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THE ROLE OF REACTIVE OXYGEN COMPOUNDS DERIVED FROM 6-HYDROXYDOPAMINE FOR BONE MARROW PURGING FROM NEUROBLASTOMA CELLS

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Abstract: 6-Hydroxydopamine(6-OHDA),a specific neurotoxin against sympathetic nerve cells,is a drug already used for purging of bone marrow from neuroblastoma cells before autologous bone marrow transplantation.However,we could not detect significant differences in the toxicity of 6-OHDA against neuroblastoma and other tumor cells under the purging conditions clinically used.In contrast, bone marrow stem cells were much more resistant .The unspecific toxic effect of 6-OHDA is caused by H_2O_2 or H_2O_2-derived products which are generated by auto-oxidation in the incubation medium before a significant amount of 6-OHDA is taken up by the cells.Withdrawal of oxygen during the incubation period and subsequent incubation with an oxygen containing medium led to a more specific destruction of neuroblastoma cells which can take up 6-OHDA selectively.

Neuroblastoma is one of the most frequent solid tumors in childhood(1).At present time,no successful treatment regimen is available for stage IV patients(generalized tumor,frequently with bone marrow metastases).Therefore, the concept of bone marrow purging from neuroblastoma cells and subsequent retransplantation after intensive chemotherapy and irradiation is favoured in recent times.Some purging procedures are already under clinical trials,among these the destruction of neuroblastoma cells with 6-OHDA (2).It is suggested that the specificity of 6-OHDA-toxicity against sympathetic nerve cells is caused by its selective uptake and the subsequent generation of toxic auto-oxidation products inside the cells(3-5).As a result of this process,two kinds of toxic products are formed: 6-OHDA-quinones,which are able to react e.g. with SH-groups of proteins(6) and reactive oxygen compounds(7,8).Addition of ascorbic acid elevates the formation of these oxygen compounds(9).Before introduction of 6-OHDA for clinical treatment,we performed experiments with tumor cell lines in order to prove the proposed incubation conditions (20 mg 6-OHDA ($1x10^{-4}$M) + 200 mg ascorbic acid ($1.1x10^{-3}$M) / 1 ,1 hour incubation time at 37°C) (21).Thereby,we could not find significant differences in the toxicity of 6-OHDA against the neuroblastoma cells and the other tumor cells tested.These unexpected findings led us to investigate the reactions occuring during the incubation period in order to develop improved and more specific incubation conditions.

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Materials and Methods

Two neuroblastoma cell lines,SK-N-SH and SK-N-LO,one osteosarcoma line,SAOS-2, one melanoma line,IgR 3,and foreskin fibroblasts(Fs-4 cells) were used.Mononuclear cells from bone marrow were prepared using lymphoprep as desribed(11). 6-OHDA·HCl,ascorbic acid,sodiumdithionite,catalase and superoxide dismutase (SOD) were purchased from Sigma,St.Louis,USA.6-OHDA was dissolved in ascorbic acid solution immediately before starting the experiments (prevention of autooxidation).
Spectrometric measurements were carried out on a Hitachi 100-80 spectrophotometer at 490 nm.
The cell experiments were carried out using the following test systems:
1) Monolayer assay:Tumor cells and fibroblasts were seminated in six well plates ($1\text{-}2\times10^5$ cells/well).One day later,the the 6-OHDA/ascorbic acid solution,and, in some cases,a solution containing catalase,SOD or sodiumdithionite was added for 1 hour.After washing,fresh incubation medium was added to the cells and the incubation was continued(incubation medium:MEM,supplemented with 5% FCS, 0.25g/l $NaHCO_3$,20 mM HEPES,5mg/l glutamine and 50 mg/l gentamycin).The cells were harvested by trypsin treatment on the 5th day and counted.
2)Human tumor stem cell assay(12,13):Tumor cells were incubated in suspension for one hour with the same substance concentrations as used for the monolayer assay.After washing,5×10^4 cells/well were plated for the colony assay.The number of colonies grown per well was counted 2-3 weeks later.
3)Mononuclear cells of bone marrow were treated in an analogous way and plated in 0.3% agar.The capacity to form colonies was followed by performing a stem cell assay (GM-CFC,granulocyte macrophage colony forming cell) as described in (14).

Results

Concentration dependent cytotoxicity of 6-OHDA/ascorbic acid

Cells were incubated with 6-OHDA/ascorbic acid as described in materials and methods.Up to a 6-OHDA-concentration of 1mg/l,all cells,except the neuroblastoma cells SK-N-SH were unaffected(Fig.1).At 20 mg/l,the recommended concentration for bone marrow purging,all tumor cell lines were destroyed to a large extent.Less than 1% tumor cell colonies compared with controls (incubation without 6-OHDA)were detectable using the tumor stem cell assay (table 1).In contrast,the colony number obtained by GM-CFC was 70 ±5% (n=5) compared with controls,i.e. bone marrow stem cells are much more resistant

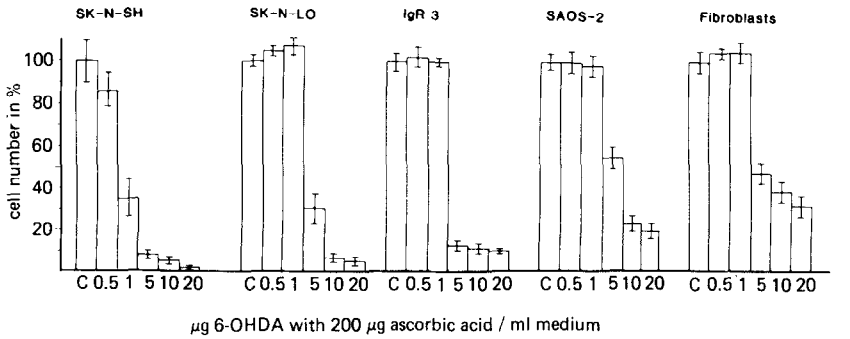

Figure 1: Dose dependent cytotoxicity of 6-OHDA/ascorbic acid against different cell lines(monolayer assay).1 hour incubation time at 37°C. Number of surviving cells is expressed in % of controls(incubation without 6-OHDA,but with ascorbic acid).Results are the mean ± S.D.(n=3).

Table 1

Surviving cells after treatment with 6-OHDA/ascorbic acid in the presence and absence of catalase and/or superoxide dismutase

Treatment	surviving cells (% of controls)			
	SK-N-SH	SK-N-LO	IgR3	SAOS-2
6-OHDA(20mg/l)/ascorbic acid (200mg/l)	6±3 [a] <1 [b]	7±3 <1	9±5 <1	26±9 <1
+ SOD (60 U/ml)	8±4 <1	8±4 <1	12±3 <1	16±2 <1
+ Catalase (2000 U/ml)	100±12 96±6	102±11 92±4	101±14 88±14	96±12 101±8
+ Catalase (2000 U/ml) + SOD (60 U/ml)	104±18 98±12	82±13 100±8	105±17 92±21	90±17 95±12

[a]Monolayer assay [b]Tumor stem cell assay
Results are the mean ±S.D.(n=4)
Controls: Incubation without 6-OHDA, but with all other compounds of each test system. Control values in the presence of catalase and/or SOD were always higher than controls without them(105-150%).

against 6-OHDA. The small diferences concerning the cytotoxicity of 6-OHDA against neuroblastoma and other tumor cells(and fibroblasts) were not expected. In comparison to data reported elsewhere(15), it was suggested, that 6-OHDA is partly already autooxidized in the incubation medium, before a significant amount is taken up by neuroblastoma cells. In order to prove this assumption, the following experiments were carried out:

Time course of 6-OHDA autooxidation in the presence of ascorbic acid

6-OHDA is quite stable in ascorbic acid solution (pH between 2-3). After the transfer of 6-OHDA/ascorbic acid into PBS (final pH:7.2), autooxidation of 6-OHDA starts immediatly as can be followed by the formation of quinones at 490 nm(16). This process is catalyzed by superoxide anion(17), therefore SOD- but not catalase-can decelerate the reaction. As depicted in Fig.2, however, it is not possible to exclude the autooxidation by this way during the one hour incubation time generally used for bone marrow purging. On the other hand, sodiumdithionite, which is able to remove oxygen from the incubation medium, can prevent completely this process when it is added in small excess.

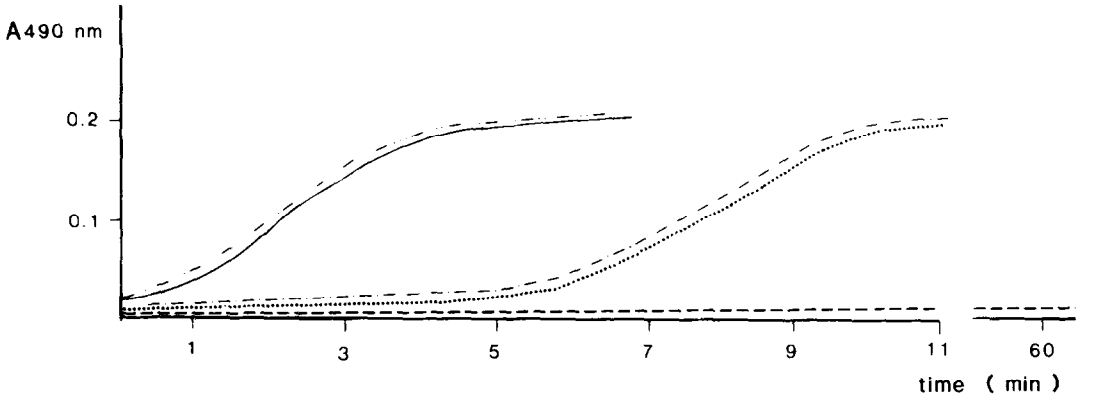

Figure 2: Time course of 6-OHDA-autooxidation measured as generation of 6-OHDA-quinones at 490 nm.Measurements were carried out in PBS with 20mg 6-OHDA + 200mg ascorbic acid/l,pH 7.2,in the absence or presence of SOD(60U/ml),catalase(2000U/ml) or sodiumdithionite(1.4×10^{-3} M).
—— :control, —·—·—:+catalase, ····· :+SOD, —·——:+catalase+SOD
---- :+sodiumdithionite.

Influence of SOD,catalase and sodiumdithionite on the survival of cells treated with 6-OHDA

A mixture of SOD and catalase,added to the incubation medium,led to a complete reduction of the cytotoxicity of 6-OHDA.Catalase alone was as effective as a mixture of SOD and catalase whereas SOD alone had no protective effect(table 1). Shortening the incubation time(10-30 minutes) led to a strong reduction of the cytotoxicity of 6-OHDA,both in the presence and absence of SOD,therefore, these experiments were not further followed(data not shown).
By coincubation of 6-OHDA/ascorbic acid with sodiumdithionite,cells were protected to a high degree.Only the neuroblastoma line SK-N-SH is destroyed to a large extend (Fig.3).

Discussion

Using the clinically recommended bone marrow purging system described in this article,no significant differences concerning the cytotoxicity of 6-OHDA against neuroblastoma and other tumor cells as well as fibroblasts could be detected.The reason for this finding is that the autooxidation products responsible for the toxicity of 6-OHDA are formed in most part already in the incubation medium before a significant amount of unoxidized 6-OHDA can be taken up specifically by the neuroblastoma cells.This could be demonstrated since addition of catalase to the incubation mixture led to a complete reduction of the cytotoxic effects of 6-OHDA.This further indicates,that among the toxic compounds formed,H_2O_2 plays an important role in the cell destruction process.Although H_2O_2 is less reactive than other oxygen compounds which can also be generated during the autooxidation of 6-OHDA, its greater stability may allow it to enter the cells after its formation in

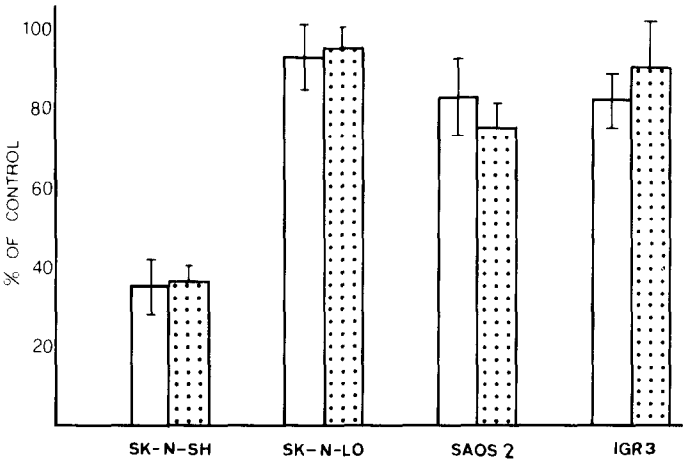

Figure 3: Survival rate of cells after treatment with 20mg 6-OHDA + 200 mg ascorbic acid /l in the presence of sodiumdithionite(1.4×10^{-3} M) expressed in % of controls(200mg ascorbic acid/l + 1.4×10^{-3} M sodiumdithionite). Results are the mean ± S.D.(n=3).Control values were about 80-90% compared with controls incubated without sodiumdithionite.
□:Monolayer assay, ⊡:Tumor stem cell assay.

the incubation medium,thereby causing a more effective cell killing.The ultimate toxic agent may be H_2O_2 itself,or more probably,some derivates generated of it(19).These unspecific effects obtained with the purging system described here are at first sight in disagreement with results obtained by Reynolds(10) who showed that there were significant differences in the toxicity of 6-OHDA against many neuroblastoma and other cells.However,the results described in our article relate to experiments performed in the presence of larger amounts of ascorbic acid(200 mg/l instead of 20 mg/l). This elevation of the ascorbic acid concentration in the purging medium was recommended for more effective cell killing,because ascorbic acid can potentiate the formation of reactive oxygen species by acting as redox-cycler:It is able to reduce 6-OHDA-quinones,which are formed during the autooxidation of 6-OHDA,back to 6-OHDA.Depending on the ascorbic acid concentration used,several cycles of this reaction can be performed.However, because most part of the reactive oxygen compounds are formed outside the cells,the more powerful killing capacity in the presence of elevated levels of ascorbic acid is at the cost of a reduced selectivity against neuroblastoma cells.

Although there were no big differences between neuroblastoma-and other tumor cells(and fibroblasts),bone marrow stem cells were much more resistent against the autooxidation products of 6-OHDA formed in the incubation medium. The method for bone marrow cell investigation described here(GM-CFS) is used as a reliable parameter for prediction of hematopoetic stem cell survival after bone marrow treatment(18).The more pronounced resistance of bone

marrow stem cells may be caused by their better equipment with cellular protection mechanisms against reactive oxygen compounds.

By reducing the concentration of 6-OHDA down to 1mg/l,only the neuroblastoma cell line SK-N-SH is significantly destroyed.As we could show elsewhere(20), this neuroblastoma cell line is able to take up catecholamines and catecholamines-analougus compounds by an active transport process,in contrast to SK-N-LO,the other neuroblastoma cell line used in these experiments.A comparable situation is described in (10):neuroblastoma cells which are able to produce catecholamines are much more sensitive to 6-OHDA than neuroblastoma cells which can not produce these substances.We suggest therefore, that the cytotoxic effects of 1mg/l 6-OHDA against the SK-N-SH cells are caused by two factors:Through reactive oxygen compounds formed in the incubation medium and additionally through a small amount of 6-OHDA which was taken up still in its unoxidized form.Once inside the cell,the subsequent autooxidation products($O_2^{\cdot -}$,H_2O_2,$OH^{\cdot}$,Singlet-oxygen and quinones,which could amplify the toxic effects of reactive oxygen compounds by lowering the GSH-concentration)can destroy the cells in a very efficient way.

Therefore,the aim for a selective purging procedure of bone marrow from neuroblastoma cells should be to establish purging conditions which allow a maximal specific uptake of 6-OHDA into the cells before its autooxidation. In order to perform such approaches,sodiumdithionite was used for removing oxygen from the incubation mixture whereby autooxidation of 6-OHDA can be avoided.After replacing this medium by a medium containing oxygen,the incorporated 6-OHDA is autooxidized inside the cells.As expected,SK-N-SH cells were damaged more significantly by this procedure than all other cell lines investigated.There is another reason why prevention of autooxidation of 6-OHDA in the incubation medium is strongly recommended:H_2O_2 inhibits the uptake of biogenic amines(15),e.g. of 6-OHDA.As a consequence,a reduction of the killing capacity of 6-OHDA against neuroblastoma cells can be expected.

Summarizing the results of our experiments,two conclusions can be drawn:

1)6-OHDA is-under the conditions so far recommended for clinical applications-not a specific reagent for bone marrow purging from neuroblastoma cells because its toxicity is caused by autooxidation products generated for the most part already in the incubation medium.However,the greater sensitivity of many tumor cells against toxic oxygen compounds may be employed under optimized conditions as a general principle for bone marrow purging from various tumor cells before autologous bone marrow transplantation.

2)Using 6-OHDA as purging reagent,a more selective destruction of neuroblastoma cells can be achieved,if its rapid autooxidation in the incubation medium is prevented until a significant amount of 6-OHDA is specifically

incorporated by neuroblastoma cells which can take up catecholamines.For clinical application,however,purging conditions have still to be improved because 100% removal of neuroblastoma cells should be reached in order to achieve cure.

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