Acta Radiologica Oncology 21 (1982) Fasc. 6

FROM THE ISTITUTO DI CHIMICA BIOLOGICA DELL'UNIVERSITÀ DI PADOVA, DIVISIONE DI RADIOTERAPIA E MEDICINA NUCLEARE AND SERVIZIO DI FISICA SANITARIA DELL'OSPEDALE CIVILE DI PADOVA, ITALY.

SENSITIVITY TO RADIATIONS OF Mg^{2+} AND $(Ca^{2+}+Mg^{2+})$ ATPase ACTIVITIES ASSOCIATED WITH ERYTHROCYTE MEMBRANE FRAGMENTS

G. SCUTARI, G. BALLESTRIN, E. VENTURELLI, P. L. ZORAT, L. TOMIO, F. CALZAVARA, C. CAUZZO, A. BONINSEGNA and D. BRANCA

The erythrocyte plasma membrane is known to possess, besides the (Na^+-K^+) ATPase, divalent cation-dependent ATPases whose activities appear to be associated with several essential cellular functions such as the outwardly directed calcium pump and the maintenance of the red cell shape (SCHATZMANN & VINCENZI 1969, ROSENTHAL et coll. 1970, GUIDOTTI 1972, REGA & GARRAHAN 1975, SCHATZMANN 1975, LYNCH & CHEUN 1979, ROUFOGALIS 1979).

Previously (SCUTARI et coll. 1980) it was shown that the activity of both the $(Ca^{2+}+Mg^{2+})$ and the Mg^{2+} ATPase is related to the redox state of the membrane thiol groups; in fact these enzymatic activities are substantially inhibited after exposure of red cell disrupted membranes to chemical agents capable of oxidizing pairs of thiols close to each other. This effect can be ascribed both to a general disturbance of the membrane assembly (and thus of the ATPases' microenvironment) and to the oxidation of pairs of thiols involved in the activity of these ATPases.

One of the best known agents capable of affecting the redox state of membrane thiols is represented by ionizing radiation, which has been demonstrated to induce significant alterations of several cellular functions (MYERS & LEVY 1964, MYERS & BIDE 1966, SHAPIRO et coll. 1966, SUTHERLAND & PIHL 1967), but the effect on the activity of the membrane-bound divalent cation-dependent ATPases has not yet been assessed. In view of the widespread use of ionizing radiation as therapeutic means and of the well recognized injuries caused to red blood cells by the inhibition of the divalent cation-dependent ATPases (SCHATZMANN & VINCENZI, ROSENTHAL et coll., GUIDOTTI, REGA & GARRAHAN, SCHATZMANN, LYNCH & CHEUNG, ROUFOGALIS) it appeared of interest to investigate the effects of irradiation of disrupted human erythrocyte membranes on the activity of these enzymes.

Methods

Preparation of disrupted erythrocyte membranes. Outdated human blood in sterile bottles containing acid-citrate-dextrose was kindly donated by the Hospital Blood Bank (Padova, Italy). Erythrocytes were separated from plasma by centrifugation at 1600×g for 20 min at 2°C; the buffy coat was removed by careful suction and the erythrocytes were then washed 4 times in 20 volumes of 125 mmol/l NaCl, 30 mmol/l 4-(2-hydroxyethyl)-1-piperazinethanesulphonate (Na-Hepes) pH 7.8. Following hemolysis in 10 volumes of ice-cold 6 mmol/l Na-

Accepted for publication 27 July 1982.

Hepes, pH 7.4, disrupted membranes were sedimented at 50000×g for 30 min at 0°C, incubated for 5 h at room temperature in the same hypotonic buffer and finally washed 4 times. The essentially hemoglobin-free membranes obtained were then assayed for protein content according to LOWRY et coll. (1951) using bovine serum albumin as a standard, suspended in the hypotonic Hepes buffer at a concentration of 10 mg protein/ml and kept frozen at −20°C until used. The membrane fragments spontaneously seal to form a population of vesicles which appear impermeable to sucrose upon centrifugation on 50 per cent w/v sucrose.

The percentage of inside-out vesicles in the mixed population was assayed, before and after irradiation, by using the acetylcholinesterase accessibility according to WAISMAN et coll. (1981) and was in the range of 45 to 55 per cent for all the membrane preparations used.

Irradiation of erythrocyte membranes. Disrupted erythrocyte membranes, suspended in the Hepes-buffer used for isolation at a concentration of 10 mg protein/ml, were placed in a pyrex container so as to obtain a thin and uniform layer of approximately 2 mm. During the irradiation the membrane suspension was constantly kept at 0°C by means of an ice bath.

Membranes were irradiated using a direct beam of radiation from a ^{60}Co source (activity 3.7×10^{13} Bq; 1000 Ci) placed under the sample container in order to avoid scattering by the edge of the container itself. The radiation beam was perpendicular to the sample surface and wide enough to allow the whole suspension to receive a homogeneous radiation dose. The apparatus used was of the clinical type and was arranged so as to obtain a minimum fire-target distance (40 cm), and consequently a maximum dose rate. It should be noted that before reaching the sample the radiation beam had to pass through a 0.5 mm thick Mylar bed, the PVC bottom of the thermostating bath (density 1, 1.8 mm thick), the ice bath (2 cm) and the bottom of the pyrex sample container (2.4 mm). However, in terms of irradiation efficiency these materials may be considered to be of homogeneous density approximately equal to 1, and the resulting total thickness was higher than that of the build-up region.

Different membrane suspensions were treated with 50 Gy and 100 Gy at a dose rate of 1.76 Gy/min. The irradiation was confirmed on line by an ionization dosimeter.

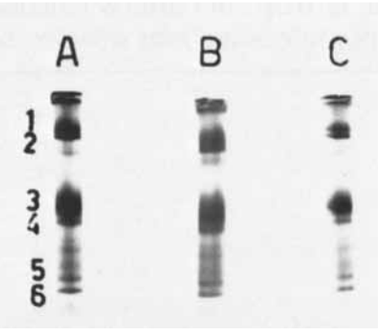

Fig. 1. Polyacrylamide gel electrophoresis of control (A), 50 Gy (B) and 100 Gy (C) irradiated erythrocyte membranes (50 μg of membrane proteins). The numerical designation of the major polypeptides is adopted from FAIRBANKS et coll. (1971).

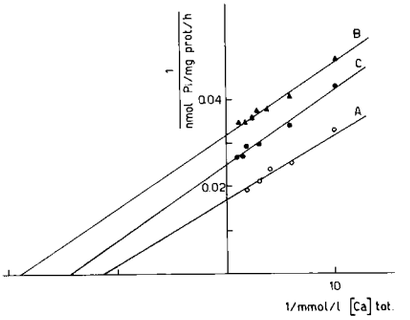

Fig. 2. Double reciprocal plot of the $(Ca^{2+}+Mg^{2+})$ ATPase activity of control (A), 50 Gy (B) and 100 Gy (C) irradiated erythrocyte membranes as a function of the total calcium concentration. Incubation medium: 120 mmol/l NaCl, 40 mmol/l Na-Hepes, pH 7.4, 4.5 mmol/l ouabain, 0.5 mmol/l ATP-Tris, 2 mmol/l $MgCl_2$ plus the indicated $CaCl_2$ concentrations.

Measurement of ATPase activities. Disrupted erythrocyte membranes (2 mg protein/ml) were incubated under constant stirring at 30°C in a reaction mixture containing 120 mmol/l NaCl, 40 mmol/l Na-Hepes, pH 7.4, 4.5 mmol/l ouabain, and different concentrations of $MgCl_2$, $CaCl_2$ and ATP neutralized with tris (hydroxymethyl)-methylamine (ATP-tris). The Mg^{2+}ATPase activity was monitored by following the release of inorganic phosphate (P_i) in the absence of calcium, while the $(Ca^{2+}+Mg^{2+})$ ATPase activity was calculated by subtracting the Mg^{2+}-dependent activity from the total hydrolytic activity observed in the presence of both calcium and magnesium.

At fixed intervals aliquots of the incubation suspension were deproteinized with 15% trichloroacetic acid and centrifuged, and inorganic phosphate

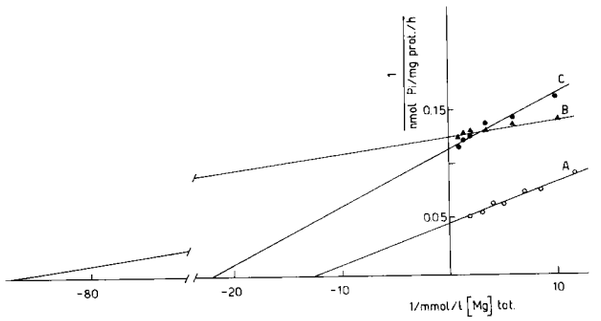

Fig. 3. Double reciprocal plot of the Mg^{2+}ATPase activity of control (A), 50 Gy (B) and 100 Gy (C) irradiated erythrocyte membranes as a function of the total magnesium concentration. Medium composition: 120 mmol/l NaCl, 40 mmol/l Na-Hepes, pH 7.4, 4.5 mmol/l ouabain, 0.5 mmol/l ATP-Tris plus the indicated $MgCl_2$ concentrations.

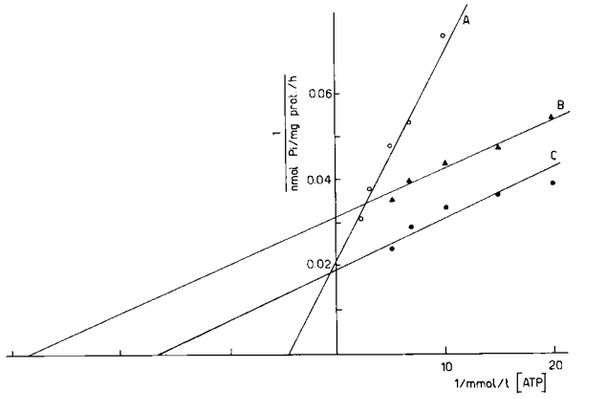

Fig. 4. Double reciprocal plot of the (Ca^{2+}+Mg^{2+}) ATPase activity of control (A), 50 Gy (B) and 100 Gy (C) irradiated erythrocyte membranes as a function of ATP concentration. Incubation medium: 120 mmol/l NaCl, 40 mmol/l Na-Hepes, pH 7.4, 4.5 mmol/l ouabain, 2 mmol/l $MgCl_2$, 0.8 mmol/l $CaCl_2$ plus the indicated concentrations of ATP-Tris.

was measured in the supernatants by the method of FISKE & SUBBAROW (1925). Occasionally the results were controlled by enzymatic assay of the ADP concentration (BERGMEYER 1974). Blanks in the absence of disrupted membranes were run to correct for non-enzymatic ATP hydrolysis.

The ATPase activities of such membrane fragments appear rather insensitive towards low calcium concentrations (calcium present as a contaminant was always less than 1.6 nmol/ml suspension as determined by atomic absorption). In fact the addition of 5×10^{-2} mmol/l EGTA did not cause any measurable change in the total ATPase activity.

In spite of standardization of the membrane isolation procedure absolute ATPase activities were not constant among different membrane batches, due to the variability in age and quality of the blood samples and the membrane fragments' vesiculation. The reported values have been derived from a fully computerized procedure using the double reciprocal plot and the non-parametric criteria to obtain statistically the best fitting (this method will be described elsewhere). On each batch of membranes all assays were performed in duplicate and individual experiments were reported four times.

Membrane thiol and amino group content was assayed after solubilization of the disrupted membranes (2 mg/ml) with 0.5% sodium dodecyl sulphate (SDS). Thiols were measured by the method of ELLMANN (1959) in 0.2 mmol/l Tris-HCl, pH 8.1, 5 mmol/l ethylene diamino-tetraacetic acid, 10 mmol/l 2, 2'-dinitro-5, 5'-dithiobenzoic acid. Amino groups were assayed according to FIELDS (1972).

SDS-polyacrylamide gel electrophoresis (PAGE) was performed at 20°C in a Shandon apparatus according to FAIRBANKS et coll. (1971). The acrylamide concentration was 5.6% crosslinked with 0.21% N, N'-methylene-bis-acrylamide. Proteins were demonstrated using Coomassie brilliant blue.

Results and Discussion

In order to assess the degree of injury induced in the erythrocyte membranes by irradiation, membrane proteins were analysed before and after the irradiation by polyacrylamide gel electrophoresis. By comparing the electrophoretic behaviour of control membranes and of membranes given 50 or 100 Gy (Fig. 1) it was observed that no dramatic variation had been introduced in the membrane protein electrophoretic behaviour by such treatments.

This result apparently is contrary to the significant decrease of membrane –SH content induced by 50 and 100 Gy which in both cases involved approximately one third of the total thiols titratable in untreated membranes. However, the observed thiol oxidation was not unexpected, since it was known that irradiation of biologic membranes leads to the formation of –S–S– bridges (MYERS & LEVY).

In the present experimental conditions, a concomitant significant decrease in the membrane amino group content, involving for both radiation intensities approximately 25 per cent of the total membrane amino groups, was observed in addition to the irradiation effect on thiol groups. Since it appears

extremely unlikely that these chemical groups might be directly affected by ionizing radiation, the most likely explanation for this effect is that during the exposure to radiation the disrupted erythrocyte membranes lose some of their components. Thus, the decrease of amino groups may well be accounted for by the release of some of the membrane phospholipids, particularly of phosphatidylethanolamine, which is known to be abundant in the inner portion of the erythrocyte native membrane.

The radiation induced decrease of the divalent cation-dependent ATPase activities (Figs 2, 3) was expected as a consequence of –SH oxidation, and confirms the results obtained using a thiol-oxidizing agent such as diamide (SCUTARI et coll.). An unexpected finding was, however, represented by the apparent increase caused by ionizing radiation in the affinity of the ATPases' binding sites towards divalent cations (Figs 2, 3) and, in the case of the $(Ca^{2+}+Mg^{2+})$ ATPase, also towards ATP (Fig. 4). Furthermore the effects of irradiation were more evident in the membranes that received 50 Gy than in those given 100 Gy. In fact the K_{diss} for calcium of the $(Ca^{2+}+Mg^{2+})$ ATPase appears to decrease in irradiated membranes from approximately 86 μmol/l (untreated membranes) to 50 μmol/l (50 Gy) and 70 μmol/l (100 Gy), while the K_{diss} for magnesium of the Mg^{2+} ATPase is lowered from 84 μmol/l (untreated membranes) to 11 μmol/l (50 Gy) and 45 μmol/l (100 Gy). Moreover, following irradiation the K_m for ATP of the $(Ca^{2+}+Mg^{2+})$ ATPase appears to be shifted from approximately 210 μmol/l (control membranes) to 35 μmol/l (50 Gy) and 60 μmol/l (100 Gy) and the Mg^{2+} ATPase activity is so inhibited that it becomes extremely difficult to appreciate changes in the rate of ATP hydrolysis at low ATP concentrations, and thus to determine significant K_m values.

The apparent increase of the affinity of the cation-binding and catalytic sites associated with a decrease of the V_{max} represents a kinetic behaviour usually encountered for uncompetitive inhibition or for substrate inhibition when the enzyme operates with a ping-pong mechanism. However, it should be emphasized that in the experiments no classic enzymatic inhibitor is present, and the results obtained probably reflect the effect on the ATPase activities of both the inhibition due to –SH oxidation and the new environmental situation caused by the loss of membrane amino groups. A possible explanation of the data presented is that the sum of these factors results in the formation of dead-end enzyme complexes which, in spite of having an apparently increased affinity for divalent cations and ATP, are unable to complete the ATP splitting cycle. Consequently in irradiated membranes a certain amount of enzyme molecules does not contribute to the total hydrolytic activity, leading to the appearance of the kind of double reciprocal plot shown in Figs 2 and 3.

On the other hand the more marked effect of 50 Gy (compared with 100 Gy) is not easy to explain, especially if it is considered that the electrophoretic behaviour of the membrane proteins and the thiol and amino groups assays seem to indicate a similar extent of membrane injury for both irradiation intensities. A possible explanation of this phenomenon is that the higher radiation intensity might turn the enzymes, inhibited by 50 Gy, to a completely uncoupled or deregulated form, possibly by causing further minor changes in their microenvironment.

SUMMARY

Treatment of disrupted erythrocyte membranes with ionizing radiation induces a partial oxidation of –SH groups (as expected from reported data) and a loss of membrane phospholipids as confirmed by the decrease of membrane amino groups. The resulting disturbance of the membrane assembly strongly affects the membrane bound divalent cation-dependent ATPase activities, possibly by causing the formation of a dead-end enzyme complex unable to complete the ATP splitting cycle.

Request for reprints: Dr Guido Scutari, Istituto di Chimica Biologica, Via F. Marzolo, 3, I-35100 Padova, Italy.

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