Effects of 5-Fluorouracil on Erythrocytes in Relation to Its Cardiotoxicity: Membrane Structure and Functioning

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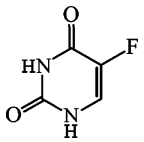
In the present study, we showed that the antineoplastic drug 5-fluorouracil (5-FU) induces in vitro exposure-time/dose-dependent changes on the level of an erythrocyte's morphology, ionic balance, and membrane fluidity. These changes are partially or fully irreversible, and we suggest that they are provoked by an irreversible depletion of ATP. Because of these changes that could also occur in vivo during 5-FU infusion, a certain amount of erythrocytes with echinocytic shape and diminished ability to deliver oxygen is present in blood for longer periods of time. This renders oxygen transport and delivery more difficult, leaving the heart with an insufficient supply of oxygen, thus leading to cardiotoxicity.

INTRODUCTION

5-Fluorouracil (5-FU) is a synthetic pyrimidine analogue whose metabolites produced during its intracellular metabolism exhibit anticancer effects by inhibition of the enzyme thymidylate synthetase or through the effects on RNA and DNA metabolism.[1] It is frequently used in high doses for the therapeutic treatment of various malignancies such as cancer of the breast, gastrointestinal tract, head, and neck. In addition to well-known negative effects such as myelosuppression, mucositis, nausea, and vomiting, cardiotoxicity has been observed to occur during the administration of this drug.[1−3] The reported frequency of incidence of 5-FU cardiotoxicity is up to 18%, with a lethality rate as high as 13.3%.[1] Although it is of great interest to elucidate the biophysical mechanisms behind this effect, we found no convincing reports. It seems that the 5-FU cardiotoxicity is an ischemic manifestation,[2,4] which could not be explained by preexisting or acute coronary heart disease or by an increased susceptibility to vasospasm.[4] Therefore, it has been proposed that an inadequate supply of oxygen to the myocardium may result from changes in the rheological properties of erythrocytes and a subsequent increase of blood viscosity.[5] Changes in rheology were attributed to the reversible echinocytosis of erythrocytes, which has been reported to occur in vitro as a result of the preferential intercalation of 5-FU into the outer hemileaflet of the erythrocyte membrane.[5] However, in vivo studies showed a decrease rather than an increase in blood viscosity during the 5-FU infusion,[3] indicating that more complex changes in the erythrocyte metabolism should be investigated. For example, contrary to the statement that 5-FU does not affect the ATP level,[5] our previous study demonstrated a significant and relatively rapid decrease in the intracellular level of ATP in 5-FU-treated erythrocytes.[6] In addition, it has been shown that 5-FU affects several intracellular components of the oxygen delivery system in erythrocytes, inducing a decrease in the oxygen tension,[6] which concurs with the hypoxemia of arterial blood observed in patients that experienced cardiotoxicity during treatment with 5-FU.[7] A decrease in ATP level can affect the normal functioning of the erythrocyte membrane, since ATP is needed for the preservation of membrane fluidity,[8] normal shape,[9] and the normal functioning of membrane ionic channels.[10] We have already reported that 5-FU affects the erythrocyte membrane band 3 macrocomplex, which is involved in the delivery of oxygen.[11] Since stable shape, structure, and functionality of the membrane are crucial for the normal functioning of erythrocytes, changes on the membrane level could lead to a decreased ability of erythrocytes to deliver oxygen. Therefore, in the present study, we investigated the effects of 5-FU on the structure and functioning of the erythrocyte membrane, as a continuation of our previous study on the effects of 5-FU on energetic and oxygen metabolism in erythrocytes.[6,11]

Chart 1. Structural Formula of 5-FU



MATERIALS AND METHODS

Chemicals. 5-FU (Chart 1) was obtained from ICN Galenika (Belgrade, Serbia and Montenegro).

Nitroxide spin-probe 7-DS [2-(5-carboxypentyl)-2-undecyl-4,4-dimethyloxazolidine-3-oxyl] was purchased from Molecular Probes (Junction City, OR). May−Grünwald stain and Giemsa stain were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade and purchased from commercial providers.

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Sample Preparation. Fresh blood was obtained from five healthy volunteers between the ages of 20 and 30, using tubes containing 0.072 mL of 7.5% K_3EDTA as the anticoagulant per 3 mL of blood (Vacuette EDTA, Greiner Bio-One, Austria). Blood was incubated at 37 °C, immediately after collecting or after the washing procedure, depending on the demands of the method in question. The hematocrit in fresh blood was ~40%, and the same hematocrit was adjusted in all samples before incubation, except for those used in EPR measurements. In all of the experiments, 5-FU was added in final concentrations of 3, 6.5, or 10 mg/mL, with the exception of the ^{31}P NMR study (10 mg/mL) and control samples, in which no 5-FU was used.

High-Performance Liquid Chromatography Assay of 5-FU in Erythrocytes. Fresh blood was centrifuged at 1500 rpm/10 min/4 °C, and the supernatant was removed. Erythrocytes were washed two more times with a phosphate buffer solution (PBS) (NaCl 8.8 g/L, Na_2HPO_4 1.2 g/L, NaH_2PO_4 0.43 g/L, pH 7.4) at the same conditions. Then, the erythrocytes were resuspended in PBS, and 5-FU was added. After 30, 60, and 120 min of incubation, the erythrocytes were washed three times with PBS at 1500 rpm/10 min in order to remove 5-FU from the solution and outer hemileaflet of the erythrocyte membrane. The final supernatant was removed, and an extraction of 5-FU from the erythrocytes was performed according to a procedure described elsewhere.[12] Obtained extracts of erythrocytes were then applied to the high-performance liquid chromatography (HPLC) column. Measurements were conducted by a Hewlett-Packard HP1100 chromatograph (Palo Alto, CA) composed of an in-line degasser, autosampler, and thermostated compartment, with a detector (HP 1100 PDA) adjusted at 267 nm. Separations were performed on a Waters Symmetry C-18 RP column (Waters, Milford, MA) that was 125 × 4 mm with a 5 Φm particle size. The temperature was 35 °C. The mobile phase consisted of 0.1% phosphoric acid and acetonitrile (90:10). A standard calibration curve was prepared by adding different quantities of 5-FU to an extract of untreated erythrocytes.

Changes in Erythrocyte Morphology. Whole blood was immediately incubated for 5, 60, and 120 min. Following incubation, a small quantity of each sample was obtained, and the erythrocytes were fixed according to the May−Grünwald Giemsa procedure. The rest of the sample was centrifuged at 1500 rpm/10 min. The plasma and the buffy coat were carefully removed, and the retrieved erythrocytes were washed two times with PBS. Washed erythrocytes were resuspended in PBS, and the same fixation procedure was performed. The morphology of the erythrocytes was analyzed by light microscopy. The percentage of echinocytes was determined according to the following criteria:[13] echinocytes I, irregularly contoured discocytes; echinocytes II, flat erythrocytes with multiple spicules; echinocytes III, spherical erythrocytes with multiple spicules; and echinocytes IV, spheres with multiple short and thin spicules.

Potassium Efflux and Level of Hemolysis. After 5, 60, and 120 min of incubation with 5-FU, whole blood was centrifuged at 3000 rpm/10 min to separate the plasma and erythrocytes. Plasma was diluted 50 times, and extracellular potassium was measured using a Jenway PFP7 flame photometer (Dunmow, England). To determine the relative degree of hemolysis, plasma was diluted 10 times and absorbencies at 540 nm were measured to determine the potential release of hemoglobin from the erythrocytes.[14]

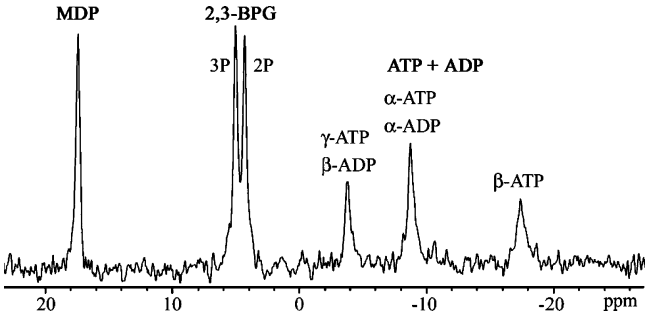

Figure 1. ^{31}P NMR spectrum of packed erythrocytes. MDP: methylenediphosphonate. 2,3-BPG: 2,3-biphosphoglycerate. ATP has three peaks (γ, α, and β) with γ and α resonances overlapping the β and α resonances of ADP.[6]

^{31}P NMR Measurements. NMR measurements were performed using a Bruker MSL 400 NMR spectrometer. Whole blood was incubated for 30, 60, and 120 min with 5-FU (10 mg/mL). Following incubation, erythrocytes were washed two times with PBS, packed by centrifugation at 3000 rpm/10 min (final hematocrit value ≈ 70%), and placed in a 10-mm quartz tube.

A capillary containing 25 mM methylenediphosphonate was placed in the center of the NMR tube as a peak intensity standard.[6] Other experimental conditions were repetition time = 250 ms, pulse angle = 40°, and the signal was accumulated for approximately 16 min (4000 accumulations). The concentration of ATP was determined using the area of the β-ATP peak (see Figure 1). The temperature was controlled at 37 °C during measurements.

EPR Measurements of Erythrocyte Membrane Fluidity. Spin-labeling of the erythrocytes (erythrocyte membranes) was performed as described earlier[15] with some modifications. In our experience, it is very important to precisely follow the procedure in order to obtain reproducible results. Fresh blood was washed two times with a NaCl−Tris isotonic buffer (140 mM NaCl, 20 mM Tris, pH adjusted to 7.4 with 1M HCl) by centrifugation at 1500 rpm/10 min/4 °C. Washed erythrocytes (250 μL) were suspended in the equal volume of NaCl−Tris buffer. An ethanol solution of fatty acid spin-probe 7-DS was supplemented on the walls of another tube. After the ethanol evaporated, the NaCl−Tris buffer was added and the solution was vortex-mixed. Then, 250 μL of this solution was added to the erythrocytes to obtain the optimal spin-label/membrane-lipid ratio of approximately 1:100.[16] Samples were gently shaken at 37 °C for 2 h, and small amounts of 5-FU solution were added to the sample, to obtain final 5-FU concentrations of 3, 6.5, and 10 mg/mL. Following a certain incubation time, a portion of a sample was taken for EPR measurements, while the other portion was washed (according to the procedure described in the study of morphology), resuspended in the NaCl−Tris solution, and then measured. EPR spectra were recorded using a Varian E104-A EPR spectrometer operating at X-band (~9.1 GHz) and adjusted to the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 5 mW; scan range, 100 G; and scan time, 8 min.

The temperature was controlled at 37 °C during the measurements. Spectra were recorded and analyzed using

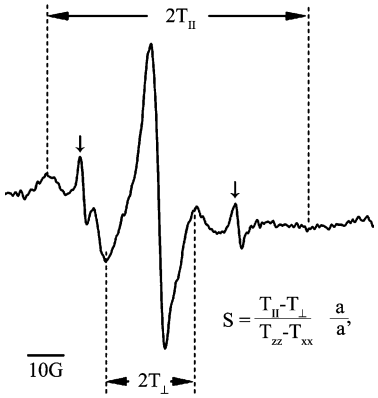

Figure 2. Typical EPR spectrum of erythrocytes labeled with 7-DS. *S*: order parameter. $2T_{||}$: outer hyperfine splitting. $2T_{\perp}$: inner hyperfine splitting. *a*: isotropic hyperfine coupling constant in crystal [$a = {}^1/_3(T_{xx} + T_{yy} + T_{zz})$]. a': isotropic hyperfine coupling constant in membrane [$a' = {}^1/_3(T_{||} + 2T_{\perp})$]. T_{xx}, T_{yy}, T_{zz}: hyperfine constants (for 7-DS, they were taken to be $T_{xx} = T_{yy} = 6.1$ G, $T_{zz} = 32.4$ G). Two narrow lines (arrows) originate from the 7-DS in solution.

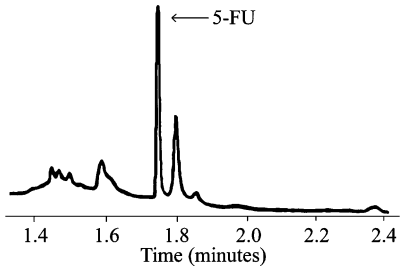

Figure 3. Typical chromatogram of the erythrocytes extract with added 5-FU at a final concentration of 0.01 mg/mL.

EW software (Scientific Software). The order parameter (*S*),[15] calculated as shown in Figure 2, was used as an indication of membrane fluidity.

Statistical Analysis. The data are presented as the means ± the standard deviation of at least three separate experiments. Significances of differences were calculated using Student's *t* test. Means were considered significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

Intracellular Level of 5-FU. Figure 3 shows a characteristic chromatogram of an extract of erythrocytes incubated with 5-FU and subsequently washed with PBS. According to a similar study,[12] the 5-FU peak in the HPLC chromatogram represents the intracellular amount of 5-FU, which rules out the assumptions that 5-FU only intercalates itself into the outer hemileaflet of the membrane and can be easily washed out.[5] Figure 4 shows concentrations of 5-FU in treated erythrocytes, implying that the maximal intracellular concentration of 5-FU is reached within the first 30 min of incubation or even faster than that, since it has been reported that the influx of 5-FU under in vivo conditions occurs within 5 min after the administration of 5-FU.[12] Estimated intracellular concentrations of 5-FU were about ${}^1/_{10}$ of the final 5-FU concentration in the sample, similar to concentrations determined in the in vivo study.[12]

Note that such low concentrations of 5-FU in an intracellular milieu can be detected because of the high sensitivity

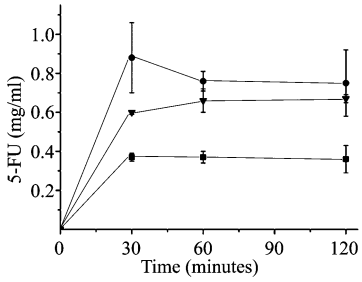

Figure 4. Intracellular concentrations of 5-FU in erythrocytes treated with 5-FU and subjected to repetitive washing. The concentrations of 5-FU in the samples before washing were (●) 10, (▼) 6.5, and (■) 3 mg/mL.

Table 1. Decrease in ATP Level in the Erythrocytes and the Reversibility of the Changes after Washing[a]

concentration of 5-FU (mg/mL)	time of incubation (minutes)		
	30	60	120
	relative ATP concentration		
10	51% ± 12%	47% ± 13%	34% ± 13%
10 + washing	79% ± 12%	62% ± 16%	31% ± 17%

[a] Concentrations of ATP in treated erythrocytes are presented as a percentage of the concentration of ATP in untreated erythrocytes (~1.45 mmol/L of erythrocytes)[6] ± standard deviation.

of HPLC (detection limit = 10 ng/mL[12]). Although these results imply that the majority of 5-FU is extracellular or embedded in the erythrocyte membrane, the portion of 5-FU that remains intracellular and cannot be removed by simple washing could be sufficient to produce significant changes in metabolism, such as a rapid depletion of oxygen tension and the consequent decrease of ATP level in erythrocytes,[6] which may be explained by the effects of intracellular 5-FU. These changes lead to other partially irreversible changes in the functioning of erythrocytes (see below).

Effects of 5-FU on the ATP Level in Erythrocytes. A ^{31}P NMR study. Table 1 shows the decrease of the intracellular concentration of ATP induced by 5-FU as a function of the time of incubation (also see ref 6). The amount of ATP decreases by 50% within the first 30 min of incubation with 5-FU, followed by a further decrease to as little as 35% of the original ATP level during the next 90 min. Repetitive washing of 5-FU-treated erythrocytes led to a partial restoration of the ATP level, but only for erythrocytes treated for 30 and 60 min, while no such recovery was detected for the incubation period of 120 min (Table 1). This shows that exposure of the erythrocytes to 5-FU irreversibly affects their energetic metabolism.

A decrease in the level of ATP most likely occurs because of increased consumption, since ATP is needed for the preservation of membrane fluidity,[8] normal shape,[9] and normal functioning of membrane ionic channels[10] (see below). Also, a decreased level of phosphorylation of ADP to ATP in the process of glycolysis could occur, since 5-FU induces increased production of 2,3-biphosphoglycerate.[6]

Effects of 5-FU on Erythrocyte Morphology. 5-FU led to an exposure-time/dose-dependent transformation of erythrocytes into the echinocytic shape (Figure 5, Table 2). The lowest concentration of 5-FU (3 mg/mL) used in this study did not induce any significant change in erythrocyte shape relative to the control samples, as reported earlier for similar

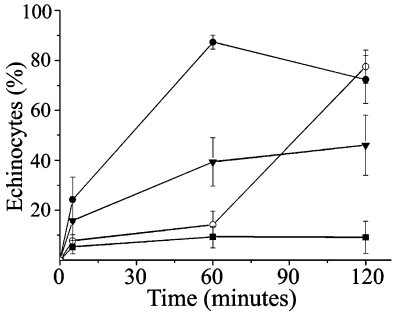

Figure 5. Percentage of echinocytes (all four types) induced by 5-FU in the erythrocyte population and the reversibility of echinocytosis. Samples were incubated with 5-FU: (●) 10 mg/mL, (○)10 mg/mL + washing of samples, (▼) 6.5 mg/mL, and (■) 3 mg/mL. The percentage of echinocytes in the control sample was constant (∼5%).

Table 2. Degree of Echinocytosis of Erythrocytes Treated with 5-FU[a]

	time of incubation (minutes)		
5-FU (mg/mL)	5	60	120
	types of echinocytes		
0	I (100%)	I (100%)	I (100%)
3	I (100%)	I (100%)	I (90%) II (10%)
6.5	I (100%)	I (30%) II (70%)	II (40%) III (60%)
10	I (80%) II (20%)	II (70%) III (30%)	III (90%) IV (10%)
10 + washing	I (100%)	II (100%)	II (50%) III (50%)

[a] Qualitative changes in the shape of the erythrocytes are described through a percentage of different types of echinocytes (types I, II, III, and IV) for different concentrations of 5-FU and incubation periods. Also, the reversibility of echinocytosis after washing of the erythrocytes is presented (10 + washing).

concentrations of 5-FU (2 mg/mL).[5] However, at 5-FU concentrations of 6.5 and 10 mg/mL, echinocytosis was apparent even after 5 min of incubation (Figure 5) and reached its full extent within the first hour of incubation (further changes are not statistically significant). It has been reported that budding of the membrane at the tips of spicules and exovesiculation can occur during the 5-FU-induced echinocytosis; however, that has been observed only for higher 5-FU concentrations (25 mg/mL).[5]

Although echinocytosis apparently reflects simple concentration dependence (Figure 5), the situation is much more complicated when the relative contribution of different types of echinocytes is taken into consideration (Table 2). For example, the formation of type III echinocytes is induced by 6.5 mg/mL of 5-FU only after 2 h, while the concentration of 10 mg/mL induced formation of this type at a relatively high degree after only 1 h of incubation. Although the percentage of echinocytes remained virtually the same during the second hour of incubation with 10 mg/mL of 5-FU, the formation of spherical erythrocytes with multiple short and thin spicules (type IV echinocytes)[13] occurred, which could be explained by a prolonged depletion of ATP (see Table 1). This shows the importance of assessing not only the overall percentage of echinocytes but also the relative contribution of different types. For instance, the spherical form of echinocytes is the ultimate form of echinocytosis and usually occurs prior to hemolysis,[17] indicating the severity of the 5-FU-induced echinocytosis. In addition, echinocytosis is known to affect the oxygen delivery system, leading to both a decreased uptake of oxygen and its release by erythrocytes.[18] The reversibility of echinocytosis was studied for the 5-FU concentration of 10 mg/mL, and it showed exposure-time dependence.

At the end of the 60 min incubation period, the changes were almost fully reversible and the percentage of echinocytes was the same as in the control sample (Figure 5). However, instead of the type I echinocytes present in the control samples, washed erythrocytes showed a higher degree of echinocytosis, namely, type II echinocytes (Table 2). This indicates that preferential intercalation of 5-FU into the outer hemileaflet of the membrane, causing an increase in the outer/inner membrane surface area ratio and the subsequent formation of the spicules, which are further stabilized by the elasticity of spectrin-based membrane skeleton,[19] could be the main mechanism for 5-FU-induced echinocytosis at this point. However, to some extent, a partially irreversible decrease in ATP, which is known to lead to echinocytosis (crenation),[9] also could be responsible, since the degree of crenation continued to be slightly increased. After 2 h of incubation, the removal of 5-FU did not change the level of echinocytosis (Figure 5). The degree was only slightly decreased, which indicates that the echinocytosis was predominantly provoked by irreversible ATP depletion (see Table 1). However, a slight decrease of the degree of crenation (the absence of echinocytes IV and reappearance of type II, Table 2) indicates that preferential intercalation of 5-FU into the outer hemileaflet is still a part of the mechanism of echinocytosis. In addition, some other mechanisms of echinocytosis should be discussed. For example, the band 3 protein is known to be involved in the regulation of the shape of erythrocytes, through interactions with spectrin.[20] However, no changes in the level of the membrane-bound spectrin have been observed during the incubation of erythrocytes with 5-FU.[11] Also, it has been reported that an increase in intracellular pH can lead to echinocytosis, but that mechanism requires an increase of pH above 7.9.[21] Measurements of the changes of chemical shift of the ^{31}P NMR signal of 2,3-BPG during the incubation of erythrocytes with 5-FU[6] demonstrated that no such changes in pH could occur. Regardless of the exact cause of echinocytosis, rapid and irreversible changes induced by high concentrations of 5-FU bear importance for in vivo applications of 5-FU, since the in vivo counterpart of washing is the dilution of the initial dose of 5-FU within blood.

Effects of 5-FU on Potassium Transport in Erythrocytes. 5-FU induced an increase of K^+ efflux from the erythrocytes into the plasma (Figure 6). An increased efflux of K^+ has been demonstrated to be good evidence of disarrangements of the erythrocyte membrane structure;[22] however, this has been considered only to be an indicator of the extent of hemolysis. Our measurements showed that 5-FU does not induce any increase in the extracellular level of hemoglobin and that even ATP was not released following the treatment with 5-FU.[6] Moreover, an examination of the changes in the morphology also show that little or no hemolysis occurred in our experimental setup, since the observed degree of echinocytosis (Table 2) is known not to precede significant hemolysis.[17] Since the evidence rules out the possibility that hemolysis is a cause of the K^+ efflux,

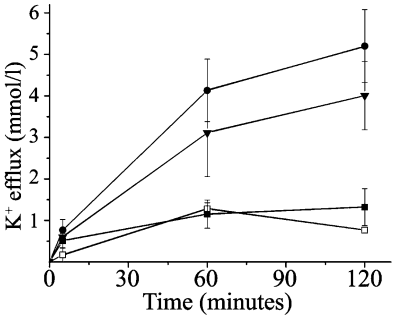

Figure 6. Efflux of potassium from erythrocytes treated with 5-FU: (●) 10, (▼) 6.5, (■) 3 mg/mL, and (□) control sample. Efflux is presented as the difference between the extracellular K^+ concentration in the sample and the concentration of K^+ in the plasma of fresh untreated blood (around 4.5 mmol/L).

Table 3. Effects of 5-FU on Membrane Fluidity and the Reversibility of Changes after 60 Minutes of Incubation and Removal of Extracellular 5-FU by Washing, Described through Order Parameter (*S*)[a]

period of incubation (minutes)	60	120	60 + washing
samples	order parameter (*S*)		
control	0.691 ± 0.008	0.692 ± 0.007	0.691 ± 0.001
5-FU 3 mg/mL	0.692 ± 0.010	0.692 ± 0.012	0.695 ± 0.015
5-FU 6.5 mg/mL	0.694 ± 0.009	0.700 ± 0.006	0.694 ± 0.006
5-FU 10 mg/mL	0.665 ± 0.012	0.667 ± 0.010	0.662 ± 0.008

[a] Values are presented as means of order parameter (*S*) ± standard deviation.

the increased efflux of K^+ points to more subtle changes of membrane integrity.

Most likely, the observed increase in K^+ efflux is induced by the depletion of ATP (Table 1), which leads to lower activity of the Na^+/K^+ pump, since this membrane ionic pump derives energy from ATP.[10] In addition, changes of erythrocyte membrane fluidity (see below) can also affect membrane permeability and transport systems[23] and should be taken into consideration.

Effects of 5-FU on the Membrane Fluidity of Erythrocytes. 5-FU concentrations of 3 and 6.5 mg/mL seem not to significantly change membrane fluidity (Table 3). However, at a concentration of 10 mg/mL of 5-FU, the order parameter (*S*) of the erythrocyte membrane decreases, showing increased membrane fluidity. The decrease of the *S* value induced by 5-FU at 10 mg/mL, which developed during the first 60 min of incubation, did not change after 120 min of incubation and is not altered upon washing of the erythrocytes. Although the removal of 5-FU leads to a decrease of the level and degree of echinocytosis (Table 2), no changes in the membrane fluidity occurred, indicating that actions of 5-FU on the erythrocyte membrane are much more complex than simple intercalation into the outer hemileaflet. The irreversibility of *S* can only be explained through intracellular changes, most likely an irreversible decrease of the ATP level, since it is known that membrane fluidity is dependent on ATP.[9]

In addition, the detachment of some membrane-associated proteins can also affect membrane fluidity,[24] and in relation to this, it has been reported that 5-FU induces changes in the protein content of the erythrocyte membrane.[11] Altered membrane fluidity can affect membrane permeability or transport systems;[23] thus, it could be an additional explanation for the increase in K^+ efflux. If the deformability of the erythrocytes is dependent on the membrane fluidity, which is still questionable,[25] the observed increase in erythrocyte membrane fluidity could explain the decrease in blood viscosity during 5-FU infusion, observed in vivo.[3]

To extrapolate the results of the in vitro study to in vivo conditions, realistic concentrations must be taken into consideration. The concentrations of 5-FU used in this study are higher than systemic concentrations in the blood of patients (usually around 0.15 mg/mL[5]); however, they are much lower than the maximal concentration at the site of application (50 mg/mL[5]). Since most of the observed changes are shown to be rapid, they should also occur locally in in vivo conditions, before the dilution of the drug occurs. In addition, because of the observed irreversibility, it can be concluded that most of the erythrocytes once significantly affected by 5-FU remain changed and, therefore, less functional and capable of normal oxygen transport and delivery.

CONCLUSIONS

Cardiotoxicity is a major problem in anticancer therapy using cytostatic drugs. Although the cardiotoxicity of cytostatic drugs is frequently a dose-limiting factor of the therapy and can lead to severe morbidity, the mechanisms are more-or-less unknown and remain to be elucidated. To the best of our knowledge, the main reason for this situation is that most of the conclusions are based on clinical studies and case reports, rather than strong experimental evidence. This study, as well as a previous one,[6] offers good experimental evidence of changes induced in erythrocytes by a cytostatic drug (5-FU), which is the most probable cause of cardiotoxicity, and such an approach can be a modus of how the cardiotoxicity of some other cytostatic drugs should be investigated.

5-FU cardiotoxicity is induced by the actions of 5-FU on erythrocytes as the main oxygen carriers. 5-FU rapidly passes the erythrocyte membrane and directly affects intracellular metabolism, inducing several major changes in the oxygen delivery system and provoking a rapid decrease of oxygen tension, which, if it occurs in vivo, can lead to ischemia and subsequent cardiotoxicity. However, in our opinion, the most important effect of 5-FU on erythrocytes is a drastic and relatively rapid decrease in the level of ATP, which leads to a number of other irreversible changes in erythrocyte structure and functioning, such as echinocytosis, an increased efflux of potassium, and an increase in membrane fluidity. In erythrocytes, cell shape change can be an indicator of major metabolic dysfunctions. We observed that 5-FU induces echinocytosis through two different mechanisms. Crenation due to the preferential intercalation of 5-FU into the outer hemileaflet of the erythrocyte membrane is an early and reversible process, while echinocytosis provoked by the decrease in ATP level dominates during prolonged incubation with 5-FU and is irreversible. Crenation is known to decrease the uptake and release of oxygen by erythrocytes,[18] so this change affects the erythrocyte's ability to deliver oxygen. 5-FU induces an increase in the efflux of potassium, which could be provoked by lower activity of the Na^+/K^+ pump that derives energy from ATP. K^+ efflux is also a sign of membrane structure disarrangements, and in relation to this,

we determined that 5-FU induces an irreversible increase in erythrocyte membrane fluidity, which is known to affect membrane permeability and transport systems. The increase in membrane fluidity can occur as a result of the drop in the ATP level or the detachment of membrane-associated proteins, which is an additional proof that changes induced by 5-FU in the erythrocyte membrane and on the intracellular level are strongly interrelated. In addition, the observed increase of fluidity of the erythrocyte membrane speaks against changes in blood rheology as the basis of 5-FU-induced cardiotoxicity and is in agreement with the decrease in blood viscosity during 5-FU infusion, observed in vivo. To conclude, 5-FU induces a set of fully or partially irreversible, interdependent changes on the intracellular level and on the level of erythrocyte membrane structure and functionality. Because of this set of changes that most likely also occur in vivo during 5-FU infusion, a certain amount of erythrocytes with an echinocytic shape and a diminished ability to deliver oxygen is present in the blood for longer periods of time. This renders oxygen transport and delivery more difficult, leading to events such as arterial hypoxemia in patients treated with 5-FU, thus leaving active tissues and organs such as the heart and brain with an insufficient supply of oxygen, so cardiotoxic effects in the form of ischemic damage occur.

Although, as yet, we do not have firm proof that the proposed mechanisms are responsible for the in vivo cardiotoxicity and an additional in vivo investigation of 5-FU cardiotoxicity should be made, we believe that the outlined set of methods is a potential scheme for investigating the mechanisms leading to cardiotoxicity caused by drugs that are already in clinical use, as well as novel cytostatic drugs.

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