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The combined effect of IDA and glutaraldehyde on the erythrocyte membrane proteins

A. Marczak *, M. Walczak, Z. Jóźwiak

Department of Thermobiology, University of Łódź, Banacha 12/16 Str, 90-237 Łódź, Poland

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Abstract

A number of investigators have been focusing their attention on the encapsulation of antineoplastic drugs within erythrocytes to diminish their side-effects. Glutaraldehyde is often used as crosslinking agent to link the drugs (including idarubicin, IDA) to the cells. The previous studies indicated that in glutaraldehyde-treated human erythrocytes the elevated level of drugs was observed but also the various changes in the organization of the red cells were noted.

In this study, we continue our investigations on the interaction of IDA and glutaraldehyde on the erythrocytes and now we concentrate on the effect of these compounds with the erythrocyte membrane proteins. For this purpose, SDS-gel electrophoresis of the cell proteins was carried out. Additionally, analysis of the disturbances of erythrocytes shape and size, accompanied by the application of flow cytometry and microscopy examination, were undertaken.

The fluorimetric method was used to estimate content of IDA in supernatants, after erythrocyte membranes incubation with different glutaraldehyde concentrations. It was observed that glutaraldehyde caused in gradually dependent manner an increase of percent of IDA linked to the cell membrane proteins. After this incorporation, perturbations in the content of the proteins in the cell membrane were observed. The protein aggregates and changes in the level of spectrin, band 3 protein and small mass proteins were noted. The use of flow cytometry and microscopy technique demonstrated also disturbances in the shape and size of erythrocytes. For all tested concentrations of glutaraldehyde, the changes were statistically significant.

Keywords: IDA; Glutaraldehyde; Erythrocyte; Cell membrane; Flow cytometry; Gel electrophoresis

1. Introduction

4-Demethoxydaunorubicin, also known as idarubicin (IDA) is an anthracycline antibiotic of the second generation. It is widely used in treatment of acute myelogenous leukemias (AMLs) as well as breast cancers (Minotti et al., 2004; Tallman et al., 2005; Marczak et al., 2006). Both, in vitro and in vivo studies indicate that IDA is more potent than daunorubicin, which is its parent compound (Lotfi et al., 2002). Moreover, side-effects such as cardiotoxicity or hair loss are indeed less frequent in comparison with other anthracyclines (Crivellari et al., 2004) but there are still some drawbacks about using this drug.

One of the most important approaches that overcome this limitation is application of drug carriers, which allows active targeting to required cells. The employment of these systems maximizes the therapeutic efficacy of the drug and reduces its systemic side-effects (Marcucci and Lefoulon, 2004).

The drug carriers can be divided into two classes: macromolecular conjugates such as polymers, dendrimers or monoclonal antibodies (Mabs) and particulate drug carriers such as liposomes, polymeric nanoparticles as well as dendrimers. In the first group, the drug is chemically linked to a macromolecule. In the second, the particulate drug carrier entraps the active chemical compound in a loading space (generally the core of the particulate). In comparison with macromolecular conjugates it isolates the drug from environment and provides a higher degree of protection from enzymatic inactivation. Moreover, the drug does not have to be linked to carrier, so one carrier can be encapsulated with different compounds (Marcucci and Lefoulon, 2004).

Erythrocytes can be also accepted as a particulate drug carrier, since they prevent drugs from degradation or inactivation.

* Corresponding author. Tel.: +48 42 6354481; fax: +48 42 6354473.
E-mail address: aszwar@biol.uni.lodz.pl (A. Marczak).

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As a part of our organisms they possess high biocompatibility, biodegradability as well as long circulation half-life (Gothoskar, 2004; Millan et al., 2004). Some of the encapsulated drugs, like IDA leak rapidly from red blood cells, but there is a chance to use crosslinkers such as glutaraldehyde, α-tocopherol, BS^3 or DTSSP (Jordan et al., 1997; Fan and Dash, 2001). We have been interested in encapsulation of anthracycline antibiotics into erythrocytes for a few years. We used the glutaraldehyde in our experiments as a crosslinker because of its small compound in comparison to other crosslinkers and because its not complicated structure. Glutaraldehyde as a bifunctional reagent can link anthracycline antibiotics to the protein. Our results of the effect of interaction of IDA or DNR and glutaraldehyde with intact erythrocytes were publicated earlier (Szwarocka and Jóźwiak, 1999; Szwarocka et al., 2001). In this article, we continue studies on the usage of glutaraldehyde to link more drug to the cell. Because our earlier work showed that after interaction of anthracycline antibiotics and glutaraldehyde with RBCs the alterations were observed in cell membrane lipids as well as in proteins, we wanted to look closer on the interaction of this compounds with isolated cell membrane proteins and compare these results with data obtained with ESR method.

The alterations in the content of proteins were monitored by SDS-gel electrophoresis. Because the proteins of the cytoskeleton and integral membrane proteins are responsible for the shape of red blood cells, after treatment erythrocytes with IDA and glutaraldehyde the changes in the shape of these cells were also measured.

2. Materials and methods

2.1. Preparation of erythrocyte suspensions and cell membrane

Human peripheral blood from healthy donors was collected in ACD solution and centrifuged at 600 × *g* for 10 min. Erythrocytes were separated from blood plasma and buffy coat by centrifugation (600 × *g*, 10 min) at 4 °C and washed three times with phosphate buffered saline, PBS (5 mM sodium phosphate buffer, containing 0.15 M NaCl, pH 7.4) and suspended in the same medium. Erythrocytes were used immediately after isolation. Erythrocyte membrane ghosts were obtained by hypotonic lysis according to the procedure of Dodge et al. (1963). The erythrocytes were hemolyzed in 20 mM phosphate buffer, pH 7.4, and washed several times with 10 mM and then 5 mM phosphate buffer containing 0.1 mM EDTA and 0.1 mM PMSF (phenylmethylsulfonyl fluoride) till hemoglobin was free. All operations were carried out at about 4 °C. Protein concentration was estimated using the method of Lowry et al. (1951) with bovine serum albumin as the standard.

2.2. The procedure of IDA encapsulation

Five percent of erythrocyte suspensions in PBS, pH 7.4, or cell membranes (100 μg of protein/ml) were incubated with idarubicin at a final concentration of 10 μg/ml (for erythrocytes) or 10 μg/mg of proteins (for cell membrane) in the dark for 30 min in a shaking bath at 37 °C. After incubation erythrocytes or cell membranes were treated with glutaraldehyde (final concentrations from 0.0005 to 0.005%) and were incubated at room temperature, 20–22 °C, for 30 min.

2.3. The content of drug in the extracellular medium

The content of idarubicin in the extracellular medium after incubation of drug pretreated erythrocyte membrane proteins with glutaraldehyde was determined by fluorescence analysis (emission at 545 nm, excitation at 490 nm). The fluorescence of idarubicin (10 μg/ml) in PBS was taken as 100%.

2.4. SDS-gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of erythrocyte membrane proteins was performed on a 5–20% running and 3% stacking gel by the method of Laemmli (1970) with slight modifications. Forty milligrams of solubilized with β-mercaptoethanol cell membrane proteins was applied onto polyacrylamide gels. The gels were stained Coomassie Brilliant Blue R-250 by the method of Fairbanks et al. (1971) and analysed with DESAGA CD-60 densitometer (Heidelberg, Germany).

2.5. Flow cytometry

Control cells and erythrocytes preincubated with drug and glutaraldehyde were analysed with a flow cytometer Becton Dickinson, LSR II. Cell size and shape were appreciated with simultaneous separate detection of low angle (FSC-A) and right angle (SSC-A) light scattering. The data obtained were displayed in the form of a diagram of cell number versus light scatter and were analysed by standard computer program WinMDI 2.8. The light scattered near the forward direction (low angle) is expected to be proportional to size (volume) of the particle and is independent on the cell refractive index and shape, whereas scattering at the right angle depends on cell shape and internal properties of the scattering particles (Salzman et al., 1990). FSC/SSC is a dual parameter contour plot histogram proportional to the total cell diversity. Data were displayed in the form of diagrams of cell numbers versus light scatter. For each set of histograms, the percentage of altered cells and peak position (*P*) was obtained from values collected by using a standard computer program for the cytometer.

2.6. Phase contrast microscopy

Erythrocytes incubated with drug and glutaraldehyde were fixed on a glass surface and the samples were observed using a phase contrast microscope (Olympus, Japan) at 600× magnification.

2.7. Statistical analysis

For statistical analysis Student's paired *t*-test had to be used. It was obligatory for data presented in this work because of

inter-individual differences. The difference was considered to be significant for $p < 0.05$. For each experiment, blood samples (control and incubated with chemical compounds) were taken from the same individual. Mean value was calculated from six donors, whereas for each donor an experimental point was a mean value from three replications (Zgirski and Gondko, 1998).

3. Results

3.1. The effect of glutaraldehyde on the binding of IDA with erythrocyte membrane proteins

The fluorimetric method was used to estimate the content of IDA in supernatants, after incubation of erythrocyte membranes with different glutaraldehyde concentrations.

The obtained results are presented in Fig. 1. To calculate the amount of IDA in supernatants, drug fluorescence in 5 mM sodium phosphate buffer (pH 7.4) was accepted as a 100%. The concentration of IDA and membrane proteins was the same in every sample, that is 10 μg/ml and 1.5 mg/ml, respectively.

Analysis of the obtained data indicates that erythrocyte membranes incubated with drug linked only about 30% of IDA. Content of anthracycline antibiotic in the medium decreases after treatment of the cell membranes with glutaraldehyde. Noticeable, a decrease of drug concentration in supernatant is observed even for the smallest concentration of glutaraldehyde. However, the results are statistically significant when its concentration is higher than 0.0005%. The application of glutaraldehyde in concentration of 0.0025% and 0.005% increases the content of linked IDA by 18% and 45%, respectively in comparison with the membranes incubated with IDA only (Sample 0).

3.2. The effect of IDA and glutaraldehyde on the content of the cell membrane proteins

Erythrocyte membranes were incubated with IDA (10 μg/ml) and variable concentrations of glutaraldehyde. Content estimation of main protein fractions had been done after SDS-PAGE and densitometred of received gels. The following protein fractions were identified: spectrin 1 and 2, ankyrin, protein band 3, band 4.1, band 4.2, band 4.9, actin, stomatin, small mass proteins and hemoglobin (Hb). The protein aggregates could be also easily marked on the gel. They appeared in samples incubated with glutaraldehyde, even at the smallest concentration (0.0005%).

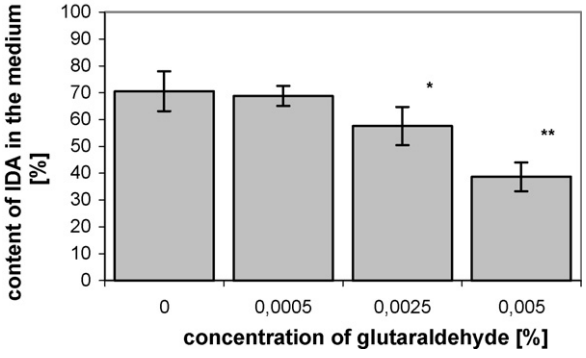

Fig. 1. The changes in the content of idarubicin in the medium after incubation of drug-treated cell membrane proteins with glutaraldehyde. The fluorescence of IDA (10 μg/ml) in PBS was taken as 100%. Values statistically significant in comparison to the samples incubated with drug only: $^{*}p < 0.05$; $^{**}p < 0.0001$.

The control sample densitogram is showed in Fig. 2(I). It contains protein fractions, which were analysed further. In this case there was no protein aggregates observed.

Fig. 2(A–C) shows exemplary densitograms for erythrocyte membranes incubated with IDA (A), glutaraldehyde (0.005%; B) and IDA with glutaraldehyde (0.005%; C). The characteristic fraction of protein aggregates was observed both in glutaraldehyde and drug plus glutaraldehyde-treated samples. However, the highest peak value was noticed when the membranes were incubated with IDA and glutaraldehyde.

The results together with standard deviations (S.D.) are presented in Fig. 3. It was shown that IDA (10 μg/mg of proteins) caused only slight changes in the level of proteins. Statistically significant decrease was noticed only in the spectrin content. Decrease in the level of this protein is also clearly noticeable in the densitograms (Fig. 2B and C). These data clearly show the correlation between concentration of glutaraldehyde and level of protein aggregates. The percentage of protein aggregates becomes higher due to increase in glutaraldehyde content and is 5.19%, 6.65% and 9.68% for concentration of glutaraldehyde 0.0005%, 0.0025% and 0.005%, respectively.

There is no statistically significant difference between the membranes incubated with only glutaraldehyde and with glutaraldehyde and IDA.

Fig. 3 shows that the biggest changes in percentage concentrations are observed for spectrin, band 3 protein and small mass proteins.

3.3. Estimation of morphological changes in erythrocytes incubated with IDA and glutaraldehyde

Light microscope and flow cytometry techniques were used to analyse erythrocytes shape and size. The results obtained for control samples are presented in Fig. 4.

It can be noticed that native human red blood cells have biconcave disc shapes. Histograms in Fig. 4B and C show that population of control cells is homogenous in respect of size and shape. There is no perturbation observed in external cell membrane. Typical scattering curves plotted on FSC-A and SSC-A diagrams are smooth, sharp and almost symmetric and surface contours plotted on FSC-A/SSC-A diagrams are greatly concentrated.

Figs. 5–7 present histograms, which were prepared by the usage of flow cytometric to estimate the morphological changes in red cells. After FSC-A histograms analysis, which gives information about cell size it can be noticed that IDA alone causes small changes in this parameter (Fig. 5; Table 1). However, the presence of glutaraldehyde, even at the lowest concentration induces formation of numerous irregular cells. Smaller cells are also observed and their amount is becoming higher due to increase in glutaraldehyde concentration. When the erythrocytes were incubated with glutaraldehyde, two different peaks in their

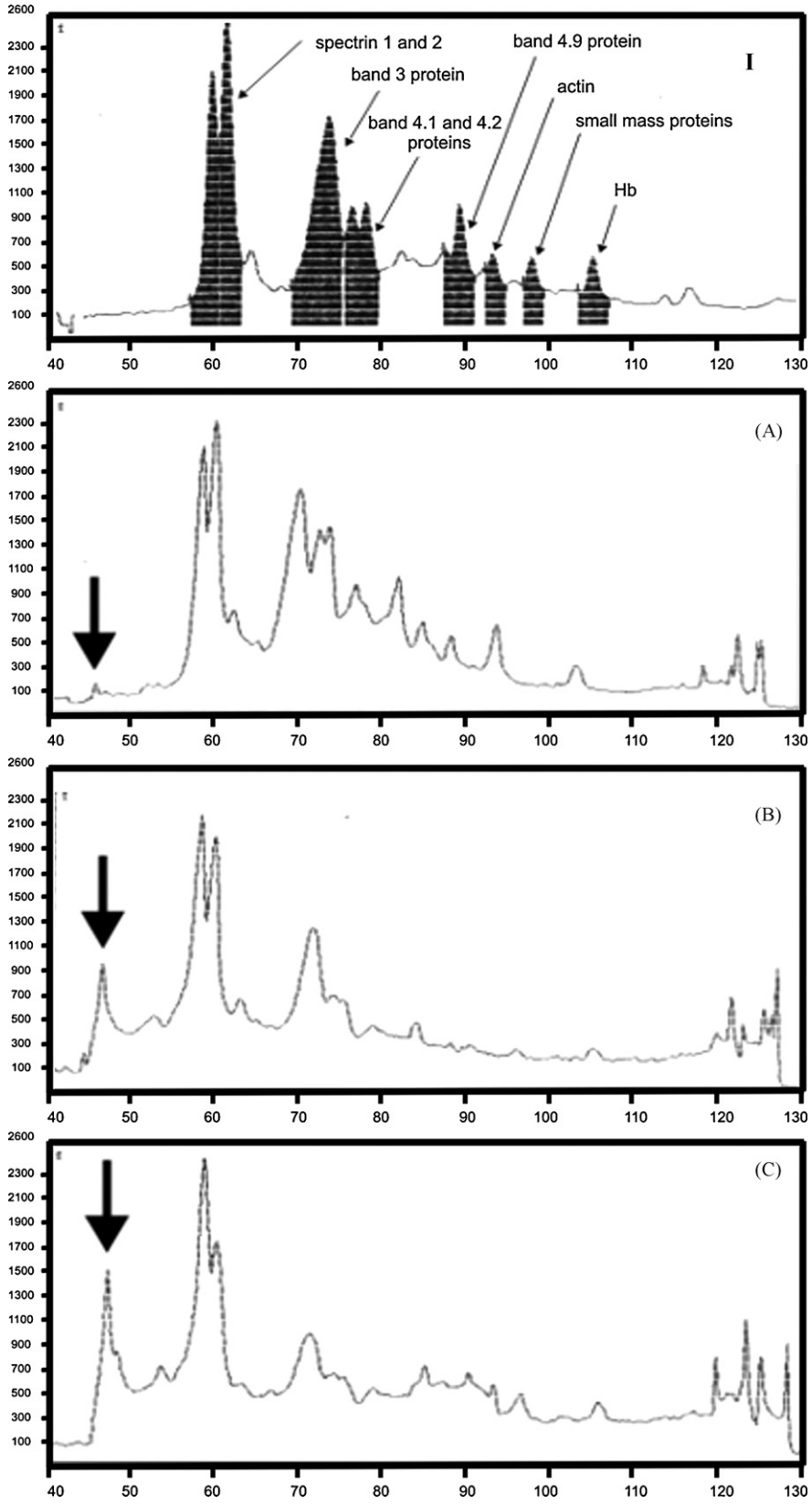

Fig. 2. The control sample densitogram with marked protein fractions used for analysis (I) and densitograms obtained from: (A) erythrocyte membranes + IDA (10 μg/mg of proteins); (B) erythrocyte membranes + glutaraldehyde (0.005%); (C) erythrocyte membranes + IDA (10 μg/mg of proteins) + glutaraldehyde (0.005%). The arrow indicates peak, which is characteristic for protein aggregates.

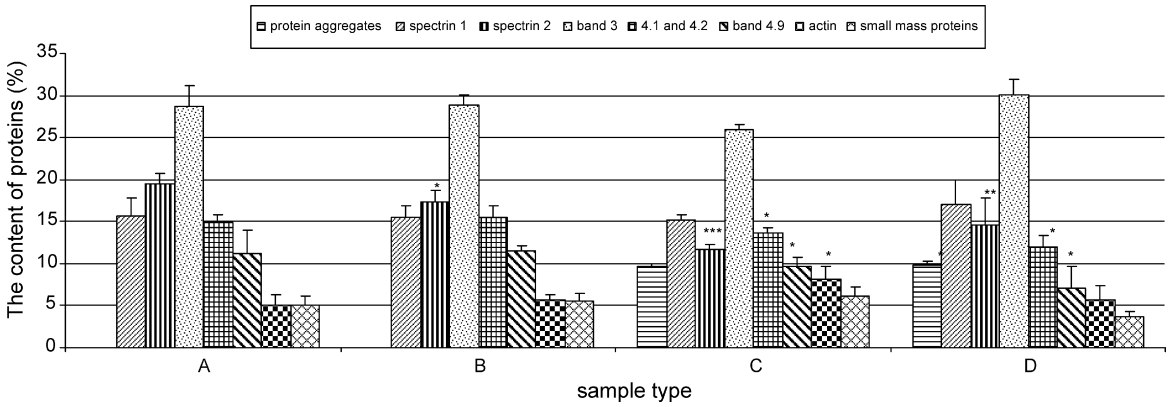

Fig. 3. The effect of IDA and glutaraldehyde on the percent content of the cell membrane proteins. (A) Control membrane proteins; (B) membrane proteins + IDA (10 μg/mg of protein); (C) membrane proteins + glutaraldehyde (0.005%); (D) membrane proteins + IDA (10 μg/mg of protein) + glutaraldehyde (0.005%). Significantly different when compared to values obtained for control erythrocytes: $^{*}p < 0.05$.

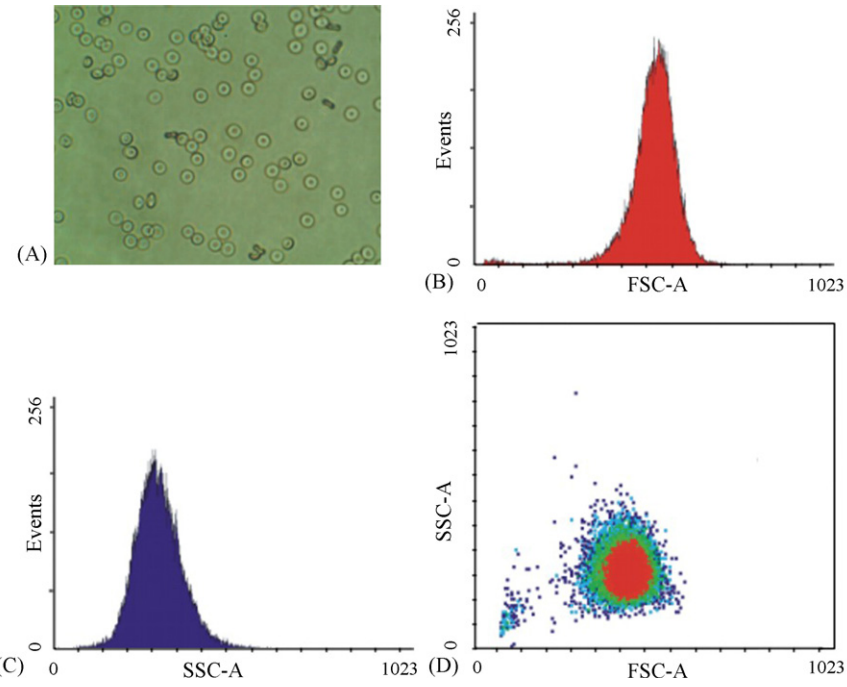

Fig. 4. Micrographs (A) and scattering diagrams of human control erythrocytes incubated in isotonic solution. The FSC-A diagrams (B) represent the light scattered near the forward direction (proportional to the value of the particles). The SSC-A diagrams (C) represent scattering at the right angle (depended on cell shape and internal properties). The FSC-A/SSC-A diagram (D) is a dual parameter contour plot proportional to the total cell diversity.

FSC-A histograms were be noticed. One of them describes native red cells population. The other, situated near *y*-axis, comes from abnormal population of cells (Fig. 6; Table 1).

The combined effect of IDA and glutaraldehyde causes as well change of cell sizes (Fig. 7; Table 1). The second peak, which originates from smaller cells, becomes stronger due to increase in glutaraldehyde content. The data included in Table 1 show that glutaraldehyde alone causes similar alterations in cellular sizes as in connection with drug. It suggests that glutaraldehyde is mainly responsible for these changes.

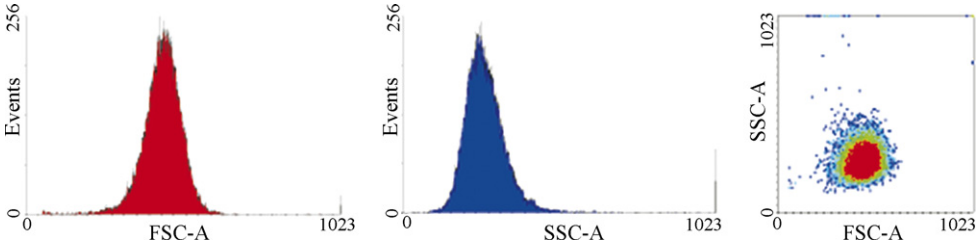

Fig. 5. Scattering diagrams of human erythrocytes incubated with IDA (10 μg/ml).

Table 1
Flow cytometric analysis of the changes in the size and shape of erythrocytes incubated with IDA and glutaraldehyde

Sample	% of unaltered cells	Peak position	
		First peak	Second peak
Control erythrocytes	97.0 ± 0.8	440.21 ± 30.02	–
Erythrocytes plus IDA	94.0 ± 4.5*	395.40 ± 43.57*	–
Erythrocytes incubated with glutaraldehyde (%)			
0.0005	83.0 ± 11.0*	371.88 ± 30.67*	231.06 ± 13.54
0.0025	78.5 ± 14.9**	368.15 ± 29.10*	223.90 ± 13.35
0.005	76.1 ± 11.9**	370.23 ± 25.77*	215.84 ± 22.32
Erythrocytes incubated with IDA and glutaraldehyde (%)			
0.0005	83.0 ± 12.1*	382.55 ± 47.69*	220.43 ± 15.83
0.0025	78.3 ± 16.0*	359.53 ± 22.80*	249.86 ± 2.33
0.005	75.6 ± 17.9*	384.06 ± 16.30*	230.33 ± 3.66

Data are expressed as a percentage of erythrocytes between the cursors relative to total cell amount (%) and the peak position in arbitrary units from histograms FSC-A. Significantly different when compared to values obtained for control erythrocytes: $^{*}p<0.05$; $^{**}p<0.001$.

SSC-A histograms provide information about cell shape and the structure of outside cell membrane surface. Both, analysis of percentage content of normal cells and location of the highest peak of histograms show that IDA alone only slightly modifies these parameters (Fig. 5; Table 2). However, our results suggest that glutaraldehyde significantly reduced the number of native erythrocytes. The alterations in shape and the structure of membrane surface enlarge, when the concentration of glutaraldehyde increases (Fig. 6). Similar perturbations are observed in erythrocytes incubated with IDA combined with glutaraldehyde (Fig. 7). In this case, glutaraldehyde is the major agent inducing these alterations.

Fig. 8 presents microscopic photographs of red blood cells, which were incubated in different conditions (magnification 600×). There are no changes observed in cells shape after incubation with IDA at concentration of 10 μg/ml. However, erythrocytes incubated with glutaraldehyde alone show some irregularities. In these samples, stomatocytes are observed. Number of changed cells increases due to enlargement of glutaraldehyde concentration. The combined effect of IDA and

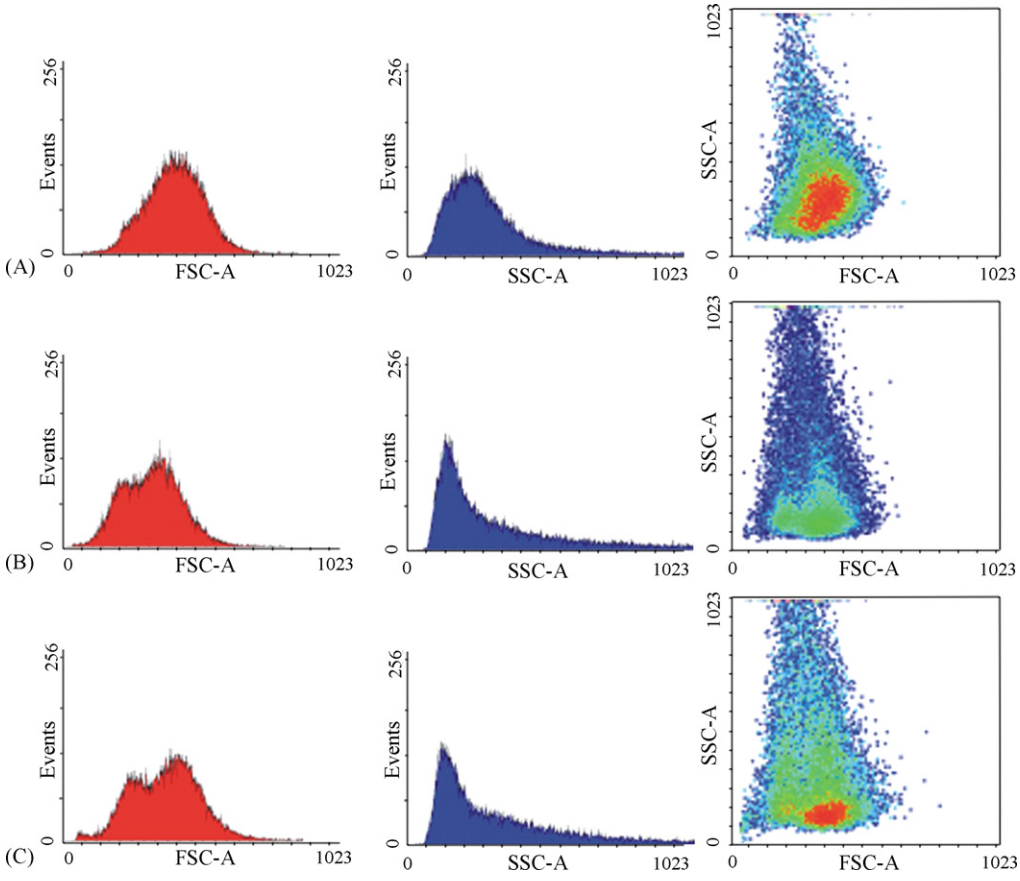

Fig. 6. Scattering diagrams of human erythrocytes incubated with glutaraldehyde at concentrations, respectively: (A) 0.0005%; (B) 0.0025%; (C) 0.005%.

Table 2
Flow cytometric analysis of the properties of the erythrocyte cell membrane surface after incubation with IDA and glutaraldehyde

Sample	% of unaltered cells	Peak position
Control erythrocytes	98.1 ± 0.1	271.22 ± 47.56
Erythrocytes plus IDA	96.2 ± 2.5	256.83 ± 20.77
Erythrocytes incubated with glutaraldehyde (%)		
0.0005	83.1 ± 11.4*	171.72 ± 48.22*
0.0025	75.2 ± 12.6*	146.63 ± 21.15*
0.005	67.2 ± 9.1**	142.48 ± 30.50**
Erythrocytes incubated with IDA and glutaraldehyde (%)		
0.0005	78.7 ± 8.3*	218.31 ± 67.86
0.0025	80.1 ± 6.2*	178.16 ± 41.66*
0.005	78.3 ± 8.9*	226.70 ± 42.21

Data are expressed as a percentage of erythrocytes between the cursors relative to total cell amount (%) and the peak position in arbitrary units from histograms SSC-A. Significantly different when compared to values obtained for control erythrocytes: $^{*}p < 0.05$; $^{**}p < 0.001$.

glutaraldehyde intensifies perturbations and increases quantity of agglutinated erythrocytes. These results confirm data obtained by flow cytometry usage.

4. Discussion

Cancer chemotherapy remains still largely non-specific. The drugs administered mainly intravenous are toxic for tumor cells as well as for normal cells. To diminish the toxic side-effects of the drugs various carriers are used. We are interested in encapsulation of antineoplastic drugs as idarubicin (IDA) within erythrocytes. To enhance its content in the erythrocytes the glutaraldehyde was used as the crosslinker.

Our previous studies (Szwarocka and Jóźwiak, 1999; Szwarocka et al., 2001) and results of other workers (Matherne et al., 1994; Ataullakhanov et al., 1996) have revealed that the application of glutaraldehyde increases the entrapping of drugs (daunorubicin, idarubicin or doxorubicin) by intact erythrocytes in comparison with sample incubated with drug only. Results obtained in this paper confirm foregoing data. Tests on isolated erythrocyte membranes show positive effect of glutaraldehyde on amount of IDA linked to membranes. Glutaraldehyde at concentration of 0.0025% and 0.005% increases the content of IDA in erythrocyte membranes by 18% and 45%, respectively. The samples were compared to control membranes incubated with drug only. SDS-PAGE of the proteins obtained from plasma membrane of glutaraldehyde-treated cells and following densitometric assay showed changes in the particular membrane fractions, especially spectrin 2, band 3 and small mass proteins. Moreover, obtained gels and densitograms indicated formation of protein aggregates in presence of glutaraldehyde, even in the smallest concentration (0.0005%). About 10% of aggregates were observed, when the concentration of aldehyde was 0.005%. The changes were slightly stronger in samples incu-

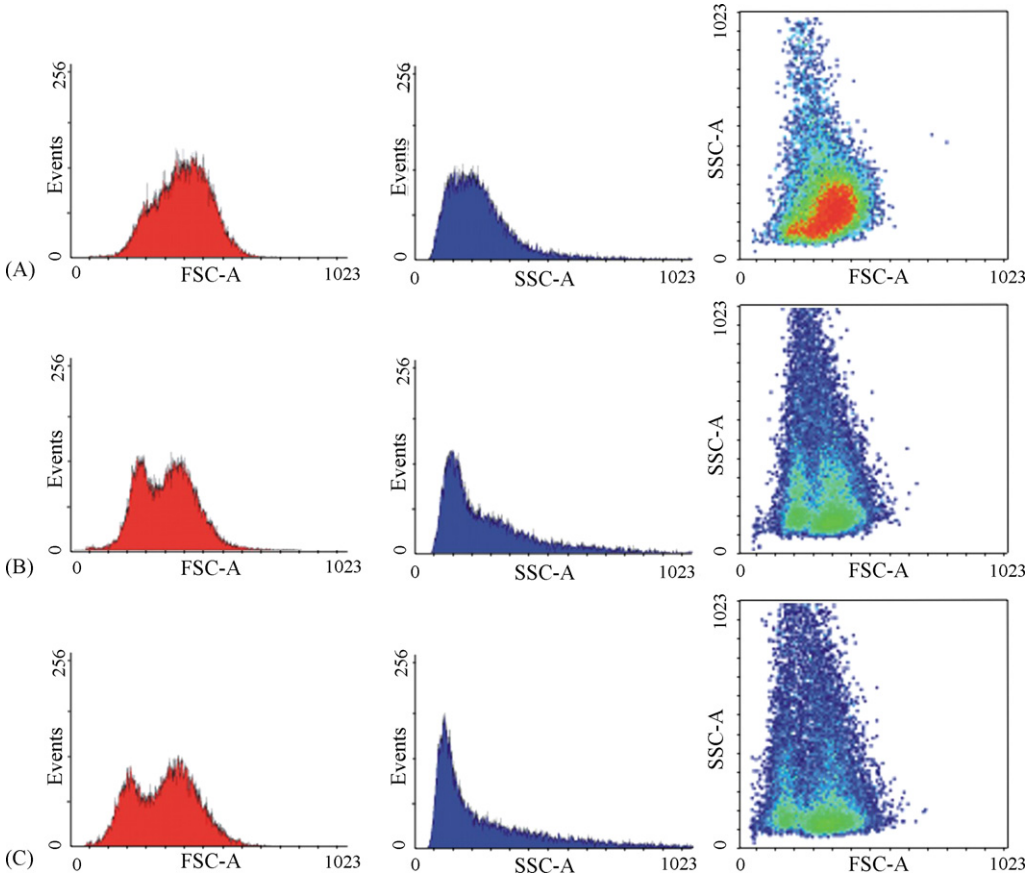

Fig. 7. Scattering diagrams of human erythrocytes incubated with IDA (10 μg/ml) and glutaraldehyde at the concentrations, respectively: (A) 0.0005%; (B) 0.0025%; (C) 0.005%.

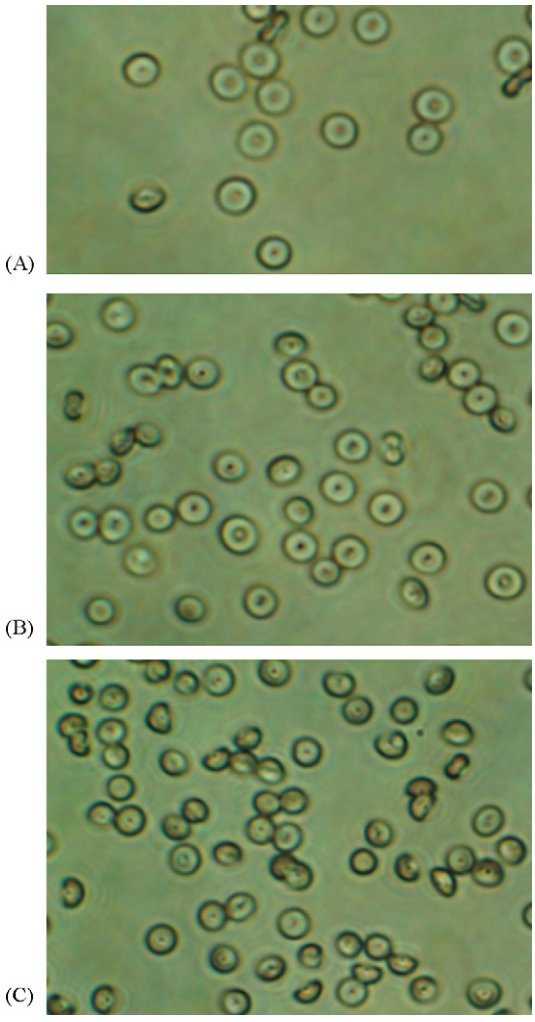

Fig. 8. Microscopic pictures of erythrocytes incubated with tested agents: (A) IDA (10 μg/ml); (B) glutaraldehyde (0.005%); (C) IDA (10 μg/ml) and glutaraldehyde (0.005%).

bated with IDA and glutaraldehyde. IDA alone caused only small perturbation in concentration of spectrin.

The above information and the previous studies suggest that both IDA alone and the combined effect of drug and glutaraldehyde induced changes in membrane proteins, which led to increased protein–protein interaction. It was also observed in erythrocytes of healthy donors and patients with acute myeloid leukemia where idarubicin caused conformational changes in membrane proteins (Szwarocka et al., 1996, 2001; Marczak et al., 2006).

In the presence of glutaraldehyde, a decrease in the W/S ratio of maleimide spin probe was observed in probes both with glutaraldehyde only and preincubated with IDA. The results imply a stronger immobilization of maleimide spin label in the erythrocyte membranes after incubation with glutaraldehyde. After treatment erythrocytes with 0.005% glutaraldehyde the changes were about 40% of control cells. (Szwarocka et al., 2001). It is correlated with present data.

Every change able to produce a loss of membrane integrity especially changes in the content of spectrin will provoke a transformation in the cell shape leading to a decreased cellular deformability and survival (Farsad and de Camilli, 2003; Pawlikowska-Pawlega et al., 2003; Suwalsky et al., 1996; Pascual et al., 1997).

Therefore, because we observed changes in the spectrin and other proteins of cytoskeleton network we studied whether the interaction of IDA and glutaraldehyde with membrane proteins may lead to changes of the erythrocyte shape and size.

The flow cytometry method was used to confirm modifications in cell shapes and sizes, which were suggested by changes in the spectrin content. Analysis of FSC-A histograms, both surface under curves and peak locations proved that native erythrocyte population decreases and appeared the stomatocytes with increasing of glutaraldehyde concentration. The cells are smaller in comparison with normal discocytes. When the highest concentration of glutaraldehyde (0.005%) was used, the population of regular cells diminished in comparison with control by 24%. Moreover, the appearance of additional peak, which was situated near *y*-axis and came from smaller cells was noticed. The combined effect of IDA and glutaraldehyde had similar negative influence on this parameter. However, drug alone caused only negligible changes in cell sizes. It suggests that glutaraldehyde is mainly responsible for these perturbations, when it is linked to IDA.

SSC-A histograms, which provide information about cell shape and the structure of outside cell membrane surface indicate that glutaraldehyde negatively modifies as well these erythrocyte factors. The alterations are becoming stronger due to increase in glutaraldehyde concentration. When 0.005% concentration of this compound was used, approximately 38% of cells were described by changed parameters. Also the peak displacement in SSC-A histogram is observed.

The above observations are confirmed by optical microscopy assay. We observed that in contrast to the biconcave disc shape typical for normal red cells, erythrocytes incubated with glutaraldehyde displayed remarkable perturbations in their morphology. Numerous irregular cells were found. The stomatocytes and agglutinated cells were observed. Quantity of changed cells increased due to enlargement of glutaraldehyde concentration. There were only small modifications in erythrocyte shapes and size noticed after incubation with IDA only.

The featured data suggest that application of glutaraldehyde provides the possibility to entrap much more of IDA by erythrocyte membranes. However, the results also show that aldehyde in the drug-pretreated erythrocytes can produce significant perturbations in the structure and function of the cells. On the other hand, suitable selection of glutaraldehyde concentration could make this compound very useful in application of erythrocytes as carriers of drugs, especially anthracyclines as idarubicin.

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