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Preclinical study

Induction of apoptotic cell death in human hepatocellular carcinoma SK-HEP-1 cells by a polyamine synthesis inhibitor, methylglyoxal bis(cyclopentylamidinohydrazone)

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The antitumor effects of a polyamine biosynthetic pathway inhibitor methylglyoxal bis(cyclopentylamidinohydrazone) (MGBCP) on the human hepatocellular carcinoma SK-HEP-1 cell line have been investigated. The growth of these cultured hepatocellular carcinoma cells was inhibited by MGBCP in a dose-dependent manner. Spermidine and spermine levels were dose-dependently depressed, and morphological changes due to programmed cell death (apoptosis) were observed in these MGBCP-treated hepatocellular carcinoma cells. These results suggest that in addition to reducing the growth rates, MGBCP can induce apoptotic cell death in this human hepatocellular carcinoma cell line. [© 1999 Lippincott Williams & Wilkins.]

Key words: Apoptotic cell death (apoptosis), DNA fragmentation, hepatocellular carcinoma cells, MGBCP, polyamines.

Introduction

The polyamines, putrescine, spermidine and spermine, are intracellular cationic aliphatic molecules essential for cellular proliferation and differentiation.[1,2] Recent studies suggest that the regulation of intracellular polyamine levels plays a pivotal role not only in cellular proliferation but also in the apoptotic process.[3–9] In various types of cancer cells, the production of polyamines is greatly increased.[10–12] Depletion of intracellular polyamines by inhibition of the polyamine biosynthesis pathway is generally associated with a decrease in the proliferation of the cells and thus it has been the primary focus in anticancer investigation.[13]

In this report, we demonstrate that the induction of apoptosis and polyamine depletion by a polyamine synthesis inhibitor, methylglyoxal bis(cyclopentylamidinohydrazone) (MGBCP), results in the inhibition of the proliferation of SK-HEP-1 human hepatocellular carcinoma cells.

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Material and methods

Chemicals

MGBCP was synthesized as described previously[14] and purity was checked by nuclear magnetic resonance and infrared spectoro-photometric analysis. The Apoptosis Detection System, Fluorescein, was obtained from Promega (Madison, WI).

Cell culture

Human hepatocellular SK-HEP-1 carcinoma cells[15] were grown in Dulbecco's modified essential medium (Gibco/BRL, New York, NY) supplemented with 10% fetal bovine serum (Gibco/BRL), non-essential amino acid (Gibco/BRL), penicillin (Gibco/BRL) and streptomycin (Gibco/BRL) at 37°C under a humidified 95% air–5% CO_2 atmosphere. MGBCP dissolved in physiological saline was added to the culture medium 24 h after seeding.

Determination of intracellular polyamine contents

Polyamines (putrescine, spermidine and spermine) were determined by HPLC as described previously.[16] Hepatoma cells were harvested by low-speed centrifugation (1000 *g* for 5 min), washed with 0.15 M NaCl, suspended in 0.4 N perchloric acid and disintegrated by freeze-thawing 3 times. The samples were centrifugated at 10 000 *g* for 30 min and the supernatants were analyzed directly by HPLC. For the determination of cellular protein, the precipitates obtained by the above centrifugation were dissolved in 0.1 N NaOH and the solution was determined by the method of Lowry *et al.*,[17] using bovine serum albumin as a standard.

Assay for DNA fragmentation

After cultivation in the presence of MGBCP for 3 days, the cells were pelleted by centrifugation. DNA was isolated from the cell pellets as described by Maniatis *et al.*[18] Equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid, pH 7.5, containing 2 mM EDTA.

Results

Effects of exposure times of MGBCP on the growth of SK-HEP-1 cells

As shown in Figure 1, the longer exposure to MGBCP yielded more suppression of the proliferation of hepatocellular carcinoma SK-HEP-1 cells. These findings suggested that apoptosis might be induced in hepatocellular carcinoma cells treated with a higher concentration and/or a longer exposure time to MGBCP.

Effect of MGBCP on polyamine contents in SK-HEP-1 cells

As shown in Table 1, MGBCP depressed the levels of spermidine and spermine in SK-HEP-1 cells in a dose-dependent manner. The level of putrescine was slightly decreased in these MGBCP-treated cells.

Morphological changes in SK-HEP-1 cells

Morphological changes of hepatoma SK-HEP-1 cells treated with MGBCP were examined under a light microscope. The morphology of the SK-HEP-1 cells treated with the drug showed blebbing and condensation of the chromatin as shown in Figure 2.

Induction of apoptosis by MGBCP

Fragmentation of genomic DNA into oligonucleosomal-sized fragments is a characterestic of the occur-

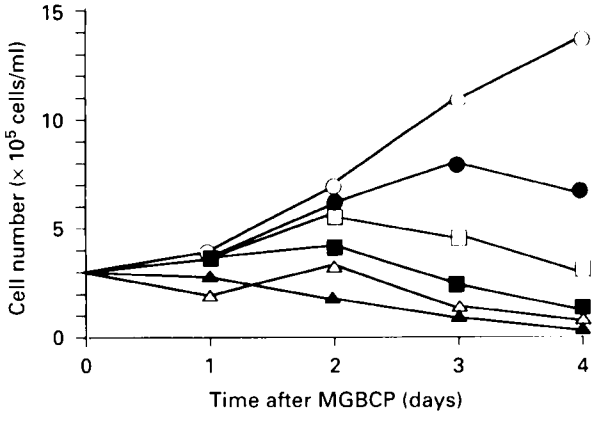

Figure 1. Effect of MGBCP on the growth of hepatocellular carcinoma SK-HEP-1 cells. The cells were grown in the absence (○) or presence of 10 (●), 20 (□), 40 (■), 50 (△), and 60 (▲) μM MGBCP.

Table 1. Effect of MGBCP on cellular polyamine contents in SK-HEP-1 cells

Treatments	Concentration (μM)	Putrescine (nmol/mg protein)	Spermidine (nmol/mg protein)	Spermine (nmol/mg protein)
Control	–	0.19 (100)	7.27 (100)	7.87 (100)
MGBCP	20	0.18 (94)	6.20 (85)	6.26 (80)
	30	0.18 (94)	5.36 (74)	5.17 (66)
	40	0.17 (89)	4.71 (65)	4.32 (55)
	50	0.17 (89)	3.90 (54)	3.95 (50)

After SK-HEP-1 cells were exposed to MGBCP at the indicated concentrations for 3 days, the cell number was counted. Then the cells were harvested to determine the intracellular polyamine and protein contents. The percent of the control without the drugs is shown in parentheses. Each value is the mean of triplicate experiments.

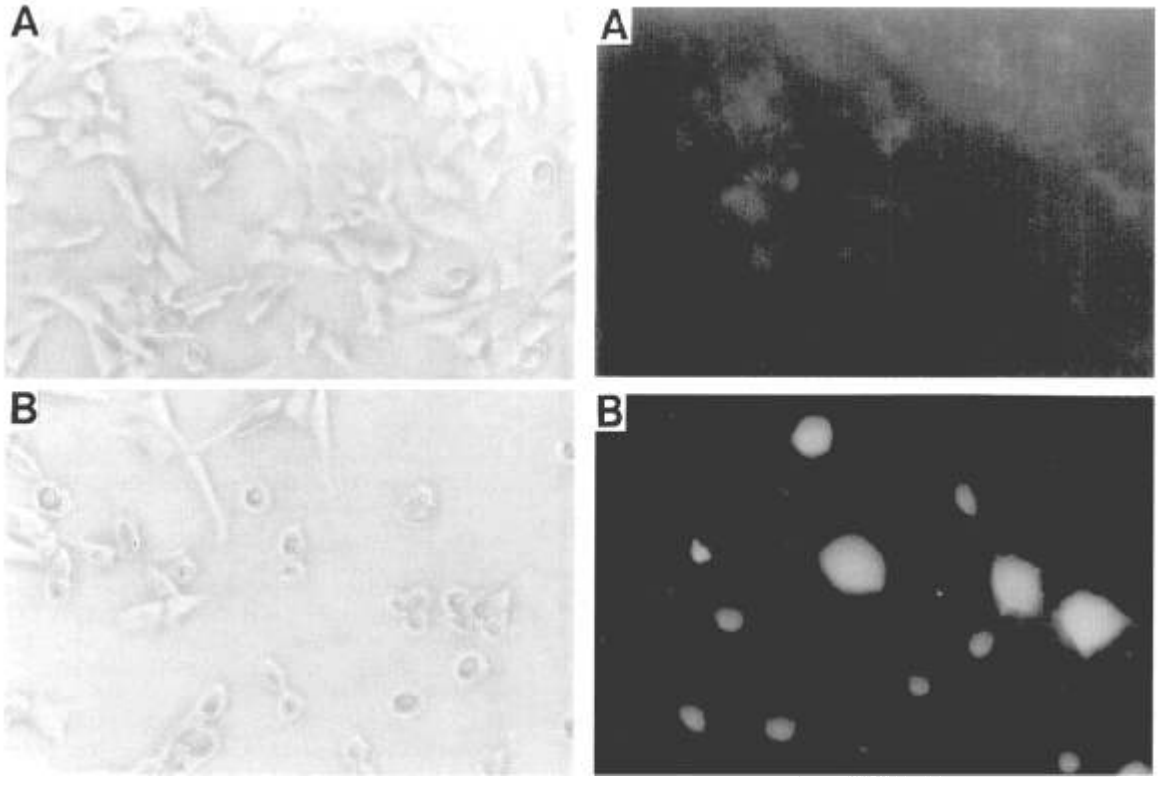

Figure 2. Morphological changes in hepatocellular carcinoma SK-HEP-1 cells. (A) Non-treated SK-HEP-1 cells. (B) SK-HEP-1 cells treated with MGBCP. The cells were cultivated with 40 μM MGBCP for 3 days and then intact monolayer cells were observed under a light microscope at magnification $\times$200 (left column). After exposure to MGBCP, the cells were fixed with 4% formaldehyde, washed with PBS, incubated with fluorescein-12UTP and terminal deoxynucleotidyl transferase, and observed under a fluorescent microscope at magnification $\times$1000 (right column).

rence of apoptosis. The apoptotic cells were observed in the MGBCP-treated cells under a light microscope (Figure 2).

As shown in Figure 3, the amounts of oligonucleosomal-sized fragments in the SK-HEP-1 cells were increased as the concentration of MGBCP was increased. These data suggested that the apoptotic pathway was activated by the MGBCP treatment in these human hepatocellular carcinoma cells.

Discussion

At present, there are several options for hepatocellular carcinoma therapy. Surgical resection offers the best chance of cure of hepatocellular carcinoma, but seldom is possible when the disease is symptomatic.[19,20] The reccurence rate after resection is high. Small tumors not suitable for resection, either because they are multiple, because of their position in the liver or because of severe hepatic dysfunction, have been treated by intralesional injection with alcohol.[21] This technique does, however, carry the risk of disseminating the tumor by facilitating the passage of malignant cells into the blood stream.[22] Embolization or chemoembolization has been used to reduce the viable tumor mass before surgery. However, it has not yet been clearly established that the advantage gained is offset by the disadvantages.[23] Liver transplantation was accompanied by a surprisingly short survival time after the recurrence.[24] It needs life-long immunosuppression and high cost. A large number of anticancer agents, including alkylating agents, antitumor antibiotics, antimetabolites, plant alkyloids, and various other agents such as platinum derivatives, procarbazine and ematine, have been tried, alone and in a variety of combinations and by different routes of administation, to treat hepatocellular carcinoma, but the predictable response rate has always been less than 20%.[25] Since single agents have limited value in the treatment of hepatocellular carcinoma, it is now desirable to develop a new type of anticancer drug.

Inhibitors of polyamine biosynthetic enzymes are known to exhibit antitumor activity against various

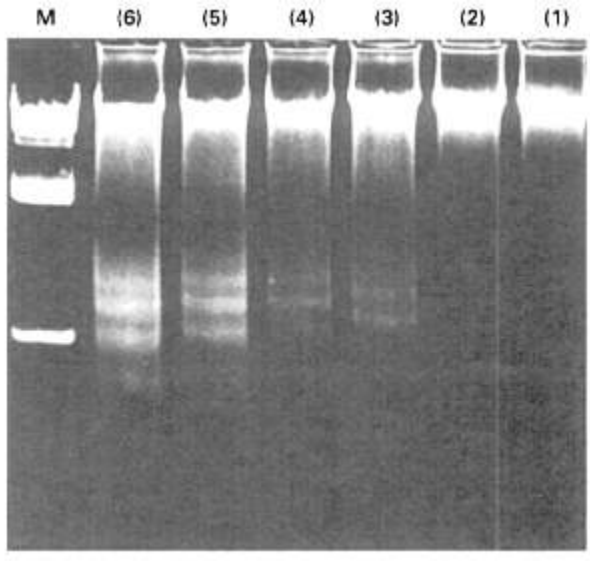

Figure 3. DNA fragmentation resulting from the exposure of hepatocellular carcinoma SK-HEP-1 cells to 0 (1), 10 (2), 20 (3), 30 (4), 40 (5) and 50 (6) μM MGBCP for 3 days. After the isolation of DNA from the cells, equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris–acetic acid, pH 7.5, containing 2 mM EDTA. M: λ DNA digested with *Hin*dIII.

cancer cells.[26,27] MGBCP is a multienzyme inhibitor for AdoMetDC, spermine and spermidine synthases. In this study, we found that the concentrations of spermine and spermidine in MGBCP-treated human hepatocellular carcinoma cells were dose-dependently suppressed and that MGBCP treatment could induce apoptosis in these cells. Oligonucleosomal-sized DNA fragmentation, which is associated with apoptosis in many cell systems, was also observed in these MGBCP-treated cells.

In conclusion, the present results suggest that the depletion by MGBCP of intracellular polyamines induces apoptosis in hepatocellular carcinoma cells. Therefore, inhibitors for polyamine biosynthesis may be useful as therapeutic agents in hepatocellular carcinoma SK-HEP-1 cells.

Conclusion

The growth of SK-HEP-1 hepatocellular carcinoma cells was inhibited by MGBCP in a dose-dependent manner. Spermidine and spermine levels were dose-dependently depressed and morphological changes due to programmed cell death (apoptosis) were observed in these MGBCP-treated hepatocellular carcinoma cells.

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