assessed by flow cytometry (annexin V/PI staining). b) *ABs increase intracellular PGE_2 and COX-2 expression in HK-2 cells.* Upper panel left PGE_2-dependent immunofluorescence merged with nuclear staining with DAPI (*original magnification, 40X). Right:* Quantitative approach to the images presented in left panel using Image J software. Lower panel Time-course of COX-2 expression. Equal protein loading was confirmed by probing with an anti-β-actin antibody. c) *ABs increase the expression of COX-2 mRNA and the activity of a human COX-2 promoter construct transfected in HK-2 cells*. In real-time RT-PCR analysis, equal RNA loading was confirmed by assessing the expression of β-actin mRNA. d) *Internalization of ABs in HK-2 cells is only slightly dependent on the COX-2-iPGE$_2$-EP receptor pathway.* Cells were pre-incubated with inhibitors/antagonists of the COX-2-iPGE$_2$-EP receptor pathway as in Fig. 3 a. Then, they were incubated with Cell-Tracker™-labelled ABs for 4 h and internalization of ABs was assessed as the increase in fluorescence intensity using image J software. e) *Intervention in the COX-2-iPGE$_2$-EP receptor pathway* after *the internalization of ABs by HK-2 cells prevents ABs-induced cell death*. Cells were incubated with ABs for 4 h. Then, inhibitors/antagonists of the COX-2-iPGE$_2$-EP receptor pathway and cell death was determined after 24 h incubation. The bars show the sum, normalized to control values, of the percent of apoptotic and necrotic cells, as assessed by flow cytometry.

General information: The ratio ABs-producer cells to ABs-recipient cells was always 2:1. For immunofluorescence studies (i.e. PGE_2 expression and internalization of ABs) ABs, which were isolated from ≅ 80,000 cells treated with 50 µM cisplatin for 24 h, were added to ≅ 40,000 intact HK-2 cells. For the remaining studies, ABs were isolated from ≅ 400,000 cisplatin-treated cells and added to ≅ 200,000 intact HK-2 cells. Pharmacological inhibitors/antagonists: 10 µM AH6809 (EP1-3 receptor antagonist), 10 µM GW627368 (EP4 receptor antagonist), 50 µM BG (inhibitor of the prostaglandin uptake transporter), 30 µM diclofenac (COX-1/COX-2 inhibitor), 3 µM etoricoxib (COX-2 inhibitor) or 25 µM Z-VAD-FMK (pan-caspase inhibitor). Western blot autoradiographs are representative examples of at least three independent experiments. Bars and error bars in graphs: Each bar represents the mean ± SD of 3 different experiments *P < 0.01 vs control; #P < 0.01 vs ABs.

Fig.4. Further studies on the ABs released by cisplatin-treated HK-2 cells. a) *Characterization of the nature of the informational macromolecules harboured in ABs*. ABs were first incubated with trypsine for 2 min or with RNaseA or DNaseI for 60 min, or were incubated at 95 ºC for 1 h. Treatments were terminated by addition of complete medium from which ABs were again isolated by centrifugation. Cells were then incubated with ABs for 24 h and cell viability was determined by MTT assay b) *ABs generated by HK-2 cells subjected to ultraviolet (UV) exposure or nutrient deprivation also induce apoptosis in intact HK-2 cells.* Cells were incubated for 8 h with ABs; then, they were washed and incubated in complete medium for 16 h. The bars show the sum, normalized to control values, of the percent of apoptotic and necrotic cells, as assessed by flow cytometry (annexin V/PI staining) c) *Role of the COX-2-iPGE$_2$-EP receptor pathway in the reduction of cell viability induced by ABs generated by UV exposure or nutrient*

deprivation. Cells were pre-incubated with inhibitors/antagonists of the COX-2-iPGE_2-EP receptor pathway as in Fig. 3 a). Then, they were incubated for 8 h with ABs, washed and incubated for 16 h in medium which was replenished again with the inhibitors/antagonists. d) *ABs generated by cisplatin-treated HK-2 cells inhibit cell proliferation and induce the release of 2nd generation ABs that stimulate cell proliferation in ABs-recipient HK-2 cells.* Left: HK-2 cells were incubated for up to 48 h with ABs isolated from cisplatin-treated. Thereafter, cell proliferation was assessed as the percentage of BrdU-positive nuclei (which was determined through manual count in five fields in a blind manner). Right. ABs, isolated from ≅ 320,000 cells exposed to 50 μM cisplatin for 24 h were added to ≅ 160,000 intact HK-2 cells (i.e. ratio ABs-producer cells to ABs-recipient cells = 2:1). After 8 h incubation, cell were washed and incubated in complete medium for 16 h to allow the induction of apoptosis and the generation of ABs. Then, ≅ 40,000 intact HK-2 cells were incubated with these 2nd generation ABs (ratio ABs-producer cells to ABs-recipient cells = 2:1) and cell proliferation was assessed. e) *Intervention in the COX-2-iPGE2-EP receptor pathway prevents ABs-induced changes in cell proliferation.* Cells were pre-incubated with inhibitors/antagonists of the COX-2-iPGE_2-EP receptor pathway as in Fig. 3 a). Then, they were incubated with either 1st generation ABs (upper panel) or 2nd generation ABs (lower panel) for 48 h or 24 h, respectively.

General information: Apoptotic treatments were as follows: 50 μM cisplatin for 24 h; UV exposure for 120 sec, followed by 24 h incubation in complete medium or 7 days in 0 % FBS and 0 % ITS. The ratio ABs-producer cells to ABs-recipient cells was always 2:1. For flow cytometry studies, ABs were isolated from ≅ 400,000 cisplatin-treated cells and added to ≅ 200,000 intact HK-2 cells. For the remaining studies ABs, isolated from ≅ 80,000 cells, were added to ≅ 40,000 intact HK-2 cells. Pharmacological inhibitors/antagonists: 10 μM AH6809 (EP1-3 receptor antagonist), 10 μM GW627368 (EP4 receptor antagonist), 50 μM BG (inhibitor of the prostaglandin uptake transporter), 30 μM diclofenac (COX-1/COX-2 inhibitor) or 3 μM etoricoxib (COX-2 inhibitor). Microphotographs are representative examples of at least three independent experiments. Bars and error bars in graphs: Each bar represents the mean ± SD of 3 different experiments $^*P < 0.01$ vs control; $\#P < 0.01$ vs ABs.