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Lipid Peroxidation and Lipid Antioxidants in Normal and Tumor Cells*

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ABSTRACT

Lipid peroxidation is often low in tumor tissue as compared to the corresponding normal tissue and it has been postulated that lipid peroxidation may be associated with cell division. In this paper the various contributory factors which control the rate of microsomal lipid peroxidation in normal rat liver and in the Novikoff hepatoma have been carefully analyzed. The low rate of lipid peroxidation in the hepatoma seems to be due to a combination of factors: low levels of polyunsaturated fatty acids and of cytochrome P-450 and elevated levels of lipid-soluble antioxidant. This lipid-soluble antioxidant is principally α-tocopherol.

INTRODUCTION

Lipid peroxidation is the oxidative deterioration of unsaturated fatty acids and is an autocatalytic chain reaction initiated and propagated by free radicals. The first step is the formation of a lipid radical by the abstraction of a hydrogen atom by an initiating radical. The most susceptible substrates for this radical attack in biomembranes are the polyunsaturated fatty acids (PUFAs) containing 2 or more double bonds. The groups between these pairs of double bonds, bis-allylic methylene groups, are particularly susceptible to hydrogen abstraction. The lipid radicals so formed react rapidly with oxygen to form lipid hydroperoxy free radicals that break down to a wide variety of carbonyl products and to further radicals that propagate the process.

The presence of PUFAs in cell membranes makes cells susceptible to damage by lipid peroxidation. This damaging process is now accepted as an important factor in certain types of tissue injury. The significance and even the occurrence of lipid peroxidation in undisturbed cells is, however, a subject of intense debate.

It has long been known that the rate of lipid peroxidation is often low in tumor tissue as compared to the corresponding normal tissue. These observations date back at least some 30 years to when Donnan (1) reported a low rate of lipid peroxidation in hepatomas that had been induced with the dye butter-yellow. Shortly after, Shuster (2) noted the same phenomenon in Ehrlich ascites tumor cells. Perceptively, Shuster pointed out that these cells would thus be relieved of the effects of the oxidation products of fatty acids which would otherwise inhibit cell division. This was perhaps the first time that lipid peroxidation had been proposed to be a regulator of cell division. In the intervening years these reports of low lipid peroxidation in tumor tissue have been confirmed and extended (3–7). At the same time other pertinent properties of tumor cells have been studied, notably their lipid composition and their content of enzymes that can stimulate or inhibit lipid peroxidation. While these studies have not always been carried out in a co-ordinated way and have studied many

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different parameters in many different types of tumor, a reasonably clear pattern has emerged, as follows. Firstly, as already stated, the rate of lipid peroxidation, both spontaneous and forced, is lower in tumor tissue than in corresponding normal tissue. Secondly, tumor cells and organelles often have an abnormal lipid composition: usually a decreased phospholipid content and a low level of polyunsaturated fatty acids with occasionally, especially in hepatomas, an increased proportion of cholesterol (2, 3, 8, 9). Thirdly, tumor cells (including many hepatoma cell lines) generally contain low, often undetectable, levels of the enzymes of the cytochrome P-450 system which can initiate and propagate lipid peroxidation (3, 10, 11). Finally, tumor tissue has been reported to contain elevated levels of antioxidant compounds, of indeterminate identity, usually assayed by ability of tumor tissue extracts to inhibit lipid peroxidation in normal tissue preparations (3, 4, 12).

In addition to these studies on cancerous tissue, related observations have been made in dividing normal cells (13, 14). Generally, a low rate of lipid peroxidation and low cytochrome P-450-mediated activity is associated with an increased rate of cell division. In the liver, normally high in lipid peroxidation activity, the rate is much lower at times of high cell division, e.g., in fetal and neonatal liver (5). Lipid peroxidation activity has been found to decrease in regenerating liver following partial hepatectomy (5, 15).

At the same time, a large amount of work stemming from Shuster's paper (2) has established that the products of lipid peroxidation can inhibit the synthesis of DNA and cell division. As already mentioned the major products are carbonyls and considerable advances have been made in identifying the carbonyl products of cellular lipid peroxidation and studying their biological activity (16–18). The most biologically active of these carbonyls have been found to be the hydroxyalkenals which inhibit many cell activities notably adenyl cyclase, S-adenosylmethionine decarboxylase, and the synthesis of protein and DNA.

Drawing these aspects together, it has been proposed, notably by Burlakova et al (13) that lipid peroxidation occurs in normal cells under physiological conditions and that it acts as a 'coarse' regulator of cell division, possibly via the effects of the products of lipid peroxidation (19). According to this hypothesis, the increased rate of cell division in cancerous tissue may be due to a decreased level of lipid peroxidation activity. In order to test this hypothesis we have initiated a program of work aimed at elucidating the contribution of various factors in the decreased level of lipid peroxidation in tumor tissue. As mentioned earlier, these factors have been measured in the past but not in a co-ordinated fashion as we have aimed to do. This program will eventually encompass a range of tumors and normal tissues with varying rates of cell division. We have started by taking the Novikoff ascites hepatoma as our tumor model in order to develop the necessary methodology and to use a model that is rapidly growing. This is a maximal deviation hepatoma with a short generation time (ca. 7 days) which is readily grown in rats and we have compared it to normal liver from identical animals.

METHODS

The Novikoff hepatoma was maintained in male Wistar rats (200–300g body weight) which were allowed access to normal laboratory food and water *ad libitum*. The cells were passaged at approximately 7-day intervals by taking 1 ml of ascitic fluid and injecting it into the peritoneum of the recipient rat. Microsomal fractions were prepared from the livers of control animals and from hepatoma cells as previously described (20), without prior starvation.

It was first necessary to establish that Novikoff hepatoma microsomes conformed to the established pattern of other tumor preparations in possessing low lipid peroxidation activity. Three systems were tested in which lipid peroxidation was stimulated either by (a) NADPH/CCl_4 (20), (b) NADPH/ADP/Fe^{2+} (21) or (c) Ascorbate/Fe (22). Systems (a) and (b) require microsomal enzymes: system (b) requires NADPH:cytochrome c reductase to maintain ADP-Fe^{2+} in a reduced state, system (a) probably requires both the reductase and cytochrome P-450. The ascorbate-iron-induced lipid peroxidation is essentially non-enzymic. In each case lipid peroxidation was assayed by the standard thiobarbituric acid test for malondialdehyde.

As expected Novikoff hepatoma microsomes were found to have very low lipid peroxidation activity (Table I). In the case of the enzymic systems, the low level of lipid

TABLE I—Levels of Various Parameters of Lipid Peroxidation Activity and Microsomal Enzyme Activity in Control Rat Liver and Novikoff Hepatoma Preparations

Parameter	Percent value relative to liver microsomes
NADPH/CCl_4-dependent MDA production	Not detectable
NADPH/ADP-Fe-dependent MDA production	9
Ascorbate/iron-dependent MDA production	5
NADPH: cytochrome c reductase activity	10
Cytochrome P-450 content	Not detectable

peroxidation could have been due to low enzymic activity. Indeed, the enzymes of the microsomal mixed function oxidase system were found to be present at much lower levels in the hepatoma preparation than in normal liver microsomes:NADPH:cytochrome c reductase in the hepatoma was found to be only 10% of the control level and cytochrome P-450 was not detectable in the hepatoma (Table I). However, the exceedingly low level of ascorbate-iron-induced lipid peroxidation in hepatoma microsomes could not be wholly ascribed to low enzyme levels. This suggests that these membrane preparations contained low levels of the PUFA substrates for lipid peroxidation and/or elevated levels of free radical scavengers acting as antioxidants.

Hepatoma and control liver microsomes were subject to chloroform:methanol (2:1, v/v) extraction and the lipid extract used to assess the fatty acid composition, the content of cholesterol, total lipid-soluble antioxidant and α-tocopherol. The fatty acid composition was assayed by conventional procedures: the extract was concentrated and subject to saponification and methylation steps in preparation for gas-liquid chromatography.

The total antioxidant assay has been described (22) and based on peroxyl radical titration. An aliquot of the lipid extract is added to a purely chemical system in which styrene undergoes autoxidation in a process analogous to lipid peroxidation. The autoxidation of styrene is also a free radical chain reaction and is initiated by the thermal decomposition of azobis(isobutyronitrile) (AIBN). Styrene oxidation is measured as oxygen utilization under carefully controlled conditions. The presence of antioxidant in the lipid extract inhibits autoxidation for a well-defined time period, the induction period. The concentration of antioxidant is calculated from the length of this induction period (τ in Fig. 1). While this method accurately measures the lipid-soluble antioxidant content of our biological samples in quantitative terms it does not identify the nature of the antioxidant(s). Each lipid extract was therefore subject to an HPLC assay which measured the concentration of vitamin E as α-, β-, γ-, and δ-tocopherols (see Ref. 22). Vitamin E was chosen for determination for two main reasons. Firstly, it is classically considered as the major lipid-soluble biological antioxidant. Secondly, it has been found actually to be the major, possibly the sole lipid-soluble chain-breaking antioxidant in human blood (22). It was natural, therefore, to determine if this was also the case in microsomes from normal liver and from hepatoma cells.

Results and Discussion

The microsomal lipid extract analyses are presented in Table II. It is readily apparent that the hepatoma microsomes contain a much lower proportion of the PUFAs which are most susceptible to peroxidation, arachidonic acid (20:4) and docosahexaenoic acid (22:6). The balance is made up by increased proportions of oleic acid (18:1) and linoleic (18:2) which are much less susceptible to oxidative attack. The content of PUFAs in each sample can be represented by the so-called double-bond index, which is derived

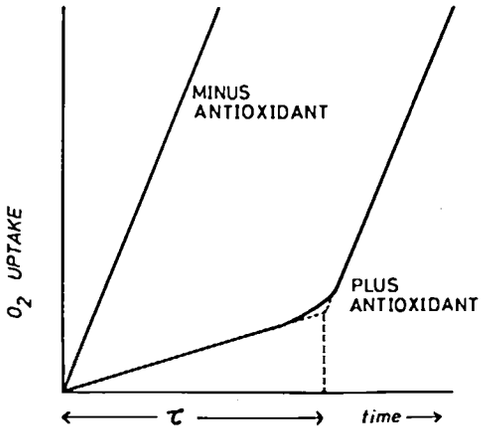

FIG. 1—Total antioxidant assayed by induction period method. The system is based on the oxidation of styrene initiated by the thermal decomposition of AIBN.

TABLE II—Typical Values for Fatty Acid Composition and Content of Antioxidant and Cholesterol in Microsomes of Normal Rat Liver and Novikoff Hepatoma

Parameter	Normal	Hepatoma
Fatty acid composition[a] (% of total)		
16:0	20	16
18:0	23	21
18:1	7	18
18:2	17	24
20:4	24	12
22:6	7	2
Double-bond index	100	70
Bis-allylic methylenes	100	57
Cholesterol	100	254
Total antioxidant/lipid	100	208
α-tocopherol/lipid	100	340
α-tocopherol/bisallylic methylene	100	368

[a] Expressed as % total; all other parameters as % of normal.

by multiplying the number of double bonds in each fatty acid by their respective percentage concentration and summing the products. The Novikoff hepatoma microsomes are found to have a double-bond index only 30% lower than that of the control microsomes. However, this index places too much weight on the proportion of oleic acid, which has only one double bond and therefore no bisallylic methylene group. A more realistic index of the susceptibility to peroxidation, ignoring antioxidant concentrations, should be given by calculating the number of bisallylic methylenes. The hepatoma microsomes are found to have approximately 40% less of the susceptible bisallylic methylene groups in relation to the controls. This difference, though large, does not seem enough to account for the high resistance to lipid peroxidation of hepatoma microsomes and so account must be taken of the antioxidant concentration. When the concentration of total antioxidants in the lipid of control and hepatoma microsomal extracts was ascertained, it was found to be approximately double in the lipid of hepatoma microsomes as compared to controls. The lipid concentration of α-tocopherol in the hepatoma microsomes was found to be approximately 3.5 times that in controls. The total lipid antioxidant in each case is largely α-tocopherol. If the ratio of α-tocopherol to bisallylic methylene groups is calculated for the hepatoma and control microsomes (Table II) then it is found to be nearly 4 times higher in the hepatoma microsomes. It seems probable that it is this ratio of inhibitor concentration to substrate concentration which determines the relative susceptibility or resistance to lipid peroxidation of the microsomal membranes.

We have made similar analyses of whole Novikoff ascites hepatoma cells and whole normal liver with comparable results to those found with the respective microsomal fractions (Table III). Again, the analysis of the fatty acid composition of the lipids shows a significantly decreased PUFA content in the hepatoma cells. This is reflected to some extent in the double bond index but rather more accurately by counting the number of bisallylic methylene groups. The concentration of α-tocopherol is found to be elevated in the whole hepatoma cells, as in the isolated hepatoma microsomes. Consequently, these cells also have a much higher α-tocopherol:bisallylic methylene group ratio than does normal liver. We are in the process of studying lipid peroxidation in these whole cells and already know them to be highly resistant. However, the whole cell possesses several other antioxidant mechanisms, such as those dependent on glutathione, which must be considered alongside the concentration of α-tocopherol. Nevertheless, for the microsomal preparations at least, we have a clear idea of the various contributory factors that result in a very low rate of lipid peroxi-

TABLE III—Typical Values for Lipid and Antioxidant Analysis of Whole Normal Rat Liver and Whole Novikoff Ascites Hepatoma Cells

Parameter	Normal	Hepatoma
Fatty acid composition[a]		
16:0	23	16
18:0	20	20
18:1	12	22
18:2	21	32
20:4	17	9
22:6	7	2
Double-bond index	100	80
Bis-allylic methylenes	100	66
Cholesterol	100	200
Total antioxidant/lipid	100	282
α-tocopherol/lipid	100	350
α-tocopherol/bisallylic methylene	100	560

[a] Expressed as % of total; all other parameters as % of normal.

dation in the Novikoff hepatoma. The significance of these findings remains to be elucidated. For example: is the low rate of lipid peroxidation a contributory mechanism of significance in the high rate of cell division of this hepatoma or merely a consequence of the extreme deviation from normal which the Novikoff hepatoma represents? We are currently extending this work to other hepatoma models and to dividing normal cells and testing for a correlation between the rate of cell division and the lipid peroxidation activity. In this way it is hoped that the true significance of lipid peroxidation to cell proliferation can be assessed.

Acknowledgments

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