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AN UNUSUAL NAD(P)H-DEPENDENT O_2^--GENERATING REDOX SYSTEM IN HEPATOMA 22a NUCLEI

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Nuclear membranes from many tumors contain an unusual redox chain discovered originally in the Hepatoma 22a nuclear membranes[7] which catalyzes superoxide dismutase-sensitive adrenaline oxidation to adrenochrome in the presence of either NADPH or NADH as electron donor, the reaction being inhibited by cyanide and azide. This redox chain can reduce anthracycline antitumor antibiotics adriamycin and carminomycin to their free radical states under anaerobic conditions. Evidence has been obtained for a higher stability of the carminomycin radical as compared to that of adriamycin. Operation of the nuclear membrane-bound redox chain can be a source of oxygen radical-mediated single strand breaks in DNA. The role of the nuclear membrane-associated electron transfer chain in augmenting the anticancer action of the anthracycline antibiotics is discussed.

KEY WORDS: Superoxide radicals, nuclear membranes, adriamycin, DNA oxidative damage, tumor therapy, NAD(P)H-oxidation.

ABBREVIATIONS: SOD, superoxide dismutase.

INTRODUCTION

It is becoming generally recognized that oxygen radical production interferes with a great number of biochemical and physiological processes in the aerobic organisms. In particular, superoxide generation mediates many normal and pathological processes in the cells.

Since low cytoplasmic superoxide dismutase activity had been established as a specific feature of most tumors[1–6] there were many efforts to evaluate a potential usefulness of this circumstance for cancer therapy and to find out whether the balance between the O_2^--producing and O_2^--scavenging reactions may be shifted in the tumor cells as compared to the normal ones.

Investigations carried out in this group showed the presence of a highly active redox chain in the nuclear membranes from Hepatoma 22a ascites cells[7] which contained NADH- and NADPH-specific dehydrogenase,[8] a b_5-type cytochrome with E_m of -70 mV and an unidentified cyanide- and azide-sensitive component.[9] This redox

chain was found to catalyze SOD-sensitive adrenaline oxidation to adrenochrome and hence is potentially active in O_2^- generation.

During our subsequent experiments we were concerned with the following questions. First, whether the novel KCN-sensitive NAD(P)H-dependent electron transfer system is present in the nuclear membranes from tumors other than Hepatoma 22a. Second, whether this redox chain can catalyze one-electron activation of the anticancer anthracycline antibiotics like adriamycin to their semiquinone state, a process believed to play an important role in the cytotoxic effect of these drugs.[10,11] Another interesting problem is whether oxygen radicals generated by the membrane-bound redox chain of nuclei can be hazardous for DNA integrity and contribute to oxygen toxicity.

Here we briefly report on some of the results obtained in our recent studies along these lines of investigations.

MATERIALS AND METHODS

Adriamycin and carminomycin were kindly supplied by Dr. V.B. Zbarsky. DNA polymerase from *Bacillus stearothermophilus* was a generous gift of Dr. Kaboev.[12] Other reagents were commercial products largely from "Sigma" and "Serva".

Isolation of nuclei, nuclear membranes and microsomes was carried out as described earlier.[7] For experimental tumor growth conditions see.[9] Adrenaline oxidation to adrenochrome was measured in a Unicam SP-8000 spectrophotometer at 480 nm.[7] Anthracycline antibiotic reduction to semiquinone was monitored by ESR spectroscopy as specified in the legends to figures. Assays of DNA nicking by alkaline gel electrophoresis were described earlier.[13]

RESULTS

The cyanide-sensitive NAD(P)H-dependent oxidation of adrenaline to adrenochrome discovered originally in the nuclear membranes from Hepatoma 22a cells[7] proved to

TABLE I

O_2^--dependent oxidation of adrenaline to adrenonchrome by microsomes and nuclear membranes from normal and tumor tissues (nmol adrenochrome/min/mg prot.)

Tissue	Microsomes		Nuclear membranes	
	NADPH-dependent	NADH-dependent	NADPH-dependent	NADH-dependent
Wistar rat liver	40 ± 6 (20)	< 1 (10)	4 ± 1 (5)	< 1 (5)
C3H/Sn murine liver	52 ± 5 (5)	< 1 (5)	9 ± 2 (5)	< 1 (5)
BALB/C murine liver	45 ± 3 (5)	< 1 (5)	7 ± 1 (3)	< 1 (3)
Wistar rat brain	6 ± 1 (4)	< 1 (4)	1.6 ± 0.1 (4)	0.7 ± 0.1 (4)
Glioma 35	1.7 ± 0.1 (3)	< 1 (3)	8 ± 1 (3)	4 ± 1 (4)
Glioma 223	2.3 ± 0.1 (3)	< 1 (3)	10 ± 1 (3)	5 ± 1 (3)
Shvetz erythromyeloma				
solid form	2.7 ± 0.1 (3)	< 1 (3)	3.5 ± 0.3 (3)	1.6 ± 0.2 (3)
ascite form	2.9 ± 0.2 (3)	< 1 (3)	3.6 ± 0.5 (3)	2.1 ± 0.3 (3)
Pliss lymphosarcoma				
solid form	2.9 ± 0.3 (4)	< 1 (4)	3.2 ± 0.8 (4)	1.1 ± 0.4 (4)
ascite form	2.2 ± 0.3 (3)	< 1 (3)	2.9 ± 0.1 (4)	1.4 ± 0.2 (4)
Zajdela Hepatoma	—	—	5.3 ± 1.2 (3)	3.3 ± 1.2 (3)

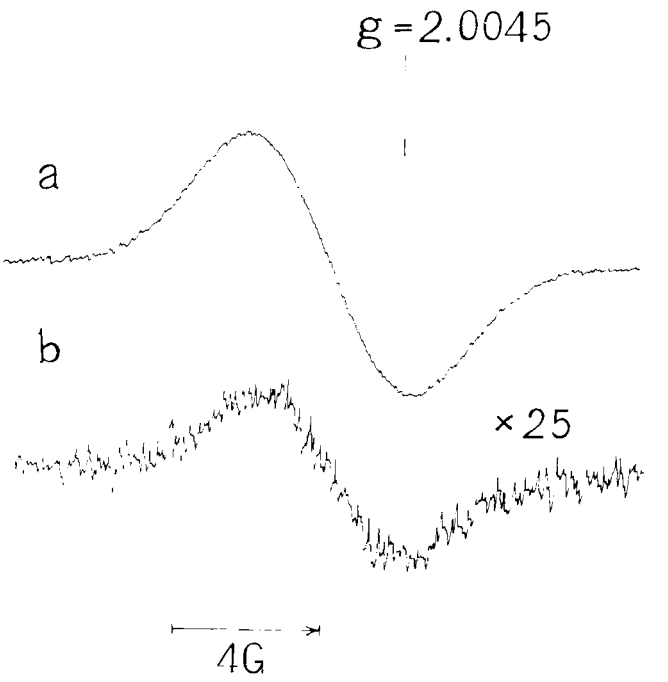

FIGURE 1 The ESR spectra of the carminomycin (a) and adriamycin (b) radicals generated upon anaerobic incubation of the antibiotics with the Hepatoma 22a nuclei in the presence of NADPH. The He-flushed reaction mixture contained 0.2 M phosphate buffer pH 7.0, 1 mM anthracycline and the nuclei (0.12 mg protein/ml); after addition of 5 mM NADPH the sample was transferred to a stoppered flat quartz cell for ESR spectroscopy. The spectra shown have been recorded 0.5 h (a) and 1 h (b) after initiation of the reaction with NADPH in a Varian E-109E X-band spectrometer under the following conditions; modulation frequency, 100 KHz; modulation amplitude, 5G; microwave power, 12.5 mW; scanning rate, 20 G/min; time constant, 0.064s; $t = 30^{\circ}C$.

be inherent in the nuclear membranes from many other experimental tumors (Table I). This activity was fully sensitive to SOD. Interestingly, whereas different specific activities have been obtained for the nuclear membranes from various tumors, the ratio of the NADPH- to NADH-dependent reaction rates of ~ 2 is fairly constant and agrees well with the data on Hepatoma 22a.[7-9]

TABLE II
The ratio of the adriamycin and carminomycin semiquinone yields during anaerobic enzymatic reduction†

Redox system	[carminomycin semiquinone] / [adriamycin semiquinone]
rat liver nuclei + NADPH	20
rat liver microsomes + NADPH	19
Hepatoma 22a nuclei + NADPH	44
xanthine oxidase + xanthine	50

† Basic condition as in Fig. 1

Whereas in the normal nuclei membranes the adrenaline co-oxidase activity can be actually ascribed to microsomal contamination, this cannot be the case with the tumor preparations in which the specific activities observed in microsomes are lower than in the nuclear membranes (Table I). Moreover, the low adrenaline co-oxidase activity of tumor microsomes was similar to that of the normal tissue preparations in that it was not inhibited by cyanide whereas both the NADH- and NADPH-dependent reactions in all the tumor nuclear membranes tested were completely inhibited by 2 mM cyanide. Analogous results have been obtained with the nuclear membranes isolated from human brain tumors.[14] Notably, the redox system of tumor nuclear membranes responsible for the adrenaline-to-adrenochrome oxidation is very temperature-labile, e.g., there occurs complete inactivation upon heating of the preparations at 60°C for 1 min.

The unusual redox chain of tumor nuclei was further assayed for redox interaction with the antitumor anthracycline antibiotics.

NAD(P)H-dependent reduction of adriamycin and carminomycin to their radical forms

Figure 1 shows typical EPR spectra observed upon an anaerobic incubation of carminomycin (a) and adriamycin (b) with the NADPH-supplemented Hepatoma 22a

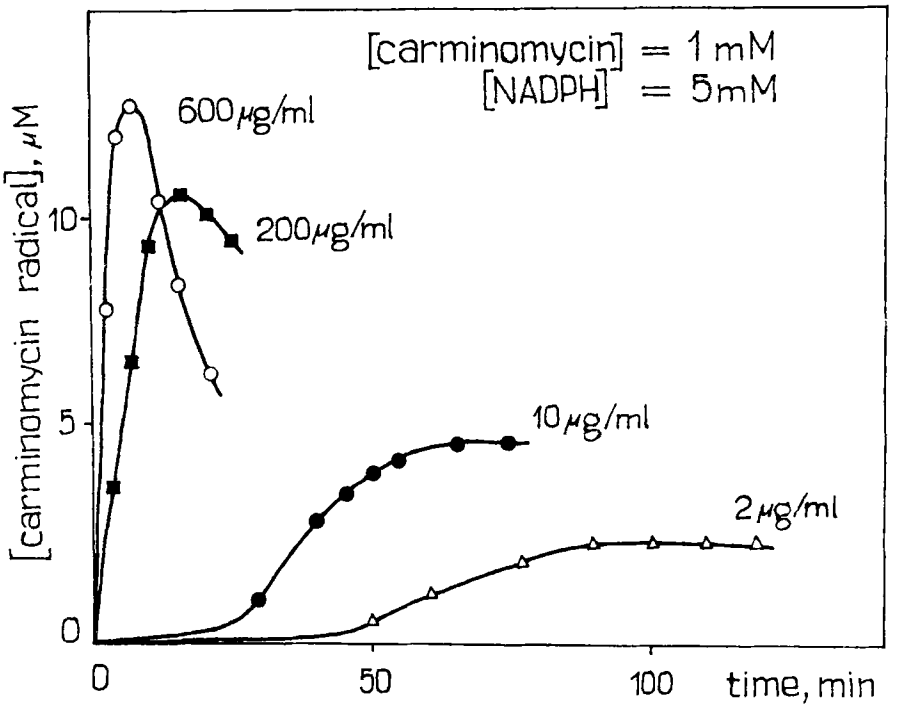

FIGURE 2 Time-course of carminomycin semiquinone formation in the presence of NADPH-supplemented rat liver nuclei. Basic conditions, as in Fig. 1. At the zero time 5 mM NADPH was added and ESR spectra of the sample were recorded one after one. Semiquinone concentrations were determined by double integration of the first derivative spectra.

nuclei. It can be seen that the radical yield is much higher for carminomycin (about 40-fold). The ratios of the radical yield for the two antibiotics observed with rat liver nuclei and microsomes as well as for xanthine oxidase-catalyzed reduction of the anthracyclines are presented in Table II. It is likely that the antibiotic semiquinone is much more stable in case of carminomycin. This possibly can be explained by the presence of a free hydroxy-group at the C-4 position of the carminomycin molecule which can stabilize the free radical by virtue of hydrogen-bonding to the unpaired electron-containing oxygen in the redox-active quinone ring of the antibiotic.[15] Notably, this hydroxy-group is substituted in adriamycin rendering the free-radical stabilization impossible.

It has to be mentioned that the concentration of the anthracycline antibiotic radicals revealed a rather complex kinetics which was similar for all the membrane preparations tested with either NADH or NADPH as electron donors. The results of a typical experiment with carminomycin incubated with various concentrations of NADPH-supplemented rat liver nuclei are given in Fig. 2. It can be seen that, qualitatively, the maximal yield of the radical and the rate at which this maximal yield is attained increase with increased concentration of the membranes. At the same time, quantitative relationships between the parameters of the radical progress curves and the membrane-bound redox chain activity may be quite complex. In the light of these observations, a comment is due that in the preceding text (Fig. 1, Table II) and in Table III below the maximal concentrations of the anthracycline antibiotics under specified conditions which are referred to.

With Hepatoma 22a nuclei carminomycin radical generation in the presence of NADPH was in most cases virtually the same as with NADPH (Table III), which is in keeping with the ability of Hepatoma 22a nuclear membranes to use NADH actively as electron donor in the adrenaline co-oxidation.[7-9] In the case of nuclei or microsomes from rat liver the yield of the antibiotic semiquinone with NADPH was about 4-fold higher (Table III).

NAD(P)H-dependent DNA damage

Oxygen radicals are known to induce breaks in DNA.[16,17] Therefore it is interesting, whether operation of the nuclear membrane-bound redox chain capable of O_2^- production can be dangerous for DNA and, hence, genome integrity. We found that upon incubation of supercoiled pBR 322 DNA with NADPH-supplemented rat liver nuclei membranes there indeed appeared a fraction of slowly moving relaxed DNA in an agarose gel electrophoresis (not shown). However, virtualy the same effect was observed in the absence of NADPH. This DNA relaxation is not likely to be due to the action of nucleases since EDTA was without effect on the process, but rather could

TABLE III
NADH effectiveness in supporting carminomycin radical generation by various membrane-bound redox chains†

Preparation	Maximal semiquinone formation (in % to that observed with NADPH)
rat liver microsomes	25
rat liver nuclei	25
Hepatoma 22a nuclei	80

† Basic conditions as in Fig. 1. NADH, 5 mM

originate in the topoisomerase activity present in the insoluble fraction of liver nuclei.[18] Association of this activity with the nuclear membranes makes it difficult to use the transition of supercoiled circular DNA to the relaxed form as a probe for oxygen radical generation by the membranes. Also stimulation of the endogenous DNA polymerase activity is not a suitable test because the radicals involved in the DNA nicking can inhibit the reparation enzymes.[19]

We have therefore attempted to elicit endogenous DNA damage with the use of exogenous DNA polymerase preferably utilizing nicked DNA. Results of a typical experiment are shown in Table IV. It can be seen that incubation with NADPH stimulates DNA labelling by ~40%. The effect is prevented completely by SOD and catalase which proves oxygen radical involvement in the nuclear DNA nicking.

Subsequent experiments showed that the oxygen radical-dependent nuclear DNA degradation is dramatically enhanced by the iron chelates such as EDTA-Fe^{3+}. Figure 3 shows rat liver DNA agarose electrophoresis pattern under denturating conditions. NADPH addition to the nuclei does not affect the sample (lane 2). However, in the presence of EDTA-Fe^{3+} there occurs a dramatic damage of the nuclear DNA as manifested by a complete disappearance of the staining material from the high molecular weight region (lane 4). EDTA-Fe^{3+} without NADPH exerted no such effect (lane 3). No significant protection was observed with SOD (lane 6), whereas catalase prevented the DNA damage completely (lane 7). Significant prevention of the DNA damage was observed also with mannitol (lane 9). The same results have been obtained with NADH used instead of NADPH, the damaging effect being even more pronounced (data not included).

These results point to involvement of H_2O_2 and probably of H_2O_2-derived $\cdot OH$ radicals in the degradation of nuclear DNA during the operation of the nuclear membrane-bound redox chain. Whether $O_2^{\cdot}$ radicals serve as a source of H_2O_2 under these conditions is not clear at present. Incidentally, SOD-sensitive reduction of

TABLE IV

Effect of the NADPH-dependent $O_2^{\cdot}$ generation in the nuclei on the nuclear DNA nicking

Additions	cpm†
none	1901
NADPH (1 mM)	2596
NADPH (1 mM), catalase (25 μg/ml) and SOD (50 μg/ml)	1939

† Background radioactivity in the absence of the added polymerase (605 cpm) was subtracted.

3.2×10^6 rat liver nuclei were incubated for 30 min at 30°C in 100 μl of an aerobic medium containing 20 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 150 mM KCl, 0.01% albumin and other additions as indicated. Subsequently, the sample was heated for 10 min at 65°C to inactivate the membrane-bound redox chain and endogenous DNA polymerases and incubated for additional 5 min at 30°C with catalase (25 μg/ml) to remove H_2O_2 accumulated. Then dNTP (50 μM each) and 1 μM [^{3}H] TTP (22×10^3 Ci/mol) and 1 U of DNA polymerase from *B. stearothermophilus* were added and after 30 min incubation at 60°C acid insoluble radioactivity was determined. For more details see.[13]

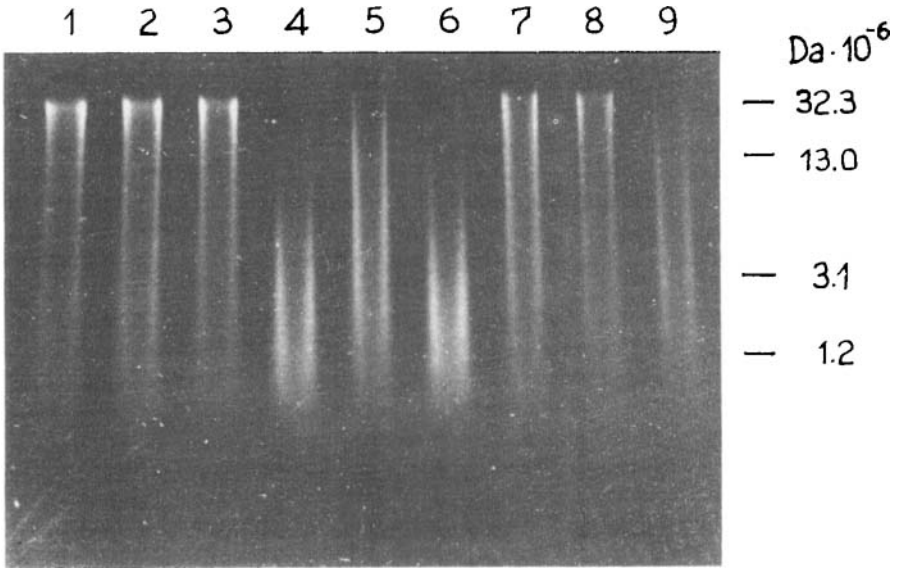

FIGURE 3 Nuclear DNA electrophoresis in 0.5% alkaline agarose gel.
Lanes: 1, nuclei;
2, nuclei + NADPH, 1 mM;
3, nuclei + EDTA-Fe^{3+}, 250 μM;
4, nuclei + NADPH, 1 mM + EDTA-Fe^{3+}, 250 μM;
5, nuclei + NADPH, 100 μM + EDTA-Fe^{3+}, 250 μM;
6, nuclei + SOD, 50 μg/ml + NADPH, 1 mM + EDTA-Fe^{3+}, 250 μM;
7, nuclei + catalase, 25 μg/ml + NADPH, 1 mM + EDTA-Fe^{3+}, 250 μM;
8, nuclei + SOD, 50 μg/ml + catalase, 25 μg/ml + NADPH, 1 mM + EDTA-Fe^{3+}, 250 μM;
9, nuclei + mannitol, 100 μM + NADPH, 1 mM + EDTA-Fe^{3+}, 250 μM; 2 × 10^6 nuclei were incubated in 100 μl of the medium containing 150 mM KH_2PO_4 pH 7.0, 5 mM EDTA and 1 mM EGTA at 30°C for 30 min. The molecular weight standards are intact λ DNA and λ DNA digested by Eco RI + Hind III.

succinylated cytochrome *c* by porcine lung and liver nuclei in the presence of NADH as well as of NADPH was reported by Yusa *et al.*[20]

As to the role of Fe^{3+}-EDTA complex, direct reduction of the latter to Fe^{2+}-EDTA by the membrane-bound redox chain can be envisaged.[21] Subsequently the reduced complex serves as an electron donor in a conventional reductive H_2O_2 cleavage to OH^- + $OH^{\cdot}$, the latter playing the major role in the DNA degradation.

DISCUSSION

The results reported in this work show that the unusual NAD(P)H-dependent cyanide-sensitive redox chain first described in the nuclear membranes from Heptoma 22a ascites cells,[7] may be a distinctive feature of many tumors including human cancers.[14] The biochemical identity of this redox system remains to be established. Notably, the sensitivity to cyanide and ability to use both NADPH and NADH as electron donors resemble the characteristics of the fatty acid desaturase as discussed earlier.[7]

The presence of an active redox chain in the nuclear membranes of tumor cells may have important implications for the strategy of cancer therapy and provides a basis for better understanding of the selectivity of action of many anticancer drugs.

In particular, redox activation of the anthracycline antibiotics is believed to mediate their cytotoxic effects. It is conceivable, that the pattern of action of the antibiotics would differ in the normal cell, where most of the reduction of the anthracyclines occurs in microsomes, and in the tumor tissues in which the drug interaction with the microsomes is insignificant and most of the reaction will take place right in the nuclei, i.e. in close proximity to DNA which is believed to be the final target of the antibiotic action.

It would be interesting to investigate whether DNA of the tumor nuclei is prone to the oxidative attack by the oxygen radicals generated by the nuclear membrane-bound redox chain. The present experiments performed so far with the liver nuclei indicate that even in this case where the membrane-associated redox activities are very low, there can occur DNA damage. Consequently, much more dramatic effects can be envisaged, say, for Heptoma 22a cells, where the nuclear membrane-bound redox chain is exceptionally active.

One can speculate that oxygen radical-dependent introduction of nicks into DNA and concomitant stimulation of the endogenous DNA polymerase activities in the nuclei may augment the action of the DNA intercalating anticancer drugs which are likely to act at the stage of DNA processing.[22] This may be another important contribution of the tumor nuclei membrane-bound redox chain to the selectivity of the adriamycin-type antibiotic action.

Acknowledgments

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