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The interaction of DNR and glutaraldehyde with cell membrane proteins leads to morphological changes in erythrocytes

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Abstract

In this study, the effects of DNR and glutaraldehyde on isolated erythrocyte membrane proteins were examined. For this purpose, SDS–gel electrophoresis was carried out. Additionally, analyses of the disturbances in erythrocyte shape and size, accompanied by the application of flow cytometry and microscopy examination, were undertaken. The amount of DNR linked to erythrocyte cell membranes was measured by a fluorimetric technique. It was observed that glutaraldehyde caused in concentration dependent manner an increase of percent of DNR linked to cell membrane proteins. After this incorporation, perturbations in the protein content of cell membranes were observed. The protein aggregates and changes in the level of spectrin, actin and band 3 protein were noted. Due to the changes in spectrin, which is mainly responsible for maintenance of the discocyte shape of erythrocytes, flow cytometry and microscopy techniques were used to control the size and shape of erythrocytes after treatment with DNR and glutaraldehyde. The disturbances in the shape and size of erythrocytes were observed for all tested concentrations of glutaraldehyde. For all tested concentrations of glutaraldehyde, the changes were statistically significant.

Keywords: DNR; Glutaraldehyde; Erythrocyte; Drug delivery

1. Introduction

Daunorubicin (DNR) is an anticancer drug which belongs to the anthracycline group of antibiotics. Even though DNR is one of the oldest drugs in this group, it is still preferred in mono- or combined therapy, in the treatment of patients with some types of malignances, such as acute myelogenous leukemias and breast cancers [1–5]. Its anticancer properties are linked to the formation of intercalative complexes with DNA, inhibition of topoisomerase II activity, generation of reactive oxygen species, and induction of apoptosis [2,6,7]. Unfortunately, its usage, just like that of many other anticancer drugs, is restricted by severe side-effects: mainly cardiotoxicity, nephrotoxicity and suppression of bone marrow function [8–11]. These side-effects are a big problem in anticancer therapy and stimulate a search for effective solutions.

One of the most important approaches that overcome this limitation is the application of drug carriers, which allow active targeting at required

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cells. The employment of these systems maximizes the therapeutic efficiency of the drug and reduces its systemic side-effects [12]. The use of erythrocytes as biological carriers offers an alternative to other carrier systems such as liposomes or nanoparticles that have been used to encapsulate different drugs, enzyme systems and therapeutic peptides. Being part of our bodies, erythrocytes possess high biocompatibilities, biodegradability, as well as long circulation half-life. They are also easy to handle. Moreover, carrier erythrocytes circulate well in vivo, can be targeted at specific organs and can carry large quantities of encapsulated drugs [13–17].

Some encapsulated drugs, such as anthracycline antibiotics leak rapidly from red blood cells, but there is a possibility to use cross-linkers to prevent drug efflux from erythrocytes and enhance the quantity of the drug in the cells [18,19]. In our study, we used glutaraldehyde as a cross-linker. We had been interested in encapsulation of anthracycline antibiotics in erythrocytes for a few years. We used glutaraldehyde as a cross-linker in our experiments due to its small particles in comparison to other cross-linkers and because it does not have a complicated structure. Glutaraldehyde as a bifunctional reagent can link anthracycline antibiotics to proteins [20]. The results of our study on the effects of interaction of IDA or DNR and glutaraldehyde with intact erythrocytes were published earlier [21,22]. In this manuscript, we continue studies on the usage of glutaraldehyde to link more drugs to cells. In our earlier studies, we observed that with this cross-linking agent a lot more drugs were entrapped in red cells. However, glutaraldehyde, in drug-pretreated erythrocytes produces significant perturbations in the organization of plasma membrane lipids and proteins. After treating erythrocytes with DNR and glutaraldehyde, alterations in fluidity were observed in the polar regions as well as in the deeper regions of cell membranes. Using the ESR method, we estimated that the incorporation of drug and glutaraldehyde into human erythrocytes also caused conformational alterations in membrane cytoskeletal proteins and changes in the internal viscosity of cells [21–23].

In this work, we wanted to look closer on the interaction of daunorubicin (DNR) and glutaraldehyde with isolated erythrocyte membrane proteins. We wanted to compare these results with data obtained with the ESR method. We monitored alterations in the content of proteins by SDS–gel electrophoresis. As proteins of the cytoskeleton and integral membrane are responsible for the shape of red blood cells, changes in the shape of these cells were also measured.

2. Materials and methods

2.1. Preparation of erythrocyte suspensions

Human peripheral freshly drawn (maximally 5-h-old) blood from healthy donors in ACD solution was centrifuged at 600*g* for 10 min at 4 °C. Blood was supplied from six donors and blood samples were studied separately for each donor. After removal of plasma and buffy coat, erythrocytes were washed three times in phosphate buffered saline, PBS, (5 mM sodium phosphate buffer, containing 0.15 M NaCl, pH 7.4) and suspended in the same medium. Erythrocyte membrane ghosts were obtained by hypotonic lysis according to the procedure of Dodge et al. [24]. 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 (phenylmethyl-sulfonyl fluoride) till hemoglobin was free. Protein concentration was estimated using the method of Lowry et al. [25] with bovine serum albumin as the standard.

2.2. The procedure of interaction of DNR with erythrocytes or cell membranes

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

2.3. The content of drug in extracellular medium

The content of daunorubicin in the extracellular medium after incubation of drug-pretreated erythrocyte membrane proteins with glutaraldehyde was determined by fluorescence analysis (emission at 595 nm, excitation at 488 nm). The fluorescence of daunorubicin (10 μg/ml) in PBS was taken as 100%.

2.4. SDS–gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of erythrocyte proteins was carried out by the method of Laemmli [26] with slight modifications. After the solubilization of ghost proteins in an SDS-buffered solution with β-mercaptoethanol, 40 mg of