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CYTOTOXIC EFFECTS OF 6-HYDROXYDOPAMINE, MEROCYANINE-540 AND RELATED COMPOUNDS ON HUMAN NEUROBLASTOMA-AND HEMATOPOIETIC STEM CELLS

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6-Hydroxydopamine(6-OHDA) and Merocyanine-540(MC-540) have been used clinically for purging of neuroblastoma cells prior to autologous bone marrow transplantation. Both substances were found to be more toxic against neuroblastoma cells than against hematopoietic stem cells. The more pronounced cytotoxic effects of 6-OHDA against neuroblastoma cells were not caused by its selective uptake; the rapid autooxidation at physiological pH leads to the formation of H_2O_2 already in the incubation medium. Cytotoxic effects were not detected in short-time test systems (4 hour chromium-51 release assay) but only after longer incubation periods. In contrast, MC-540 proved to be toxic almost equally in short- and long-time test systems. 4-Hydroxynonenal(4-HNE) that may be formed in the plasma membrane subsequently to photoactivation of MC-540 was only slightly more toxic to neuroblastoma cells than to hematopoietic cells. Although the use of 6-OHDA and MC-540 in bone marrow purging has some limitations, the sensitivity of neuroblastoma cells against reactive oxygen compounds may be exploited more generally for therapy of this tumor.

KEY WORDS: Bone marrow transplantation, neuroblastoma, 6-Hydroxydopamine, Merocyanin 540, 4-Hydroxynonenal.

INTRODUCTION

Neuroblastoma is a tumor of the sympathetic nervous system in childhood.[1] Because its very poor prognosis in stage IV (disseminated disease) several new concepts have been introduced in clinical therapy in last years, among them allogenic and autologous bone marrow transplantation.[2,3] Since bone marrow of neuroblastoma patients is often contaminated with tumor cells, purging is recommended prior to autologous transplantation. Table 1 summarizes some purging methods that have been used partly in clinical therapy. Among the methods are purging in neuroblastoma with 6-hydroxydopmaine (6-OHDA) and Merocyanine 540 (MC 540). These substances are cytotoxic, at least partly, via generation of reactive oxygen compounds: in case of 6-OHDA it is assumed that it would be taken up selectively (being a catecholamine-analogous substrate) in neuroblastoma cells and, subsequently, forms reactive oxygen compounds by autooxidation.[12,13] MC 540 is enriched in the outer leaflet of the plasma membrane more abundantly in neuroblastoma cells than

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TABLE 1
Methods for bone marrow purging prior to autologous bone marrow transplantation

1.	Methods using monoclonal antibodies in connection with: complement lysis[4] antibody-dependent cell killing using lymphokine-activated killer cells (LAK-cells)[5] magnetic microspheres[6]
2.	Separation of tumor cells from bone marrow cells using differential lectine binding[7]
3.	Drug dependent killing procedures, e.g with 4-Hydroperoxycyclophosphamide[8] 4-Hydroxazaphosphorine (mafosfamide)[9] 6-Hydroxydopamine (6-OHDA)[10] Merocyanine-540 (MC-540)[11]

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in hematopoietic stem cells: after illumination with white or green light, cytotoxic compounds like singlet oxygen are formed.[14,15] Subsequently, other cytotoxic compounds such as 4-hydroxynonenal that may act as a "second toxic messenger" is considered to be generated.[16,17]

A substance clinically suitable for bone marrow purging should be able to destroy tumor cells quantitatively under conditions being significantly less toxic to hematopoietic stem cells. The purpose of our study was therefore to compare the cytotoxic effects of 6-OHDA, MC 540, and of substances secondarily formed, against human neuroblastoma – and hematotoietic stem cells.

MATERIALS AND METHODS

Cells

The human neuroblastoma cell lines SK-N-SH, SK-N-LO and IMR-5 were grown as monolayers in minimal essential medium, supplemented with 5% foetal calf serum(FCS), HEPES(20 mM), $NaHCO_3$ (0.25 g/l), gentamycin(0.05 g.l).

Mononuclear hematopoietic cells were prepared either from heparinized whole blood or from bone marrow samples of healthy donors using lymphoprep (Nygaard, Oslo) density gradient centrifugation.[18]

Test systems

The chromium-51 release assay was performed in round bottom 96 well plates using labeled neuroblastoma cells in suspension. 100 μl ^{51}Cr (100 μCi, New England Nuclear, Dreieich, W. Germany) + 100 μl neuroblastoma cells (10^7/ml) were incubated for 1 hour, washed and the specific lysis was determined after a 4 hour incubation period. The test is described in more detail in.[19] The MTT-test described in[20] was performed in 96 well plates two days after application of drugs. (MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]). The colony forming assay of tumor cells was carried out according to the method described by Hamburger and Salmon[21] the colony formation assay of hematopoietic cells (CFU-c) was performed as described in.[22] Generally, the toxic substances were added in aliquots about 1/10 of the total volume of the test system for the time intervals indicated.

The illumination source for photo-activation studies was a HQI-TS, 150 W/NDL

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Hg-high pressure lamp (150 W;75 Im/W; color temperature: 4300 K, Osram, Munich); illumination was carried out in a distance of 40 cm from the cell plates.

Chemicals

MC-540, 6-OHDA, ascorbic acid (sodium salt) and H_2O_2 were obtained from Sigma, Munich. 4-Hydroxynonenal (4-HNE) was prepared as described in.[23]

RESULTS

Cytotoxicity of 6-OHDA and H_2O_2 on neuroblastoma and hematopoictic stem cells

The cytotoxic effects of 6-OHDA (20 μg/ml; 10^{-4}M) + ascorbate (200 μg/ml; 10^{-3}M) (clinically recommended for purging) on neuroblastoma- and hematopoietic stem cells (CFU-c) are shown in Figure 1. As expected, hematopoietic stems cells proved to be more resistant than the neuroblastoma cell lines SK-N-LO and SK-N-LO. However, it was not expected that SK-N-LO cells were almost as sensitive as SK-N-SH cells, since only SK-N-SH cells take up catecholamines and their analogous compounds selectively, as shown in Table 2. For these experiments, iodine(125)meta-iodobenzylguanidine [(^{125}I)mIBG] was used as catecholamine-analogous substrate; neuroblastoma cells internalize mIBG by the same mechanism as dopamine and noradrenaline.[24] The reason for almost the same sensitivity of SK-N-LO- and SK-N-SH cells to 6-OHDA is that, at physiological pH, autooxidation occurs more rapidly than the uptake of 6-OHDA in its unoxidized form into SK-N-SH cells. The most important cytotoxic substance formed during autooxidation in the incubation medium is H_2O_2.[25,26] H_2O_2 was almost equally cytotoxic to both neuroblastoma cell lines as 6-OHDA (Fig. 2A/2B). Using the short-time test system (4 hour chromium-51 release assy), the cytotoxic effects of both substances were found to be very small:

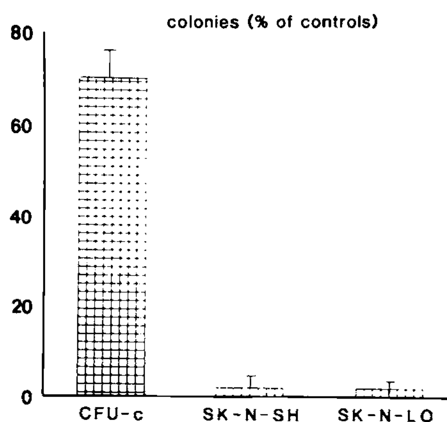

FIGURE 1 Cytotoxic effects of 6-OHDA (10^{-4}M) + ascorbic acid (10^{-3}M) on hematopoietic stem cells (CFU-c) and neuroblastoma cells (colony forming assay). 1 hour incubation time at 37°C; 3 experiments in duplicate (CFU-c) and triplicate (neuro-blastoma cells): mean ± S.D.

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TABLE 2
Uptake of the catecholamine-analogous compound meta-iodobenzylguanidine [(^{125}I) mIBG, (10^{-7}M)] in SK-N-SH and SK-N-LO cells (pmol/3 × 10^5 cells) in the absence or presence of a 100-fold excess of dopamine.

cell line	10^{-7}M (^{125}I)mIBG	10^{-7}M (^{125}I)mIBG + 10^{-5}M dopamine
SK-N-SH	$7.1 \pm 0.052 \times 10^{-12}$	$0.87 \pm 0.2 \times 10^{-12}$
SK-N-LO	$0.4 \pm 0.05 \times 10^{-12}$	$0.38 \pm 0.01 \times 10^{-12}$

1 hour incubation time at 37°C; experimental data from one typical experiment in triplicate.

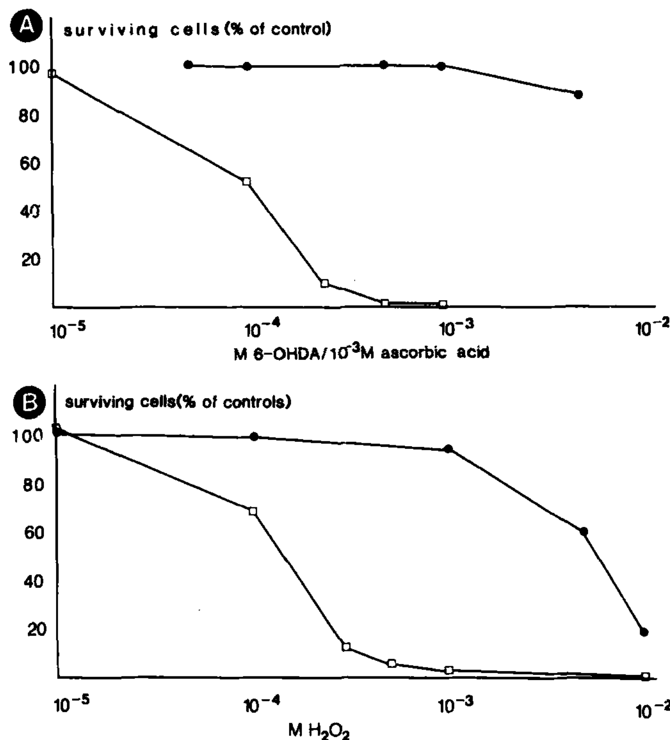

FIGURE 2 Comparison of the cytotoxic effects of 6-OHDA/ascorbic acid (A) and H_2O_2 (B): —●—●—: 4 hour chromium-51 release assay —□—□—: MTT-assay (2 days culture time). One experiment of several others in triplicate using SK-N-SH cells as example. VC: 2–8% in both test systems.

however, after longer observation periods (MTT-test two days after application of 6-OHDA and H_2O_2), significant effects were observed. In contrast, MC 540 was equally cytotoxic in both test systems (see below).

Cytotoxicity of Merocyanine 540 and 4-hydroxynonenal on neuroblastoma- and hematopoietic stem cells

Figure 3 shows the effects of MC 540 + illumination and of illumination alone on human neuroblastoma cell lines as well as on human hematopoietic stem cells (CFU-

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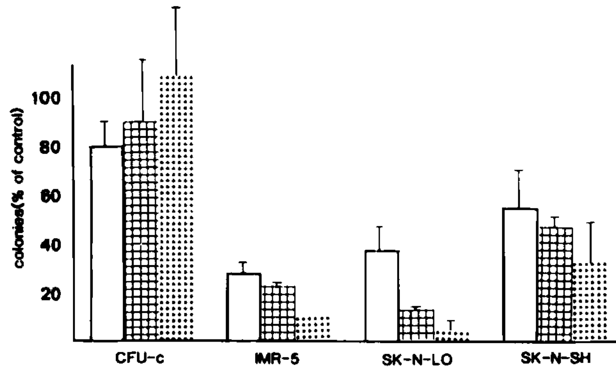

FIGURE 3 Cytotoxic effects of illumination (10 minutes) alone and in the presence of MC-540 on hematopoietic stem cells (CFU-c) and on neuroblastoma cells (colony forming assay). CFU-c: 2 experiments in triplicate; neuroblastoma cells: 3 experiments in triplicate; mean ± S.D. □: illumination alone; □: illumination + 15 μg/ml MC-540; □: illumination + 30 μg/ml MC-540

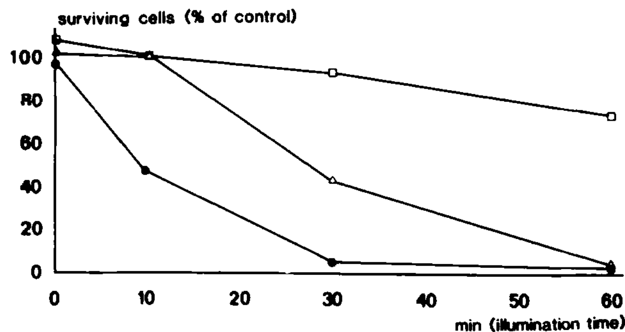

FIGURE 4 Influence of illumination time and the FCS-concentration on the survival rate of IMR-5 cells measured by the MTT-test (2 days culture time; 1 experiment in triplicate; VC between 2–8%). —●—●— 0% FCS: —△—△— 7.5% FCS; —□—□— 50% FCS

c) by conditions also used in clinical therapy. Neuroblastoma cells were more sensitive than hematopoietic stem cells although a significant portion of malignant cells survived. Illumination alone in the absence of MC 540 was rather toxic to neuroblastoma cells, whereas incubation with MC 540 without illumination was not.

Prolongation of illumination time using MC 540 treated neuroblastoma cells increased the cytotoxic effects: increasing amounts of serum in the incubation mixture abolished the cytotoxic effects (Figure 4). The cytotoxic effects are rapidly detectable: similar results were obtained using the 4 hour chromium-51 release assay and the MTT test measured two days after application of MC 540 (Figure 5A/5B). In addition to MC 540, the effects of 4-HNE on hematopoietic stem cells and the neuroblastoma cell line IMR-5 were measured comparatively in the corresponding colony forming test systems (Figure 6). The neuroblastoma cell line was only slightly more sensitive against 4-HNE compared to hematopoietic stem cells. Similarly to MC 540 (but in

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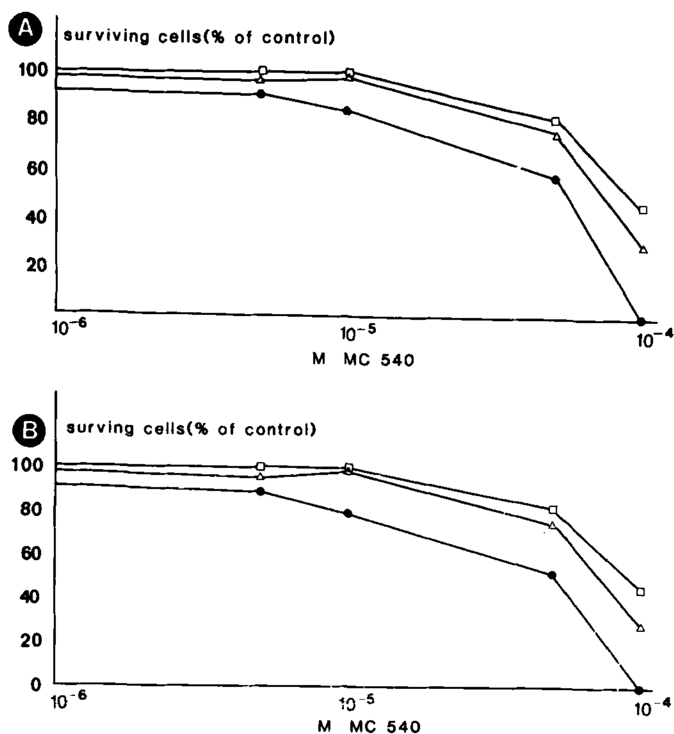

FIGURE 5 Comparison of the cytotoxic effects of MC-540 (10 minutes illumination time) on neuroblastoma cells using A: 4 hour chromium-51 release assay and B: 2 day MTT assay —□—□—: IMR-5; —●—●—: SK-N-LO —△—△—: SK-N-SH VC for all measurements: 2–6%

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contrast to 6-OHDA and H_2O_2), the cytotoxic effects caused by 4-HNE were rapidly observed in the chromium-51 release assay (data not shown).

DISCUSSION

Some years of clinical experiences with bone marrow transplantation in neuroblastoma indicate that the survival rate was not improved significantly, either in the allogenic or in the autologous transplantation system (with and without purging). The relapse rate remains high and it is not always clear whether this is due to an insufficient purging procedure or due to problems in destroying small tumor areas in other body sides. Purging procedures often lack specifity; in the case of 6-OHDA two problems are to be considered: First, autooxidation occurs very fast at physiological pH, so a significant part of unoxidized 6-OHDA can not be incorporated into neuroblastoma cells, and secondly, only a small number of neuroblastoma cells are able to internalize catecholamines selectively. Nevertheless, hematopoietic cells proved to be much more resistent against 6-OHDA/H_2O_2 than neuroblastoma cells. The fact that in short time test systems almost no effects were observed indicate that other cell compounds than the plasma membrane are the main primary targets, most probably DNA.[27] In

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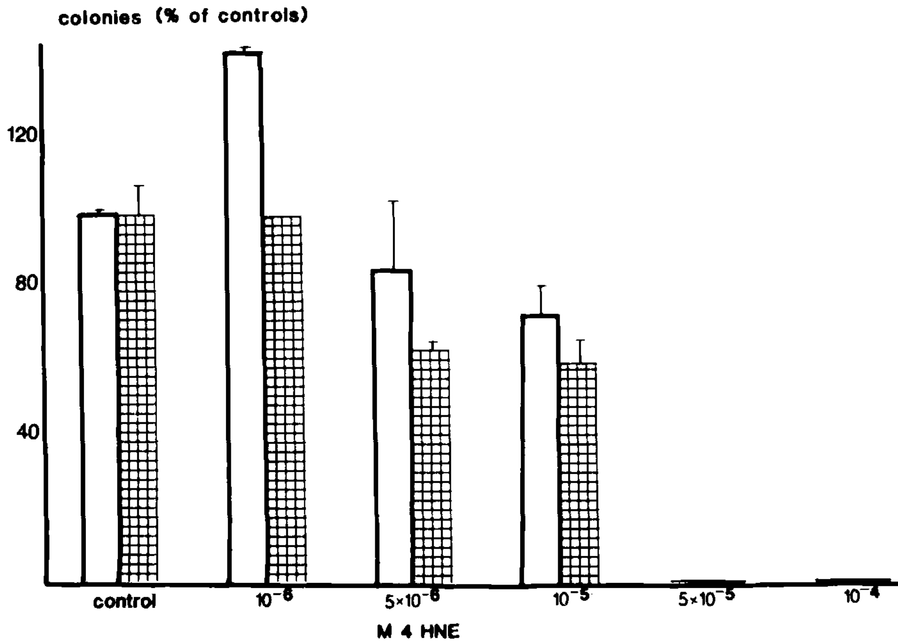

FIGURE 6 Cytotoxic effects of 4-HNE on hematopoietic stem cells (CFU-c) (□) and on the neuroblastoma cell line IMR-5 (□); n = 3; mean ± S.D.

contrast, using MC 540 and 4-HNE, cytotoxic effects can be observed already after a short incubation time being typical for membrane damage. The data obtained using MC 540 are generally in agreement with those of Sieber,[11] but quantitatively there are some differeneces; this may be due to the use of different sources for illumination. A striking observation was that colony formation of hematopoietic stem cells slightly increased compared to controls after incubation with small amounts of MC 540 and 4-HNE. Obviously, toxic stress enhances defense mechanisms in hematopoietic cells.

SUMMARY

Several problems remain unsolved in bone marrow purging; also the concept of specific destruction of neuroblastoma cells in bone marrow samples using 6-OHDA and MC 540 has several limitations. Nevertheless, the different sensitivity of neuroblastoma cells compared to hematopoietic stem cells to reactive oxygen compounds may be exploited generally in therapy of this tumor: neuroblastoma cells have some properties that could be responsible for their sensitivity against reactive oxygen compounds: (1) cystathioninuria, perhaps causing a reduction in GSH-levels, is often observed in neuroblastoma patients;[28] (2) being a cell of the sympathetic nervous system, neuroblastoma should be able to accumulate ascorbic acid; (3) ferritin is accumulated in neuroblastoma cells.[29] Although ferritin is primarily protective, liberation of iron e.g. by ascorbic acid may allow the formation of highly cytotoxic

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hydroxyl radicals from systems generating other reactive oxygen compounds (e.g. from H_2O_2 directly or from cytostatic drugs producing H_2O_2). These investigations are currently under way.

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