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ANTIOXIDANT SYSTEMS IN TUMOUR CELLS: THE LEVELS OF ANTIOXIDANT ENZYMES, FERRITIN, AND TOTAL IRON IN A HUMAN HEPATOMA CELL LINE

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The human hepatoma cell line Hep 3B, which has the hepatitis B virus genome, shows over 80% decrease of copper/zinc superoxide dismutase activity, over 90% decrease of manganese superoxide dismutase activity, over 70% decrease of catalase activity, absence of glutathione peroxidase and glutathione S-transferase activities, over 270-fold increase of ferritin content and 25-fold increase of total iron compared to normal autopsy liver. These conditions of low antioxidant enzyme activities and iron overload are those which support the accumulation of oxygen free-radicals and DNA damage commonly considered to be carcinogenic mechanisms.

Key words: Human Hepatoma, Superoxide Dismutase, Catalase, Glutathione Peroxidase, Glutathione S-Transferase, Ferritin, Iron.

INTRODUCTION

The enzymes copper/zinc and manganese superoxide dismutase (SOD) together with catalase, glutathione peroxidase, and glutathione S-transferase form a defence system against oxygen free-radicals, while non-protein-bound, non-heme, and ferritin iron are potential generators of these radicals in biological systems[1,2]. Measurements of SOD activity in tumour cells have tended to show depression of MnSOD activity and less strict depression of Cu/ZnSOD activity. MnSOD activity has been found to be significantly reduced in 70 different types of cancer including human, rat, mouse, spontaneous, transplanted, virally-induced, chemically-induced, *in vivo* and *in vitro* tumour cells[3]. This supports the hypothesis that the cancer phenotype is characterized by loss of MnSOD activity[4]. There are however discordant observations in human tumours and neoplastic cell lines[5,6,7,8,9].

Marklund *et al.*[7] have reported the SOD activity of 31 human cell lines of different origins. Cu/ZnSOD activity was less variable than MnSOD activity and showed a

tendency to decrease in neoplastic cell lines except for lymphoma and leukemia cells in which it was elevated, conforming to previous observations,[4,10,11]. Decrease in MnSOD activity appeared to be unusual in human neoplastic cell lines. The tendency was one of no change or slight increase of MnSOD activity except in malignant mesothelioma cells which appeared to have very high MnSOD and moderately high Cu/ZnSOD activity compared to other cell lines and normal tissues.

In Morris hepatomas, which show decrease of SOD activity compared to normal rat liver, total SOD and MnSOD activity correlate inversely with growth rate. MnSOD activity in isolated mitochondria is apparently decreased in medium- and fast-growing, and increased in slow-growing hepatoma compared to normal liver. Electron micrographs show mitochondrial membrane damage in medium- and fast-growing hepatomas. Superoxide production by submitochondrial particles does not differ significantly in slow- or fast-growing hepatoma compared to normal liver[12]. The behaviour of MnSOD activity in regenerating rat liver shows that decrease of activity is not a simple consequence of rapid cell division[13].

We report here observations on the activity of SOD and other antioxidant enzymes, ferritin content and total iron in the human heptoma Hep 3B cell line compared to normal autopsy liver.

MATERIALS AND METHODS

Autopsy liver samples were obtained shortly after death. The livers showed no visible signs of disease. Hep 3B cells were cultured as described elsewhere[14,15]. Liver specimens were cut into thin slices which were washed exhaustively with ice-cold phosphate buffered saline and homogenized by hand with 9 vol. of 0.1 M phosphate-buffer, containing 0.1 mM EDTA, pH 7.8 in a Potter-Elvehjem homogenizer. The homogenate was frozen to −20°C, thawed, and sonicated to ensure enzyme release. The supernatant remaining after centrifuging at 20,000 g was stored at −20°C before use as assay material. Hep 3B cells collected by centrifuging were washed with ice-cold phosphate buffered saline and processed as for liver slices.

Superoxide dismutase activity was measured according to McCord and Fridovich[16]. The activity remaining in reaction mixtures containing 2 mM KCN was taken as due to MnSOD. SOD units were calculated as described by Ysbaert-Vanneste and Vanneste[17]. Catalase, glutathione peroxidase, and glutathione S-transferase activities were measured as described elsewhere[18,19,20]. SOD, catalase and glutathione peroxidase activities of the liver samples were corrected for contamination by red blood cells on the basis of residual haemoglobin in the extracts. The corrections applied did not exceed 5% of the measured activities. Ferritin was measured by an R1A kit (Micromedic Systems, Inc.). Total iron was determined by atomic absorption spectrophotometry after extraction with perchloric acid, and corrected for zero residual haemoglobin for autopsy liver samples. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951).

The statistical significance of observed values for the Hep 3B cells was assessed in relation to the observed range of autopsy liver values by means of a test statistic given by Natrella[2].

TABLE I
Antioxidant enzymes, ferritin, and total iron in autopsy liver and Hep 3B cells

	Autopsy liver[a]	Hep 3B[b]	Δ	P[c]
CuZnSOD (U/mg protein)	20.1 ± 9.31	3.9 ± 0.18	−81%	$P < 0.005$
MnSOD (U/mg protein)	21.8 ± 4.29	1.7 ± 0.09	−92%	$P < 0.005$
Catalase (U/mg protein)	64.5 ± 15.89	16.5 ± 0.81	−74%	$P < 0.005$
Glutathione peroxidase (U/mg protein)	0.25 ± 0.11	0	−100%	$P < 0.025$
Glutathione S-transferase (U/mg protein)	0.91 ± 0.61	0	−100%	$P < 0.05$
Ferritin (μg/mg protein)	2.4 ± 0.38	656.2 ± 41.06	+272-fold	$P < 0.0001$
Total iron (μg/mg protein)	1.5 ± 0.61	39.5 ± 1.94	+25-fold	$P < 0.0001$

[a] Mean ± SD of 4 samples
[b] Determined value ± SE of replicates
[c] Significance of difference

RESULTS AND DISCUSSION

The data obtained are given in Table I which shows over 80% decrease of Cu/ZnSOD activity, over 90% decrease of MnSOD activity, over 70% decrease of catalase activity, absence of glutathione peroxidase and glutathione S-transferase activities, over 270-fold increase of ferritin content, and 25-fold increase of total iron in the Hep 3B cells compared to autopsy liver. The decrease in CuZnSOD and MnSOD activity is comparable to that apparent in fast-growth rate Morris hepatomas, as can be seen in Table II.

Although measurement of superoxide production by washed submitochondrial particles in the presence of succinate and antimycin A has suggested that this does not

TABLE II
Comparison of superoxide dismutase activity in Hep 3B with that in Morris hepatomas[a]

Hepatoma	Growth rate	CuZnSOD (%)	MnSOD (%)
Morris 96218A	Slow	57	60
Morris 5123D	Medium	38	12
Morris 7288ctc	Fast	23	11
Hep 3B	—	19	6

[a] Data for Morris hepatomas from Bize *et al.*[12]. Measured activities for normal rat liver (100%) were CuZnSOD 148 ± 20(SE)U/mg protein; MnSOD 52 ± 9(SE)U/mg protein: Xanthine-xanthine oxidase-nitro blue tetrazolium assay of SOD was used by these authors.

differ significantly in slow- (9618A) or fast-growing (7288ctc) Morris hepatomas compared to normal rat liver[12], the potential to produce superoxide in the cytosol, measured as realizable xanthine oxidase activity, is 27% of that of normal liver in the slow-growing 9618A hepatoma, 31% in the medium-growing 5123D hepatoma, and on average 9% in fast-growing Morris hepatomas[23]. It is, therefore, dangerous to conclude that normal superoxide production is associated with decreased SOD activity in hepatomas. On the contrary, it is possible that superoxide production and SOD activity are not dissociated in these tumours and the level of SOD activity could be coupled to the level of superoxide production. There has been some evidence that hyperoxia does not induce MnSOD activity in tumour cell lines[24]. However, Petkau *et al.*[25] observed induction of SOD activity by hyperoxia in 7,12-dimethylbenz[a]anthracene-initiated rat mammary carcinoma.

Hep 3B cells have the hepatitis B virus (HBV) genome like primary hepatocellular carcinoma, as shown by secretion of HBsAg[15]. Kulkarni *et al.*[26] showed decreased ethanol/chloroform-extractable SOD activity in monkey liver infected with HBV especially after monkey liver passage of the virus. The magnitude of the decrease (−74%) was close to that observed in the present work for Cu/ZnSOD activity in Hep 3B (−81%) cells. These workers did not study MnSOD activity.

It is possible for MnSOD activity to decrease at the transforming stage of tumourigenesis. In an immortalized cell line, the W1-38 embryonic lung fibroblast, Yamanaka and Deamer[27] found somewhat higher SOD activity than in normal embryonic lung tissue, while in SV-40 transformed cells MnSOD activity was greatly diminished but total SOD activity was somewhat higher than in untransformed W1-38 cells. These results are confirmed in the data of Marklund *et al.*[7] which show increase of Cu/ZnSOD (+57%) and decrease of MnSOD activity (−97%) in transformed W1-38 fibroblasts with respect to embryonic lung fibroblasts.

Recent work has confirmed the association of high SOD activity and induction of SOD with the normal phenotype, in contrast to the cancer phenotype, though no distinction was made between Cu/ZnSOD and MnSOD activities. Fernandez-Pol *et al.*[28] showed that normal rat kidney (NRK) cells are tolerant to 12.5 μM paraquat and 100 μM paraquat induced SOD activity (+50%) in the cells. On the other hand, 12.5 μM paraquat is cytotoxic to cells transformed by Kirsten sarcoma (K-NRK) or SV-40 virus (SV-NRK) and 100 μM paraquat does not induce SOD in these cells. This correlates with higher SOD activity in NRK compared to K-NRK (+118%) or SV-NRK cells. In K-NRK cells exposed to 12.5 μM paraquat revertant cells (less than 1 in 100) appear (e.g. RE8G3) which show increased SOD activity (+482% with respect to K-NRK and +167% with respect to NRK cells) and 100 μM paraquat induces further SOD activity (+17%) in these revertants. The normal phenotype in revertant cells depends on continued presence of paraquat. However, loss of SOD activity in revertants in absence of paraquat was not tested.

As regards other oxidant enzymes, the general view is that catalase activity may be low in tumour cells[7,29,30], as observed in Hep 3B cells, but not always[7]. Glutathione peroxidase activity may be within the range of normal tissues[7] or even high[10,31], in contrast to total absence in Hep 3B cells. Low glutathione peroxidase activity has been observed in a mouse bladder carcinoma[30]. As for glutathione S-transferase, this requires investigation in tumour cells.

Low levels of antioxidant enzyme activities go hand in hand with the abnormally-low rates of lipid peroxidation often observed in tumour cells *in vitro*[32,33,34]. There

may, however, be other forms of antioxidant activity[35,36,37]. Oberley *et al.*[38] have proposed that the lack of differentiation in cancer cells is caused by a relative lack of free-radical scavengers, particularly MnSOD, coupled with production of free radicals, especially superoxide. Oxygen free-radicals may accumulate in Hep 3B cells because of low SOD activity and high ferritin and iron content. Superoxide probably mobilizes iron from ferritin which catalyzes the Haber-Weiss reaction and hydroxyl radical production[39,40]. Production of oxygen free-radicals is potentially carcinogenic because of DNA damage[37,41]. Extensive DNA damage brought about by reactive oxygen intermediates is observed in rat liver nuclei incubated with Fe(II) salts, but it remains to be seen whether or not nuclear DNA is damaged in iron overload[42]. Normal cellular mechanisms can be considered to segregate DNA and iron.

Ferritin and total iron are greatly increased in Hep 3B cells. The rapid proliferation of tumour cells is dependent on their ability to take up iron. In three rat hepatoma cell lines, studied by Lloyd *et al.*,[43] the rate of iron uptake was less than in fetal hepatocytes and similar to that in adult hepatocytes. Transferrin binding by the hepatoma cells was less than in adult or fetal hepatocytes. Thus the efficiency of iron uptake, in terms of the iron: transferrin uptake ratio, was equivalent to fetal hepatocytes and greater than for adult hepatocytes. However, the proportion of iron incorporated into ferritin was less in the hepatoma cells than in fetal and adult hepatocytes. In the present work, the total iron determined for autopsy liver was compatible with normal saturation of the estimated ferritin. On the other hand, the total iron determined for Hep 3B cells suggests less-than-normal saturation of the estimated ferritin. Low iron content has been reported for ferritin isolated from human hepatocellular carcinoma[44].

Exogenous transferrin is not required for the growth of $H_4A_2C_2$ Reuber rat hepatoma cells nor for the delivery of iron to these cells. Moreover, iron incorporation by the hepatoma cells and corresponding increases in ferritin levels (over 50-fold) are greatly facilitated in cells grown in serum-free medium[45]. These cells are able to utilize ferric ammonium citrate very effectively to stimulate increases in ferritin levels with predominance of L rich (spleen type) ferritins in cell cultures maintained for two weeks or more. The iron uptake may involve an active endocytosis of hydrolyzed iron polymers[45]. The fact that bovine transferrin does not bind to human transferrin receptor suggests that cells grown in medium supplemented with fetal calf serum, as in the present work, meet their iron requirements by other mechanisms than receptor-mediated uptake from transferrin, as already noted by Hradilek *et al.*[46] for HeLa cells.

Fernandez-Pol *et al.*[47] obtained mutants of K-NRK cells which did not have measurable transferrin receptors though still able to maintain rapid proliferation rates. The apparently high efficiency of iron uptake in relation to transferrin binding in rat hepatoma cells[43] may reflect the fact that certain cell lines depend on iron-carrier compounds other than serum transferrin for their iron requirements.[48]

The intracellular levels of ferritin are thought to be regulated by the levels of non-heme iron. Much of the stimulatory effect of iron on ferritin synthesis, where observed, appears to be due to a post-translational effect leading to shell assembly[49]. An inverse relationship between stimulation of ferritin synthesis and ferritin iron content has been reported for rat hepatoma[50]. The present results and inferences for Hep 3B cells are consistent with this observation.

Liver and reticulocytes accumulate ferritin mRNA in excess of that required for constitutive ferritin synthesis, while excess iron can greatly increase ferritin synthesis and accumulation without detectably changing the concentration of translatable

ferritin mRNA in a tadpole model. While storing mRNA adds an additional burden to the economy of the cell, the toxicity of iron for DNA may explain the advantage of transcribing ferritin genes in advance of iron accumulation by the cell, as suggested by Shull *et al.*[51] This is an attractive hypothesis to apply to Hep 3B and other tumour cells which concentrate iron and synthesize disproportionately large amounts of ferritin.

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