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Short communication

Decreased autolysis of dead cells in Adriamycin-resistant lines of the sarcoma 180 of mouse

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Summary. Adriamycin-resistant cell lines of the sarcoma 180 of mouse reveal, besides the known resistance mechanism, a decrease in cellular autolysis compared with the original line. This decrease has been demonstrated by comparative analysis of cell-free supernatants isolated from 7-day-old sensitive and resistant ascites tumors in mice. In order to demonstrate these changes we isolated and quantified the DNA, detecting higher amounts within the supernatants of resistant lines. Furthermore, unspecific DNA-cleaving activity within raw homogenates of the cells is substantially lower in the resistant lines. Residual parts of chromatin may trap the drug and in this way lower its effective concentration. However, the results may also reflect changes in enzymatic complement playing a hitherto unknown role within living cells.

Introduction

It is commonly accepted that anthracycline-resistant cells demonstrate an increased drug extrusion in vitro [1]. This can also be demonstrated with Adriamycin-resistant S 180 [8]. However, the clarity of this concept may have suppressed the search for other selective effects of the anthracycline therapy. Undegraded residues of nucleic acids and chromatin in the extracellular compartment of the ascites tumor might reduce the effective concentration of intercalating drugs. Therefore, we investigated the occurrence of changes in cellular autolysis in Adriamycin-resistant cells of S 180 by monitoring residual deoxyribonucleic acid in supernatants of ascites tumors.

Materials and methods

Sensitive and Adriamycin-resistant cells of S 180 were transplanted i.p. weekly to NMRI mice (2×10^6 cells). Total ascites was punctured on day 7 after transplantation. Adriamycin resistance was maintained by weekly treatment with Adriamycin (4 mg/kg body weight) over 100 passages. Adriamycin treatment was omitted for at least one passage prior to the experiment. The degree of resistance was determined by a short-term test using the incorporation of uridine [9]. The resistant cells demonstrated a 300-fold increase in the concentration of Adriamycin needed for 50% inhibition of ^{3}H-uridine incorporation. Concomitantly, they can grow in the cell culture in the constant presence of Adriamycin 30 mg/l, indicating a similar resistance factor.

Cell-free supernatants of the ascites fluid were extracted three times with TRIS-buffer-saturated phenol. The resulting solution was run for 60 h in a cesium sulfate density gradient (swinging bucket rotor SW 60, Beckman) to isolate the DNA. After a dialysis against 0.02 *M* potassium phosphate buffer at pH 7 the UV spectrum was determined and the quantification was performed according to the absorbance at 260 nm. The 260:280 ratio was about 2 (SD of the mean = 0.4). The DNA was further analyzed by electrophoresis in 1% agarose in TRIS/borate/EDTA buffer at pH 7 in the presence of a trace of ethidium bromide.

To determine the DNA-cleaving activity in the raw homogenates, equal cell amounts were homogenized in a Potter-Elvehjem device, subsequently frozen and thawed, and finally centrifuged at 5000 *g* for 20 min in a fixed-angle rotor. The unspecific DNA-cleaving activity was determined according to Kunitz [4] using salmon sperm DNA and with continuous recording of the increase in absorbancy. The initial slope of the curve was used for determination of the enzymatic units present within the sample.

Results

We compared the residual DNA within the ascites supernatants of S 180 and supernatants of its three independently developed Adriamycin-resistant descendants. Table 1 shows that the resistant lines developed supernatants with increased amounts of DNA. The variability of the values may be caused by the individual growth of the tumors in the NMRI mice. Since, despite the constant growth period, the ascites volume varies widely and the characteristics described depend on the stage of the tumor rather than on the actual duration of the growth period, we have omitted the results of statistical testing (which, however, revealed significant differences) from this collection of data and present the individual replicates of the experiment.

Despite an equilibrium centrifugation in the density gradients, the isolated DNA banded broadly. This indicates a low molecular weight of the banded DNA (Fig. 1 a) [5]. Electrophoretic separation of the samples after the gradient centrifugation resulted in a cleavage pattern typical for nucleosomal residues (Fig. 1 b) [7].

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Table 1. Residual DNA in the supernatants of Adriamycin-sensitive and -resistant S 180

Sensitive (original)	Resistant lines		
	1	2	3
0.009	0.151	0.061	0.065
0.035	0.245	0.290	0.204
0.055	0.160	0.430	0.121
0.053	0.115	0.076	0.042
–	0.521	–	0.104
–	0.243	–	–
0.066	–	2.130	1.180
–	0.800	0.600	1.180

Absorbance (OD/ml ascites supernatant) of samples after phenol extraction, cesium sulphate gradient, and dialysis. Replicate parallel analyses of 7-day-old tumors

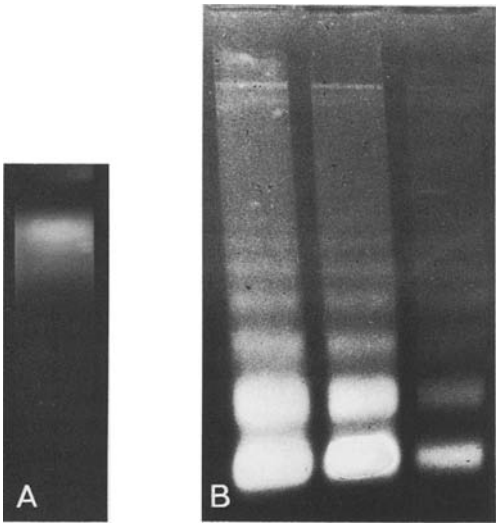

Fig. 1 A, B. DNA from the ascites supernatant of S 180 of mouse (Adriamycin-resistant line). **A** Cesium sulfate density gradient (average density 1.46 g/cm^3. Note the wide band despite the equilibrium centrifugation (60 h, 33000 upm, SW 60, Beckman). (0.001 mg/ml ethidium bromide). **B** Electrophoretic separation of the DNA in 1% agarose gel (TRIS/borate/acetate buffer, pH 7). Different amounts of one sample. Note the small amount of high-molecular-weight DNA and the cleavage pattern (stained by ethidiumbromide)

We also compared the enzymatic activities of homogenates of the cells, exemplified by the DNA-cleaving activity. DNA-cleaving activity was generally increased in frozne-thawed samples, indicating localization of a considerable proportion of the activity in compartments which are not fully destroyed by the Potter homogenizer. The DNA-cleaving activity of homogenized and frozen-thawed samples containing constant cell counts was 322 ± 124 and 59 ± 40 units in sensitive and resistant cells, respectively. This difference is significant at $\alpha = 0.05$ (Kruskal-Wallis test).

Viability determinations using propidium iodide uptake [3] demonstrated a smaller amount of dead cells in resistant tumors than in sensitive ones. The dead-to-viable ratios were 24% for sensitive and 10% for resistant tumors at the 7th day after transplantation. This difference was determined in six independent measurements ($n > 20000$ cells each) and was significant at $\alpha = 0.05$ (Kruskal-Wallis test).

Discussion

We demonstrated a reduced rate of dead cells in an Adriamycin-resistant descendant of the S 180. If identical autolytic abilities are assumed for sensitive and resistant cells the supernatants in the resistant line would be expected to have a reduced amount of cellular debris. Contrary to this expectation, we demonstrated an increased amount of cellular fragments in supernatants of three independently developed Adriamycin-resistant descendants of the S 180. We chose the DNA to demonstrate the character of the residues, because of the easy extraction procedure and because of the characteristic autolytic cleavage pattern (cf. Fig. 1) [7]. An identical pattern was observed in both sensitive and resistant lines. However, an *increased* amoount of DNA was seen in supernatants of resistant cells (Table 1). The cleavage pattern led us to conclude a chromatin-associated state of the DNA in the supernatant.

To confirm these results we additionally investigated the potential DNA-degrading activity in the sensitive and resistant lines. In frozen-thawed homogenates of resistant cells there was considerably decreased DNA-cleaving activity as measured according to Kunitz [4]. Since this is certainly not a specific DNAase activity it is not surprising that similar differences were observed in an acid phosphatase assay (data not shown). We assume that the reduced autolytic activity in Adriamycin-resistant cells of S 180 might result from a selection pressure towards *increased drug-trapping capability* of the cellular fragments. However, the phenomenon described might also be the result of changes in nuclear enzymes involved in DNA synthesis. These enzymes differ between sensitive and resistant cells [6] and may also be involved in the lysis of the DNA after cell death.

The drug-trapping effect cannot be further specified by investigations of unfractionated cell-free supernatants of ascites tumors (data collected by this approach are not shown). There are three possible reasons for the poor success in experiments using centrifugation and filtration methods:

1. As Eksborg et al. [2] have demonstrated, there is prominent binding of anthracyclines to proteins, especially to albumin, which is one of the main components in the ascites fluid.
2. The DNA fragments demonstrated are integrated within a wide range of particle sizes. Any possible method of determining the drug-trapping capability refers only to a part of this scale.
3. All substances needed for the suppression of clotting of the fresh ascites fluid interfere with both the drug solubility and the trapping characteristics of the particles.

For these reasons we believe that immediate extraction of the ascites fluid is the only method that makes it possible to demonstrate the differences described. From the basic data on the intercalation of anthracyclines [10] we conclude a correaltion between the demonstrated intercalation of ethidium bromide and the potential intercalation of the Adriamycin.

Similar investigations in L 1210 of the mouse and is Adriamycin-resistant descendant failed to demonstrate

DNA residues within the sensitivity limits of the used methods. Differences in the enzymatic complement of the cells and in the manner of cell death may account for the negative result of the analysis. The comparison of resistant and sensitive lines of S 180 and L 1210 tumors excludes the possibility of significant participation of host-originated enzymatic activity.

This aspect, so far not considered, demonstrates multiple effects of selection pressure in the cytostatic therapy.

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References

1. Danø K (1976) Experimentally developed cellular resistance to daunomycin. Acta Pathol Microbiol Scand [A] Suppl 256
2. Eksborg SH, Ehrsson H, Ekquist B (1982) Protein binding of anthraquinone glycosides with special reference to Adriamycin. Cancer Chemother Pharmacol 10: 7
3. Horan PK, Kappler JW (1977) Automated fluorescent analysis for cytotoxicity assays. J Immunol Methods 18: 309
4. Kunitz M (1950) Crystalline desoxyribonuclease I. Isolation and general properties, spectrophotometric method for the measurements of the desoxyribonuclease activity. J Gen Physiol 33: 349
5. Meselson MF, Stahl W, Vinograd J (1957) Equilibrium sedimentation of macromolecules in density gradients. Proc Natl Acad Sci (USA) 43: 581
6. Nelson EM, Tewey KM, Liu LF (1984) Mechanism of antitumor drug action: Poisoning of mammalian DNA topoisomerase II on DNA by 4'-(9-acridinylamino)-methanesulfon-*M*-anisidide. Proc Natl Acad Sci USA 81: 1361
7. Noll M (1974) Subunit structure of chromatin. Nature 251: 249
8. Šonka J, Stoehr M, Vogt-Schaden M, Volm M (1985) Anthracycline resistance and consequences of the in situ – in vitro transfer. Cytometry 6: 437
9. Volm M, Lindner C (1978) Detection of induced resistance in short-term tests. Adriamycin-resistant sarcoma 180. Z Krebsforsch 91: 1
10. Zunino F, Gambetta R, DiMarco A, Zaccara A (1972) Interaction of daunomycin and its derivatives with DNA. Biochim Biophys Acta 277: 489

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