Ascorbic Acid Enhances the Effects of 6-Hydroxydopamine and H$_2$O$_2$ on Iron-dependent DNA Strand Breaks and Related Processes in the Neuroblastoma Cell Line SK-N-SH

Gernot Bruchelt,$ Ingrid U. Schraufstätter, Dietrich Niethammer, and Charles G. Cochrane

Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037 [G. B., I. U. S., C. G. C.], and Department of Hematology and Oncology, Children’s Hospital, University of Tuebingen, Ruemelinstrasse 23, D-7400 Tuebingen, Federal Republic of Germany [D. N.]

ABSTRACT

Neuroblastoma cells accumulate ascorbic acid and iron. It was hypothesized that these features could be exploited for sensitizing neuroblastoma cells for therapy in combination with reactive oxygen intermediates. In the present study the effects of 6-hydroxydopamine (6-OHDA) and H$_2$O$_2$ on metabolic parameters critical for cell survival were investigated in cells with low and high ferritin content in the presence and absence of ascorbate. Human neuroblastoma SK-N-SH cells were pretreated with 100 $\mu$M FeSO$_4$ and 10 $\mu$M desferrioxamine, respectively, for 24 h yielding cells with different ferritin contents. The effects of 6-OHDA and H$_2$O$_2$ (25 $\mu$M-250 $\mu$M) in the absence and presence of 1 mM ascorbic acid on DNA strand break formation, activation of poly(ADP-ribose) polymerase, and finally decrease in NAD$^+$ and ATP concentration were investigated. All these parameters were influenced by 6-OHDA and H$_2$O$_2$ in a concentration-dependent manner in a similar way. The effects were most pronounced in ferritin-rich cells and in the presence of ascorbic acid. Using isolated CCC PM2 DNA, 6-OHDA and ascorbic acid caused strand breaks that were prevented in the presence of mannitol or desferrioxamine. H$_2$O$_2$-mediated strand breaks were observed only in the presence of ascorbic acid. Based on these data and data published by others a model explaining the deleterious effects of ascorbic acid on neuroblastoma cells is presented. It is suggested that continuous application of a high dosage of ascorbic acid might be a useful approach in neuroblastoma therapy.

INTRODUCTION

Neuroblastoma stage IV has a poor prognosis (1). Therefore, new therapeutic approaches are currently under clinical investigation. Among them is autologous bone marrow transplantation in combination with different purging procedures in order to remove contaminating neuroblastoma cells (2–4). In one of these purging systems bone marrow cells were incubated with 100 $\mu$M 6-OHDA in combination with 1 mM AA. It was assumed that neuroblastoma but not bone marrow cells would take up the catecholamine-analogous compound 6-OHDA (5). Inside the cells, H$_2$O$_2$ formed during the oxidation of 6-OHDA would destroy the neuroblastoma cells specifically (7, 8). Furthermore, AA included in a 10-fold molar excess would enhance these effects by acting as redoxycler (9, 10). However, some of these theoretical considerations as arguments for purging procedures proved to be incorrect. Since the oxygen concentration present in the purging mixture (about 250 $\mu$M) is limited, most of the AA is not consumed by the redox cycling process. Nevertheless, the cytotoxic effects of the combination 6-OHDA/AA were the more pronounced the more AA was present (9). This means that AA might have caused some toxic effects irrespective of its function as redoxycler. Furthermore, oxidation of 6-OHDA occurs rapidly so that most of the reactive oxygen intermediates are already formed in the incubation medium before a significant amount of 6-OHDA is taken up selectively by neuroblastoma cells (11). Moreover, only a small percentage of neuroblastoma cells can take up catecholamines selectively (12); therefore both neuroblastoma and hematopoietic stem cells are equally exposed to the reactive oxygen compounds. Neuroblastoma cells proved to be more vulnerable to 6-OHDA/AA than hematopoietic cells (9, 11) although the reason for this is unknown. We supposed that AA might sensitize neuroblastoma cells in concert with iron. Iron is found in these cells in large amounts incorporated in ferritin (13, 14). AA is concentrated in sympathetic nervous tissue (15, 16) and should therefore accumulate in neuroblastoma cells if provided in significant amounts. The potential cytotoxic effects of AA alone or in combination with cytostatic drugs have been described for neuroblastoma and other cells (17–19). However, some confusion exists since AA can act either as antioxidants (e.g. Refs. 20 and 21) or as prooxidants, the latter especially in combination with iron (22–24). Iron stored as ferritin is deprived of its potential cytotoxic effect, but it can be released from ferritin yielding ferrous iron (Fe$^{2+}$) by many agents, among them 6-OHDA and AA (25–28). Fe$^{2+}$ catalyzes the formation of the highly toxic OH$^-$ radicals from H$_2$O$_2$ in the Fenton reaction (29). In accordance with these findings, a mixture of H$_2$O$_2$ and ferritin was shown to produce OH$^-$ radicals in the presence but not in the absence of AA (30). AA and ferritin, acting together, could therefore be an effective enhancer system for therapeutic regimens that are operative via generation of H$_2$O$_2$. The most sensitive cellular target structure for H$_2$O$_2$ is obviously the DNA (31–34). H$_2$O$_2$ causes DNA strand breaks that are followed by the activation of poly(ADP-ribose) polymerase; this enzyme consumes NAD$^+$, a process that is subsequently associated with a loss of ATP (35, 36).

The aim of our study was to investigate the influence of AA on the effects of 6-OHDA and H$_2$O$_2$ on these DNA-dependent pathways using neuroblastoma cells with high and low ferritin content in order to find a better rational basis for a potential application of AA in the therapy of this tumor.

MATERIALS AND METHODS

Materials

Ethidium bromide, 6-hydroxydopamine, ascorbic acid, and 1,10-phenanthroline were from Sigma, St. Louis, MO; CCC PM2 DNA was from Boehringer Mannheim, Mannheim, Federal Republic of Germany; mannitol was from Calbiochem-Behring Corp., La Jolla, CA; desferrioxamine and desferriothiocin were from Ciba-Geigy, Basle, Switzerland; $[^{15}N]$NAD$^+$ (specific activity, 25 Ci/mmole) was from ICN,
Irvine, CA; L-[carboxyl-14C] ascorbic acid (specific activity, 10–30 mCi/mmole) was from Amersham-Buchler, Braunschweig, Federal Republic of Germany.

Determination of DNA Strand Breaks (Cell-free System)

(a) Ethidium-binding Assay According to the Method of Lown (37).

Briefly, CCC PM2 DNA (10 μl; 2 μg) was incubated with 10 μl PBS or with additives dissolved in PBS. After addition of 180 μl of ethidium bromide-containing alkaline buffer the samples were heated for 4 min at 96°C. Twenty μl of this solution were used for electrophoresis (see below); the remaining 180 μl were mixed with an additional 1 ml alkaline buffer before measurement of ethidium bromide fluorescence on a Perkin-Elmer model 650–15 (Hitachi-Perkin Elmer Instruments, Mountain View, CA).

(b) Electrophoresis. Twenty μl of DNA containing solution (200 ng, see α) were mixed with 4 μl loading buffer. Electrophoresis was carried out using 15 μl of this solution on a 0.75% agarose gel (375 mg of agarose were melted in 50 ml Tris-boric acid-EDTA buffer (8.1 g Tris base plus 4.125 g boric acid plus 0.7 g disodium EDTA-2 H2O/liter) containing 2.5 μl of ethidium bromide (10 mg/ml)). Electrophoresis was carried out using Tris-boric acid-EDTA buffer containing 0.5 mg ethidium bromide/liter at 100 V for about 2 h.

Determination of Ferritin Content in SK-N-SH Cells

Cells (1 × 10⁶/ml) were resuspended in 6-well plates (Greiner, Nuertingen, Federal Republic of Germany). After 24 h, 40 μl PBS (control), 40 μl DFO (10 μM), and 40 μl FeSO₄ (100 μM), respectively, were added. Ferritin was determined in cell lysates after 24 h using a commercial immunoassay (Tandem-Fer; Hybritech, Hürth-Effern, Federal Republic of Germany).

Uptake of [14C]Ascorbic Acid

SK-N-SH cells, grown as monolayers in 12-well plates (Greiner) at a density of 1 × 10⁶ cells, were incubated with 5–100 μM [14C] ascorbic acid in phosphate-buffered saline plus 1% bovine serum albumin for 30 min at 37°C. Cells were then washed three times with cold buffer solution, and cell pellets were lysed with 500 μl 0.3 M NaOH and mixed with 5 ml scintillation cocktail (Unisolve; Zinsser, Frankfurt, Federal Republic of Germany) before counting. For calculation of the intracellular AA concentration, cell volume was determined by centrifugation of SK-N-SH cells in a hemocrit centrifuge; 10⁶ SK-N-SH cells correspond to 2.14 μl.

Generation of SK-N-SH Cells with Different Ferritin Content

SK-N-SH cells were grown as monolayers using RPMI 1640 containing glutamine (2 mM), gentamicin (50 mg/liter), and 10% fetal calf serum (Gibco) in 75-cm² flasks. At about 80–90% confluency, flasks were incubated for 12 min.

RESULTS

SK-N-SH cells grown as monolayers were split and treated either with 10 μM DFO, 100 μM FeSO₄, or no further additions for 24 h. As shown in Fig. 1, DFO treatment reduced and FeSO₄ treatment strongly enhanced the ferritin content in these cells. The mean ferritin content of untreated cells was 19.1 ± 7.0 (SD) ng/10⁶ cells. After treatment with DFO, ferritin content dropped to 6.10 ± 3.67 ng/10⁶ (P < 0.01), after treatment with FeSO₄ it increased to 62.62 ± 15 ng/10⁶ cells (P < 0.01; 4 independent experiments in duplicate, Student’s t test). Ferritin content in iron-pretreated cells was 13.5 ± 8.7 times higher than in the correspondent DFO-pretreated cells.

DNA Strand Breaks in Isolated PM2 Phage DNA. In order to characterize the effects of H₂O₂, 6-OHDA, and AA on DNA, first experiments were carried out on isolated CCC PM2 DNA. Fig. 3A shows that 6-OHDA as well as AA generate single strand breaks in CCC PM2 DNA. H₂O₂ caused DNA strand

High Performance Liquid Chromatography Analysis of NAD⁺ and ATP

Cells (about 2 × 10⁶/ml) were incubated for 30 min with 6-OHDA and H₂O₂. Supernatants were removed and the cell pellets were treated with 220 μl 8% perchloric acid. After centrifugation, 200 μl of each supernatant were neutralized with 200 μl KOH/Tris. Supernatants were collected and frozen at −20°C for high performance liquid chromatography analysis. Two hundred μl were injected and separation was carried out on a Du Pont C₅ column. A gradient of 100% 100 mM phosphate buffer, pH 6.0, to 60% buffer plus 40% methanol was applied with a flow rate of 1.3 ml/min. Absorbance was measured at 256 nm; flow rate was 1.3 ml/min. The ATP signal appeared at about 5.4 min; the NAD⁺ signal appeared at about 17 min.

![Fig. 1. Ferritin levels in SK-N-SH cells after 24 h incubation with 10 μM DFO and 100 μM FeSO₄](cancerres.aacrjournals.org)
ASCORBATE, FERRITIN, AND REACTIVE OXYGEN COMPOUNDS IN NEUROBLASTOMA

Fig. 2. Uptake of [14C] ascorbic acid into SK-N-SH cells. About 10^6 cells were incubated at 37°C in 12-well plates for 30 min with 5–100 μM [14C] ascorbic acid. Mean of triplicate measurements; bars, SEM.

Fig. 3. A, strand break formation of CCC PM2 DNA. Lane 1, control; Lane 2, H2O2; Lane 3, 6-OHDA; Lane 4, ascorbic acid; Lane 5, H2O2 plus ascorbic acid; Lane 6, 6-OHDA plus ascorbic acid. Incubation was carried out for 1 h using 250 μM H2O2 or 6-OHDA and 10 mM ascorbic acid. B, determination of CCC PM2 DNA strand breaks after 20 min incubation with 1 mM ascorbic acid and 100 μM 6-OHDA in the absence and presence of mannitol (100 mM) and desferrithiocine (10 mM). In parallel, DNA strand breaks were determined using the fluorescence assay according to the method of Lown (numbers in parentheses, percentage of DNA strand breaks measured in the fluorescence assay). Lane 1, control (0%); Lane 2, 1 mM ascorbic acid (45.7%); Lane 3, 6-OHDA (100%); Lane 4, 6-OHDA plus mannitol (22.9%); Lane 5, 6-OHDA plus desferrithiocine (10.5%); Lane 6, ascorbic acid plus mannitol (5.8%); Lane 7, ascorbic acid plus desferrithiocine (5.8%).

breakage only in the presence of AA. Fig. 3B shows that DNA strand breaks caused by 6-OHDA or AA could be prevented by adding mannitol (an OH radical scavenger) or desferrithiocine (an iron chelator), suggesting that in both cases DNA strand breakage is caused by ferrous iron-dependent formation of OH radicals. Superoxide dismutase, previously purged by dialysis, reduced strand break rates of AA and 6-OHDA (data not shown). Essentially the same results could be observed using the fluorescence assay (see legend to Fig. 3B).

DNA Strand Breaks in SK-N-SH Cells after Treatment with 6-OHDA and H2O2. Treatment of SK-N-SH cells with H2O2 or 6-OHDA led to dose-dependent DNA strand break formation (Fig. 4). FeSO4-pretreated SK-N-SH cells were more sensitive against 6-OHDA and H2O2 than DFO-pretreated cells. In contrast to the cell-free system, AA alone had no effects in this test system. However, AA enhanced the effects of 6-OHDA as well as of H2O2 in DFO and in FeSO4-pretreated cells. The most distinct differences were observed in DFO-pretreated cells using intermediate concentrations (100 μM) of H2O2 (P < 0.01) and 6-OHDA (P < 0.05); 3 independent experiments, 2–4-fold. The amount of alkaline stable DNA ("control") was 70 ± 5% in DFO-pretreated cells compared to 61.9 ± 11% in the FeSO4-pretreated group (6 independent experiments, each 2–4-fold; P > 0.05). Strand break rate after incubation with 75 μM H2O2 was greatly reduced in both groups when the incubation was carried out in the presence of the iron chelator 1,10-phenanthroline (100 μM). It dropped from 37% to 10% in the DFO group and from 42% to 5% in the FeSO4 group.

Poly (ADP-Ribose) Polymerase Activity after Treatment with 6-OHDA and H2O2. Fig. 5 shows the effects of H2O2 and 6-OHDA on poly(ADP-ribose) polymerase activity in DFO- and FeSO4-pretreated SK-N-SH cells. AA enhanced the effects in both groups. Similar to DNA strand break experiments, the differences between incubations with and without AA were more pronounced in DFO-pretreated cells. The basal enzyme activity in FeSO4-pretreated cells was higher than in DFO-pretreated cells (3.34 ± 0.55 versus 2.49 ± 0.93 pmol protein-bound poly(ADP-ribose)/min/10^6 cells (5 independent experiments, 2 fold, P > 0.05).

Decrease of NAD+ and ATP in SK-N-SH cells after Treatment with 6-OHDA and H2O2. Fig. 6 shows the decrease of NAD+ levels in SK-N-SH cells after incubation with 6-OHDA and H2O2. The effects were more intense in FeSO4-pretreated cells.
Fig. 5. Poly(ADP-ribose) polymerase activity in DFO- and FeSO₄-pretreated SK-N-SH cells after treatment with 6-OHDA and H₂O₂ in the absence (•) or presence (■) of 1 mM ascorbate, expressed as percentage of controls (set as 100%; n = 5). Single experiments in duplicate for 25 μM and 100 μM; two experiments in duplicate for 250 μM (SEM bars, 2.1–8.3%).

AA clearly enhanced the effects of H₂O₂ in DFO-pretreated cells whereas the effects of 6-OHDA were amplified in DFO- as well as in FeSO₄-pretreated cells. The basal levels of NAD⁺ of DFO-pretreated cells were 1.53 ± 0.31 nmol/10⁶ cells; those of FeSO₄-pretreated cells were 1.62 ± 0.16 nmol/10⁶ cells (4 independent experiments; P > 0.05).

The decrease of ATP levels in SK-N-SH cells after exposure to H₂O₂ and 6-OHDA is shown in Fig. 7. Again, the effects were more pronounced in FeSO₄-pretreated cells. Similarly as shown for NAD⁺, AA enhanced the ATP-depleting effects of 6-OHDA more strongly than those of H₂O₂. The control levels of DFO-treated cells were 6.96 ± 1.54 nmol/10⁶ cells and of FeSO₄-treated cells 7.90 ± 0.70 nmol/10⁶ cells (4 experiments; P > 0.05).

DISCUSSION

The aim of the present study was to investigate the effects of AA on iron-dependent DNA strand break formation and related processes in neuroblastoma cells after incubation with H₂O₂ and 6-OHDA. Numerous investigations document the interactions and cytotoxic effects of these substances in different biological systems (e.g. Refs. 42–47). The biochemical features of neuroblastoma cells (high ferritin content, endogenous H₂O₂ production) seem to render them especially vulnerable by AA.

Experiments with 6-OHDA, H₂O₂, and AA in a cell-free system using isolated CCC PM2 DNA showed that 6-OHDA and AA induced strand breaks, whereas H₂O₂ was effective only in the presence of AA. In agreement with other reports, DNA strand break formation is apparently caused by iron-dependent formation of OH⁻ radicals (48). Analogous processes may occur within neuroblastoma cells. These cells contain iron-rich ferritin which is dispersed within the whole cell areas (13). Therefore, iron release by substances such as AA and 6-OHDA should be feasible, in principle, on multiple regions. We supposed that AA could inflect damage upon these iron-rich cells in the presence of H₂O₂, which could be delivered by appropriate drugs (e.g. 6-OHDA) or formed endogenously in critical amounts under certain conditions (see below). The ability of neuroblastoma cells to accumulate AA would be a presupposition for this concept. SK-N-SH cells indeed incorporate it effectively (Fig. 2). The range of AA in normal serum is about 50–150 μM but is often markedly reduced in cancer patients (49). Uptake of large amounts of AA can enhance serum levels 2–3-fold (49). Under cell culture conditions, these amounts lead to an intracellular AA concentration in neuroblastoma cells in the millimolar range. Calculated from the uptake experiment shown in Fig. 2, the intracellular ascorbate concentration in SK-N-SH cells raised from 0.45 mM after incubation with 50 μM AA to 1.14 mM after 30 min incubation with 200 μM AA. The basal levels in SK-N-SH cells are negligibly low since the culture media commonly used do not contain this vitamin.

The present study showed that 6-OHDA and H₂O₂ caused similar toxic effects in a concentration-dependent manner on DNA and the metabolic pathways related to DNA strand break.
The enhancing effect of elevated cellular iron content on oxidative damaging processes is not restricted on DNA; e.g., the DNA strand break rates caused by \( \text{H}_2\text{O}_2 \) in other cells (31). Elevated amounts of cystathionine will be found in urine in about 50–80% of neuroblastoma patients (56). Consequently, only a reduced intracellular cysteine production occurs and could limit GSH supply necessary for peroxidase activity and other pathways of the cellular defense system (57). Furthermore, AA is known being a catatase inhibitor in vitro (58). This might also be true in vivo. Finally, an important aspect of ascorbic acid toxicity is probably its capacity to release iron from ferritin as shown in the cell-free system (27). Although not yet proved experimentally, this reaction is assumed to occur intracellularly and should facilitate the formation of OH radicals by the Fenton reaction. Thus, in neuroblastoma several biochemical processes influenced by AA could act in concert and be responsible for the \( \text{H}_2\text{O}_2 \)-mediated destructive processes described.

In order to support this interpretation, it is worth mentioning that a very similar biochemical pattern is believed to be responsible for tissue destruction in Parkinson disease. According to the "endogenous toxin hypothesis" (59, 60), an elevated dopamine production occurs in some areas of the substantia nigra which may compensate for loss of dopamine production in already destroyed tissue compartments. This leads to an enhanced intracellular \( \text{H}_2\text{O}_2 \) formation due to an enhanced dopamine turnover by monoamine oxidase. Since the substantia nigra in this disease is characterized by an enhanced iron level (61) and reduced GSH, catatase, and GSH peroxidase activity (62), oxidative damaging processes are favored. Indeed, lipid peroxidation products can be detected in the substantia nigra of Parkinson's patients (63). Similar pathways may play a role in neuroblastoma cells in the presence of ascorbic acid.

Neuroblastoma cells, like other tumor cells, are in a somewhat unstable metabolic condition. Permanent high dose application of AA may continually irritate neuroblastoma cells by forcing the described biochemical processes and finally allow a break in this fragile metabolic balance in those cells that had survived a previous therapy. Therefore, application of high dosages of AA for an unlimited period of time after the end of the conventional therapy may be a mild, beneficial, and easily performable attempt to prevent relapses in neuroblastoma.

REFERENCES


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