HISTOCHEMICAL AND ELECTRON SPIN RESONANCE STUDY
OF SERUM METALLOPROTEINS IN DAB HEPATOMA

HIROSHI KAWASAKI, TOSHIRO NAKASHIMA, HIDEHIKO TANAKA*,
SHIGEYUKI TERADA* AND EIJI KIMOTO*

1st Department of Pathology, Kurume University, School of Medicine, Kurume 830
and Department of Chemistry*, Faculty of Science, Fukuoka University,
Fukuoka 814

Received for publication September 21, 1981

The metalloproteins in the liver of p-dimethylaminoazobenzene (DAB)-
treated rats were analyzed by means of immunohistochemical and electron
spin resonance (ESR) techniques in comparison with normal or carbon tetra-
chloride-damaged livers. Immunohistological method revealed intensive
distribution of serum ceruloplasmin and transferrin in DAB induced tumor
tissues. Concomitantly, a remarkable increase was also noted in the ESR
signals of both cupric ion and non-heme ferric ion of rhombic high-spin form,
which were ascribed to these metalloproteins. Their biological implications
in malignancy are discussed.

As shown by different biochemical and histochemical approaches, the liver
produces most of the plasma proteins except for the immunoglobulins (9). Cerulo-
plasmin (Cp) and transferrin (Tr), copper and iron-containing plasma metallopro-
teins, respectively, are also products of the liver and circulate in the body.

High levels of plasma Cp activity were found in a variety of acute and chronic
diseases including cancer (12). Rice (26) concluded that Cp may play a role of an
acute phase reactant. ESR studies by Foster et al. (11) also demonstrated that the
magnitude of the g=2.05 signal due to the copper ion bound to Cp increased signi-
ficantly in sera of cancer patients to above that of normal individuals though no
qualitative difference in ESR signals could be found between them. Recently, we
reported that the plasma of advanced cancer patients showed relatively lower levels
of Fe(III) signals as well as higher Cu(II) signals than those of the normal and,
thus, the Cu(II)/Fe(III) signal ratio was higher in most cancer patients (18). In-
creased plasma Cp should be considered as a reflection of an overproduction in the
liver.

In the present study, attention was paid to the immunohistochemical demon-
stration of hepatic Cp and Tr, and the characterization of the states of copper and
iron bound to these proteins by ESR measurements in DAB hepatomas of rats.
MATERIALS AND METHODS

Wistar strain rats, 8 weeks of age, were fed a commercial diet (CLEA-CE 2) containing 0.06% DAB. Rats were sacrificed for autopsies at about 80 weeks from the beginning of the experiment. Twenty livers with hepatic tumors were examined for ESR spectra, of which ten tumor masses were bigger than 3 cm in diameter and the other ten were smaller.

Several days after a subcutaneous injection of a 1:1 mixture (1 ml) of carbon tetrachloride and olive oil to normal rats, necrotic livers with severe fatty metamorphosis were produced. They were examined in comparison with DAB hepatomas.

For immunohistological examination, liver specimens were fixed in a 1:1 mixture of cold ethanol-acetone and embedded in paraffin. Tissue sections were subjected to indirect immunofluorescence stain. Rabbit's antisera against rat Cp and Tr were purchased from Miles Labs. Inc., Elkhart, Ind. On Ouchterlony assay, they gave a single precipitin line against the respective rat serum proteins. The tissue sections, pretreated with these antisera, were stained with FITC-labeled goat IgG against rabbit's IgG (Seikagaku Kogyo, Tokyo). The immunofluoresced preparations were examined under a fluorescence microscope.

For ESR measurements, liver slices (about 0.5 g) were transferred, with a syringe attached to a long plastic tube, into the bottom of a 3.5 mm I.D. quartz tube. ESR spectra were recorded at $-196^\circ$C in liquid nitrogen in a Dewar as the first derivative of an absorption, using a JES-FE1X spectrometer, JEOL, Tokyo, at about 9.15 GHz with 100 KHz modulation frequency. The incident microwave power was 8 mW, except during power saturation studies. The magnetic field was calibrated by the splitting of Mn$^{2+}$ in MgO, and the g-values were standardized using Li-TCNQ ($g=2.00252$).

RESULTS

Hepatic Cp and Tr were detected by the fluorescent antibody technique using either anti-Tr or anti-Cp antiserum and FITC-labelled anti-globulin. Though both Cp and Tr should be synthesized in hepatic cells and circulating in blood vessels, immunofluorescent staining of normal livers gave only weak fluorescence for these proteins. Carbon tetrachloride-damaged livers were stained quite faintly by these antisera as well. On the contrary, every hepatic tissue from DAB hepatoma-bearing rats exhibited very high fluorescence for both of these proteins under the same conditions (Figs. 1, 2). The stained tumor cells were clustered in small groups. These serum proteins were seen in the cytoplasm and the plasma membrane region of tumor cells.

The intensive distribution of Cp and Tr associated with DAB hepatoma was also demonstrated by ESR spectra of tumor tissues. DAB hepatoma in the terminal stage gave much simpler ESR spectrum than that of normal livers, as shown in Figs. 3 (A) and (B). Signals of cytochrome P-450 (anisotropic signal of low-spin heme iron at $g=1.91, 2.25$ and $2.41$), xanthine oxidase molybdenum ($g=1.97$) and mitochondrial S-iron ($g=1.94$) were completely lost in tumor tissues, in agreement with the results of Nebert and Mason (23) and Emanuel (8). The sextet signal of manganous ions, indicator of a normally functioning state of hepatic mitochondria...
was also absent in DAB hepatoma. In various stages of tumor development, intermediate patterns between these two spectra could be seen. In carbon tetrachloride-damaged livers (Fig. 3 (C)), these signals also disappeared probably due to an extensive degradation of hepatic cells.

An intensive ESR signal around $g = 2.00$, corresponding to that of organic free radicals, was seen in both normal and malignant hepatic tissues. Swartz (28) has
suggested that this signal is associated with paramagnetic metal ion complexes rather than a true free radical of low molecular weight. Power saturation studies indicated that this signal did not change appreciably between 0.1 and 90 mW, though the free radical was saturated at a low microwave power.

In this region, a characteristic three-line signal was seen in most but not all DAB hepatomas. This complex signal could be assigned to the triplet hyperfine splitting of NO coordinate of Fe²⁺-heme, as reported by Maruyama et al. (22). This triplet signal was, however, not specific to the tumor, but observable even in decayed tissues when left standing for a couple of days at room temperature.

DAB hepatoma showed a profound signal at $g=2.05$ which was ascribed to the perpendicular component of cupric ions. The parallel components at higher $g$-values were too small to be demonstrable. Normal livers did not show the copper signal at all. In carbon tetrachloride-damaged livers, the cupric ion signal was seen in a moderate height.
At \( g = 4.2 \), the signal of non-heme ferric ion was seen having the hyperfine structure characteristic of serum Tr (Fig. 3 (B)) (1, 6). Again, normal livers did not show such a signal. In carbon tetrachloride-damaged livers, a signal of \( g = 4.2 \) appeared but was different in view of the hyperfine structure from that of the typical Tr.

The signal at \( g = 6.0 \) increased significantly in carbon tetrachloride-damaged liver, but only slightly in DAB hepatomas. This signal seems to represent the high-spin, heme-ferric ions such as methemoglobin (15).

**DISCUSSION**

Lane (19, 20) reported that the normal hepatic tissues of humans and rats could be stained for serum Tr by the fluorescent antibody technique and the stained cells were evenly distributed throughout the tissues. Other immunohistological methods have also revealed the random distribution of serum Cp in the normal hepatic cells (10). Under our experimental conditions, the fluorescence staining for Tr or Cp of normal or carbon tetrachloride-damaged rat livers was only marginal. The discrepancy could be explained by technical differences in the preparation of the liver specimens or of the antibodies. In contrast to these tissues, both Cp and Tr have been demonstrated abundantly in DAB hepatomas by immunohistochemical staining (Fig. 1).

Our ESR study on DAB hepatoma has confirmed the previous observations (8, 23) indicating the decline and disappearance of the normal products of differentiated hepatic cells during the development of tumors. This was shown to support the view of a low differentiation and an antigenic deletion in malignancy (17). In turn, the spectra of decayed products occurred in tumors.

In addition, the ESR analysis of hepatic metal ions has added further proof for the immunohistological results. DAB hepatoma exhibited considerably high signals of cupric and ferric ions which were primarily bound to the apoproteins of Cp and Tr, respectively. Although there is a large amount of copper in normal liver, most of the copper ions exist in the form of cuprous ions or ESR non-detectable cupric ions due to a dipole pairing as stated by Malkin and Malmström (21). Homogenate of normal liver was found to possess a large capacity to reduce the added cupric ions to cuprous ions (29). Even in the peroxidative state of carbon tetrachloride-damaged livers, cupric ion signal was only barely demonstrable.

There is also a large amount of ferric ions normal in liver. These ions, which might exist mostly in the core of iron-storing protein, ferritin (5), did not give an ESR signal. Occurrence of the \( g = 4.2 \) signal without a hyperfine structure in carbon tetrachloride-demaged livers might be due to a decompartmentalization of ferritin molecules and a consequent non-specific binding of liberated ferric ions to certain complex-forming substances such as organic acids, amino acids and proteins.

Both the cupric and ferric ion are the representative ESR-demonstrable paramagnetic components of the serum. Though these free ions are highly toxic, they exist in an innocuous form, i.e., in the form of Cp and Tr. The present observations seem to give evidence for these serum proteins acting as 'symbodies' which are designated by Apffel and Peters (2) in contradistinction to antibodies and play a role in a masking of all the surface antigens of tumor cells.
Cells and tissues, even in the malignant state, might be protected against oxidizing free radicals by a complex antioxidant mechanism. As a primary factor in the cellular defense against oxidative damage, the presence of superoxide dismutase is of important necessity. Lowered levels of this enzyme activity, as compared to normal livers, have been found in rat and mouse hepatomas by Bozzi et al. (3), Oberley et al. (24) and Sahu et al. (27). These results seem to be in good agreement with the well-known observation that high energy radiation and hyperbaric oxygenation have injurious effects on the viability of tumor cells.

According to Dormandy (7) and Cranfield et al. (4), serum behaves as a powerful antioxidant and the particular activity is correlated with Cp and the iron-free fraction of Tr. Cp, possessing a ferroxidase activity, serves as a powerful lipid-autooxidation inhibitor (14), a scavenger of superoxide anion radicals (13) and also a neutralizing agent against the toxohormone activity (16). The free iron, the most important lipid autooxidation catalyst, is readily mopped up and immobilized by Tr through the ferroxidase activity of Cp. Cp increases sharply in the sera of cancer bearing hosts (12). We found the elevated ESR signal of cupric ions in sera of DAB hepatoma-bearing rats, as had been recognized in various cancer patients (11). Although the scavenging activity of Cp against superoxide anion radicals is substantially less than that of erythrocyte superoxide dismutase (21), it may still play a significant role as circulating scavenger of oxygen-derived free radicals.

Even if poorly differentiated tumor cells lack their own defense mechanism against oxygen toxicity, they seem to survive by taking advantage of widely distributed serum metalloproteins for this purpose.

REFERENCES


