Stimulation of Erythrocyte Phosphatidylserine Exposure by Paclitaxel

Philipp A. Lang¹, Jens Huober², Cornelia Bachmann², Daniela S. Kempe¹, Malgorzata Sobiesiak¹, Ahmad Akel¹, Olivier M. Niemoeller¹, Peter Dreischer¹, Kerstin Eisele¹, Barbara A. Klarl¹, Erich Gulbins³, Florian Lang¹ and Thomas Wieder¹

Department of Physiology¹ and Department of Obstetrics and Gynaecology², University of Tübingen and Department of Molecular Biology, University of Essen³

Key Words

Annexin-V • Eryptosis • Calcium • Ceramide • Eryptosis

Abstract

Side effects of cytostatic treatment include development of anemia resulting from either decreased generation or accelerated clearance of circulating erythrocytes. Recent experiments revealed a novel kind of stress-induced erythrocyte death, i.e. eryptosis, which is characterized by enhanced cytosolic Ca^{2+} levels, increased ceramide formation and exposure of phosphatidylserine at the cell surface. The present study explored whether cytostatic treatment with paclitaxel (Taxol®) triggers eryptosis. Blood was drawn from cancer patients before and after infusion of 175 mg/m^2 Taxol®. The treatment significantly decreased the hematocrit and significantly increased the percentage of annexin-V-binding erythrocytes *in vivo* (by 37%). *In vitro* incubation of human erythrocytes with $10 \mu\text{M}$ paclitaxel again significantly increased annexin-V-binding (by 129%) and augmented the increase of annexin-V-binding following cellular stress. The enhanced phosphatidylserine exposure was not dependent on

caspase-activity but paralleled by erythrocyte shrinkage, increase of cytosolic Ca^{2+} activity, ceramide formation and activation of calpain. Phosphatidylserine exposure was similarly induced by docetaxel but not by carboplatin or doxorubicin. Moreover, eryptosis was triggered by the Ca^{2+} ionophore ionomycin ($10 \mu\text{M}$). In mice, ionomycin-treated eryptotic erythrocytes were rapidly cleared from circulating blood and sequestered into the spleen. In conclusion, our data strongly suggest that paclitaxel-induced anemia is at least partially due to induction of eryptosis.

Copyright © 2006 S. Karger AG, Basel

Introduction

Taxanes, e. g. paclitaxel (Taxol®) and docetaxel (Taxotere®), are effective anticancer drugs [1] widely used in the treatment of a variety of malignancies [2, 3]. The primary targets of taxanes comprise components of the cytoskeleton [4], and impaired regulation of

cytoskeletal organization is considered a crucial step in cancer cell proliferation [5]. While non-tumorigenic cells, such as smooth muscle cells, undergo paclitaxel-induced cell cycle arrest rather than cell death [6], treatment of cancer cells with paclitaxel leads to activation of suicidal death pathways, i.e. programmed cell death or apoptosis [7-9]. The mechanisms of paclitaxel-induced apoptosis may depend on the cell type but involve p53 signaling [10] and in most cells caspases [11, 12].

The reported side effects of cytostatic treatment with paclitaxel (Taxol®) include anemia [13-15], which may result from decreased formation of new erythrocytes or from accelerated clearance of circulating erythrocytes. Accelerated clearance, in turn, may be the result of stress-induced programmed erythrocyte death or eryptosis, which is characterized by cellular shrinkage [16-19], externalization of phosphatidylserine [16-22] and activation of cellular proteases [16, 22-24]. Eryptosis has similarities to but may be distinct from erythrocyte senescence and neocytolysis [25-29].

Recent experiments disclosed that an increase of erythrocyte Ca^{2+} activity leads to the generation of “eat me” signals, i. e. exposure of phosphatidylserine at the cell surface [16, 17, 30] due to stimulation of Ca^{2+} sensitive scrambling of the cell membrane [31]. As macrophages are equipped with receptors specific for phosphatidylserine [32], erythrocytes exposing phosphatidylserine at their surface will be rapidly recognized, engulfed and degraded [33] and are thus expected to be rapidly eliminated from circulating blood. Accordingly, the decreased life span of erythrocytes with sickle cell disease, thalassemia and glucose-phosphate dehydrogenase deficiency has been shown to be paralleled by enhanced phosphatidylserine exposure [34].

Ca^{2+} activity of erythrocytes may be increased by activation of a Ca^{2+} -permeable cation conductance which is activated by hyperosmotic shock, oxidative stress and glucose depletion [18]. Besides increasing cell membrane scrambling, Ca^{2+} activates Ca^{2+} -sensitive K^+ channels [35] which similarly favors erythrocyte programmed cell death [36]. In addition, erythrocyte scrambling is sensitized to Ca^{2+} by the lipid-derived second messenger ceramide which is formed by sphingomyelinase [19].

The present study has been performed to explore whether exposure to paclitaxel triggers phosphatidylserine exposure in erythrocytes and to elucidate the underlying signaling mechanisms, i.e. Ca^{2+} entry, acid sphingomyelinase activation, ceramide formation and activation of proteases.

Materials and Methods

Patients

To test for *in vivo* effects of paclitaxel, eryptosis was determined in eight patients before and after cytostatic therapy with paclitaxel (Taxol®). In six cases paclitaxel was given in combination with carboplatin and in two cases in combination with carboplatin and gemcitabine. Doses were 175 mg/m^2 for paclitaxel, 800 mg/m^2 for gemcitabine and AUC 5 for carboplatin. For comparison, eryptosis was determined in 20 healthy donors. Patients and healthy donors gave their informed consent and the study was approved by the Ethic Committee of the University of Tübingen (project number: 184/2003V). The patients blood was drawn immediately prior to and immediately following a 3 hours infusion period of 175 mg/m^2 paclitaxel and subsequently analyzed *in vitro*.

Animal experimentation

To further test for *in vivo* effects of paclitaxel and for *in vivo* clearance of eryptotic erythrocytes, animal experiments were performed according to the German animal protection law and approved by the local authorities (registration number PY 6/04). Erythrocytes were drawn from C57BL/6 mice (female aged 4 - 6 months) by retroorbital venopuncture. Experiments on mouse erythrocytes were performed at 37°C in standard Ringer solution as described below. Where indicated, the Ca^{2+} ionophore ionomycin (Sigma, Taufkirchen, Germany) was diluted into Ringer solution from a 1 mM dimethylsulfoxide (DMSO) stock solution.

To test whether paclitaxel induces the release of ceramide *in vivo* by activation of the acid sphingomyelinase, we injected 4.5 $\mu\text{g}/\text{g}$ paclitaxel (Sigma) intravenously into C57BL/6 mice. Whole blood and serum samples were collected prior to and 10 min after injection of paclitaxel. To determine the activity of the acid sphingomyelinase activity in the serum, the samples were diluted 1:50 in 250 mM sodium acetate (pH 5.0), 1.3 mM EDTA and 0.1% NP40, 0.05 μCi per sample [^{14}C]sphingomyelin (52 mCi/mmol; MP-Biomedicals, Eschwege, Germany) were added and the samples were incubated for 30 min at 37°C. Lipids were then extracted in 4 volumes of $\text{CHCl}_3:\text{CH}_3\text{OH}$ (2:1, v/v), an aliquot of the upper aqueous phase was collected and the release of [^{14}C]choline chloride from [^{14}C]sphingomyelin was measured by scintillation counting. Whole blood samples were first lysed for 10 min in 4 volumes of 250 mM sodium acetate (pH 5.0), 1.3 mM EDTA and 1% NP40, diluted in 250 mM sodium acetate (pH 5.0), 1.3 mM EDTA, and 0.1% NP40 and then processed as above.

Erythrocyte concentrates and cell culture

To explore the induction of eryptosis *in vitro*, leukocyte-free erythrocyte concentrates [37] from healthy donors were exposed to 5 - 100 μM paclitaxel, to other chemodrugs, or to paclitaxel in combination with other cellular stressors (i.e. hyperosmotic shock, glucose depletion) as indicated.

To compare the effects of paclitaxel on erythrocytes to those in nucleated cells, Jurkat T-cells were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS), 0.56 g/l L-glutamine, 100 000 units/l penicillin and 0.1 g/l

streptomycin. Where indicated, paclitaxel (10 μ M) was added for subsequent determination of annexin binding or processing and activation of caspase-3 [9].

Solutions

In vitro experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl₂. Paclitaxel from Sigma was added to standard Ringer solution at final concentrations of 5 - 100 μ M by addition of appropriate volumes of a 10 mM stock solution in DMSO. Ionomycin was dissolved in DMSO and diluted into Ringer solution at a final concentration of 1 μ M. 10 mM aqueous stock solutions of carboplatin ((1,1-cyclobutanedicarboxylato) platinum) from Sigma, 50 mM docetaxel in DMSO or 10 mM aqueous doxorubicin-hydrochloride from Fluka (Buchs SG, Switzerland) were diluted into standard Ringer to achieve 10 μ M final concentration. Appropriate concentrations of vehicle (DMSO or water) in Ringer solution served as vehicle controls. Where indicated, extracellular osmolarity of the Ringer solution was increased by addition of sucrose (550 mM), or glucose was replaced by isoosmolar concentrations of NaCl. Where indicated, experiments were performed in the presence of the cell-permeable pan-caspase inhibitor zVAD-fmk (in which z stands for benzyloxycarbonyl and fmk for fluoromethyl ketone) from Calbiochem (Bad Soden, Germany) which was added to the solutions from a 20 mM DMSO stock solution 30 min prior to addition of paclitaxel. This inhibitor was synthesized as a methyl ester to enhance cell permeability.

FACS analysis

FACS analysis was performed as described [38]. After incubation in the presence or absence of paclitaxel or other cytostatic drugs, cells were washed in annexin-V-binding buffer containing (in mM) 125 NaCl, 10 HEPES/NaOH (pH 7.4), and 5 CaCl₂. Erythrocytes were suspended in a solution composed of Annexin-V-Fluos (Roche Diagnostics, Mannheim, Germany) and annexin-V buffer (dilution of 1:50). After 10 min of incubation, samples were finally diluted 1:5 in annexin-V-binding buffer and measured by flow cytometric analysis on a FACS-Calibur from Becton Dickinson (Heidelberg, Germany). Cells were analysed by forward scatter and annexin-V-fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of ceramide

After incubation in the presence or absence of paclitaxel or docetaxel, cellular ceramide was determined by an antibody-based, flow cytometric method as described previously [19]. To this end, cells were stained for 1 hour at 4°C with anti-ceramide antibody (clone MID 15B4, isotype IgM) from Alexis (Grünberg, Germany) or isotype-matched pure mouse antibody (Alexis) in phosphate buffered saline (PBS) containing 1 % FCS at a dilution of 1:5. After three washes with PBS/1 % FCS, cells were stained with polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig specific antibody (Pharmingen, Hamburg, Germany) in PBS/1 % FCS at a dilution

of 1:50 for 30 min. Unbound secondary antibody was removed by washing the cells two times with PBS/1 % FCS and samples were analyzed by flow cytometric analysis on a FACS-Calibur. FITC-fluorescence intensity was measured in FL-1. As a positive control 1 unit/ml *Streptomyces* sp. sphingomyelinase (from Sigma) was added.

The results were confirmed by a biochemical method using diacylglycerol kinase and [³²P] gamma-ATP as described [37]. After stimulation of erythrocytes with 10 μ M paclitaxel for 24 h, the samples were extracted in chloroform:methanol:1N HCl (100:100:1), the lower phase was collected and dried. Diacylglycerol was then degraded by alkaline hydrolysis of the samples in 100 μ l of 0.1 N methanolic KOH at 37°C for 60 min. The samples were re-extracted, dried and resuspended in 20 μ l of detergent solution (7.5% (w/v) n-octylglucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid (DETAPAC)). The samples were then sonicated for 10 min in a bath sonicator and the kinase reaction was initiated by addition of 70 μ l reaction mix consisting of 10 μ l DAG-kinase (GE healthcare; München, Germany) (in 5 mM potassium phosphate buffer (pH 7.0), 10% glycerol, 1 mM 2-mercaptoethanol, 0.005 M imidazole/HCl, 0.5 mM DETAPAC (pH 6.6)), 50 μ l assay buffer (0.1 M imidazole/HCl (pH 6.6), 0.1 M NaCl, 25 mM MgCl₂ and 2mM EGTA), 2.8 mM DTT, 5 μ M ATP and 10 μ Ci [³²P]gamma-ATP. The kinase reaction was performed for 30 min at room temperature. The reaction was terminated by addition of 1 ml of chloroform: methanol: 1 N HCl (100:100:1). 170 μ l of a buffered saline solution (135 mM NaCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES pH 7.2) and 30 μ l of a 100 mM EDTA-solution were added. The samples were extracted, the lower phases collected, dried, dissolved in 20 μ l of chloroform:methanol (1:1) and separated on a Silica G60 TLC plate with chloroform:methanol:acetic acid (65:15:5). The plate was exposed, ceramide identified by co-migration with an identical standard, scraped from the plate and quantified by liquid scintillation counting. The amount of ceramide was determined by comparison with a ceramide standard curve using C₁₆-ceramide (Axxora; Grünberg, Germany).

Measurement of intracellular Ca²⁺

Intracellular Ca²⁺ measurements were performed as described [39]. Briefly, erythrocytes were loaded with Fluo-3/AM (Calbiochem) by addition of 2 μ l of a Fluo-3/AM stock solution (2.0 mM in DMSO) to 1 ml erythrocyte suspension (0.16 % packed cell volume in Ringer). The cells were incubated at 37 °C for 15 min under protection from light. An additional 2 μ l aliquot of Fluo-3/AM was added, with incubation carried out for 25 min. Fluo-3-AM-loaded erythrocytes were centrifuged at 1000 g for 5 min at 22 °C and washed two times with Ringer solution containing 0.5 % bovine serum albumin (Sigma) and one time with Ringer. For flow cytometry, Fluo-3/AM-loaded erythrocytes were re-suspended in 1 ml Ringer (0.16 % packed cell volume) containing paclitaxel (10 μ M) or the Ca²⁺ ionophore ionomycin (1 μ M) or vehicle alone (DMSO) and incubated for different time periods at 37°C. Then, Ca²⁺-dependent fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Cell shrinkage and thus increased concentration of Fluo-3 as a possible cause for enhanced Fluo-3-dependent fluorescence was ruled out by two series of control experiments: (i) osmotic shrinkage of erythrocytes in the presence of 1 mM amiloride to block the cation channel did not significantly alter the Fluo-3-dependent fluorescence and (ii) the ionomycin- and paclitaxel-induced increase of Fluo-3-dependent fluorescence was not blunted in the presence of 125 mM extracellular K⁺, i.e. in the absence of cellular shrinkage (data not shown).

Immunoblotting

Immunoblotting was done as reported previously [23]. Enriched and washed erythrocytes (0.3 % hematocrit) were incubated at 37°C for 24 hours in standard Ringer solution or glucose-free Ringer solution containing either paclitaxel (10 µM) or vehicle as control. Thereafter, erythrocytes were centrifuged, and the cell pellets (100 µl) hypotonically lysed in 50 ml of 20 mM HEPES/NaOH (pH 7.4) containing a cocktail of protease inhibitors composed of 2.5 mM EDTA, 10 µg/ml pepstatin A, 10 µg/ml leupeptin, 5 µg/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) from Roche Diagnostics. Ghost membranes were pelleted (15000 rpm for 20 min at 4°C), re-suspended in 10 mM HEPES/NaOH (pH 7.4) containing the protease inhibitor cocktail and then re-pelleted. In the following, white ghost membranes were solubilized in 125 mM NaCl, 25 mM HEPES/NaOH (pH 7.3), 10 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1% SDS, 0.5% deoxycholic acid, 1% Triton-X-100, 10 µl β-mercaptoethanol. The protein concentration of the samples was determined with the Bradford method (Biorad, München, Germany) with bovine serum albumin (Sigma) as standard. Equal amounts of lysate protein (50 µg per lane) were separated by 6% SDS-PAGE, and proteins were transferred to Protan BA83 nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Protein transfer was controlled by Ponceau red staining. After blocking with 5 % nonfat milk at 4°C overnight, the blots were probed for 1 h at room temperature either with a polyclonal rabbit anti-human µ-calpain (domain IV) antibody (Sigma, affinity isolated antibody C5611) or with a polyclonal rabbit anti-caspase-3 antibody (Biomol, Hamburg, Germany) at a 1:1000 dilution each. After washing, the blots were incubated for 1 h at 21°C with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (Amersham, Freiburg, Germany) at a 1:1000 dilution. Antibody binding was detected with the enhanced chemoluminescence (ECL) kit from Amersham.

Measurement of the half-life of fluorescence-labeled mouse erythrocytes

Fluorescence-labeled mouse erythrocytes were obtained by staining Ringer-treated or ionomycin-treated (1 µM for 1 h) erythrocytes with carboxyfluorescein diacetate, succinimidyl ester (CFSE) from Molecular Probes (Leiden, The Netherlands). To this end, the cells were incubated with labeling solution (50 µM CFSE in PBS) for 10 min at 37°C. The cells were pelleted at 2000 g for 5 min and resuspended in fresh, prewarmed PBS. The fluorescence-labeled erythrocytes were then injected into the tail veins of healthy C57BL/6 mice (female aged 4 months). After 2 hours, blood was drawn from the mice, the mice were

sacrificed and the spleens were dissected and carefully mashed through a net. Finally, CFSE-dependent fluorescence intensity of the erythrocytes in whole blood or in spleen suspensions was measured in the fluorescence channel FL-1 as described above. The percentage of CFSE-positive erythrocytes was calculated in % of the whole population.

Determination of the hematocrit

Erythrocytes (8 % hematocrit) were treated for 48 hours with 10 - 100 µM paclitaxel. After incubation, cell suspensions were transferred to microhematocrit tubes (Brand GmbH, Wertheim, Germany) and one end of the tube was sealed with clay. The filled tube was centrifuged at 12000 g for 5 min in a microhematocrit centrifuge (Hettich, Tuttlingen, Germany) and the hematocrit values were read from a microhematocrit reader.

Cell counts

Hematocrit, erythrocyte, platelet and leukocyte densities were counted using a hemocytometer (Paul Marienfeld GmbH, Lauda-Königshofen, Germany) and standard methods (Central laboratory unit of the University Medical Center Tübingen).

Statistics

Data are expressed as means ± SEM. Statistical analysis was made by paired or unpaired t-test, one sample t-test or ANOVA, as appropriate. $p < 0.05$ was considered as statistically significant.

Results

To explore whether cancer treatment using paclitaxel (Taxol®) leads to anemic complications, blood samples were taken from ovarian and endometrial cancer patients receiving paclitaxel therapy. Cell counts and other blood parameters of the patients immediately before and after treatment with paclitaxel (3 hrs, 175 mg/m²) are provided in table 1. Prior to infusion, the patients were anemic but reticulocyte counts were enhanced. As listed in table 1, the treatment led to a significant further decrease of erythrocyte number (by 6.3 %), packed cell volume (by 7.5 %) and hemoglobin concentration (by 7.5 %). Furthermore, the mean corpuscular volume (MCV) after treatment tended to be reduced, a result, however, not reaching statistical significance. The decrease of mature erythrocyte number was not paralleled by a decrease in reticulocyte count which instead tended to increase. Taken together, these data pointed to the involvement of a process leading to enhanced clearance of circulating erythrocytes.

Additional experiments were performed to elucidate whether the decrease of mature erythrocytes after paclitaxel treatment was the result of phosphatidylserine exposure. As illustrated in Figs. 1A and 1B, paclitaxel

Table 1. Blood count in samples from patients before and after *in vivo* treatment with 175 mg/m² paclitaxel (Taxol®). Values are means ± SEM (n=8). P values of significant differences between the respective values before treatment and after treatment with paclitaxel are given in column 4. means ± SEM, n.s. = not significant.

	Blood parameter		
	before	after	paired t-test
Leukocytes (μl ⁻¹)	6120 ± 682	6536 ± 1092	n.s.
Neutrophils (%)	85 ± 3	89 ± 1	n.s.
Eosinophils (%)	0.5 ± 0.2	0.5 ± 0.3	n.s.
Basophiles (%)	0.20 ± 0.05	0.13 ± 0.03	n.s.
Lymphocytes (%)	10 ± 1	9 ± 1	n.s.
Monocytes (%)	3.8 ± 1.7	1.3 ± 0.3	n.s.
Unclassified (%)	0.9 ± 0.3	0.7 ± 0.2	n.s.
Erythrocytes (10 ⁶ /μl)	3.51 ± 0.16	3.29 ± 0.10	0.033
Hematocrit (%)	33.2 ± 0.8	30.7 ± 0.6	0.008
Hemoglobin (g/dl)	10.63 ± 0.37	9.83 ± 0.27	0.0009
MCH (pg)	29.9 ± 0.8	29.3 ± 0.6	n.s.
MCHC (g/dl)	31.5 ± 0.8	31.5 ± 1.0	n.s.
Hypochrom. Ery (%)	17.9 ± 9.7	11.8 ± 5.6	n.s.
MCV (fl)	95 ± 4	93 ± 4	n.s.
Reticulocytes (%)	1.9 ± 0.3	2.4 ± 0.5	n.s.
HB/reti (g/dl)	33.9 ± 0.8	33.8 ± 0.7	n.s.
Platelets (1000/μl)	326 ± 34	317 ± 37	n.s.

infusion indeed led to a significant ($p < 0.05$) increase of annexin-V binding erythrocytes *in vivo* (from 1.11 ± 0.18 to 1.51 ± 0.35 %; $n = 8$). The forward scatter of annexin-V binding cells was significantly smaller than in the remaining erythrocytes, pointing to a decreased cell volume of annexin-V binding erythrocytes (Fig. 1C). This further indicates that a small part of the erythrocyte population indeed undergoes programmed erythrocyte death after paclitaxel treatment characterized by phosphatidylserine exposure and cellular shrinkage.

In a previous study, peak levels of 6 μM and AUC (area under curve) levels of 19 μM·h after infusion of 175 mg/m² paclitaxel for 3 h were detected in patient's plasma [40]. Thus, *in vitro* experiments using paclitaxel concentrations in the low μmolar range were performed to elucidate the mechanisms leading to enhanced annexin-V binding of erythrocytes following exposure to paclitaxel. In accordance with the *in vivo* data, human erythrocytes from healthy donors showed significantly enhanced annexin-V binding after paclitaxel treatment (10 μM for 48 h) as compared with vehicle-treated control erythrocytes (Table 2). Interestingly, only microtubuli-targeting drugs, i.e. paclitaxel and docetaxel, induced significant erythrocyte annexin-V binding, whereas nucleus-targeting cytostatics, i.e. carboplatin and doxorubicin, at the same concentration were ineffective (Table 2). Paclitaxel-induced annexin-V binding was concentration-dependent with an effective concentration range from 10 to 100 μM (Fig. 2A-C). Enhanced phosphatidylserine exposure in the presence of 10 μM paclitaxel was already observed after 3 hours of

incubation, and the effect reached a plateau after approximately 10 hours (Fig. 2D). We also checked whether paclitaxel might interfere with stress-induced erythrocyte death. Indeed, *in vitro* incubation of erythrocytes from healthy individuals for 6 hours with paclitaxel significantly enhanced annexin-V binding of hyperosmotically stressed (addition of 550 mM sucrose to standard Ringer) erythrocytes (Fig. 2A, B). Thus, exposure to paclitaxel triggers annexin-V binding and sensitizes erythrocytes to the "proeryptotic" effect of hyperosmotic shock.

In another set of experiments, paclitaxel further sensitized the erythrocytes to the "proeryptotic" effects of energy depletion. Glucose depletion significantly increased the percentage of annexin-V binding erythrocytes, an effect significantly enhanced by simultaneous treatment with paclitaxel (Fig. 3A, B).

Decrease of cell volume is one of the hallmarks of programmed erythrocyte death [19]. Indeed, paclitaxel (10, 40 and 100 μM) treatment led within 48 hours to a significant decrease of the hematocrit (by 6.4 ± 2.3 %, $n=5$, 11.2 ± 4.3 %, $n=5$, and 16.4 ± 4.8 %, $n=5$, respectively). Furthermore, FACS analysis revealed that *in vitro* paclitaxel treatment significantly decreased the forward scatter (Fig. 3C), a finding at least partially due to decrease of the erythrocyte volume. Similar to annexin binding, the decrease of forward scatter following paclitaxel-treatment was concentration-dependent (Fig. 3D).

Programmed erythrocyte death is mainly mediated by rapid Ca²⁺- and ceramide-dependent signaling pathways (for review see [41, 42]). Thus, Fluo3-dependent

Fig. 1. Stimulation of erythrocyte phosphatidylserine exposure and cell shrinkage in patients before and after *in vivo* treatment with paclitaxel (Taxol®). **A.** Two-dimensional flow cytometry histograms of forward scatter reflecting the cellular volume (x-axis) and annexin-V binding reflecting phosphatidylserine exposure (y-axis) in a representative experiment of erythrocytes drawn from cancer patients before (*left panel*) and after (*right panel*) treatment with 175 mg/m² paclitaxel. The squares enframe the portion of annexin-V binding cells. **B.** Annexin-V binding of erythrocytes drawn from cancer patients before (closed columns) and after (open columns) treatment with paclitaxel (Means $\pm$ SEM; n=8). * indicates significant difference (p<0.05; one sample t-test and paired t-test) from control (before treatment with paclitaxel). **C.** Forward scatter of erythrocytes drawn from cancer patients before (closed columns) and after (open columns) treatment with paclitaxel. Lower segments of two-dimensional flow cytometry histograms shown in A) are annexin-V negative, and upper segments are annexin-V positive cells. Data are means $\pm$ SEM (n=8). * indicates significant difference (p<0.05; unpaired t-test) from the respective values of annexin-V negative cells (segments).

Table 2. Effect of different cytostatic drugs on erythrocyte phosphatidylserine exposure *in vitro*. Means $\pm$ SEM of annexin-V binding erythrocytes after 48 h of incubation are given in % of the respective controls. P values of significant differences between the respective values in the presence or in the absence (control) of the cytostatic drug are given in column 4. n.s. = not significant. Annexin-V binding of the respective control erythrocytes was ^a4.0 $\pm$ 1.0 % of the total population, ^b2.1 $\pm$ 0.5 % of the total population, ^c2.1 $\pm$ 0.4 % of the total population, ^d6.6 $\pm$ 1.3 % of the total population.

Cytostatic drug	Annexin-V binding cells (% of control)	N	Paired t-test
Paclitaxel (10 μ M)	229 $\pm$ 30 ^a	15	0.01
Docetaxel (10 μ M)	345 $\pm$ 45 ^b	14	0.0001
Carboplatin (10 μ M)	138 $\pm$ 15 ^c	12	n.s.
Doxorubicin (10 μ M)	93 $\pm$ 8 ^d	8	n.s.

fluorescence was applied to determine whether an increase of cytosolic Ca²⁺ activity could participate in the triggering of the erythrocyte membrane scrambling following paclitaxel exposure. As a matter of fact, the exposure to 10 μ M paclitaxel led within 20 min to an increase of the percentage of erythrocytes with enhanced Fluo3 fluorescence (Fig. 4A, B). Similar to paclitaxel, 10 μ M docetaxel significantly increased the percentage of erythrocytes with enhanced intracellular Ca²⁺ concentration (by 312 $\pm$ 99 % (p<0.05; n=4) as compared with vehicle-treated controls).

To investigate ceramide signaling, we measured ceramide generation in paclitaxel treated and control erythrocytes by an anti-ceramide antibody-based flow cytometric assay. As illustrated in Fig. 5A and Fig. 5B, the percentage of ceramide-positive cells was significantly

increased following exposure to 10 μ M paclitaxel. A similar increase of ceramide was observed following exposure to purified bacterial sphingomyelinase which served as a positive control in our assay system (see also Figs. 5A, B). Docetaxel (10 μ M) similarly enhanced the percentage of erythrocytes with elevated intracellular ceramide levels (by 103 $\pm$ 13 %; p<0.05 (n=4) as compared with vehicle-treated controls). The flow cytometric data were confirmed by a biochemical assay using diacylglycerol kinase to quantify cellular ceramide. As shown in Fig. 5C, treatment of erythrocytes with 10 μ M paclitaxel for 24 h led to an approximately two-fold increase of intracellular ceramide. To test whether paclitaxel induces ceramide by *in vivo* activation of the acid sphingomyelinase, we injected 4.5 μ g/g paclitaxel into C57/BL/6 mice and determined the activity of the acid

Fig. 2. Enhanced erythrocyte annexin-V binding following in vitro exposure to hypertonic shock and paclitaxel. A. Histograms of annexin-V binding reflecting phosphatidylserine exposure in a representative experiment of erythrocytes incubated for 6 hours either in isotonic Ringer solution (*left panels*), or in Ringer solution containing 550 mM sucrose (*right panels*) in the absence (*upper panels*) or presence (*lower panels*) of 10 μM paclitaxel. The numbers depict the percentage of annexin-V-positive cells. B. Annexin-V binding of erythrocytes (means ± SEM; n=14-16) after a 6 hour incubation in isotonic Ringer (300 mOsm) and in Ringer solution containing 550 mM sucrose (850 mOsm) in the absence (closed columns) or presence (open columns) of 10 μM paclitaxel. * indicates significant difference (p<0.05; ANOVA) from respective values in the absence of paclitaxel, # indicates significant difference from isotonic Ringer (p<0.05; ANOVA). C. Concentration-dependent effect of paclitaxel on annexin-V binding of erythrocytes (means ± SEM; n=10-26) after 48 hours of incubation in isotonic Ringer. * indicates significant difference (p<0.05; ANOVA) from control erythrocytes (in the absence of paclitaxel). D. Effect of 10 μM paclitaxel on annexin-V binding of erythrocytes (means ± SEM; n=8-16) after 3 - 48 hours of incubation in isotonic Ringer. * indicates significant difference (p<0.05; ANOVA) from the respective control erythrocytes (in the absence of paclitaxel), # indicates significant difference from the respective value after 24 hours of incubation (p<0.05; ANOVA).

sphingomyelinase in whole blood samples as well as in the serum, since the acid sphingomyelinase is also secreted upon activation [43]. The results demonstrate an activation of the acid sphingomyelinase in blood samples and a significant release of the enzyme into the serum already 10 min after injection of paclitaxel (Fig. 5D). Taken together, those data show that taxane-induced

programmed erythrocyte death involves increase of cytosolic Ca^{2+} activity and formation of ceramide.

To explore the role of caspases in paclitaxel-induced eryptosis, we treated erythrocytes for 24 hours with 10 μM paclitaxel and investigated the processing of procaspase-3 by immunoblotting. As shown in Fig. 6A, erythrocytes contained significant amounts of

Fig. 3. Enhanced erythrocyte annexin-V binding following in vitro glucose depletion and paclitaxel exposure and effect of paclitaxel on erythrocyte cell volume. A. Histograms of annexin-V binding reflecting phosphatidylserine exposure in a representative experiment of erythrocytes incubated for 48 hours either in Ringer solution (*left panels*), or in Ringer solution without glucose (*right panels*) in the absence (*upper panels*) or presence (*lower panels*) of 10 μ M paclitaxel. The numbers depict the percentage of annexin-V-positive cells. B. Annexin-V binding of erythrocytes (means $\pm$ SEM; n=10-18) after a 48 hour incubation in glucose containing Ringer (5 mM) and in Ringer solution without glucose (0 mM) in the absence (closed columns) or presence (open columns) of 10 μ M paclitaxel. *indicates significant difference (p<0.05; ANOVA) from respective values in absence of paclitaxel; # indicates significant difference from standard Ringer with 5 mM glucose (p<0.05; ANOVA). C. Histograms of forward scatter reflecting the cellular volume of erythrocytes after 48 hours of incubation in standard Ringer solution (control) or in Ringer solution containing 10 μ M paclitaxel (paclitaxel). Note that the forward scatter of paclitaxel-treated cells is shifted to the left, i.e. to a smaller cell volume. D. Concentration-dependent effect of paclitaxel on the forward scatter of erythrocytes after 48 hours of incubation in standard Ringer solution. Data are means $\pm$ SEM (n=8-18). * indicates significant difference (p<0.05; ANOVA) from control erythrocytes (in the absence of paclitaxel).

in the absence (closed columns) or presence (open columns) of 10 μ M paclitaxel. *indicates significant difference (p<0.05; ANOVA) from respective values in absence of paclitaxel; # indicates significant difference from standard Ringer with 5 mM glucose (p<0.05; ANOVA). C. Histograms of forward scatter reflecting the cellular volume of erythrocytes after 48 hours of incubation in standard Ringer solution (control) or in Ringer solution containing 10 μ M paclitaxel (paclitaxel). Note that the forward scatter of paclitaxel-treated cells is shifted to the left, i.e. to a smaller cell volume. D. Concentration-dependent effect of paclitaxel on the forward scatter of erythrocytes after 48 hours of incubation in standard Ringer solution. Data are means $\pm$ SEM (n=8-18). * indicates significant difference (p<0.05; ANOVA) from control erythrocytes (in the absence of paclitaxel).

procaspase-3. However, processing of the enzyme by limited proteolysis after paclitaxel treatment was not observed, even when the cells were additionally stressed by removal of glucose (Fig. 6A). In contrast, paclitaxel-treated Jurkat T-cells clearly showed activation of caspase-3 as evident from the appearance of the immunoreactive 21, 20 and 17 kDa active subunits of the protease (Fig. 6A, second lane). In another set of experiments, we used the pan-caspase inhibitor zVAD-fmk to functionally link caspase activation to phosphatidylserine exposure. Whereas zVAD-fmk significantly inhibited annexin-V binding in Jurkat T-cells, the same concentration of the inhibitor did not blunt phosphatidylserine exposure in erythrocytes (Fig. 6B), again pointing to caspase-independent activation of

erythrocyte death by paclitaxel.

Calpains are Ca^{2+} -dependent proteases playing a role in erythrocyte senescence [44] and eryptosis [16, 23]. We thus checked for activation of μ -calpain after paclitaxel treatment utilizing Western blot analysis. Paclitaxel (10 μ M for 24 hours) stimulated in erythrocytes the cleavage of the latent calpain protein (p80) into its proteolytic active form (p76) and into another specific cleavage product migrating at about 40 kDa (Fig. 6C). Processing of μ -calpain was also observed in the absence of glucose. Taken together, paclitaxel-induced programmed erythrocyte death does not depend on the activation of caspases but involves μ -calpain.

To explore the physiological significance of the appearance of phosphatidylserine on the surface of

Fig. 4. Increase of erythrocyte free Ca^{2+} concentration following in vitro exposure to paclitaxel. A. Representative two-dimensional flow cytometry histograms showing the forward scatter reflecting the cellular volume (x-axis) and the Fluo3-dependent fluorescence reflecting the intracellular Ca^{2+} concentration (y-axis) of erythrocytes incubated for 20 min. either in Ringer solution (left), in Ringer containing 10 μM paclitaxel (middle), or in Ringer containing 1 μM of the Ca^{2+} ionophore ionomycin (right) as a positive control. The squares enframe the portion of Fluo3-positive cells. B. Mean percentage of erythrocytes $\pm$ SEM ($n=8$) with enhanced Fluo3-dependent fluorescence after incubation for 20 min. in the absence (C) or presence of 10 μM paclitaxel (Pacl.), or in the presence of 1 μM ionomycin (Iono) as a positive control. * indicates significant difference ($p<0.05$, ANOVA) from control erythrocytes (in the absence of paclitaxel or ionomycin).

erythrocytes, we measured the clearance of erythrocytes from peripheral blood in an *in vivo* mouse model system. First, we treated mouse erythrocytes with the Ca^{2+} ionophore ionomycin (1 μM) to trigger phosphatidylserine exposure in $60 \pm 4\%$ ($n=8$) of the erythrocytes. In a second step, we labeled these erythrocytes with carboxy-fluorescein-diacetate-succinimidyl ester (CFSE) leading to nearly complete labeling of the cell population. Third, we injected the erythrocytes intravenously into mice. As shown in Fig. 7, clearance of ionomycin-treated, phosphatidylserine-exposing erythrocytes from peripheral blood was significantly enhanced (by 129 %) as compared with vehicle-treated controls. On the other hand, the cleared, CFSE-labeled cells accumulated in the spleen, a process which was significantly increased (by 77 %) by prior treatment of the erythrocytes with ionomycin (Fig. 7B, right columns).

Discussion

The present experiments disclose a novel action of the cytostatic drugs paclitaxel and docetaxel, i.e. stimulation of erythrocyte membrane scrambling leading to phosphatidylserine exposure at the erythrocyte surface. Following exposure to those cytostatics, a small percentage of erythrocytes increases cytosolic Ca^{2+} activity, shrink, stimulate ceramide formation and expose phosphatidylserine. The effect is specific for drugs known to interfere with microtubuli (for review see [45]) whereas DNA-targeting chemotherapeutics, e. g.

carboplatin and doxorubicin (for review see [46]), were ineffective. The heterogeneity of the response of erythrocytes in a given population to paclitaxel might be attributed to their different age, an effect which has already been described in an earlier study dealing with prostaglandin E_2 -induced erythrocyte death [23]. Furthermore, the present results clearly show that paclitaxel treatment sensitizes the affected erythrocytes for other stressors, namely hyperosmotic shock and glucose removal. Thus, paclitaxel treatment could additionally prime the affected erythrocytes for stress-induced cell death.

The increase of cytosolic Ca^{2+} activity following treatment of erythrocytes with paclitaxel could be due to enhanced entry or impaired extrusion of Ca^{2+} . However, it is interesting to note that a connection between microtubule organization and calcium transport has already been described earlier [47]. Increased cytosolic Ca^{2+} activity, in turn, is expected to stimulate Ca^{2+} sensitive K^+ channels [35]. The activation of those channels is followed by erythrocyte hyperpolarization, which drives Cl^- out of the cell [36, 48]. The loss of KCl with the osmotically obliged water then leads to the observed cell shrinkage [36, 48, 49]. Accordingly, previous studies have shown that ionomycin-induced cell shrinkage and annexin-V binding are inhibited by an increase of extracellular K^+ concentration or by addition of the K^+ channel blockers charybdotoxin or clotrimazol [36]. Along those lines, it is noteworthy that cell shrinkage or loss of cytosolic K^+ are well known triggers of apoptosis in nucleated cells as well [50].

Fig. 5. Induction of ceramide formation in erythrocytes by paclitaxel. **A.** Two-dimensional flow cytometry histograms of the forward scatter reflecting the cellular volume (x-axis) and anti-ceramide FITC-coupled antibody binding reflecting ceramide levels (y-axis) as obtained by FACS analysis in a representative experiment of erythrocytes incubated for 24 hours in Ringer solution (left panel), in Ringer solution + 10 μ M paclitaxel (middle panel), or in Ringer solution + 1 U/ml SMase for 5 min. (right panel) as a positive control. The squares enframe the portion of anti-ceramide-positive cells. **B.** Anti-ceramide FITC-coupled antibody binding (means $\pm$ SEM; $n = 6$) as obtained by FACS analysis of erythrocytes after a 24 hours treatment with Ringer solution in the absence (Co.) or presence of 10 μ M paclitaxel (Pacl.), or after 5 min incubation with 1 U/ml SMase (SMase) as a positive control. * indicates significant difference from control ($p < 0.05$, ANOVA). **C.** Arithmetic means $\pm$ SEM ($n = 3$) of ceramide formation in pmol/ 10^7 cells as obtained by the diacylglycerol kinase assay in erythrocytes incubated for 24 hours in Ringer solution (Control) or in Ringer solution containing 10 μ M paclitaxel (Pacl.). * indicates significant difference from Ringer-treated cells (unpaired t-test, $P < 0.05$). **D.** Paclitaxel activates the acid sphingomyelinase *in vivo*. Paclitaxel (4.5 μ g/g) was intravenously injected into C57/BL/6 mice. Activity of the acid sphingomyelinase was analysed in whole blood samples and serum immediately prior to and 10 min after injection of paclitaxel. Shown are the means $\pm$ SEM. * indicates significant difference from control ($p < 0.05$, ANOVA).

Paclitaxel further activates the acid sphingomyelinase with subsequent formation of ceramide. This observation discloses another novel effect of paclitaxel in erythrocytes, which could well contribute to its toxicity. This cytotoxic mechanism may not only be operative in human erythrocytes but as well in nucleated cells. Indeed, paclitaxel has been shown previously to induce ceramide formation in human cancer cells [51]. Ceramide, in turn, is a well known inducer of apoptosis in a wide variety of nucleated cells [52, 53]. Furthermore, activation of human erythrocyte sphingomyelinase, leading to enhanced ceramide levels, has also been observed following exposure of erythrocytes to hyperosmotic shock [19] or platelet activating factor [37]. In those studies, the Ca^{2+}

sensitizing effect of ceramide on erythrocyte membrane scrambling has been demonstrated.

Enhanced phosphatidylserine exposure is expected to accelerate the clearance of the affected erythrocytes from circulating blood, as they are recognized by phosphatidylserine receptors on macrophages [32], engulfed and degraded [33]. Notably, the reticulocyte number of the patients was enhanced pointing to stimulation of erythrocyte formation. Accordingly, the anemia in those patients should be due to accelerated clearance and not due to impaired formation of erythrocytes. In order to detect differences in the clearance of erythrocytes in the first hours after injection, more than 50 % of the erythrocytes should be annexin-V

Fig. 6. Involvement of calpain but not caspase-3 in paclitaxel-induced programmed erythrocyte death. **A.** Jurkat T-cells were treated for 24 hours either with control medium (C) or with medium containing 10 μ M paclitaxel (Pacl.) as a positive control. In parallel, erythrocytes were treated for 24 hours either with standard Ringer solution (C), with standard Ringer solution containing 10 μ M paclitaxel (Pacl.), with Ringer solution in the absence of glucose (0 Glc.) or with Ringer solution in the absence of glucose containing 10 μ M paclitaxel (Pacl. + 0 Glc.). In the following, 25 μ g (Jurkat T-cells) or 300 μ g (erythrocytes) of the cellular extracts were loaded per lane and activation of caspase-3 was analyzed by Western blotting. Positions of molecular mass markers are indicated at the left. The arrow indicates the position of procaspase-3 (ProC-3) and the stars indicate the position of the 21 kDa (*, p21), the 20 kDa (**, p20) and the 17 kDa (***, p17) active subunit of caspase-3. **B.** Annexin binding of Jurkat T-cells (Jurkat, upper diagram) or erythrocytes (Erythrocytes, lower diagram) after 24 hours of incubation with 10 μ M paclitaxel (Pacl.) either in the absence (- z-VAD-FMK) or in the presence of 25 μ M z-VAD-FMK (+ z-VAD-FMK). Control refers to Jurkat T-cells or erythrocytes exposed for 24 hours to culture medium (C, left diagram) or standard Ringer solution (C, right diagram), respectively. Arithmetic means $\pm$ SEM (n = 4-10) of annexin-positive cells are given in % of the total population. *, indicates significant difference from control (p<0.05, ANOVA). #, indicates significant difference from paclitaxel-treated cells in the absence of z-VAD-FMK (p<0.05, ANOVA). **C.** Immunoblot showing μ -calpain-specific immunoreactive protein bands of erythrocytes incubated for 24 hours in standard Ringer solution in the presence or absence of 10 μ M paclitaxel (Pacl. or C, respectively) or in glucose-free Ringer solution in the presence or absence of 10 μ M paclitaxel (Pacl. + 0 Glc or 0 Glc, respectively). Note that paclitaxel stimulates the proteolytic cleavage of the latent form of calpain (*, p80/78) into its active form (**, p76) and into a cleavage product migrating at approx. 42 kDa (***). Positions of molecular mass markers are indicated at the left.

positive, a value which cannot be reached by paclitaxel treatment *in vitro*. However, the rapid clearance of ionomycin-treated, CFSE-labelled erythrocytes shown in the present study, indeed illustrates the short survival of phosphatidylserine-exposing erythrocytes in circulating blood.

Paclitaxel-induced exposure of phosphatidylserine may further stimulate the adherence of erythrocytes to the vascular wall [33] and may thus contribute to the

impairment of microcirculation [54, 55], especially at the site of infusion. Enhanced adhesiveness of erythrocytes could as well participate in hemostasis [56]. Only recently, altered membrane phospholipid asymmetry has been shown to be involved in the risk for thrombosis in patients with hereditary hydrocytosis [57].

Paclitaxel-induced programmed death of nucleated cells mainly proceeds in a caspase-dependent manner [11, 12] while, with the exception of oxidative stress [24]

Fig. 7. Enhanced clearance from peripheral blood and trapping in the spleen of phosphatidylserine-exposing erythrocytes. A. Histograms of CFSE-dependent fluorescence of erythrocytes in peripheral blood (*upper histograms*) or in spleen suspensions (*lower histograms*) 2 hours after injection of CFSE-labeled, vehicle- (*left histograms*) or ionomycin-treated (*right histograms*) cells. Annexin-binding of vehicle-treated control cells was $6 \pm 1\%$ ($n=8$) and ionomycin-induced annexin-binding reached $60 \pm 4\%$ ($n=8$) of the total population. The numbers depict the percentage of CFSE-positive cells. B. CFSE-positive, vehicle-treated erythrocytes (*closed columns*) or CFSE-positive, ionomycin-treated erythrocytes (*open columns*) in % of the total population (means $\pm$ SEM; $n=8$) in peripheral blood (*left columns*) or in spleen suspensions (*right columns*) 2 hours after injection into wild type mice. * indicates significant difference ($p<0.05$; ANOVA) from the respective values in the absence of ionomycin.

and Fas-dependent erythrocyte death [22], procaspases remain inactive during stress-induced eryptosis [16, 17, 19]. Here, we demonstrate that paclitaxel-induced eryptosis does not involve processing of procaspase-3. Furthermore, caspase inhibition remained without appreciable effect on paclitaxel-induced phosphatidylserine exposure of erythrocytes. Thus, caspase-independence clearly distinguishes paclitaxel-induced eryptosis from apoptosis of nucleated cells after treatment with this anticancer drug.

In conclusion, the anemia following paclitaxel treatment is at least in part secondary to an increase of the erythrocytic Ca^{2+} activity and to a parallel activation of sphingomyelinase with subsequent formation of ceramide. Ceramide then sensitizes the erythrocytes for the stimulating effects of Ca^{2+} on membrane scrambling. Thus, Ca^{2+} and ceramide concert to trigger cell membrane scrambling and lead to phosphatidylserine exposure of affected erythrocytes. The affected erythrocytes are finally cleared from circulating blood leading to anemia. Moreover, phosphatidylserine exposure favors the

adhesion of erythrocytes to the vascular wall and is thus prone to interfere with microcirculation.

Abbreviations

ANOVA (analysis of variance); CFSE (carboxyfluorescein diacetate, succinimidyl ester); DMSO (dimethylsulfoxide); ECL (enhanced chemoluminescence); EDTA (ethylenediaminetetraacetic acid); FACS (fluorescence activated cell sorting); FCS (fetal calf serum); FITC (fluorescein isothiocyanate); fmk (fluoromethyl ketone); Fluo-3/AM (Fluo-3 acetoxymethyl ester); HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid); PBS (phosphate buffered saline); PMSF (phenylmethylsulfonyl fluoride); RPMI (Roswell Park Memorial Institute); SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis); SEM (standard error of the mean); SMase (sphingomyelinase); z (benzyloxycarbonyl).

Acknowledgements

The authors acknowledge the technical assistance of E. Faber and S. Heuser and the meticulous preparation of the manuscript by Lejla Subasic and Tanja Loch. This study was supported by the Deutsche Forschungsgemeinschaft, Nr. La 315/6-1, La 315/13-1, Gu 335/13-1,

Le 792/3-3, by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Center for Interdisciplinary Clinical Research) IZKF 01 KS 9602 and by the Else Übelmesser-Stiftung (University of Tübingen). P.A.L. and D.S.K. have been supported by a stipend from the IZKF (Fö. 01KS9602).

References

- Pietras K, Rubin K, Sjoblom T, Buchdunger E, Sjoquist M, Heldin CH, Ostman A: Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. *Cancer Res* 2002;62:5476-5484.
- Chang JC, Wooten EC, Tsimelzon A, Hilsenbeck SG, Gutierrez MC, Tham YL, Kalidas M, Elledge R, Mohsin S, Osborne CK, Chamness GC, Allred DC, Lewis MT, Wong H, O'Connell P: Patterns of resistance and incomplete response to docetaxel by gene expression profiling in breast cancer patients. *J Clin Oncol* 2005;23:1169-1177.
- McGuire WP, Hoskins WJ, Brady MF, Kucera PR, Partridge EE, Look KY, Clarke-Pearson DL, Davidson M: Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. *N Engl J Med* 1996;334:1-6.
- Ferlini C, Raspaglio G, Mozzetti S, Cicchillitti L, Filippetti F, Gallo D, Fattorusso C, Campiani G, Scambia G: The seco-taxane IDN5390 is able to target class III beta-tubulin and to overcome paclitaxel resistance. *Cancer Res* 2005;65:2397-2405.
- Besson A, Assoian RK, Roberts JM: Regulation of the cytoskeleton: an oncogenic function for CDK inhibitors? *Nat Rev Cancer* 2004;4:948-955.
- Blagosklonny MV, Darzynkiewicz Z, Halicka HD, Pozarowski P, Demidenko ZN, Barry JJ, Kamath KR, Herrmann RA: Paclitaxel induces primary and postmitotic G1 arrest in human arterial smooth muscle cells. *Cell Cycle* 2004;3:1050-1056.
- Li R, Moudgil T, Ross HJ, Hu HM: Apoptosis of non-small-cell lung cancer cell lines after paclitaxel treatment involves the BH3-only proapoptotic protein Bim. *Cell Death Differ* 2005;12:292-303.
- Ofir R, Seidman R, Rabinski T, Krup M, Yavelsky V, Weinstein Y, Wolfson M: Taxol-induced apoptosis in human SKOV3 ovarian and MCF7 breast carcinoma cells is caspase-3 and caspase-9 independent. *Cell Death Differ* 2002;9:636-642.
- Wieder T, Essmann F, Prokop A, Schmelz K, Schulze-Osthoff K, Beyaert R, Dorken B, Daniel PT: Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3. *Blood* 2001;97:1378-1387.
- Cassinelli G, Supino R, Perego P, Polizzi D, Lanzi C, Pratesi G, Zunino F: A role for loss of p53 function in sensitivity of ovarian carcinoma cells to taxanes. *Int J Cancer* 2001;92:738-747.
- Park SJ, Wu CH, Gordon JD, Zhong X, Emami A, Safa AR: Taxol induces caspase-10-dependent apoptosis. *J Biol Chem* 2004;279:51057-51067.
- Von Haefen C, Wieder T, Essmann F, Schulze-Osthoff K, Dorken B, Daniel PT: Paclitaxel-induced apoptosis in BJAB cells proceeds via a death receptor-independent, caspases-3/-8-driven mitochondrial amplification loop. *Oncogene* 2003;22:2236-2247.
- Chen YM, Perng RP, Lee YC, Shih JF, Lee CS, Tsai CM, Whang-Peng J: Paclitaxel plus carboplatin, compared with paclitaxel plus gemcitabine, shows similar efficacy while more cost-effective: a randomized phase II study of combination chemotherapy against inoperable non-small-cell lung cancer previously untreated. *Ann Oncol* 2002;13:108-115.
- Kourousis C, Kakolyris S, Androulakis N, Heras P, Vlachonicolis J, Vamvakas L, Vlata M, Hatzidaki D, Samonis G, Georgoulas V: Salvage chemotherapy with paclitaxel, vinorelbine, and cisplatin (PVC) in anthracycline-resistant advanced breast cancer. *Am J Clin Oncol* 1998;21:226-232.
- Lluch A, Ojeda B, Colomer R, Barnadas A, Massuti B, Casado A, Angeles C, Maroto P: Doxorubicin and paclitaxel in advanced breast carcinoma: importance of prior adjuvant anthracycline therapy. *Cancer* 2000;89:2169-2175.
- Berg CP, Engels IH, Rothbart A, Lauber K, Renz A, Schlosser SF, Schulze-Osthoff K, Wesselborg S: Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell Death Differ* 2001;8:1197-1206.
- Bratosin D, Estaquier J, Petit F, Arnault D, Quatannens B, Tissier JP, Slomianny C, Sartiaux C, Alonso C, Huart JJ, Montreuil J, Ameisen JC: Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. *Cell Death Differ* 2001;8:1143-1156.
- Lang KS, Duranton C, Poehlmann H, Myssina S, Bauer C, Lang F, Wieder T, Huber SM: Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ* 2003;10:249-256.
- Lang KS, Myssina S, Brand V, Sandu C, Lang PA, Berchtold S, Huber SM, Lang F, Wieder T: Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes. *Cell Death Differ* 2004;11:231-243.
- Head DJ, Lee ZE, Swallah MM, Avent ND: Ligation of CD47 mediates phosphatidylserine expression on erythrocytes and a concomitant loss of viability in vitro. *Br J Haematol* 2005;130:788-790.
- Head DJ, Lee ZE, Poole J, Avent ND: Expression of phosphatidylserine (PS) on wild-type and Gerbich variant erythrocytes following glycophorin-C (GPC) ligation. *Br J Haematol* 2005;129:130-137.
- Mandal D, Mazumder A, Das P, Kundu M, Basu J: FAS-, caspase 8- and caspase 3-dependent signaling regulate the activity of the aminophospholipid translocase and phosphatidylserine externalization in human erythrocytes. *J Biol Chem* 2005;280:39460-7.

- 23 Lang PA, Kempe DS, Myssina S, Tanneur V, Birka C, Laufer S, Lang F, Wieder T, Huber SM: PGE(2) in the regulation of programmed erythrocyte death. *Cell Death Differ* 2005;12:415-428.
- 24 Mandal D, Moitra PK, Saha S, Basu J: Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes. *FEBS Lett* 2002;513:184-188.
- 25 Barvitenko NN, Adragna NC, Weber RE: Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell Physiol Biochem* 2005;15:1-18.
- 26 Bosman GJ, Willekens FL, Werre JM: Erythrocyte aging: a more than superficial resemblance to apoptosis? *Cell Physiol Biochem* 2005;16:1-8.
- 27 Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM, Lang F: Mechanisms of suicidal erythrocyte death. *Cell Physiol Biochem* 2005;15:195-202.
- 28 Rice L, Alfrey CP: The negative regulation of red cell mass by neocytolysis: physiologic and pathophysiologic manifestations. *Cell Physiol Biochem* 2005;15:245-250.
- 29 Schwarzer E, Kühn H, Valente E, Arese P: Band 3/COMPLEMENT-mediated Recognition and Removal of Normally Senescent and Pathological Human Erythrocytes. *Cell Physiol Biochem* 2005;16:133-146.
- 30 de Jong K, Rettig MP, Low PS, Kuypers FA: Protein kinase C activation induces phosphatidylserine exposure on red blood cells. *Biochemistry* 2002;41:12562-12567.
- 31 Woon LA, Holland JW, Kable EP, Roufogalis BD: Ca²⁺ sensitivity of phospholipid scrambling in human red cell ghosts. *Cell Calcium* 1999;25:313-320.
- 32 Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM: A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 2000;405:85-90.
- 33 Eda S, Sherman IW: Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine. *Cell Physiol Biochem* 2002;12:373-384.
- 34 Lang KS, Roll B, Myssina S, Schittenhelm M, Scheel-Walter HG, Kanz L, Fritz J, Lang F, Huber SM, Wieder T: Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. *Cell Physiol Biochem* 2002;12:365-372.
- 35 Brugnara C, de Franceschi L, Alper SL: Inhibition of Ca(2+)-dependent K⁺ transport and cell dehydration in sickle erythrocytes by clotrimazole and other imidazole derivatives. *J Clin Invest* 1993;92:520-526.
- 36 Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca²⁺-activated K⁺ channels in human erythrocyte apoptosis. *Am J Physiol Cell Physiol* 2003;285:C1553-C1560.
- 37 Lang PA, Kempe DS, Tanneur V, Eisele K, Klarl BA, Myssina S, Jendrosseck V, Ishii S, Shimizu T, Waidmann M, Hessler G, Huber SM, Lang F, Wieder T: Stimulation of erythrocyte ceramide formation by platelet-activating factor. *J Cell Sci* 2005;118:1233-1243.
- 38 Andree HA, Reutelingsperger CP, Hauptmann R, Hemker HC, Hermens WT, Willems GM: Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *J Biol Chem* 1990;265:4923-4928.
- 39 Andrews DA, Yang L, Low PS: Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human red blood cells. *Blood* 2002;100:3392-3399.
- 40 Gianni L, Kearns CM, Giani A, Capri G, Vigano L, Lacatelli A, Bonadonna G, Egorin MJ: Nonlinear pharmacokinetics and metabolism of paclitaxel and its pharmacokinetic/pharmacodynamic relationships in humans. *J Clin Oncol* 1995;13:180-190.
- 41 Lang F, Lang KS, Wieder T, Myssina S, Birka C, Lang PA, Kaiser S, Kempe D, Duranton C, Huber SM: Cation channels, cell volume and the death of an erythrocyte. *Pflugers Arch* 2003;447:121-125.
- 42 Lang F, Lang PA, Lang KS, Brand V, Tanneur V, Duranton C, Wieder T, Huber SM: Channel-induced apoptosis of infected host cells-the case of malaria. *Pflugers Arch* 2004;448:319-324.
- 43 Schissel SL, Schuchman EH, Williams KJ, Tabas I: Zn²⁺-stimulated sphingomyelinase is secreted by many cell types and is a product of the acid sphingomyelinase gene. *J Biol Chem* 1996;271:18431-18436.
- 44 Glaser T, Schwarz-Benmeir N, Barnoy S, Barak S, Eshhar Z, Kosower NS: Calpain (Ca(2+)-dependent thiol protease) in erythrocytes of young and old individuals. *Proc Natl Acad Sci U S A* 1994;91:7879-7883.
- 45 Blagosklonny MV: Analysis of FDA approved anticancer drugs reveals the future of cancer therapy. *Cell Cycle* 2004;3:1035-1042.
- 46 Yang XL, Wang AH: Structural studies of atom-specific anticancer drugs acting on DNA. *Pharmacol Ther* 1999;83:181-215.
- 47 de Pennart H, Cibert C, Petzelt C, Maro B: Microtubule tracks can be detected in mouse oocytes with an antibody directed against a calcium transporter. *J Cell Sci* 1994;107 (Pt 7):1899-1908.
- 48 Myssina S, Lang PA, Kempe DS, Kaiser S, Huber SM, Wieder T, Lang F: Cl-channel blockers NPPB and niflumic acid blunt Ca(2+)-induced erythrocyte 'apoptosis'. *Cell Physiol Biochem* 2004;14:241-248.
- 49 Kucherenko Y, Browning J, Tattersall A, Ellory JC, Gibson JS: Effect of peroxynitrite on passive K⁺ transport in human red blood cells. *Cell Physiol Biochem* 2005;15:271-280.
- 50 Bortner CD, Hughes FM, Jr., Cidlowski JA: A primary role for K⁺ and Na⁺ efflux in the activation of apoptosis. *J Biol Chem* 1997;272:32436-32442.
- 51 Charles AG, Han TY, Liu YY, Hansen N, Giuliano AE, Cabot MC: Taxol-induced ceramide generation and apoptosis in human breast cancer cells. *Cancer Chemother Pharmacol* 2001;47:444-450.
- 52 Gulbins E, Dreschers S, Wilker B, Grassme H: Ceramide, membrane rafts and infections. *J Mol Med* 2004;82:357-363.
- 53 Kolesnick RN, Kronke M: Regulation of ceramide production and apoptosis. *Annu Rev Physiol* 1998;60:643-665.
- 54 Pawloski JR, Hess DT, Stamler JS: Export by red blood cells of nitric oxide bioactivity. *Nature* 2001;409:622-626.
- 55 Sprague RS, Stephenson AH, Dimmitt RA, Weintraub NL, Branch CA, McMurdo L, Lonigro AJ, Weintraub NA: Effect of L-NAME on pressure-flow relationships in isolated rabbit lungs: role of red blood cells. *Am J Physiol* 1995;269:H1941-H1948.
- 56 Andrews DA, Low PS: Role of red blood cells in thrombosis. *Curr Opin Hematol* 1999;6:76-82.
- 57 Gallagher PG, Chang SH, Rettig MP, Neely JE, Hillery CA, Smith BD, Low PS: Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis. *Blood* 2003;101:4625-4627.