On the Possible Involvement of Ascorbic Acid and Copper Proteins in Leukemia. IV. ESR Investigations on the Interaction between Ascorbic Acid and Some Copper Proteins

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The interaction between lyophilized samples of ascorbic acid and some copper proteins (ceruloplasmin, cytochrome-c-oxidase, ascorbate-oxidase) has been investigated by means of ESR spectroscopy. The spectra obtained are identical to the one obtained with leukemic blood. The consequences of this for the molecular events occuring in cancer are discussed. The model proposed can explain the experimental findings reported thus far (such as change in spin concentration with the development of cancer, the presence of a high concentration of antioxidants etc.) as well as reconsile the two existing and seemingly contradictory hypothesis. Possible implications for lipid peroxidation and for the respiratory process are discussed.

Introduction

In a preceding paper we have shown that the electron spin resonance (ESR) spectra of lyophilized blood of patients with acute leukemia exhibit an increase in spin concentration and an additional peak (at about $g = 2.005$) not being present in control samples [1]. In order to elucidate the possible causative agents as well as their prospective receptors, several substances have been added to either native blood, erythrocytes and their white ghosts, leukocytes or plasma of healthy persons. Of all the substances tested (*e. g.* vitamin A, vitamin E, vitamin C, catecholamines, such as adrenaline, noradrenaline, dopamine), only ascorbic acid produces a spectrum which is identical to the one obtained from blood or its fractions of leukemic patients [2, 3]. Since these changes were observed in both white ghosts and plasma, the receptor for vitamin C has to be searched for in membrane and plasma as well. Transition metal ions are known to interact with ascorbic acid, therefore, atomic absorption studies have been conducted on erythrocytes, their white ghosts, and plasma [3]. These experiments revealed that Cu and Fe are present in these blood constituents. ESR investigations on the interaction between ascorbic acid and Cu^{2+}, Mn^{2+}, or Fe^{3+} have shown a certain interaction, however, the typical "leukemic" signal was not observed. In the case of Cu, a decrease in spin concentration caused by a $Cu^{2+} \rightarrow Cu^{+}$ transition occured [4]. Since Cu^{2+} exerted the strongest interaction with vitamin C, it seems quite possible that copper-containing proteins might function as receptors for ascorbic acid.

In order to elucidate the mechanism of interaction between ascorbic acid and possible receptors the effect of vitamin C on a few copper proteins has been investigated by means of ESR spectroscopy.

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Materials and Methods

The copper proteins ascorbate oxidase (EC 1.10.3.3; Boehringer, Mannheim), cytochrome-c-oxidase (EC 1.9.3.1; Serva, Heidelberg), and ceruloplasmin (EC 1.12.3; Sigma, Munich) were used without further purification. Ascorbic acid was purchased from Merck, Darmstadt.

Erythrocytes were prepared according to a method described recently [2].

Ascorbic acid was dissolved in either bidistilled water or in phosphate buffer, pH 7, and added to the enzyme solutions in different concentrations. These solutions were, then, freeze-dried in order to get the identical conditions used for the investigation of the biological material.

ESR spectra of these lyophilized samples were obtained with a Varian E-9, 100-kHz modulation X-

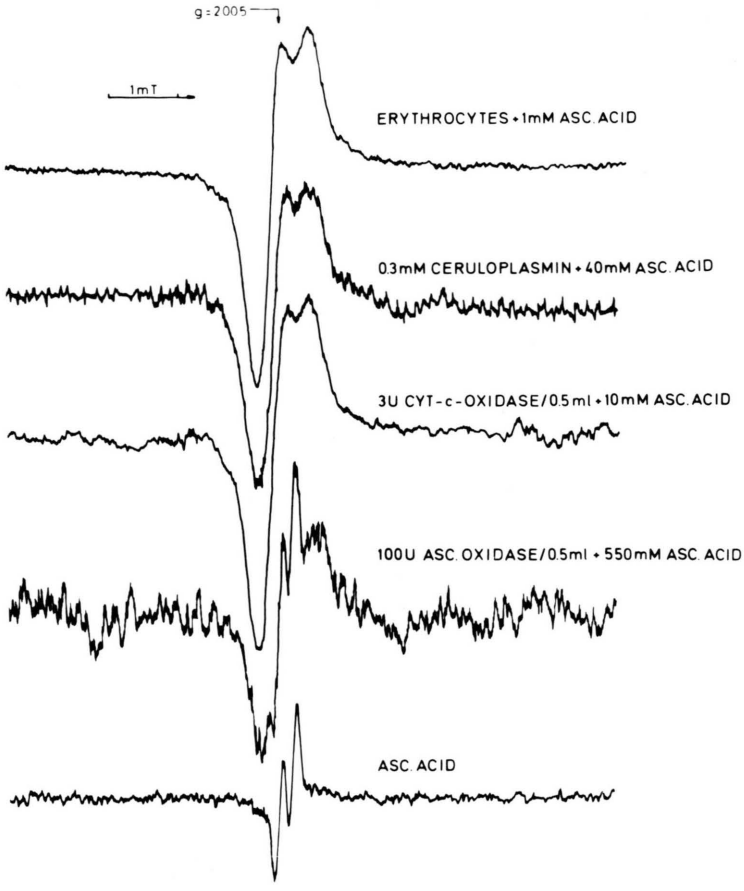

Fig. 1. The effect of ascorbic acid on different copper-proteins and erythrocytes. At the bottom: semidehydroascorbate doublet obtained after freeze-drying ascorbic acid. Note: different experimental conditions were used for the different samples. Spectra shall demonstrate qualitative response only.

band spectrometer. Details of the procedure were described recently [2].

Results and Discussion

The results obtained when ascorbic acid is added to either erythrocytes or some copper-containing proteins are shown in Fig. 1. The upper spectrum shows the ESR response of erythrocytes which resembles the spectrum obtained with leukemic blood [2]. As can be seen, similar spectra were obtained with ceruloplasmin, cytochrome-c-oxidase, and ascorbate oxidase. In the latter case, the vitamin C concentration was chosen very large in order to get the semidehydroascorbate (SDA) free radical. Only at high concentrations it can be clearly identified. This free radical obtained by freeze-drying of ascorbic acid is shown in the last spectrum. Hence, the "leukemic" peak at about $g = 2.005$ seems to be due to this free radical. It should be pointed out that in the case of all three enzymes investigated the typical "leukemic" spectrum develops gradually with increasing concentration of ascorbic acid and decreases in spin concentration above a certain concentration of vitamin C. This response is analogue to that one reported for the blood constituents [2, 3]. Experiments conducted with ascorbic acid and some iron-containing proteins, such as cytochrome-c and catalase, did not reveal any signal.

It should be pointed out that the SDA radical will not be obtained after addition of large concentrations of ascorbic acid to erythrocytes. In this case, the cooperative effect of the membrane constituents modifies the relaxation time in such a way that the hf structure of the SDA radical cannot be resolved any longer. Also, in the case of the Cu^{2+}-vitamin C interaction [4] this hf structure cannot be observed. In

this case, ascorbic acid is probably oxidized immediately to the dehydroascorbic acid state.

The experimental findings suggest that in the case of leukemia the ESR spectrum observed might be due to the interaction between copper-proteins investigated and ascorbic acid. It might be assumed that other copper-proteins show a similar behavior.

In the past, attempts have been made to elucidate the molecular nature of the ESR signal observed (s. *e. g.* ref. [5]). There is some speculation that it might be attributable to differences in degrees of lipid peroxidation [6]. The intermediate semiquinone state of oxidation of some vitamins (*e. g.* vitamins A, E, and C) is assumed to play a certain role in this peroxidation process [6 – 8]. In the case of ascorbic acid, semidehydroascorbate is readily formed both enzymically and nonenzymically, but its function is not established yet.

Walaas *et al.* [9] have reported that catecholamines can serve as substrates for ceruloplasmin. They have obtained a small ESR singlet at $g = 2.005$ from frozen samples of an incubation mixture of dopamine and ceruloplasmin. This was taken as a possible indication that the free radical of dopamine was present as the initial oxidation product [10].

Quantitatively, an early increase in free radicals during carcinogenesis has been reported [11 – 17], however, most of the results obtained show that tumor tissues usually have fewer free radicals than their normal counterparts [18 –20] and exhibit a particularly high concentration of antioxidants [20, 21]. Many of these antioxidants acting as carcinogens readily form complexes with certain biological acceptors [22, 23]. Duchesne believes that the organic free radicals normally present in healthy tissue but not identified yet serve as receptors for those antioxidants [20].

This interaction will decrease the free radical concentration of the tissue resulting in a higher tumor incidence. Such an idea was also favored more recently by Fröhlich who suggested that carcinogenic molecules transfer electrons to certain regions of the cell and that, therefore, a high density of non-localized electrons might be the underlying cause of cancer [24]. On the contrary, an increase in the intensity of abnormal free radical signals in tissue is actually associated with a decrease in tumor incidence [25]. Also, administration of stable free radicals derived from 4-substituted tetramethylpiperidine oxides to mice suffering from transplanted leukemia produced an antileukemic effect [26].

There is a contradictory point of view. Considerable evidence exists in the literature which suggests that carcinogenesis by chemical agents might result from their intracellular metabolic conversion into free radicals by one-electron oxidation processes [27, 28]. Acting as electrophiles, they are thought to be the proximate — or immediately reactive — carcinogens. This hypothesis is favored by the fact that some antioxidants exert an inhibitory effect on carcinogenesis [29, 29 a].

Several authors have tried to reconcile both contradictory points of view [29]. They argued that competition for carcinogenic free radicals can be provided most effectively by other free radicals that act as scavengers or quenchers. There is neither an experimental proof of this reasoning nor does it explain the inhibitory effect of certain antioxidants on carcinogenesis.

At present, there are still too many imponderables. In order to elucidate the mechanism of carcinogenesis, it is very important to identify the ESR spectrum described. We believe that it is caused by the interaction between ascorbic acid and copper-proteins. Its structure as well as its dependence on ascorbic acid concentration might be explained as follows.

It should be pointed out that the two peaks located at about $g = 2.005$ do not belong to the same species. ESR power saturation studies show that the "leukemic" peak saturates faster. Moreover, it is influenced stronger by oxygen. This peak located somewhat more downfield is positioned at the same g factor as the semidehydroascorbate (SDA) radical (s. Fig. 1). During the interaction between ascorbic acid and copper-proteins the protein moity is reduced to a paramagnetic species while vitamin C is oxidized to the SDA radical in a one-electron transfer reaction. This reaction is probably catalyzed by the Cu^{2+} ions present in the protein. Their ESR hf spectrum is located, as is well-known, more downfield than the two peaks under consideration. Thus, the vitamin C-copper-protein interaction results in two radicalic species, the SDA radical and the copper-containing protein radical located slightly upfield. With increasing concentration of ascorbic acid more and more copper-protein is reduced according to the one-electron transfer reaction just mentioned. This results in an increase in spin concentration [2]. An optimum in spin concentration is reached when all of the protein

has undergone this one-electron reduction. At a surplus of vitamin C, this copper-containing protein radical as well as the SDA radical are reduced and a concomittant decrease in spin concentration occurs. The final state represented by the two non-radicalic species ascorbic acid and the twofold reduced protein is only possible under anaerobic condition. At small concentrations of ascorbic acid, the effect of oxygen has to be encountered.

Oxygen affects both types of radicals. The oxidation of the SDA radical results in the non-paramagnetic dehydroascorbate entity. Under normal conditions, oxygen and ascorbic acid will exert their influence on the suitable biological receptors resulting in an equilibrium which depends, of course, on their concentrations. Thus, the concentrations of radicals observed in lung and liver, *e. g.*, should be different [44].

In the normal living state there is always a certain interaction between copper-proteins and ascorbic acid. In erythrocytes it might be inhibited by some biomolecules which protect the copper-proteins. Since normal plasma and leukocytes as well already exhibit the ESR spectrum, produced by the ascorbic acid — copper-protein interaction ("leukemic" spectrum), it might be possible that they act as scavengers or protective species for the erythrocytes against ascorbic acid. Carcinogenic substances might either facilitate this interaction also between erythrocytes and ascorbic acid or provoke an increased production of copper-proteins, possibly as an antibody reaction. The latter case might be favored since a change in the quantity of at least some transition metal ions in tumor tissue has been reported consistently [30 – 34]. In general, an increase in the copper level with a concomittant decrease in the iron level has been observed. The living system will respond to this increased copper-protein level with an increased concentration of antioxidants [20, 21, 35 – 37]. Because of its apparent specificity for copper-proteins the uptake as well as the consumption of vitamin C might be enlarged. The turn over rate of vitamin C is, then, in the case of cancer, considerably enlarged which should result in a reduction of the actually measurable ascorbic acid concentration. This, in fact, has been observed by several authors [38]. The reduction of the copper-proteins and SDA as well by ascorbic acid will contribute to the overall net increase in the concentration of antioxidants [20, 21, 35 – 37].

If such an interaction should also result in a reduction of Cu^{2+} to Cu^{+}, this might cause, then, a loss of the ferroxidase activity of *e. g.* ceruloplasmin [39]. In this case, ferrous iron cannot longer be oxidized to ferric iron which binds to free transferrin in order to be transported to the sites where it is needed. This suggests that also iron might be involved in the processes described.

The changes observed in the concentration and oxidation state modify the oxidation-reduction potentials which are accompanied by irreversible cell damage.

This cell damage might be caused primarily by lipid peroxidation, a process which destroys the structural and functional integrity of various lipoprotein membranes. In the case of erythrocytes, peroxidation will lead to hemolysis of the red cells. The exact manner in which lipid peroxidation is initiated and leads to structural and functional derangements of lipoprotein membranes is not known in detail. There are several indications that the semidehydroascorbate radical produced by the ascorbate-copper-protein interaction initiates directly this oxidation process [8]. It is interesting to note that also this process depends on the ascorbic acid concentration. At low concentrations, vitamin C is prooxidant, but antioxidant at high concentrations (ref. [8] and references therein). It is tempting to correlate this observation with our results on the dependence of the spin concentration on ascorbic acid concentration [2] and the explanation given above.

The experimental results obtained emphasize the importance of electron transfer reactions, that is oxidation-reduction processes occurring in normal tissue and their modification by a possible involvement of ascorbic acid in the case of cancer. As a consequence, lipid peroxidation might occur. The critical alteration of necessary membrane phospholipids might lead to a loss of membrane-bound enzymatic activity. Eventually, the products produced might modify the electron transport chain, as has been proposed by Szent-Györgyi [40]. Finally, they will cause the inhibition and breakdown of the respiratory process [41 – 43].

Vitamin C might interact with all copper-proteins. The specificity for a certain type of malignancy might be given by the special structure of the protein.

This proposal might probably reconcile the different hypotheses proposed for explaining carcinoge-

nesis. It agrees well with the experimental results obtained thus far. An application of these findings to malignancies in other types of tissue will be discussed in detail elsewhere [44].

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