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Acknowledgements—Supported by the Children Cancer Foundation of Sweden, Swedish Cancer Society, HKH Kronprinsessan Lovisas förening för barnasjukvård, American-Scandinavian Foundation, Hans von Krantzows and Ollie och Elof Ericssons Stiftelser.

Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 463–466, 1995

0959–8049/95 $9.50+0.00

0959-8049(95)00059-3

Cell Death by Oxidative Stress and Ascorbic Acid Regeneration in Human Neuroectodermal Cell Lines

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In this paper, we show that human neuroectodermal cells exposed to 1–5 mM hydrogen peroxide or 10 nM–1 mM ascorbate die by programmed cell death induced by oxidative stress. The cell death by peroxide occurs within 4 h and involves approximately 80% of B-mel melanoma cells, while ascorbate causes cell death of approximately 86% of B-mel cells within 24 h. SK-N-BE(2) neuroblastoma cells are more resistant, 32% and 43% cell death for peroxide and ascorbate, respectively. In all cases, cell death causes hypodiploic DNA staining, evaluated by flow cytometry. Both cell lines can efficiently metabolise ascorbate due to significant levels of NADH-dependent semidehydroascorbate reductase and glutathione-dependent dehydroascorbate reductase. The cell death observed suggests a pro-oxidant, rather than anti-oxidant, role for ascorbic acid at physiological concentrations under these experimental conditions.

Key words: apoptosis, ascorbic acid, cell death, hydrogen peroxide, melanoma, neuroblastoma
Eur J Cancer, Vol. 31A, No. 4, pp. 463–466, 1995

INTRODUCTION

CELL DEATH by apoptosis is a gene-regulated programme of cell self-deletion acting under physiological conditions in multicellular organisms, characterised by specific nuclear (chromatin condensation, DNA fragmentation) and cytoplasmic (integrity of the cell membrane with formation of stable protein cross-links catalysed by tissue-transglutaminase, EC 2.3.2.13, thus conferring resistance to breakage and chemical attack to the apoptotic body) events [1, 2]. The negative control of apoptosis is maintained by ced-9 in *C. elegans* [3], by bcl-2 [4, 5] and possibly other gene products [6–8] in vertebrates. Recently, additional members of the *BCL-2* gene family have been isolated, *BCL-X* and *BAX*, that may act synergistically or antagonistically with *BCL-2* [9, 10]. bcl-2 seems to function as a cell death suppressor protein by decreasing the generation of reactive oxygen species [11, 12]. The kinetics of cell death induced by hydrogen peroxide seem to suggest that it acts on downstream effector elements with respect to *BCL-2*.

Vitamin C (ascorbic acid) is involved in many metabolic processes where it acts as an electron donor. The reduced form, ascorbate (AA), is oxidised either by single electron transfer with

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formation of the free radical semidehydroascorbate (SDA) or by two electron transfers with formation of dehydroascorbate (DA). SDA and DA can be converted back to ascorbate through enzymatic reduction by the NADH-dependent SDA reductase (SDAR; EC 1.6.5.4) or by the glutathione (GSH)-dependent DA reductase (DAR; EC 1.8.5.1) [13, 14]. In the absence of these recycling systems, SDA spontaneously disproportionates to ascorbate and dehydroascorbate, which is lost by hydration in an irreversible reaction. Ascorbate is a potent anti-oxidant; however, in the presence of transition metals, it shows a pro-oxidative activity.

Vitamin C has been indicated to be useful in cancer prevention [15]. The potential protective role could be related to the interaction of ascorbate or SDA species with toxic free radicals. Moreover, vitamin C has been reported to be selectively toxic to some types of malignant cell—melanoma, neuroblastoma—at concentrations that are physiological in human, and a pro-oxidative, rather than anti-oxidative, role as been suggested [16, 17]. In fact, tumour cells seem more sensitive to oxidative stresses, because of their altered pattern of antioxidant enzymes when compared with their non-malignant counterparts [15]. Whether such cell death occurs by apoptosis or necrosis has not yet been established.

The present study was undertaken to investigate (i) the mechanism of cell death by oxidative/pro-oxidative stress (using hydrogen peroxide or ascorbic acid) in two human neuroblastoma and melanoma cell lines; and (ii) the ability of cancer cells to regenerate ascorbate from their oxidative products. To this end, we quantitated the DAR and SDAR activities. An effective recycling could enhance the pro-oxidative action; therefore, ascorbate may react with iron allowing the production of oxygen free radicals via the Fenton reaction [17].

MATERIALS AND METHODS

Cell cultures

The human neuroblastoma, SK-N-BE(2), and melanoma, B-mel, cell lines were grown in a 1:1 mixture of minimal essential medium (MEM) and Hanks F-12 media supplemented with 15% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 1.2 g/l bicarbonate, non-essential amino acids (1% v/v) and 15 mM Hepes in a humidified atmosphere with 5% (v/v) CO_2 at 37°C. Cell growth was evaluated by plating 50×10^3 cells per 25 cm^2 flask and counting the viable cell number daily in a Thoma haemocytometer chamber. Triplicate experiments were evaluated by two independent observers. Cell growth was also evaluated by ^{3}H-thymidine uptake [18].

Cell death was evaluated by flow cytometry, staining with 50 mg/ml propidium iodide [19] on a FACScan flow cytometer (Becton-Dickinson, California, U.S.A.). Events were triggered by the FSC signal and gated for FSC-H/FSC-A/SSC to avoid aggregates. 10,000 events were evaluated using the Lysis II Programme.

Extraction procedures

Mechanically removed cells were washed twice in phosphate-buffered saline (PBS), resuspended in 0.05 M Tris–HCl buffer, pH 7.8, containing 0.3 mannitol, 1 mM EDTA, 10 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin, 0.05% (w/v) cysteine, and disrupted by two cycles of freezing–thawing with liquid nitrogen. Lysates were centrifuged at 2500 rpm for 20 min at 4°C and supernatants were used for enzyme assays.

Pig liver extract was prepared by centrifugation at 2500 rpm for 20 min at 4°C of a homogenate in Tris–HCl buffer as described above (1:3 w/v). Peripheral blood lymphocytes (PBL) from healthy volunteers were separated by gradient centrifugation at room temperature.

Enzyme assays

DAR activity was measured by the rate of increase in absorbance at 265 nm due to ascorbate formation (E = 15 mM cm^{-1}), at 25°C [20]. The assay mixture contained 0.1 M K-phosphate buffer at pH 6.0, 1 mM EDTA, 1 mM dehydroascorbate (ICN Nutritional Biochemical), 2 mM GSH (Sigma) and aliquots (50–300 µl of 0.1–2 mg protein/ml solution) of each sample in a final volume of 1 ml. Correction was made for the non-enzymatic reaction of GSH with dehydroascorbate.

SDAR activity was measured by the rate of ascorbate free radical-dependent oxidation of NADH, by monitoring the decrease in 340 nm absorbance (E = 6.2 mM cm^{-1}), at 25°C [20]. The assay mixture contained 0.05 M Tris–HCl buffer at pH 7.8, 1 mM EDTA, 0.1 mM NADH (Sigma), 1 mM ascorbate (Sigma) and aliquots of each sample in a final volume of 1 ml. The reaction was started by adding 1 U of ascorbate oxidase, purified from green zucchini peelings according to Avigliano and colleagues [21], to generate SDA radical [22]. The concentration of ascorbate oxidase was selected to give maximal reductase activity, as determined by preliminary experiments. Correction was made for direct oxidation of NADH by homogenates. Samples heated at 100°C for 10 min were used as blanks in both assays. Protein was measured by the method of Bradford [23], using bovine serum albumin as standard.

RESULTS

Cell death by ascorbate or hydrogen peroxide in neuroblastoma and melanoma cells

Table 1 shows the effects of ascorbate at concentrations of 10 nM–1 mM on the growth rate of both SK-N-BE(2) human neuroblastoma cells and peripheral blood lymphocytes (PBL). PBL were tested to compare the effects with human non-malignant cells. The data indicate that the neuroblastoma cell line had a significant slower growth at very low concentrations (10–100 nM) while micromolar concentrations were required to obtain the same effect on PBL.

In order to investigate the possibility that this growth inhi-

Table 1. Toxicity of ascorbate in different neuroblastoma cells and lymphocytes

	SK-N-BE(2) neuroblastoma	PBL lymphocytes
Untreated	1123 ± 245*	1430 ± 89*
AA, 10^{-8} M	641 ± 109	1209 ± 97
AA, 10^{-7} M	682 ± 73	1297 ± 102
AA, 10^{-6} M	661 ± 84	694 ± 72
AA, 10^{-5} M	737 ± 85	687 ± 59
AA, 10^{-4} M	249 ± 121	540 ± 43
AA, 10^{-3} M	106 ± 40	nt

Cells were cultured as described previously in the presence of fresh medium for 48 h. The cells were then washed and plated in a 96 U-bottomed titration plate to evaluate growth for 48 h. ^{3}H-thymidine was added for the last 8 h of culture. Peripheral blood lymphocytes (PBL) were stimulated to grow in the presence of phytohaemagglutinin. * Growth is expressed as cpm/min. Data are the mean ± S.E.M. of six determinations.

bition may be related to the induction of cell death by apoptosis, we evaluated DNA hypodiploic staining by flow cytometry using propidium iodide. Figure 1 indicates that ascorbate induced hypochromic DNA fragmentation, typical of programmed cell death. Both SK-N-BE(2) and B-mel melanoma cells died within 24–36 h at 0.1–1 mM ascorbate, although B-mel cells were more sensitive than SK-N-BE(2) to cell death; 86% versus 43% death using 1 mM ascorbate at 24 h (data not shown).

Finally, we evaluated the effect of ascorbate with peroxide known to induce programmed cell death [11]. Both B-mel and SK-N-BE(2) died within 2–4 h at 1–5 mM peroxide concentration (Figure 1). By this method, B-mel cells were also more sensitive to cell death than SK-N-BE(2) cells; 80% versus 32% death using 5 mM peroxide at 4 h (data not shown).

While the induction of cell death by apoptosis by peroxide has already been described by Hockenbery and associates [11] on FL5.12-Neo cells, 1 mM ascorbate has been reported to moderately protect cell death and scavenge reactive oxygen species in GT1-7 cells [12]. However, this differs from the toxic effect of ascorbic acid on neuroblastoma and melanoma cells reported here. Since the recycling of ascorbic acid may be relevant for the pro-oxidant action, we evaluated the activity of the enzymes involved.

Ascorbic acid recycling activity

We investigated the capacity of neuroblastoma and melanoma cells to regenerate AA from their oxidative products. To this end, we evaluated the GSH-dependent DAR and NADH-dependent SDAR activities. The data are shown in Table 2. Both cell lines expressed high SDAR and DAR activities, when compared with normal pig liver, known to have elevated SDAR and DAR activities. The DAR activity was lower than that in PBLs, which completely lack any SDAR activity.

DISCUSSION

Several indirect experimental results indicate the involvement of oxygen radicals in programmed cell death. Hydrogen peroxide induces programmed cell death, and *BCL-2* seems to act by decreasing the generation of reactive oxygen species [11, 12]. Furthermore, oxygen radicals produced by ionising radiation are known to induce apoptosis. Anti-oxidants, such as N-acetylcysteine and thioredoxin, protect against apoptosis induced by tumour necrosis factor-α [24]; similarly, N-(2-mercaptoethyl)-1,3-propanediamine reduces glucocorticoid and radiation-induced apoptosis in thymocytes [25]. GST (EC 2.5.1.18) increases its transcript's expression during apoptosis in rat ventral prostate tissue, induced by androgen withdrawal [26]. GST decreases the levels of electrophilic species through their conjugation to reduced glutathione, further supporting an oxidative stress in apoptosis. The downregulation of these defences has been found during steroid hormone-mediated apoptosis of lymphoid cell lines: transcript levels of catalase (EC 1.11.1.6), manganese superoxide dismutase (Mn-SOD, EC 1.15.1.1), copper-zinc superoxide dismutase (Cu,Zn-SOD, EC 1.15.1.1), NAD(P)H DT-diaphorase (EC 1.6.99.2) and thioredoxin sharply fell within 8 h, whilst GST (mu class) increased and GSH levels fell [27]. Taken together, these data indicate that (i) oxidative stresses may cause programmed cell death; and that (ii) the overall antioxidant systems are reduced in cells undergoing programmed cell death.

Our data are in keeping with the idea that oxidative stresses (e.g. hydrogen peroxide) induce programmed cell death. We have demonstrated that ascorbic acid, generally involved in

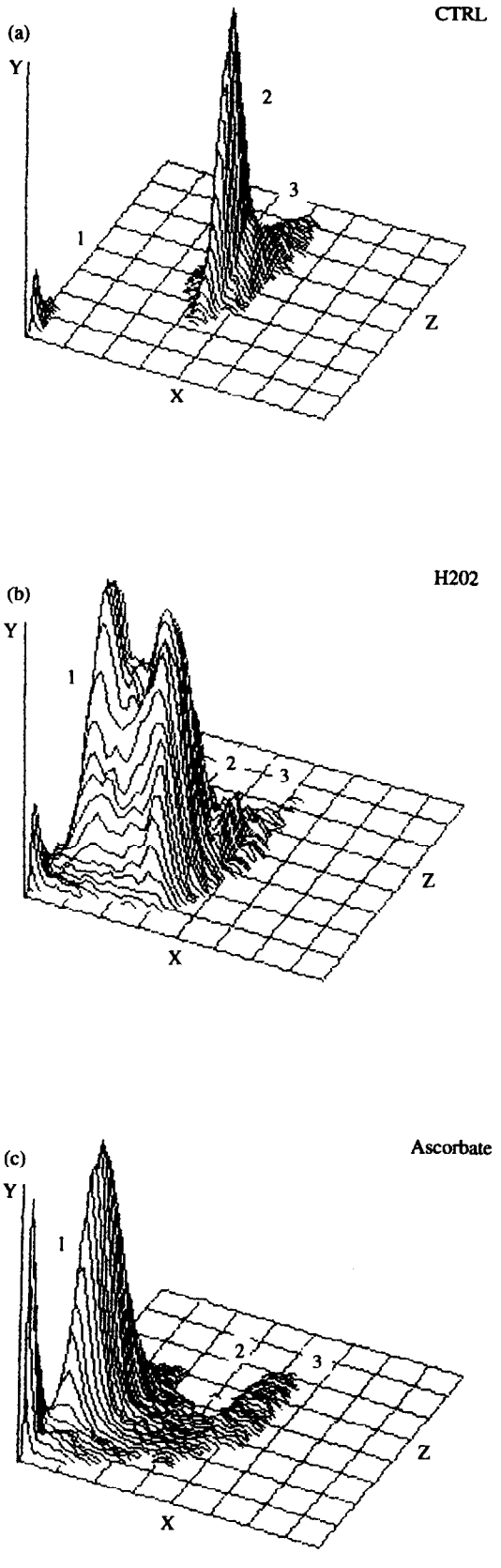

Figure 1. 3-D flow cytometry evaluation of cell death induced in B-mel cells. Axes represent 570 nm propidium iodide fluorescence (*x*), 179° forward scatter (*z*), number of events acquired (*y*). Cell deaths with fragmented DNA are in area 1, quiescent G_0/G_1 cells in area 2, and growing (G_2/M) cells in area 3. Panels show untreated cells (a), cells treated with 5 mM hydrogen peroxide for 4 h (b) or with 1 mM ascorbic acid for 24 h (c).

Table 2. Specific activity of NADH-dependent semi dehydroascorbate reductase (SDAR) and GSH-dependent dehydroascorbate reductase (DAR) in different neuroectodermal cells

	SDAR activity	DAR activity
SK-N-BE(2), neuroblastoma	18.2 ± 0.8*	11.4 ± 0.4†
B-mel, melanoma	24.2 ± 0.9	10.5 ± 0.3
PBL, lymphocytes	0.0 ± 0.1	50.7 ± 1.5
Normal pig liver	7.0 ± 0.3	9.4 ± 0.3

Cells were cultured as described previously in the presence of fresh medium for 48 h. The cells were then washed extensively in PBS and mechanically removed from flasks. Activity was measured as * nmol NADH oxidised/min/mg protein or as † nmol DA reduced/min/mg protein. Data are the mean ± S.E.M. of five determinations carried out on different experiments.

biological anti-oxidative defences mechanisms, induces programmed cell death in our model. We suggest that the effective recycling of ascorbate could enhance the pro-oxidative action of ascorbic acid. In fact, melanoma cells show a higher SDAR activity, which is possibly responsible for the higher pro-oxidant toxicity of ascorbic acid shown in Figure 1.

In conclusion, we show that neuroectodermal cancer cells may recycle ascorbic acid at physiological concentrations [28]; this leads to a pro-oxidant activation of the programmed cell death pathway, similar to that induced by typical oxidative stressants, such as hydrogen peroxide.

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Acknowledgements—The work was partially supported by grants: Progetti Finalizzati C.N.R. "ACRO" (to GM); Ministero Sanità; MURST.