Curcumin Induced Suicidal Erythrocyte Death

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Key Words

Annexin V • Apoptosis • Ceramide • Calcium • Channels

Abstract

The natural nutrient component Curcumin with anti-inflammatory and antitumor activity has previously been shown to stimulate apoptosis of several nucleated cell types. The present study has been performed to explore whether Curcumin could similarly induce suicidal death of erythrocytes or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface. Phosphatidylserine exposing cells are phagocytosed and thus rapidly cleared from circulating blood. Erythrocyte membrane scrambling may be triggered by increase of cytosolic Ca^{2+} activity or formation of ceramide. To test for eryptosis, erythrocyte phosphatidylserine exposure has been estimated from annexin V binding, and erythrocyte volume from forward scatter in FACS analysis. Exposure of erythrocytes to Curcumin ($= 1\mu\text{M}$) increased annexin V binding and decreased forward scatter, pointing to phosphatidylserine exposure at the cell

surface and cell shrinkage. According to Fluo3 fluorescence Curcumin increased cytosolic Ca^{2+} activity and according to immunofluorescence Curcumin increased ceramide formation. As shown previously, hypertonic shock (addition of 550mM sucrose), chloride removal and glucose depletion decreased the forward scatter and increased annexin V binding. The effects on annexin binding were enhanced in the presence of Curcumin. Exposure to Curcumin did, however, not significantly enhance the shrinking effect of hypertonic shock or Cl^- removal and reversed the shrinking effect of glucose withdrawal. The present observations disclose a proeryptotic effect of Curcumin which may affect the life span of circulating erythrocytes.

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Introduction

The anti-inflammatory and antitumor nutrient component Curcumin has been shown to trigger apoptosis in a wide variety of cells [1-11]. Curcumin is effective through a variety of mechanisms including influence on chromosomal organisation [12], telomerase [13], protein expression [14-16], phosphatidylinositol 3 kinase pathway

[17], mammalian target of rapamycin [18], eicosanoid formation [19] and ceramide production [20]. On the other hand, Curcumin may exert an antioxidative and antiapoptotic action [21, 22].

Controversial evidence has been reported about effects of Curcumin on cystic fibrosis conductance regulator CFTR [23]. While some studies reported that trafficking of intact or defective CFTR to the cell membrane is enhanced by Curcumin [24-28], others failed to observe altered CFTR trafficking in the presence of Curcumin [29-32]. Erythrocytes express CFTR [33-35], which is apparently involved in ATP release [36-38].

In erythrocytes Curcumin has been shown to trigger translocation of plasma membrane phospholipids and exposure of phosphatidylserine at the erythrocyte surface, effects attributed to changes of cell membrane lipid fluidity [39]. Phosphatidylserine exposure is a key event not only in apoptosis of nucleated cells [40-42] but as well part of eryptosis, the suicidal machinery of erythrocytes [43].

Eryptosis may follow activation of Ca^{2+} -permeable erythrocyte cation channels [44-46]. The subsequent entry of Ca^{2+} results in activation of Ca^{2+} -sensitive K^{+} channels, KCl exit, osmotic loss of water and cell shrinkage [47, 48]. Moreover, Ca^{2+} triggers Ca^{2+} -sensitive scrambling of the cell membrane [49] with phosphatidylserine exposure at the cell surface [45]. Cell membrane scrambling is further triggered by ceramide (acylsphingosine) [50]. Since phosphatidylserine exposing erythrocytes are rapidly engulfed and degraded by macrophages [51], they are rapidly eliminated from circulating blood [52]. Thus, similar to erythrocyte senescence [53-55] and neocytolysis [56], eryptosis leads to suicidal erythrocyte death. A number of substances or conditions have previously been shown to trigger eryptosis [43] including PGE2 [57], platelet activating factor [58], paclitaxel [59], chlorpromazine [60], mercury [61], lead [62], hemolysin [63], phosphate depletion [64], iron deficiency [52], Hemolytic Uremic Syndrome [65] malaria infection [66] and several inherited erythrocyte disorders [67]. Eryptosis is inhibited by erythropoietin [68], urea [69], catecholamines and distinct channel inhibitors [44, 48].

The present experiments have been performed to determine the effects of Curcumin on cytosolic Ca^{2+} activity, ceramide formation, cell volume and phosphatidylserine exposure in erythrocytes. The results reveal that Curcumin triggers eryptosis at least in part by increasing cytosolic Ca^{2+} activity and by triggering the generation of ceramide.

Materials and Methods

Erythrocytes, solutions and chemicals

Experiments were performed at 37°C with isolated erythrocytes drawn from healthy volunteers. The volunteers provided informed consent. The study has been approved by the ethics committee of the University of Tübingen (184/2003V).

Ringer solution contained (in mM): 125NaCl, 5KCl, 1MgSO₄, 32N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1CaCl₂; pH 7.4. Where indicated, glucose was omitted from the Ringer solution and replaced by NaCl (2.5mM), chloride was replaced by gluconate or osmolarity increased by addition of 550mM sucrose.

Curcumin was used at concentrations ranging from 0.1 to 10μM, ionomycin at a concentration of 1μM. Where applicable, the final concentration of the solvent dimethyl sulfoxide (DMSO) was 0.1%. Ionomycin and Curcumin were purchased from Sigma (Taufkirchen, Germany), the Ca^{2+} dye Fluo-3/AM from Calbiochem (Bad Soden, Germany).

FACS analysis of annexin V binding and forward scatter

FACS analysis was performed as described [70]. After incubation in the presence or absence of Curcumin, cells were washed in annexin V binding buffer containing (in mM): 125NaCl, 10HEPES/NaOH (pH 7.4), and 5CaCl₂. Erythrocytes were suspended in a solution composed of Annexin-V-Fluos (Roche Diagnostics, Mannheim, Germany) and annexin V buffer (dilution of 1:50). After 10min. 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 488nm and an emission wavelength of 530nm.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} measurements were performed as described previously [57]. Erythrocytes were loaded with Fluo-3/AM (Calbiochem) by addition of 2μl of a Fluo-3/AM stock solution (2.0mM in DMSO) to 1ml erythrocyte suspension (0.16% hematocrit in Ringer). Cells were incubated at 37°C for 15min under protection from light. Subsequently, an additional 2μl aliquot of Fluo-3/AM stock solution was added, and the cells were incubated for 25min. Fluo-3-AM-loaded erythrocytes were centrifuged at 1000g for 5min at 22°C and washed two times with Ringer solution containing 0.5% bovine serum albumin (Sigma) and one time with Ringer. Fluo-3/AM-loaded erythrocytes were re-suspended in 0.5 ml Ringer solution (0.16% hematocrit) containing different concentrations of Curcumin, and incubated for different time periods at 37°C. Then, Ca^{2+} -dependent fluorescence intensity was measured by FACS analysis in the fluorescence channel FL-1 with an excitation wavelength of 488nm and an emission wavelength of 530nm. As a positive control for enhanced Ca^{2+} activity, Fluo-3-labelled

Fig. 1. Stimulation of phosphatidylserine exposure by Curcumin. **A.** Histograms of annexin V binding in a representative experiment of erythrocytes from healthy volunteers exposed for 24h to Ringer solution without (left panel) or with (right panel) 10 μ M Curcumin. **B.** Dose dependence of the Curcumin effect on annexin V binding and hemolysis. Arithmetic means $\pm$ SEM (n=4) of the percentage annexin V binding erythrocytes (black bars) exposed for 24h to Ringer solution without (Ringer) or with Curcumin at concentrations ranging from 0.1 to 10 μ M. For comparison, the haemoglobin release (reflecting hemolysis) is shown in % of maximal haemoglobin release (after exposure to distilled water). * indicates significant difference from control values (ANOVA; $P < 0.05$). **C.** Time dependence of the Curcumin effect on phosphatidylserine exposure. Arithmetic means $\pm$ SEM (n=4) of the percentage annexin V binding erythrocytes exposed for 0.5 to 24h to Ringer solution without (Ringer) or with Curcumin at concentrations ranging from 0.1 to 10 μ M. * indicates significant difference from control values (ANOVA; $P < 0.05$).

erythrocytes were incubated in Ringer containing 1 μ M of ionomycin.

Determination of ceramide formation

For determination of ceramide, cells were stained for 1h at 4°C with an anti-ceramide antibody (Alexis corporation, Lausen, Switzerland) or isotype matched pure mouse antibody in phosphate buffered saline (PBS) containing 1% fetal calf serum (FCS) at a dilution of 1:5 as described recently [50]. After incubation of the cells with different concentrations of Curcumin, cells were washed three times with PBS/1% FCS and then stained with a 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 30min. 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.

Determination of hemolysis

In order to control for hemolysis, cells were suspended (0.16% hematocrit) and incubated for 24h in Ringer's solution containing different concentrations of curcumin. After incubation, hemoglobin concentration in the supernatant was determined by photometric measurement (Tecan Elisa Reader, at 405nm). The respective values are expressed in percentage of haemoglobin concentration following complete lysis of the cells by exposure to pure water (set as 100% hemolysis). The absorption of Curcumin has been determined concurrently and was subtracted from the absorption of the supernatant.

Statistics

Data are expressed as arithmetic means $\pm$ SEM and statistical analysis was made by paired or unpaired t-test or ANOVA, as appropriate.

Results

Annexin V binding has been utilized to identify phosphatidylserine exposing erythrocytes. The percentage of annexin V exposing erythrocytes was in average $2.53 \pm 0.37\%$ (n=4) following exposure to drug-free Ringer solution. The addition of Curcumin dose-dependently increased the percentage of annexin V binding cells (Fig. 1A,B). The effect reached statistical significance at 1µM Curcumin. At 10µM Curcumin led to significant annexin binding within 30min (Fig. 1C).

Alterations of cell volume were estimated by determination of the forward scatter in FACS analysis. Curcumin exposure was followed by a dose dependent decrease of the forward scatter pointing to erythrocyte shrinkage (Fig. 2A,B). Following exposure to 10µM Curcumin a significant decrease of forward scatter was observed within 30min (Fig. 2C).

Fluo3-dependent fluorescence was employed to explore whether Curcumin influences cytosolic Ca^{2+} activity. As illustrated in Fig. 3, Curcumin increased significantly the cytosolic free Ca^{2+} concentration in erythrocytes. For comparison, the effect of the Ca^{2+} ionophore ionomycin (1µM) on the Fluo3-dependent fluorescence was tested. The increase of the cytosolic Ca^{2+} concentration could contribute to or even account for the stimulation of phosphatidylserine exposure and cell shrinkage following Curcumin treatment.

According to immunofluorescence Curcumin further significantly increased ceramide formation (Fig. 4). Thus, in addition to increased cytosolic Ca^{2+} activity, ceramide could contribute to the stimulation of cell membrane scrambling.

Further experiments have been performed to explore whether Curcumin modified the well known eryptotic effect of hypertonic cell shrinkage. As displayed

Fig. 3. Effects of Curcumin on cytosolic Ca^{2+} activity. Arithmetic means $\pm$ SEM ($n=4$) of Fluo3-dependent fluorescence in erythrocytes incubated for 60min (white bars), 180min (grey bars) or 360min (black bars) in Ringer solution without or with Curcumin at concentrations from 0.1 to 10 μM , or with 1 μM ionomycin (Iono) as a positive control. *indicates significant difference from the respective Ringer-treated controls (ANOVA; $P<0.05$).

Fig. 4. Effects of Curcumin on ceramide formation. Arithmetic means $\pm$ SEM ($n=4$) of anti-ceramide-FITC fluorescence (reflecting ceramide formation) in erythrocytes incubated for 24h in Ringer solution without Curcumin (Ringer), or with Curcumin (0.1 μM , to 10 μM). *indicates significant difference from Ringer-treated controls (ANOVA; $P<0.05$).

in Fig. 5, hyperosmotic shock significantly increased the percentage of annexin V binding erythrocytes. The effect of hyperosmotic shock on annexin V binding was significantly augmented by addition of Curcumin. The exposure of erythrocytes to hyperosmotic solutions was further followed by the expected decrease of forward scatter pointing to cell shrinkage (Fig. 6). In hypertonic medium, the forward scatter was similar in the presence and absence of Curcumin. Thus, in hypertonic medium Curcumin did not elicit further profound cell shrinkage.

Similar to hyperosmolarity, Cl^- removal leads to cell shrinkage and eryptosis. Thus, additional experiments have been performed to explore whether Curcumin modified the effects of Cl^- removal. As shown in Fig. 7, Cl^- removal significantly increased the percentage of annexin V binding erythrocytes, an effect significantly enhanced by addition of Curcumin. Cl^- removal further decreased forward scatter, again pointing to cell shrinkage. Similar to the observations following hypertonic exposure, the forward scatter following Cl^- removal was not further decreased by Curcumin. Thus, in the absence of Cl^- , Curcumin did not further shrink the erythrocytes. Instead, in the absence of Cl^- , higher concentrations of Curcumin led to a slight but significant increase of forward scatter.

Eryptosis is further triggered by glucose removal from the medium. Thus, experiments were performed to explore, whether Curcumin modifies the eryptosis during glucose depletion. As expected, glucose removal again markedly increased the percentage of annexin V binding erythrocytes (Fig. 9) and decreased the forward scatter (Fig. 10). The increase of annexin binding of glucose depleted erythrocytes was further increased by Curcumin. In contrast, Curcumin blunted the effect of glucose depletion on forward scatter. Thus, at 10 μM Curcumin, the forward scatter was similar in glucose replete and glucose depleted erythrocytes.

Discussion

The present experiments confirm the previous observation [39] that Curcumin triggers cell membrane lipid scrambling with phosphatidylserine exposure at the cell surface. They extend those observations by the demonstration that Curcumin leads to cell shrinkage, another hallmark of eryptosis [43]. Moreover, the experiments reveal that Curcumin stimulates Ca^{2+} entry and triggers ceramide formation. The concentrations

Fig. 5. Effects on annexin V binding of hyperosmotic shock in the presence or absence of Curcumin. A. Histograms of annexin V binding in a representative experiment of erythrocytes exposed for 6 hours to either isotonic (290mOsm) Ringer solution (upper panels) or hypertonic (850mOsm) Ringer solution (lower panels) in the absence (left panels) or in the presence (right panels) of 1 μ M Curcumin. B. Arithmetic means $\pm$ SEM (n=4) of the percentage annexin V binding erythrocytes following incubation for 6h in isotonic (290mOsm; white bars) or hypertonic (850mOsm; black bars) Ringer solution either in the absence or presence of Curcumin at concentrations from 0.1 to 10 μ M. * indicates significant difference from isotonic Ringer solution (ANOVA; $P < 0.05$), # indicates significant difference from the respective control values in the absence of Curcumin (ANOVA; $P < 0.05$).

needed for a significant increase of cytosolic Ca^{2+} activity were larger than the concentrations needed to trigger significant annexin V binding. It should be kept in mind, however, that due to loss of dye, the Ca^{2+} measurements could be performed only up to 3h incubation, while the annexin V binding has been determined following 24h exposure to curcumin. Following an extended exposure

Fig. 6. Effects on forward scatter of hyperosmotic shock in the presence or absence of Curcumin. A. Histograms of forward scatter in a representative experiment of erythrocytes exposed for 6h to either isotonic (290mOsm) Ringer solution (upper panels) or hypertonic (850mOsm) Ringer solution (lower panels) in the absence (left panels) or in the presence (right panels) of 1 μ M Curcumin. B. Arithmetic means $\pm$ SEM (n=4) of forward scatter of erythrocytes incubated for 6h in isotonic (290mOsm; white bars) or hypertonic (850mOsm; black bars) Ringer solution either in the absence or presence of Curcumin at concentrations from 0.1 to 10 μ M. * indicates significant difference from isotonic Ringer solution (ANOVA; $P < 0.05$), # indicates significant difference from the respective control values in the absence of Curcumin (ANOVA; $P < 0.05$).

lower curcumin concentrations are presumably needed to increased Ca^{2+} activity.

Ca^{2+} is expected to activate Ca^{2+} -sensitive K^{+} channels. The subsequent exit of K^{+} should hyperpolarize the cell, which should in turn drive Cl^{-} out of the cell. The exit of K^{+} , Cl^{-} and osmotically obliged water should lead to cell shrinkage and thus to a decrease of forward scatter

Fig. 7. Effects on annexin V binding of Cl^- removal in the presence or absence of Curcumin. A. Histograms of annexin V binding in a representative experiment of erythrocytes exposed 24h to either Ringer solution (upper panels) or to Cl^- -free solution (lower panels) either in the absence (left panels) or presence (right panels) of $1\mu\text{M}$ Curcumin. B. Arithmetic means $\pm$ SEM ($n=4$) of annexin V binding in % of the total population of erythrocytes incubated for 24h in chloride containing (white bars) or chloride free (black bars) solution either in the absence or presence of Curcumin at concentrations from 0.1 to $10\mu\text{M}$. *indicates significant difference from glucose containing Ringer solution (ANOVA; $P<0.05$), #indicates significant difference from the respective control values in the absence of Curcumin (ANOVA; $P<0.05$).

Fig. 8. Effects on forward scatter of chloride removal in the presence or absence of Curcumin. A. Histograms of forward scatter in a representative experiment of erythrocytes exposed 24h to either Ringer solution (upper panels) or chloride-free solution (lower panels) either in the absence (left panels) or presence (right panels) of $1\mu\text{M}$ Curcumin. B. Arithmetic means $\pm$ SEM ($n=4$) of erythrocyte forward scatter following incubation for 24h in chloride containing (white bars) or chloride free (black bars) solution either in the absence or presence of Curcumin at concentrations from 0.1 to $10\mu\text{M}$. *indicates significant difference from glucose containing Ringer solution (ANOVA; $P<0.05$), # indicates significant difference from the respective control values in the absence of Curcumin (ANOVA; $P<0.05$).

[47, 48]. Increase of cytosolic Ca^{2+} activity is further expected to trigger lipid scrambling of the cell membrane leading to phosphatidylserine exposure at the cell surface [45]. Additional Ca^{2+} sensitive functions include modification of the cytoskeleton [71, 72] and activation of a wide variety of Ca^{2+} sensitive enzymes such as transglutaminase [73], phospholipases [74], calpain [73],

protein kinases and phosphatases [75, 76]. The degradation of membrane proteins by calpain fosters cell membrane blebbing, another hallmark of eryptosis [77, 78].

Ceramide formation presumably contributes to the triggering of phosphatidylserine exposure following Curcumin treatment, as ceramide has previously been

Fig. 9. Effects on annexin V binding of glucose removal in the presence or absence of Curcumin. A. Histograms of annexin V binding in a representative experiment of erythrocytes exposed 24h to either Ringer solution (upper panels) or to glucose free solution (lower panels) either in the absence (left panels) or presence (right panels) of 1μM Curcumin. B. Arithmetic means $\pm$ SEM (n=4) of annexin V binding in % of the total population of erythrocytes incubated for 24h in glucose containing (white bars) or glucose free (black bars) solution either in the absence or presence of Curcumin at concentrations from 0.1 to 10μM. *indicates significant difference from glucose containing Ringer solution (ANOVA; $P < 0.05$), #indicates significant difference from the respective control values in the absence of Curcumin (ANOVA; $P < 0.05$).

Fig. 10. Effects on forward scatter of glucose removal in the presence or absence of Curcumin. A. Histograms of forward scatter in a representative experiment of erythrocytes exposed 48h to either Ringer solution (upper panels) or glucose-free solution (lower panels) either in the absence (left panels) or presence (right panels) of 1μM Curcumin. B. Arithmetic means $\pm$ SEM (n=4) of erythrocyte forward scatter following incubation for 24h in glucose containing (white bars) or glucose free (black bars) solution either in the absence or presence of Curcumin at concentrations from 0.1 to 10μM. *indicates significant difference from glucose containing Ringer solution (ANOVA; $P < 0.05$), #indicates significant difference from the respective control values in the absence of Curcumin (ANOVA; $P < 0.05$).

shown to sensitize the cells to the scrambling effects of Ca^{2+} [50]. A stimulating effect of Curcumin on ceramide formation has similarly been observed in nucleated cells [20].

In view of a putative stimulating effect of Curcumin on CFTR [24] and the role of CFTR in ATP release [36-38], Curcumin could trigger cellular release of ATP leading to cellular ATP depletion on the one side and autocrine

purinergic stimulation on the other. Our observations do not allow postulating or excluding the contribution of this potential mechanism to Curcumin induced eryptosis.

Nevertheless, Curcumin triggers cell membrane scrambling not only by direct influence on lipid fluidity but initiates several cellular mechanisms known to cause eryptosis.

Interestingly, Curcumin reverses the decrease of forward scatter during glucose depletion. The present data do not allow any safe conclusion as to the mechanism underlying this paradox effect of Curcumin on forward scatter. It is tempting to speculate, however, that glucose depletion may lead to impairment of the Na⁺/K⁺ ATPase, which is required for the maintenance of the K⁺ and Na⁺ gradients across the cell membrane. In theory, Curcumin could favour the entry not only of Ca²⁺ but as well of Na⁺. At adequate energy supply the Na⁺ would be eliminated by the Na⁺/K⁺ ATPase and the stimulating effect of Ca²⁺ on K⁺ channels would lead to the expected KCl loss and cell shrinkage. In the absence of glucose the impairment of the Na⁺/K⁺ ATPase would preclude the elimination of Na⁺ and thus blunt the exit of K⁺ due to dissipation of gradients. However, presently there is no experimental support for this putative mechanism.

The exposure of phosphatidylserine at the cell surface is one of the signals triggering the phagocytotic removal of apoptotic or eryptotic cells, as phagocytes are

equipped with phosphatidylserine receptors [79], which mediate engulfment and intracellular degradation of the defective cells [51]. Thus, phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood [52]. Hence, at least in theory, Curcumin may decrease the erythrocyte life span and thus favour the development of anemia.

In conclusion, Curcumin does not only induce apoptosis of nucleated cells but similarly triggers suicidal death of erythrocytes. Mechanisms involved in Curcumin induced erythrocyte death include stimulation of Ca²⁺ entry and formation of ceramide. Those mechanisms may similarly participate in the effects of Curcumin on nucleated cells.

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References

- 1 Belkaid A, Copland IB, Massillon D, Annabi B: Silencing of the human microsomal glucose-6-phosphate translocase induces glioma cell death: potential new anticancer target for curcumin. *FEBS Lett* 2006;580:3746-3752.
- 2 Chan WH, Wu HY, Chang WH: Dosage effects of curcumin on cell death types in a human osteoblast cell line. *Food Chem Toxicol* 2006;44:1362-1371.
- 3 Chen HW, Huang HC: Effect of curcumin on cell cycle progression and apoptosis in vascular smooth muscle cells. *Br J Pharmacol* 1998;124:1029-1040.
- 4 Collett GP, Campbell FC: Curcumin induces c-jun N-terminal kinase-dependent apoptosis in HCT116 human colon cancer cells. *Carcinogenesis* 2004;25:2183-2189.
- 5 Jee SH, Shen SC, Tseng CR, Chiu HC, Kuo ML: Curcumin induces a p53-dependent apoptosis in human basal cell carcinoma cells. *J Invest Dermatol* 1998;111:656-661.
- 6 Jiang MC, Yang-Yen HF, Yen JJ, Lin JK: Curcumin induces apoptosis in immortalized NIH 3T3 and malignant cancer cell lines. *Nutr Cancer* 1996;26:111-120.
- 7 Kuo ML, Huang TS, Lin JK: Curcumin, an antioxidant and anti-tumor promoter, induces apoptosis in human leukemia cells. *Biochim Biophys Acta* 1996;1317:95-100.
- 8 Nogaki A, Satoh K, Iwasaka K, Takano H, Takahama M, Ida Y, Sakagami H: Radical intensity and cytotoxic activity of curcumin and gallic acid. *Anticancer Res* 1998;18:3487-3491.
- 9 Tong QS, Zheng LD, Lu P, Jiang FC, Chen FM, Zeng FQ, Wang L, Dong JH: Apoptosis-inducing effects of curcumin derivatives in human bladder cancer cells. *Anticancer Drugs* 2006;17:279-287.

- 10 Wang Z, Zhang Y, Banerjee S, Li Y, Sarkar FH: Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells. *Cancer* 2006;106:2503-2513.
- 11 Zheng LD, Tong QS, Wu CH: Growth inhibition and apoptosis inducing mechanisms of curcumin on human ovarian cancer cell line a2780. *Chin J Integr Med* 2006;12:126-131.
- 12 Wolanin K, Magalska A, Mosieniak G, Klinger R, McKenna S, Vejda S, Sikora E, Piwocka K: Curcumin affects components of the chromosomal passenger complex and induces mitotic catastrophe in apoptosis-resistant Bcr-Abl-expressing cells. *Mol Cancer Res* 2006;4:457-469.
- 13 Cui SX, Qu XJ, Xie YY, Zhou L, Nakata M, Makuuchi M, Tang W: Curcumin inhibits telomerase activity in human cancer cell lines. *Int J Mol Med* 2006;18:227-231.
- 14 Divya CS, Pillai MR: Antitumor action of curcumin in human papillomavirus associated cells involves downregulation of viral oncogenes, prevention of NFkB and AP-1 translocation, and modulation of apoptosis. *Mol Carcinog* 2006;45:320-332.
- 15 Scott DW, Loo G: Curcumin-induced GADD153 gene up-regulation in human colon cancer cells. *Carcinogenesis* 2004;25:2155-2164.
- 16 Song G, Mao YB, Cai QF, Yao LM, Ouyang GL, Bao SD: Curcumin induces human HT-29 colon adenocarcinoma cell apoptosis by activating p53 and regulating apoptosis-related protein expression. *Braz J Med Biol Res* 2005;38:1791-1798.
- 17 Hussain AR, Al Rasheed M, Manogaran PS, Al Hussein KA, Platanius LC, Al Kuraya K, Uddin S: Curcumin induces apoptosis via inhibition of PI3'-kinase/AKT pathway in acute T cell leukemias. *Apoptosis* 2006;11:245-254.
- 18 Beevers CS, Li F, Liu L, Huang S: Curcumin inhibits the mammalian target of rapamycin-mediated signaling pathways in cancer cells. *Int J Cancer* 2006;119:757-764.
- 19 Lev-Ari S, Maimon Y, Strier L, Kazanov D, Arber N: Down-regulation of prostaglandin E2 by curcumin is correlated with inhibition of cell growth and induction of apoptosis in human colon carcinoma cell lines. *J Soc Integr Oncol* 2006;4:21-26.
- 20 Moussavi M, Assi K, Gomez-Munoz A, Salh B: Curcumin mediates ceramide generation via the de novo pathway in colon cancer cells. *Carcinogenesis* 2006;27:1636-1644.
- 21 Chen J, Tang XQ, Zhi JL, Cui Y, Yu HM, Tang EH, Sun SN, Feng JQ, Chen PX: Curcumin protects PC12 cells against 1-methyl-4-phenylpyridinium ion-induced apoptosis by bcl-2-mitochondria-ROS-iNOS pathway. *Apoptosis* 2006;11:943-953.
- 22 Chan WH, Wu HJ, Hsuw YD: Curcumin inhibits ROS formation and apoptosis in methylglyoxal-treated human hepatoma G2 cells. *Ann N Y Acad Sci* 2005;1042:372-378.
- 23 Mall M, Kunzelmann K: Correction of the CF defect by curcumin: hopes and disappointments. *Bioessays* 2005;27:9-13.
- 24 Berger AL, Randak CO, Ostedgaard LS, Karp PH, Vermeer DW, Welsh MJ: Curcumin stimulates cystic fibrosis transmembrane conductance regulator Cl-channel activity. *J Biol Chem* 2005;280:5221-5226.
- 25 Egan ME, Pearson M, Weiner SA, Rajendran V, Rubin D, Glockner-Pagel J, Canny S, Du K, Lukacs GL, Caplan MJ: Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. *Science* 2004;304:600-602.
- 26 Lipecka J, Norez C, Bensalem N, Baudouin-Legros M, Planelles G, Becq F, Edelman A, Davezac N: Rescue of DeltaF508-CFTR (cystic fibrosis transmembrane conductance regulator) by curcumin: involvement of the keratin 18 network. *J Pharmacol Exp Ther* 2006;317:500-505.
- 27 Norez C, Antigny F, Becq F, Vandebrouck C: Maintaining low Ca2+ level in the endoplasmic reticulum restores abnormal endogenous F508del-CFTR trafficking in airway epithelial cells. *Traffic* 2006;7:562-573.
- 28 Wang W, Li G, Clancy JP, Kirk KL: Activating cystic fibrosis transmembrane conductance regulator channels with pore blocker analogs. *J Biol Chem* 2005;280:23622-23630.
- 29 Dragomir A, Bjorstad J, Hjelte L, Roomans GM: Curcumin does not stimulate cAMP-mediated chloride transport in cystic fibrosis airway epithelial cells. *Biochem Biophys Res Commun* 2004;322:447-451.
- 30 Grubb BR, Gabriel SE, Mengos A, Gentzsch M, Randell SH, Van Heeckeren AM, Knowles MR, Drumm ML, Riordan JR, Boucher RC: SERCA pump inhibitors do not correct biosynthetic arrest of deltaF508 CFTR in cystic fibrosis. *Am J Respir Cell Mol Biol* 2006;34:355-363.
- 31 Loo TW, Bartlett MC, Clarke DM: Thapsigargin or curcumin does not promote maturation of processing mutants of the ABC transporters, CFTR, and P-glycoprotein. *Biochem Biophys Res Commun* 2004;325:580-585.
- 32 Song Y, Sonawane ND, Salinas D, Qian L, Pedemonte N, Galletta LJ, Verkman AS: Evidence against the rescue of defective DeltaF508-CFTR cellular processing by curcumin in cell culture and mouse models. *J Biol Chem* 2004;279:40629-40633.
- 33 Lange T, Jungmann P, Haberle J, Falk S, Duebbers A, Bruns R, Ebner A, Hinterdorfer P, Oberleithner H, Schillers H: Reduced number of CFTR molecules in erythrocyte plasma membrane of cystic fibrosis patients. *Mol Membr Biol* 2006;23:317-323.
- 34 Sterling KM, Jr., Shah S, Kim RJ, Johnston NI, Salikhova AY, Abraham EH: Cystic fibrosis transmembrane conductance regulator in human and mouse red blood cell membranes and its interaction with ecto-apyrase. *J Cell Biochem* 2004;91:1174-1182.
- 35 Verloo P, Kocken CH, Van der WA, Tilly BC, Hogema BM, Sinaasappel M, Thomas AW, De Jonge HR: Plasmodium falciparum-activated chloride channels are defective in erythrocytes from cystic fibrosis patients. *J Biol Chem* 2004;279:10316-10322.
- 36 Abraham EH, Sterling KM, Kim RJ, Salikhova AY, Huffman HB, Crockett MA, Johnston N, Parker HW, Boyle WE, Jr., Hartov A, Demidenko E, Efrid J, Kahn J, Grubman SA, Jefferson DM, Robson SC, Thakar JH, Lorico A, Rappa G, Sartorelli AC, Okunieff P: Erythrocyte membrane ATP binding cassette (ABC) proteins: MRP1 and CFTR as well as CD39 (ecto-apyrase) involved in RBC ATP transport and elevated blood plasma ATP of cystic fibrosis. *Blood Cells Mol Dis* 2001;27:165-180.
- 37 Olearczyk JJ, Stephenson AH, Lonigro AJ, Sprague RS: Receptor-mediated activation of the heterotrimeric G-protein Gs results in ATP release from erythrocytes. *Med Sci Monit* 2001;7:669-674.
- 38 Sprague RS, Ellsworth ML, Stephenson AH, Lonigro AJ: Participation of cAMP in a signal-transduction pathway relating erythrocyte deformation to ATP release. *Am J Physiol Cell Physiol* 2001;281:C1158-C1164.
- 39 Jaruga E, Salvioli S, Dobrucki J, Chrul S, Bendorowicz-Pikula J, Sikora E, Franceschi C, Cossarizza A, Bartosz G: Apoptosis-like, reversible changes in plasma membrane asymmetry and permeability, and transient modifications in mitochondrial membrane potential induced by curcumin in rat thymocytes. *FEBS Lett* 1998;433:287-293.
- 40 Foller M, Kasinathan RS, Duranton C, Wieder T, Huber SM, Lang F: PGE2-induced apoptotic cell death in K562 human leukaemia cells. *Cell Physiol Biochem* 2006;17:201-210.

- 41 Gulbins E, Jekle A, Ferlinz K, Grassme H, Lang F: Physiology of apoptosis. *Am J Physiol Renal Physiol* 2000;279:F605-F615.
- 42 Zhou Q, Zhao J, Wiedmer T, Sims PJ: Normal hemostasis but defective hematopoietic response to growth factors in mice deficient in phospholipid scramblase 1. *Blood* 2002;99:4030-4038.
- 43 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.
- 44 Lang KS, Myssina S, Tanneur V, Wieder T, Huber SM, Lang F, Duranton C: Inhibition of erythrocyte cation channels and apoptosis by ethylisopropylamiloride. *Naunyn Schmiedeberg Arch Pharmacol* 2003;367:391-396.
- 45 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.
- 46 Lang PA, Kempe DS, Akel A, Klarl BA, Eisele K, Podolski M, Hermle T, Niemoeller OM, Attanasio P, Huber SM, Wieder T, Lang F, Duranton C: Inhibition of erythrocyte "apoptosis" by catecholamines. *Naunyn Schmiedeberg Arch Pharmacol* 2005;372:228-235.
- 47 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.
- 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 Woon LA, Holland JW, Kable EP, Roufogalis BD: Ca²⁺ sensitivity of phospholipid scrambling in human red cell ghosts. *Cell Calcium* 1999;25:313-320.
- 50 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.
- 51 Boas FE, Forman L, Beutler E: Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proc Natl Acad Sci U S A* 1998;95:3077-3081.
- 52 Kempe DS, Lang PA, Duranton C, Akel A, Lang KS, Huber SM, Wieder T, Lang F: Enhanced programmed cell death of iron-deficient erythrocytes. *FASEB J* 2006;20:368-370.
- 53 Bosman GJ, Willekens FL, Werre JM: Erythrocyte aging: a more than superficial resemblance to apoptosis? *Cell Physiol Biochem* 2005;16:1-8.
- 54 Arese P, Turrini F, Schwarzer E: Band 3/ complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. *Cell Physiol Biochem* 2005;16:133-146.
- 55 Barvitenko NN, Adragna NC, Weber RE: Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell Physiol Biochem* 2005;15:1-18.
- 56 Rice L, Alfrey CP: The negative regulation of red cell mass by neocytolysis: physiologic and pathophysiologic manifestations. *Cell Physiol Biochem* 2005;15:245-250.
- 57 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.
- 58 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.
- 59 Lang PA, Huober J, Bachmann C, Kempe DS, Sobiesiak M, Akel A, Niemoeller OM, Dreischer P, Eisele K, Klarl BA, Gulbins E, Lang F, Wieder T: Stimulation of erythrocyte phosphatidylserine exposure by paclitaxel. *Cell Physiol Biochem* 2006;18:151-164.
- 60 Akel A, Hermle T, Niemoeller OM, Kempe DS, Lang PA, Attanasio P, Podolski M, Wieder T, Lang F: Stimulation of erythrocyte phosphatidylserine exposure by chlorpromazine. *Eur J Pharmacol* 2006;532:11-17.
- 61 Eisele K, Lang PA, Kempe DS, Klarl BA, Niemoller O, Wieder T, Huber SM, Duranton C, Lang F: Stimulation of erythrocyte phosphatidylserine exposure by mercury ions. *Toxicol Appl Pharmacol* 2006;210:116-122.
- 62 Kempe DS, Lang PA, Eisele K, Klarl BA, Wieder T, Huber SM, Duranton C, Lang F: Stimulation of erythrocyte phosphatidylserine exposure by lead ions. *Am J Physiol Cell Physiol* 2005;288:C396-C402.
- 63 Lang PA, Kaiser S, Myssina S, Birka C, Weinstock C, Northoff H, Wieder T, Lang F, Huber SM: Effect of *Vibrio parahaemolyticus* haemolysin on human erythrocytes. *Cell Microbiol* 2004;6:391-400.
- 64 Birka C, Lang PA, Kempe DS, Hoefling L, Tanneur V, Duranton C, Nammi S, Henke G, Myssina S, Krikov M, Huber SM, Wieder T, Lang F: Enhanced susceptibility to erythrocyte "apoptosis" following phosphate depletion. *Pflugers Arch* 2004;448:471-477.
- 65 Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T, Attanasio P, Akel A, Schafer R, Friedrich B, Risler T, Baur M, Olbricht CJ, Zimmerhackl LB, Zipfel PF, Wieder T, Lang F: Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. *J Mol Med* 2006;84:378-388.
- 66 Brand VB, Sandu CD, Duranton C, Tanneur V, Lang KS, Huber SM, Lang F: Dependence of *Plasmodium falciparum* in vitro growth on the cation permeability of the human host erythrocyte. *Cell Physiol Biochem* 2003;13:347-356.
- 67 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.
- 68 Myssina S, Huber SM, Birka C, Lang PA, Lang KS, Friedrich B, Risler T, Wieder T, Lang F: Inhibition of erythrocyte cation channels by erythropoietin. *J Am Soc Nephrol* 2003;14:2750-2757.
- 69 Lang KS, Myssina S, Lang PA, Tanneur V, Kempe DS, Mack AF, Huber SM, Wieder T, Lang F, Duranton C: Inhibition of erythrocyte phosphatidylserine exposure by urea and Cl⁻. *Am J Physiol Renal Physiol* 2004;286:F1046-F1053.
- 70 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.
- 71 Nunomura W, Takakuwa Y, Tokimitsu R, Krauss SW, Kawashima M, Mohandas N: Regulation of CD44-protein 4.1 interaction by Ca²⁺ and calmodulin. Implications for modulation of CD44-ankyrin interaction. *J Biol Chem* 1997;272:30322-30328.
- 72 Takakuwa Y, Mohandas N: Modulation of erythrocyte membrane material properties by Ca²⁺ and calmodulin. Implications for their role in regulation of skeletal protein interactions. *J Clin Invest* 1988;82:394-400.
- 73 Anderson DR, Davis JL, Carraway KL: Calcium-promoted changes of the human erythrocyte membrane. Involvement of spectrin, transglutaminase, and a membrane-bound protease. *J Biol Chem* 1977;252:6617-6623.
- 74 Allan D, Billah MM, Finean JB, Michell RH: Release of diacylglycerol-enriched vesicles from erythrocytes with increased intracellular (Ca²⁺). *Nature* 1976;261:58-60.

- 75 Cohen CM, Gascard P: Regulation and post-translational modification of erythrocyte membrane and membrane-skeletal proteins. *Semin Hematol* 1992;29:244-292.
- 76 Minetti G, Piccinini G, Balduini C, Seppi C, Brovelli A: Tyrosine phosphorylation of band 3 protein in Ca²⁺/A23187-treated human erythrocytes. *Biochem J* 1996;320 (Pt 2):445-450.
- 77 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.
- 78 Bratosin D, Estaquier J, Petit F, Arnoult 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.
- 79 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.