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MIBG CAUSES OXIDATIVE STRESS AND UP-REGULATION OF ANTI-OXIDANT ENZYMES IN THE HUMAN NEUROBLASTOMA CELL LINE SK-N-BE(2C)

Jan CORNELISSEN, André B.P. VAN KUILENBURG, P.A. VOÛTE and Albert H. VAN GENNIP*

Academic Medical Centre, Departments of Paediatrics and Clinical Chemistry, PO Box 22700, 1100 DE Amsterdam, The Netherlands

We report the effects of meta-iodobenzylguanidine (MIBG), a neuroblastoma-seeking agent, on cell proliferation and several oxidative stress-related parameters in the human neuroblastoma cell line SK-N-BE(2c). MIBG inhibited the proliferation of this cell line in micromolar concentrations. Measurements of the malondialdehyde (MDA) concentrations (a measure of the extent of lipid peroxidation) of cells treated with MIBG showed that increasing concentrations of MIBG led to an increase in MDA levels of the cells. This effect was most pronounced after one day of cellular exposure to MIBG and disappeared after 3 days. Disappearance of the elevated MDA levels caused by MIBG is probably the result of increased activity of the H_2O_2 detoxifying enzymes, catalase and glutathion peroxidase (GPx). The catalase- and GPx-enzyme activity of cells exposed to MIBG steadily increased with time, reaching a maximum after 4 days. Oxidative stress caused by MIBG thus at first leads to cellular damage (lipid peroxidation) but over a longer period does not lead to decreased proliferation rate of the cells, most likely because of cellular adaptation to increased oxidative stress by up-regulation of the H_2O_2 detoxifying enzymes catalase and GPx. *Int. J. Cancer* 72:486–490, 1997.
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Meta-iodobenzylguanidine (MIBG) is a structural analogue of the neurotransmitter norepinephrine. It is recognized by the active norepinephrine transport system (uptake 1) and accumulates therefore in tissues of neuro-endocrine origin (Wieland *et al.*, 1980). In its radio-iodinated form, MIBG is used clinically as a tumour-seeking radio-pharmaceutical agent for the diagnosis and treatment of neuro-endocrine tumours such as neuroblastoma and pheochromocytoma (Hoefnagel *et al.*, 1985, 1987).

The anti-proliferative effect of the chemical component of [^{131}I] MIBG (*i.e.*, MIBG) has recently attracted more interest since a phase-II study was started in which a number of carcinoid patients was treated with high doses (34 mg/m²) of unlabelled MIBG before administration of [^{131}I] MIBG (Taal *et al.*, 1996). It is thought that part of the anti-proliferative effect exercised by MIBG on a large number of cell lines of various origin is caused by inhibition of complexes I and III of the mitochondrial respiratory chain. However, in our studies on the effect of MIBG on proliferation rate and mitochondrial ATP synthesis in the human cell lines Molt-4 and SK-N-BE(2c), we observed that complete proliferation arrest was achieved at MIBG concentrations much higher than those required for complete inhibition of mitochondrial ATP synthesis (Cornelissen *et al.*, 1995a,b).

Recently, we demonstrated in beef-heart submitochondrial particles (SMPs) that inhibition of complex I of the mitochondrial respiratory chain by MIBG leads to the formation of superoxide and to lipid peroxidation of the mitochondrial inner membrane (Cornelissen *et al.*, 1997). The extent of lipid peroxidation of the mitochondrial inner membrane correlated directly to the inhibition of complex I of the respiratory chain. The increase in superoxide formation, however, was maximal at a concentration approximately 7 times higher than the MIBG concentrations required for complete inhibition of mitochondrial ATP synthesis. This observation suggests that lipid peroxidation of the mitochondrial inner membranes and the formation of superoxide could be partly responsible for the anti-proliferative effects of MIBG at concentrations lower than those needed for complete inhibition of mitochondrial ATP synthesis, and that the formation of superoxide might be responsible for the anti-proliferative effects of MIBG at concentrations higher than those required for complete inhibition of mitochondrial ATP synthesis.

It seems likely that the proliferation rate of neuroblastoma cells can be affected by oxidative stress, to which these cells are especially sensitive. The reason for this sensitivity lies in their high abundance of iron holding protein ferritin (Iancu *et al.*, 1988), and high intra-cellular concentrations of hydrogen peroxide due to low levels of catalase and glutathion peroxidase (Steinkühler *et al.*, 1988). Because of these properties, neuroblastoma cells contain all components required for the Fenton reaction, which results in the formation of highly reactive hydroxyl radicals and thus cellular damage.

To determine whether inhibition of complex I of the respiratory chain by MIBG does in fact lead to oxidative stress in intact cells and subsequently to a decreased proliferation rate of the cells, we studied the effects of MIBG on cell proliferation and several oxidative-stress-related parameters in the human neuroblastoma cell line SK-N-BE(2c).

MATERIAL AND METHODS

Cell culture

The human neuroblastoma cell line SK-N-BE(2c) was cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂. DMEM (Bio Whittaker, Verviers, Belgium) supplemented with 10% FCS (Sebak, Aidenbach, Germany), 10 mM L-glutamine, 100 IU/ml streptomycin and 0.1 mg/ml penicillin (GIBCO, Paisley, UK) was used as culture medium. The cells were cultured in the continuous presence of adequate concentrations of MIBG (EMKA-Chemie, Markgröningen, Germany) for 1 to 5 days, in order to study the effects on cell proliferation, lipid peroxidation and catalase/GPx activities of the cells. Cell proliferation was studied by culturing the cells in the presence of MIBG, harvesting the cells every 24 h and determining of the protein content of the cultures.

Lipid peroxidation in SK-N-BE(2c) cells

Lipid peroxidation was measured by determination of the malondialdehyde (MDA) content of the cells by the thiobarbituric acid method (Wasowicz *et al.*, 1993). The effect of MIBG and radical scavengers on MDA concentrations was studied by adding MIBG (1–25 µM), vitamin E (100 µM) or reduced glutathion (GSH) (1.5 mM) to the culture medium, 1 (scavengers) or 1–3 days (MIBG) before MDA concentrations were measured.

Measurement of H_2O_2 detoxifying enzymes in SK-N-BE(2c)

Measurement of catalase and glutathion peroxidase (GPx) activities occurred with cells that were cultured for up to 5 days in the continuous presence of various concentrations of MIBG. Before measurement of the enzyme activity, cells were harvested and dissolved in PBS/0.1% Triton X-100. For each assay, 20 µg cellular protein was used.

Catalase activity in SK-N-BE(2c) cells was studied by measuring the conversion rate of H_2O_2 and ethanol to water and ethanol by

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*Correspondence to: Fax: +31-20-6962596.
E-mail: a.h.vangennip@amc.uva.nl

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catalase. The ethanal formed in this reaction was subsequently converted to acetic acid by aldehyde dehydrogenase (ADH) (Boehringer, Mannheim, Germany), which simultaneously reduces NAD^+ to NADH. Assay conditions for the reactions were: 100 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 7.4), 0.05% Triton X-100, 10% ethanol, 1 mM 4-methyl pyrazol (an inhibitor of ethanol dehydrogenase) (Sigma, St. Louis, MO), 1 mM NAD^+ and 10 U/ml ADH. The catalase activity was measured by following the increase in absorbance due to the reduction of NAD^+ at 340 nm. Measurement of GPx activity in SK-N-BE(2c) cells was performed with the use of the Ransel enzyme kit (Randox, Crumlin, UK). The conversion of cumene hydroperoxide to hydroxycumene by GPx and the simultaneous oxidation of GSH to GSSG (oxidized glutathion) could be measured by following the decrease in absorbance due to the oxidation of NADPH at 340 nm. NADPH is oxidized when the reduction of GSSG to GSH by the enzyme glutathion reductase takes place.

RESULTS

Figure 1 shows the proliferation-inhibiting effect of MIBG on the human neuroblastoma cell line SK-N-BE(2c). As observed before (Cornelissen *et al.*, 1995a), increasing concentrations of MIBG led to a decreased proliferation rate of the cells, as illustrated by the reduced increase of the protein content of the cell cultures. In order to support our hypothesis on the oxidative-stress-inducing properties of MIBG, we measured MDA concentrations (a measure of lipid peroxidation) in SK-N-BE(2c) cells exposed for one day to various concentrations of MIBG. As is shown in Figure 2, addition

FIGURE 1 – Effect of MIBG on proliferation of SK-N-BE(2c) cells. SK-N-BE(2c) cells were cultured in DMEM culture medium in the presence of various concentrations of MIBG. Every 24 hr for 4 consecutive days, cells were harvested and protein content of the cultures was measured. Results are given as the mean of 2 independent experiments. ▲, 0 μM ; ●, 1 μM ; ▼, 2.5 μM ; ◆, 5 μM ; ■, 10 μM ; +, 25 μM MIBG.

FIGURE 2 – Effect of MIBG on lipid peroxidation in SK-N-BE(2c) cells. SK-N-BE(2c) cells were cultured in the presence of various concentrations of MIBG. After 1 day, MDA concentrations (as a measure of lipid peroxidation) were measured as described in “Material and Methods”. Results are given as the mean of 4 independent experiments $\pm$ SD.

of increasing concentrations of MIBG led to a dose-dependent increase of the MDA concentration in the cells. At an MIBG concentration of 25 μM , the MDA concentration was almost maximal, and more than 2-fold higher than control values (0.46 and 0.18 nmol/mg protein respectively).

Little or no increase in the MDA concentrations could be observed when radical scavengers (100 μM vitamin E or 1.5 mM GSH) were present during overnight incubation with MIBG (Fig. 3). Of the 2 scavengers used, vitamin E proved to be the more effective. Despite the protecting effect of the scavengers against MIBG-induced increase in MDA levels, no increase in proliferation rate could be observed when cells were cultured for 5 days in the presence of 25 μM MIBG plus vitamin E or GSH (in comparison with cells that were treated with only 25 μM MIBG) (data not shown).

Measurement of the cellular MDA concentrations after 1, 2 and 3 days of exposure to MIBG (Fig. 4) revealed that the MDA levels were maximal after one day of exposure, but gradually declined to control levels during days 2 and 3.

The cellular enzymatic defence system against oxidative stress consists of superoxide dismutase (SOD), which converts superoxide to H_2O_2 (a substrate of the Fenton reaction, therefore still harmful for the cell) and GPx and catalase that convert H_2O_2 to water and oxygen. In the experiment reported in Figure 5, we measured the activity of the enzymes responsible for the last detoxifying step (GPx and catalase) in cells exposed to MIBG for 4 days. By doing this we hoped to find out whether the normalization of the MDA levels in cells exposed to MIBG was due to an increase in the activity of these enzymes. Exposure of the SK-N-BE(2c) cells to increasing concentrations of MIBG did indeed lead to a

FIGURE 3 – Effect of MIBG and radical scavengers on lipid peroxidation in SK-N-BE(2c) cells. SK-N-BE(2c) cells were cultured in the presence of 25 µM MIBG ± 100 µM vitamin E or 1.5 mM GSH. After 1 day, MDA concentrations were measured as described in “Material and Methods”. Results are given as the mean of 3 independent experiments ± SD.

dose-dependent increase in the activity of these enzymes. At a MIBG concentration of 25 µM, the increase in catalase and GPx activity was maximal (180 and 260% of control values respectively). As shown in Figure 6, the increase in activity of both enzymes starts after one day of exposure to MIBG and is nearly maximal at day 4. The GPx activity of control cells reduces slightly over the 5-day incubation period, whereas the catalase activity remains more or less constant.

DISCUSSION

In this paper we discuss the oxidative-stress-inducing properties of the neuroblastoma-seeking agent MIBG and its effects on cell proliferation and several oxidative-stress-related parameters in the human neuroblastoma cell line SK-N-BE(2c).

As reported earlier, MIBG decreases the proliferation rate of the SK-N-BE(2c) cell line (Cornelissen *et al.*, 1995a). We explained this decrease in proliferation rate by the inhibiting action of MIBG on complexes I and III of the mitochondrial respiratory chain. Since an increase in the MIBG concentration to concentrations higher (10–25 µM) than those required to cause optimal inhibition of respiratory chain activity (5–10 µM) led to an even more serious inhibition of cell proliferation, it was concluded that inhibition of the respiratory-chain activity could not be entirely responsible for the cell-proliferation-inhibiting properties of MIBG.

Experiments performed in SMPs by our group and others showed that inhibition of complex I by MPP⁺, rotenone, piericidin A (Takeshige and Minakami, 1979; Takayanagi *et al.*, 1980; Hasegawa *et al.*, 1990; Ramsay and Singer, 1992) or MIBG (Cornelissen *et al.*, 1997) led to increased lipid peroxidation and

FIGURE 4 – Time-dependence of the effect of MIBG on lipid peroxidation in SK-N-BE(2c) cells. SK-N-BE(2c) cells were cultured in the presence of various concentrations of MIBG. After 1, 2 or 3 days, MDA concentrations were measured as described in “Material and Methods”. Results are given as the mean of 2 independent experiments. ▲ 0 µM; ● 5 µM; ◆ 10 µM and ■ 25 µM MIBG.

formation of superoxide. When MIBG is used to inhibit complex I, the formation of superoxide is maximal at MIBG concentrations much higher than those required to achieve optimal inhibition of respiratory-chain activity, and might therefore be responsible for the proliferation-inhibiting effect of MIBG at high concentrations, which cannot be entirely explained by the inhibition of respiratory-chain activity.

Addition of MIBG to SK-N-BE(2c) cells indeed led to increased concentrations of MDA in these cells. The increased MDA concentration is a measure of increased lipid peroxidation. This is caused not only directly by inhibition of complex I (as is observed in SMPs), but also indirectly by the formation of superoxide (also due to inhibition of complex I). Indirect lipid peroxidation occurs when superoxide is converted to H₂O₂ by Mn SOD, located in the mitochondrial matrix. H₂O₂ can easily cross the mitochondrial membranes to enter the cytosol, and react with Fe²⁺ in the Fenton reaction, to form the highly reactive hydroxyl radicals. These radicals react non-specifically within the cell, giving rise to the formation of MDA when they react with membranes (Halliwell and Gutteridge, 1991). The MIBG-induced increase in the MDA concentration is therefore due to peroxidation, not only of mitochondrial membranes, but of all membranes. Due to the non-specific manner in which the hydroxyl radicals react, the increase in MDA concentrations should be seen as a reflection of general cellular damage rather than just a measure of membrane damage.

The increase in cellular MDA normalizes, despite the presence of MIBG, after 3 days of MIBG exposure. This decrease correlates to an increase in activity of H₂O₂-detoxifying enzymes. It seems

FIGURE 5 – Effect of MIBG on the activity of the H_2O_2 -detoxifying enzymes catalase and GPx. SK-N-BE(2c) cells were cultured in the continuous presence of various concentrations of MIBG. After 4 days, cells were harvested and dissolved in PBS/0.1% Triton X-100. Activity was measured as described in “Material and Methods”, and is given as the mean of 4 independent experiments $\pm$ S.D., and representing the percentage of control values, i.e., no MIBG added (●, catalase: 14.2 ± 1.3 nmol/mg $\cdot$ min; ▲, GPx: 9.77 ± 4.0 nmol/mg $\cdot$ min).

likely, therefore, that the increased activity of the H_2O_2 detoxifying-enzymes is responsible for normalization of the MDA levels. Increase of the activity of these enzymes will lead to a decrease in H_2O_2 concentration within the cell, depriving the Fenton reaction of substrate. Consequently, the formation of hydroxyl radicals through the Fenton reaction (and thus the formation of MDA) will occur at a lower rate.

In conclusion, the oxidative stress caused by MIBG at first leads to cellular damage (reflected in the increased concentrations of MDA), but over a longer period does not result in inhibition of cell proliferation. This is reflected by the lack of effect of the addition of radical scavengers on cell proliferation of cells treated with MIBG (data not shown), most likely because the cells adapt to the increase in oxidative stress by up-regulation of the H_2O_2 -detoxifying enzymes, catalase and GPx.

FIGURE 6 – Time-dependence of the effect of MIBG on the activity of the H_2O_2 -detoxifying enzymes catalase and GPx. SK-N-BE(2c) cells were cultured in the continuous presence of 25 μM MIBG. Every 24 hr for 5 consecutive days, cells were harvested and dissolved in PBS/0.1% Triton X-100. Activity was measured as described in “Material and Methods”, and is given as the mean of 2 independent experiments, representing the percentage of control values, i.e., no MIBG added (catalase, 15.3 nmol/mg $\cdot$ min; GPx, 7.6 nmol/mg $\cdot$ min). ○, catalase activity of control cells; △, GPx activity of control cells; ●, catalase activity of cells grown in the presence of MIBG; ▲, GPx activity of cells grown in the presence of MIBG.

Mono ADP ribosylation is another process affected by MIBG (Smets *et al.*, 1990). Inhibition of this process by MIBG could be an explanation for the inhibition of cell proliferation caused by MIBG at high concentrations. Increased oxidative stress due to MIBG in the long term does not affect cell proliferation, but merely changes the activity of the enzymatic defence system against oxidative stress.

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