FTY720-Induced Suicidal Erythrocyte Death

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Key Words

Phosphatidylserine • Scrambling • Calcium • Cell volume • Eryptosis • Apoptosis

Abstract

FTY720 is a potent anti-inflammatory drug known to trigger suicidal death or apoptosis of a variety of nucleated cells. Erythrocytes may similarly undergo suicidal erythrocyte death or eryptosis. Hallmarks of eryptosis include cell membrane scrambling and cell shrinkage, which are triggered by increase in cytosolic Ca^{2+} concentration and ceramide. The present study explored whether FTY720 stimulates eryptosis. Cell membrane scrambling was determined from annexin V-binding, cell shrinkage from forward scatter in FACS analysis, cytosolic Ca^{2+} concentration from Fluo3 fluorescence, ceramide formation from fluorescence-labeled antibody binding and hemolysis from the hemoglobin concentration in the supernatant. Within 48 hours exposure to FTY720 (10 μM) significantly increased annexin V-binding, decreased forward scatter and increased cytosolic Ca^{2+} concentration but did not significantly modify ceramide formation. The effects of FTY720 were significantly blunted in the nominal absence of extracellular Ca^{2+} . In conclusion, at toxic concentrations, FTY720 stimulates

suicidal cell death, an effect at least partially due to stimulation of Ca^{2+} entry.

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Introduction

The sphingosine analog FTY720 is a potent anti-inflammatory substance effective in autoimmune disease, transplantation, atherosclerosis and traumatic brain injury [1-3]. FTY720 is considered to be at least partially effective following conversion into FTY720-phosphate and subsequent stimulation of sphingosine phosphate receptor S1P1 by FTY720-phosphate [1, 4]. FTY720 is phosphorylated mainly in blood platelets [5], whereas erythrocytes are able to phosphorylate sphingosine but not FTY720 [4]. FTY720 has previously been shown to trigger apoptosis of several cell types [6-8]. In other cell types, FTY720 has been shown to be without effect on cell survival [9] or even protect against apoptosis [10]. The apoptotic effect of FTY720 may be due to activation of protein kinase C (PKC) [6].

Similar to apoptosis of nucleated cells, erythrocytes may undergo suicidal cell death or eryptosis, which is characterized by scrambling of the erythrocyte membrane and by cell shrinkage [11]. The cell membrane scram-

bling results in exposure of phosphatidylserine at the cell surface, which is apparent from annexin V-binding of the erythrocytes [11]. Cell shrinkage is evident from a decrease of forward scatter in FACS analysis [11]. Further triggers of eryptosis include increase in cytosolic Ca^{2+} concentration [12–14], which triggers membrane scrambling [11] and activates Ca^{2+} -sensitive K^{+} channels [15, 16]. In the following, the exit of KCl with osmotically obliged water leads to cell shrinkage [17]. The cytosolic Ca^{2+} concentration may be increased by activation of Ca^{2+} -permeable cation channels [14, 18–20]. The Ca^{2+} sensitivity of phospholipid scrambling is increased by ceramide [21].

Phosphatidylserine-exposing erythrocytes are recognized by macrophages, engulfed and thus rapidly eliminated from circulating blood [22, 23]. Nothing is known about effects of FTY720 on the function or survival of erythrocytes.

The present study thus explored whether FTY720 influences eryptosis, i.e. membrane scrambling and shrinkage of erythrocytes.

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003V).

Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl , 5 KCl , 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl_2 ; pH 7.4 at 37°C for 48 hours. Where indicated, FTY720, PKC β inhibitor, PKC Inhibitor (EGF-R Fragment 651–658, myristoylated), PKC α inhibitor Ro-32-0432 (all from Calbiochem, Bad Soden, Germany), tert-butylhydroperoxide (TBOOH), and ionomycin (both from Sigma, Schnellendorf, Germany) were added at the indicated concentrations. The concentration of the solvent DMSO was =0.1%, a concentration which does not influence eryptosis (data not shown).

FACS analysis of annexin V-binding and forward scatter

After incubation under the respective experimental condition, cells were washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-Fluos (Roche, Mannheim, Germany) in this solution for 20 min under protection from light. In the following, the forward scatter of the cells was determined and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS calibur (BD, Heidelberg, Germany).

Measurement of intracellular Ca^{2+}

After incubation erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM CaCl_2 and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Determination of ceramide formation

To determine ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 1 $\mu\text{g/ml}$ anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. After two washing steps with PBS-BSA, cells were stained for 30 minutes with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analysed by flow cytometric analysis in FL-1.

Measurement of hemolysis

After 48 hours of incubation at 37°C , the samples were centrifuged (3 min at 400 g, RT), and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Statistics

Data are expressed as arithmetic means $\pm$ SEM, and statistical analysis was made using ANOVA with Tukey's test as post-test. n denotes the number of different erythrocyte specimens studied.

Results

A hallmark of suicidal erythrocyte death or eryptosis is cell membrane scrambling, which is evidenced from binding of Fluos-labelled annexin V to phosphatidylserine at the erythrocyte surface utilizing FACS analysis. As shown in Fig. 1A,B, the percentage of annexin V binding and thus phosphatidylserine-exposing erythrocytes was low in the absence of FTY720. Exposure of erythrocytes for 48 hours to Ringer solution containing FTY720 (5–10 μM) was followed by a significant increase in annexin V-binding to the erythrocyte surface. The time course of PS exposure of erythrocytes exposed to Ringer solution without and with 10 μM FTY720 is shown in Fig. 1C.

FTY720 did not disrupt the integrity of the membrane (Fig. 2A,B). Accordingly, no significant stimulation of

Fig. 1. Effect of FTY720 on phosphatidylserine exposure of erythrocytes. A. Histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes exposed for 48 hours to Ringer solution without (-, black line) and with (+, red line) 10 μ M FTY720. B. Arithmetic means $\pm$ SEM (n = 4-8) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 48 hours to Ringer solution without (white bar) or with (black bars) FTY720. *** (p<0.001) indicates significant difference from the respective value without exposure to FTY720. C. Time dependence of the arithmetic means $\pm$ SEM (n = 5) of the percentage of PS-exposing cells following incubation in Ringer solution without and with 10 μ M FTY720.

hemolysis was observed following a 48 hours treatment with 10 μ M FTY720 (Fig. 2A,B).

The second hallmark of eryptosis is cell shrinkage, which could be revealed by a decrease of forward scatter in FACS analysis. Exposure of erythrocytes for 48 hours to Ringer solution containing FTY720 was indeed followed by a significant decrease of erythrocyte forward scatter (Fig. 3A,B).

Both, cell membrane scrambling and cell shrinkage are triggered by increase in cytosolic Ca^{2+} concentration. Thus, additional experiments were performed to determine the intracellular Ca^{2+} concentration utilizing Fluo3 fluorescence. As demonstrated in Fig. 4A,B, a 48 hours incubation of erythrocytes in the presence of FTY720 (10 μ M) was followed by a significant increase in Fluo3 fluorescence. Accordingly, FTY720 indeed increased the cytosolic Ca^{2+} concentration.

Further series of experiments were performed to explore whether the increase in cytosolic Ca^{2+} concentration accounted for the stimulation of cell membrane scrambling. To this end, FTY720 was applied in the presence and nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 4C, the removal of extracellular Ca^{2+} significantly blunted the FTY720-induced cell annexin V-binding. The time course of Fluo3 fluorescence of erythrocytes exposed to Ringer solution without and with 10 μ M FTY720 is shown in Fig. 4D. To test whether the FTY720-induced increase in Fluo3 fluorescence was due to increased dye loading, Fluo3 fluorescence was determined in erythrocytes incubated in Ringer solution without and with 10 μ M FTY720 prior to and following exposure to 5 μ M of the Ca^{2+} ionophore ionomycin for 2 min. As a result, Fluo3 fluorescence was (mean $\pm$ SEM; all n = 5) 21.0 ± 0.9 (0 μ M FTY720) and 39.5 ± 1.5 (10 μ M FTY720) prior to and 238.9 ± 14.0 (0 μ M FTY720) and 246.1 ± 10.2 (10 μ M FTY720) following addition of ionomycin. Thus, FTY720 does not influence dye loading.

Fig. 2. Effect of FTY720 on the integrity of the cellular membrane. A. Arithmetic means $\pm$ SEM (n = 4) of the percentage of hemolysed erythrocytes following exposure for 48 hours to Ringer solution without (white bar) or with (black bars) FTY720. B. Fluorescence microphotograph of an erythrocyte stained with fluorescent annexin V. Prior to microscopy, the erythrocyte was exposed for 48 h to 10 μ M FTY720 in Ringer solution.

Fig. 3. Effect of FTY720 on erythrocyte forward scatter. A. Original histogram of the forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) 10 μ M FTY720. B. Arithmetic means $\pm$ SEM (n = 4-8) of erythrocyte forward scatter following exposure for 48 hours to Ringer solution without (white bar) or with (black bar) FTY720. *** (p<0.001) indicates significant difference from the respective value without exposure to FTY720.

Fig. 4. Role of Ca^{2+} in FTY720-induced eryptosis. A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 48 hours to Ringer without (-, black line) and with (+, red line) 10 μM FTY720. B. Arithmetic means $\pm$ SEM ($n = 4-8$) of the geo means of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer without (white bar) or with (black bars) FTY720. *** ($p < 0.001$) indicates significant difference from the respective value in the absence of FTY720. C. Arithmetic means $\pm$ SEM ($n = 3$) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 48 hours to Ringer solution without (white bars) or with (black bars) FTY720 in the presence (left bars) and nominal absence (right bars) of extracellular Ca^{2+} . *** ($p < 0.001$) indicates significant difference from the respective value without exposure to FTY720. # ($p < 0.05$) indicates significant difference from the presence of extracellular Ca^{2+} . D. Time dependence of the arithmetic means $\pm$ SEM ($n = 5$) of Fluo3 fluorescence of erythrocytes following incubation in Ringer solution without and with 10 μM FTY720.

Inhibition of protein kinase C by calphostin (50 nM) did not significantly modify the stimulation of eryptosis by FTY720 (10 μM) as the percentage of annexin V-binding erythrocytes was $44.1 \pm 4.7\%$ ($n = 5$) in the absence and $47.1 \pm 3.4\%$ ($n = 5$) in the presence of calphostin. To exclude a potential role of PKC isoforms, further inhibitors besides calphostin were similarly tested. In this series, the percentage of annexin V-binding erythrocytes exposed to 10 μM FTY720 for 48 h was $51.8 \pm 4.4\%$ ($n = 12$) in the absence and $51.2 \pm 4.4\%$ ($n = 12$) in the presence of 20 nM of the PKC α inhibitor Ro-32-0432, $50.5 \pm 4.5\%$ ($n = 12$) in the presence of 50 nM of the PKC β inhibitor, and $57.0 \pm 4.5\%$ ($n = 12$) in the presence of 10 μM of the PKC inhibitor (EGF-R Fragment 651-658, myristoylated).

In a further series of experiments, a specific antibody against ceramide was employed to explore whether FTY720 stimulates ceramide formation. As a result, FTY720 (0.5-10 μM) did not significantly modify the erythrocytic ceramide abundance. The mean ceramide-dependent fluorescence intensity was 19.9 ± 0.8 ($n = 4$) prior to and 22.1 ± 1.3 ($n = 4$) following treatment with 10 μM FTY720.

Discussion

The present study reveals a novel effect of FTY720, i.e. the stimulation of erythrocyte cell membrane scrambling and of erythrocyte shrinkage, two hallmarks of

eryptosis, the suicidal death of erythrocytes. In theory, the effect could be due to the phosphorylated form, following conversion of FTY720 into FTY720-phosphate and subsequent stimulation of sphingosine phosphate receptor S1P1 by FTY720-phosphate [1, 4]. However, erythrocytes may not be able to phosphorylate FTY720 [4] and the effect may be mediated by the nonphosphorylated form of FTY720. The effect is at least partially due to increase in cytosolic Ca^{2+} concentration, a well known trigger of eryptosis [12-14, 24, 25]. Accordingly, FTY720 increases cytosolic Ca^{2+} concentration and the effect is significantly blunted by the removal of extracellular Ca^{2+} . The removal of Ca^{2+} did not completely abrogate the stimulating effect of FTY720 on cell membrane scrambling, which could point to the involvement of an additional mechanism. As FTY720 has previously been shown to stimulate protein kinase C (PKC) in nucleated cells [6], and as activation of PKC has been shown to similarly trigger cell membrane scrambling [26], the possibility was tested, that activation of PKC may have contributed to the stimulation of cell membrane scrambling by FTY720. However, FTY720-induced eryptosis was not significantly modified by the specific PKC inhibitor calphostin (50 nM). Moreover, FTY720 did not stimulate the formation of ceramide, another major stimulator of eryptosis [21].

The concentration needed for an effect is 10 μM , which is in the range of the concentrations reached in rat after i.v. injection of effective dosages of the substance [27, 28]. FTY720 may, however, be bound to plasma pro-

teins [29] and the actual therapeutic plasma concentrations of free FTY720 may be actually lower. Thus, the present observations may be relevant to FTY720 intoxication rather than for adequate dosages of the drug. At least in theory, the sensitivity to FTY720 may be enhanced by disorders or drugs similarly stimulating eryptosis. As reviewed earlier [11] eryptosis is triggered by a variety of other xenobiotics and endogenous substances [30-37]. Moreover, several clinical disorders stimulate eryptosis [11, 38-40]. At least in theory, the proeryptotic effect of FTY720 could be potentiated by simultaneous exposure to those substances or in patients suffering from those diseases.

Enhanced eryptosis may lead to anemia, as eryptotic cells bind to phosphatidylserine receptors on macrophages [41], which engulf and degrade phosphatidylserine-exposing cells [42]. As a result, eryptotic cells are rapidly cleared from circulating blood [43]. Side effects of FTY 720 indeed include anemia [44]. However, anemia is not invariably observed in patients receiving the substance [44]. As long as the accelerated loss of circulating erythrocytes is matched by a similarly increased formation of new erythrocytes, the patients may escape clinically overt anemia. An increased formation

of new erythrocytes is apparent from an increase in the reticulocyte number.

Eryptotic cells may adhere to endothelial cells [45-49] and stimulate coagulation [45, 50, 51]. Accordingly, eryptotic cells may interfere with blood flow and thus disturb microcirculation. Along those lines, eryptosis has been suggested to participate in the vascular injury of metabolic syndrome [52].

In conclusion, FTY720 stimulates suicidal death of mature erythrocytes or eryptosis. The effect is at least partially due to Ca^{2+} entry. The proeryptotic effect of FTY720 may lead to relevant anemia and/or disturbance of microcirculation in the presence of other proeryptotic substances or in particularly susceptible patients.

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