Arch. Pharm. Res. Vol.18, No.3, pp. 159-163, 1995

Enhancement of Methylene Blue-induced Cytotoxicity in Human Brain Tumor Cells by an Iron Chelator, Deferoxamine

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(Received January 25, 1995)

Previously, we have reported that methylene blue (MB) induces cytotoxicity in human brain tumor cells through the generation of free radicals. In this study the effect of deferoxamine (DFO), an iron chelator, on MB-induced cytotoxicity was investigated using SK-N-MC human neuroblastoma and U-373 MG human astrocytoma cells as model cellular systems. The cytotoxic effect of MB was potentiated by DFO. The potentiation effect of DFO was significantly blocked by either stoichiometric amounts of ferric ion, various antioxidants, hydroxyl radical scavengers or intracellular Ca^{2+} release blockers. These results suggest that hydroxyl radical and intracellular Ca^{2+} may act as important mediators of the enhanced cytotoxicity by MB and DFO. These results further suggest that the combined treatment with MB and DFO may be useful for the therapeutical applications of human brain tumors.

Key words : Methylene blue, Deferoxamine, Human brain tumors, Hydroxyl radical, Intracellular Ca^{2+}

INTRODUCTLON

An increasing body of evidence implicates that oxygen free radicals (OFRs) are involved in the pathophysiology of a variety of diseases, including atherosclerosis, iron toxicity, Parkinson's disease and ischemia followed by reperfusion (Halliwell, 1989; Traystman *et al.*, 1991; Coyle and Puttfarcken, 1993; Yu, 1994). OFRs have also been implicated in carcinogenic processes induced by radiation and some chemical carcinogens (Goldstein and Witz, 1990; Yu, 1994). Paradoxically, OFRs mediate a very important part of the mechanism of antitumor agents (Powis, 1989).

Methylene blue (MB) has been widely used in experimental and clinical areas as a guanylyl cyclase (GC) inhibitor (Ignarro *et al.*, 1984) and a diagnostic indicator (Ansari, 1968; Scanlon, 1973), respectively. Interestingly, recent observations have demonstrated that MB can generate OFRs (Kontos and Wei, 1993; Marczin *et al.*, 1992). MB has also been shown to have a cytotoxic effect on human brain tumor cells (Lee and Wurster, 1995a). In their study it has been demonstrated that the cytotoxic mechanism of MB is not due to its influence on cellular cGMP levels, but due to the generation of free radicals (Lee and Wurster, 1995a).

Deferoxamine (DFO) has been shown to have antiproliferative and cytotoxic effects on a number of tumor cells including neuroblastoma cells (Becton and Bryles, 1988; Seligman *et al.*, 1993). DFO acts like a free radical scavenger (Hershko, 1992), because DFO can chelate free iron which is the major catalyst for the generation of hydroxyl radicals (Graf *et al.*, 1984). In contrast, DFO has been shown to have a prooxidant action producing highly reactive and potentially cytotoxic hydroxyl radicals (Borg and Schaich, 1986; Lee and Wurster, 1995b).

Intracellular Ca^{2+} appears to have an important role in the processes of cell proliferation (Villereal and Byron, 1992) and cell death (Orrenius *et al.*, 1989). OFRs are shown to modulate the intracellular Ca^{2+} siganlling mechanisms (Franceschi *et al.*, 1990; Klyszcz-Nasko *et al.*, 1993; Richter and Kass, 1991).

Thus, in this study we investigated the possible interactions of MB and DFO on the growth of human brain tumor cells, and explored the mechanism of these interactions relating to free radicals and intracellular Ca^{2+}, using SK-N-MC human neuroblastoma and U-373 MG human astrocytoma cell lines as model cellular systems.

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MATERIALS AND METHODS

Materials

U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines were purchased from American Type Culture Collection (Rockville, MA). The powders for Eagle's minimum essential medium and Earle's basal salt solution, trypsin solution, trypan blue, sodium pyruvate, methylene blue (MB), dantrolene (DANT), ruthenium red (RR), mannitol (MT), thiourea (TU), 1,3-dimethylthiourea (DMTU), L-ascorbic acid (ASC), L-cysteine (CYS), reduced glutathione (GSH) and deferoxamine (DFO) were obtained from Sigma Chemical CO. (St. Louis, MO). 3,4,5-Trimethoxybenzoic acid-8-(diethylamino)octyl ester (TMB-8) was from Aldrich Chem. (Milwaukee, WI). Fetal bovine serum and antibiotics (penicilline and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). Stock solutions of the drugs were made in distilled water, and added to the culture medium.

Cell culture

Cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in an Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 200 IU/ml penicilline, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every another day. After attaining confluency the cells were subcultured by trypsinization.

Cytotoxicity test

Cells from four to five-day-old cultures were seeded in equal numbers in 35×10 mm culture dishes at the density of 2×10^5 cells/dish. The volume of the medium in the dishes was 2 ml. Drugs to be tested were added to cultures 1 day after seeding in order to ensure uniform attachment of cells at the onset of the experiments. The cells were grown for an additional 2 days. Drugs and culture medium were replaced every day. In control experiments cells were grown in the same media containing drug-free vehicle. Cell viability was assessed by trypan blue exclusion method. Total and viable cells which were stained with 0.2% trypan blue after trypsinization, were counted using a hemocytometer.

Data analysis

All experiments were performed four times. All data were displayed as percentage of control. Data were expressed as mean±standard error of the mean (SEM), and were analyzed using one way analysis of variance and Student-Newman-Keul's test for individual comparisons. P values less than 0.05 are considered to be statistically significant.

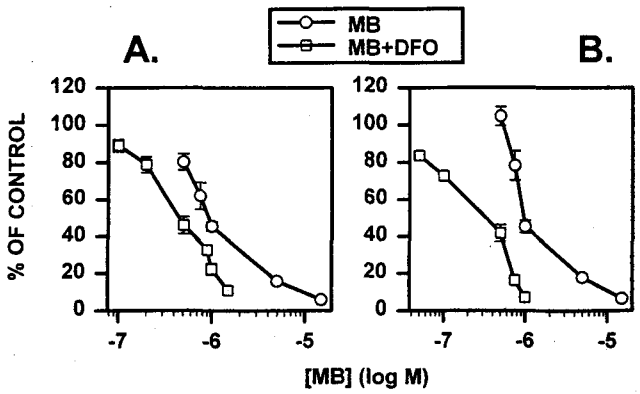

Fig 1. Potentiation of MB-induced cytotoxicity by DFO in SK-N-MC human neuroblastoma (A) and U-373 MG human satrocytoma cells (B). The concentrations of DFO were 1 and 5 μM in neuroblastoma and astocytoma cells, respectively. The results are expressed as percentage change in the number of viable cells obtained in the drug-free vehicle. The data points represent the mean±SEM.

RESULTS

As shown in Fig. 1, DFO shifted the dose-response curves of MB to the left in both cells, indicating potentiation of the MB effects. In these experiments the concentrations of DFO were 1 and 5 μM in neuroblastoma and astrocytoma cells, respectively. At these concentrations, DFO alone did not significantly alter viability of these cells.

The possible mechanism of the DFO-induced potentiation was examined and the results were depicted in Fig. 2. Addition of stoichiometric amounts of ferric ion significantly reversed the DFO-induced potentiation in both cells (Fig. 2, A and B). DFO-induced enhancement of the MB cytotoxicity was also significantly reversed by the treatments with either antioxidants (ASC, CYS or GSH) or hydroxyl radical scavengers (Fig. 2, C and D). TU, DMTU and MT which have been widely used as hydroxyl radical scavengers (Whitacre and Cathcart, 1992), were used in these experiments. DANT and TMB, known inhibitors of intracellular Ca^{2+} release (Rittenhouse-Simmons and Deykin, 1978; Zhang and Melvin, 1993) and RR, another inhibitor of intracellular Ca^{2+} release, specifically, from the ryanodine-sensitive Ca^{2+} pools (Ehrilich *et al.*, 1994), showed the similar results. (Fig. 2, C and D).

DISCUSSION

Because of its ability to inhibit vasodilation induced

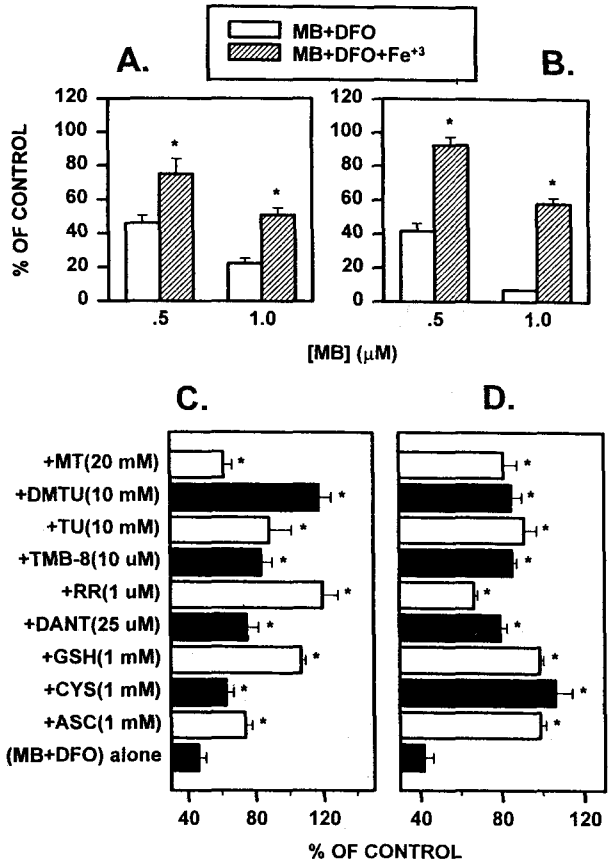

Fig 2. The mechanism of the DFO-induced potentiation of the MB cytotoxicity in SK-N-MC human neuroblatoma (A and C) and U-373 MG human astrocytoma cells (B and D). Addition of stoichiometric amounts of ferric ion can reverse the DFO-induced potentiation (A and B). The concentrations of DFO and Fe^{+3} were I and 5 μM in neuroblastoma and astrocytoma cells, respectively. Treatments with either antioxidants, hydroxyl radical scavengers or intracellular Ca^{2+} release blockers also effectively blocked the enhancement actions of DFO (C and D). The data presentation is the same as Fig. 1. *p<0.05 compared to (MB+DFO) alone.

by a variety of vasodilators, MB has been very widely used in the cardiovascular research (Ignarro *et al.*, 1984; Marczin *et al.*, 1992). MB has been also used in the clinical area as diagnostic markers; for example, in determination of endotracheal tube placement (Scanlon, 1973) and determination of prepartum leakage of amniotic fluid (Ansari, 1968). In addition, MB has been used as a photosensitizer in the photodynamic therapy in bladder cancer cells (Yu *et al.*, 1990) and has been reported to have a cytotoxic effect on brain tumor cells (Lee and Wurster, 1995a).

Iron appears to play a central role in the pathogenesis of free radical-associated diseases (Halliwell and Gutteridge, 1985). Since iron acts as a major catalyst for the formation of hydroxyl radicals (Graf *et al.*, 1984), in this study we employed DFO which has a very high and specific affinity to ferric ion (Keberle, 1964), and tested whether iron chelation by DFO can block the MB-induced cytotoxicity. Interestingly, the results showed that DFO did not inhibit but further potentiated the cytotoxic effects of MB in both cells (Fig. 1). The possible mechanism of this DFO-induced potentiation was further investigated. Addition of stoichiometric amounts of ferric ion can partly, but significantly interfere with the DFO-induced potentiation in both cells (Fig. 2, A and B), suggesting that the mechanism of this action of DFO may be related to its ability to chelate iron. However, considering the involvement of hydroxyl radicals in the MB-induced cytotoxicity (Lee and Wurster, 1995a) and the well-known role of iron in the generation of hydroxyl radicals (Graf *et al.*, 1984), iron chelation by DFO should prevent the MB-induced cytotoxicity. Moreover, various antioxidants or hydroxyl radical scavengers can inhibit the DFO-induced potentiation (Fig. 2, C and D). Furthermore, DFO has been reported to have a prooxidant action (Borg and Schaich, 1986). Recently, we have reported that hydroxyl radicals are involved in the DFO-induced cytotoxicity of human neuronal cell lines (Lee and Wurster, 1995b). Thus, these results suggest that the DFO-induced potentiation may be due to its prooxidant action. The reversing effect of ferric ion on the DFO-induced potentiation can be explained by its growth-promoting actions on these cells (Lee and Wurster, 1995b). Growth-promoting agents have been shown to prevent the cytotoxicity induced by various conditions (Knozaki *et al.*, 1993; Mattson and Bose, 1993).

Intracellular Ca^{2+} is believed to be a common mediator of chemical-induced cell death (Orrenius *et al.*, 1989). OFRs have been demonstrated to increase intracellular Ca^{2+} level through the internal store release (Franceschi *et al.*, 1990; Richter and Kass, 1991; Roveri *et al.*, 1992; Ueda and Shah, 1992). Using intracellular Ca^{2+} release blocker TMB-8, Ueda and Shah showed that it prevented both OFR-induced increased intracellular Ca^{2+} concentration and cell injury (Ueda and Shah, 1992). Significant inhibiiton of the DFO-induced potentiation by three different intracellular Ca^{2+} release blockers (DANT, TMB-8 and RR) used in the present study (Fig. 2, C and D), implies that intracellular Ca^{2+} released from the internal stores may be an important mediator of the cytotoxicity induced by agents which can produce OFRs (Orrenius *et al.*, 1989).

In conclusion, DFO can potentiate the MB-induced cytotoxicity in human brain tumor cells. The DFO-induced potentiation might be due to its prooxidant action globally. Intracellular Ca^{2+} may also be involved in these mechanisms. These results suggest that the combined treatment of MB and DFO may be useful

in the therapeutic applications of human brain tumors.

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