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INDUCTION OF APOPTOSIS IN HUMAN GASTRIC CARCINOMA CELL LINE MGC-803 BY MONOCLONAL ANTIBODY PD4

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Objective: To study the effect of McAb PD4 on the cell cycle and on cell injury in the gastric carcinoma cell line, MGC-803. Methods: The effects of McAb PD4 on cell proliferation cycle and cell injury of MGC-803 cells were examined by flow cytometry analysis, DNA electrophoresis and terminal deoxynucle otidyl transferase assay. Fas antigen was investigated by ELISA. Results: McAb PD4 inhibited tumor-growth of MGC-803 cells in nude mice by inducing apoptosis. Conclusion: P40 is a tumor-associated antigen distinct from the Fas antigen. Molecular cloning of P40 will define the pathway and mechanism of apoptosis induced by McAb PD4. Induction of apoptosis by McAb PD4 may be a useful therapeutic approach in treating cancer.

Key words: Gastric carcinoma, Apoptosis, Cell cycle, McAb.

Tumors arise not only from abnormal cellular proliferation but also from a decrease in apoptosis, which is a physiological form of cell death first described in 1972 (Kerr et al., 1972; Carson and Ribeiro, 1993). Numerous stimuli such asglucocoticoids, withdrawal of essential growth factors, drugs, radiation and antibody can induce apoptosis. Radiotherapy and chemotherapy can kill tumor cells by inducing apoptosis. The monoclonal antibody anti-Fas/APO-1 can initiate apoptosis in some kinds of lymphoma cells (Trauth et al., 1989; Yonehara et al., 1989). Fas/APO-1, the receptor for this antibody, was cloned from lymphoma cells (Itoh et al., 1991) and belongs to the superfamily of receptors of nerve growth factor/tumor growth factor. Fas/APO-1 has been confirmed as an important molecular in inducing apoptosis (Dhein et al., 1992).

Although apoptosis has been an active area of study, reports of monoclonal antibodies that induce apoptosis in solid tumor cells are few. We have prepared monoclonal antibody PD4 (McAb PD4), which can inhibit the proliferation of ras-transformed cells (Dong et al., 1994). In the present study, we have examined the effects of McAb PD4 on the cell cycle and DNA integrity in the gastric carcinoma cell MGC-803. In addition, we assessed the ability of McAb PD4 to inhibit tumor growth in nude mice. And we further investigated if the Fas/APO-1 antigen expressed on MGC-803 cells.

MATERIALS AND METHODS

Cell Cultures

The human gastric carcinoma cell line, MGC-803,

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was established in China and was maintained in RPMI-1640 supplemented with 15% fetal calf serum. Cells were incubated with McAb PD4 at 50 µg/ml.

Animals

Nude mice and Balb/c mice, 6-8 weeks old, were purchased from the Experimental Animal Center, Chinese Academy of Medical Sciences, Beijing.

Antibody and Reagents

Murine McAb PD4 of IgG2b isotype was prepared from ascitic fluid and purified by affinity chromatography on a potein A-SepharoseCL-4B column. Normal mouse IgG was purified from Balb/c mice serum. Rabbit anti-Fas antibody (IgG) (Santa Cruz) and mouse anti-Fas monoclonal antibody (IgM) (Upstate) were used as first antibodies in ELISA experiments. Both horseradish hyperoxidase (HRP) coupled goat anti-mouse IgG and anti-rabbit IgG was purchased from Beijing Zhong Shan Biotechnology Company.

Flow Cytometry Analysis

MGC-803 cells treated with McAb PD4 for 24 hours were analyzed by flow cytometry as described previously (Andrddff et al., 1986). The cells were harvested and washed twice with PBS, then resuspended and fixed in 75% ethanol at 4°C overnight. After washing, the cells were stained with propidium iodide 50µg/ml (Sigma) for 20 minutes at 4°C. The samples were passed through a 40 µ nylon mesh and analyzed with an EPICS-PROFILE II flow cytometer (Coulter), which was equipped with an argon ion laser. The excitation wavelength was 488 nm, and the 630 nm pass filter was used for detecting red fluorescence. Data were analyzed using the Multicycle software. Untreated MGC-803 cells and MGC-803 cells treated with normal mouse IgG served as controls.

DNA Cleavage

DNA fragmentation was examined by agarose gel electrophoresis as described (Shih and Stutman, 1996) with some modifications. Antibody-treated cells were collected and resuspended in 0.5 ml lysate buffer (10 mM Tris-HCL, PH 8.0; 5 mM EDTA; 0.5% SDS; and RNase 100 µg/ml, freshly added) at 37°C for 1 hour. Proteinase K was added at a concentration of 200 µg/ml and incubated at 50°C for 3 hours. The samples were then extracted respectively with equal volume of phenol, phenol/chloroform, and chloroform. DNA in water phase was precipitated with 2 volumes of 100% ethanol and 1/10 volume of 3M sodium acetate and centrifuged at 12000g for 10 minutes. The pellet was rinsed twice with 70% ethanol. After rinsing, the pellet was air dried and resuspended in TE buffer. DNA samples were used for electrophoresis on 1.5% agarose gel preimpregnated with ethidium bromide. After electrophoresis at 60V for 2 hours, DNA was visualized under ultraviolet light.

Terminal Deoxynucleotidyl Transferase Assay

We used an in situ cell death detection kit (Boehringer Mannheim) as specified by the manufacturer to determine apoptosis. Briefly, 1 cm^2 glass slides were placed into the wells of a 24-well plate, and then MGC-803 cells were pipetted into the plate (1×10^5 cells/well). We incubated the 24-well plate 3 hours at 37°C. After adding McAb PD4, we incubated the plate for additional 24 hours at 37°C. The glass slides with attached cells were taken out of the 24-well plate, air dried, and fixed with 10% freshly prepared paraformaldehyde solution for 30 minutes at room temperature. Then we rinsed the slides with PBS and incubated them in a permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 minutes on ice. Again, we rinsed the slides twice with PBS and added 50 µl freshly prepared TUNEL reaction mixture to each sample. The slides were incubated in a humidified chamber for 60 minutes at 37°C in the dark. Finally, we rinsed the slides 3 times with PBS. The samples were embedded with antifade DABCO ($C_6H_{12}N_2$, Sigma) and then analyzed under a fluorescence microscope.

We also used another immunohistochemical cell death kit with same principle to detect apoptosis under light microscope.

Measurement of Fas Expression by ELISA

We added 1×10^4 MGC-803 cells/well to 96-well plates and incubated them at 37°C overnight. Cells were fixed with ice cold 0.25% glutaraldehyde for 20 minutes and washed with PBS. After adding 200 µl of 1% BSA for a 2-hour incubation and washing cells

with PBS, we added McAb PD4, McAb PD4+anti-Fas antibdy, anti-Fas antibody (at a final concentration of 5 μg/ml) respectively. Control cells were incubated with mouse serum or rabbit serum. After a 1-hour incubation and 5 washes with PBS, we added 50 μl/well of HRP-goat α mouse IgG (1:500) and HRP-goat α rabbit IgG (1:1000) respectively. The cells were incubated for another 45 minutes and washed with PBS, and 200 μl OPD and H_2O_2 substrate solution were added. After incubation at room temperature for 20 minutes the reaction was terminated by adding a 12.5% H_2SO_4 solution. The OD492nm was then examined by an ELISA Reader (Bio-Rad).

In Vivo Experiment

MGC-803 cells were incubated with McAb PD4 for 24 hours. Then 1×10^6 cells (0.1ml volume) were injected hypodermically into one side of the 4 nude mice's back (2 points in each mouse). On the other side of the back, Control mice were injected with the same number of MGC-803 cells treated with normal mouse IgG. Tumor size was measured 12 to 33 days after injection.

RESULTS

Effects of McAb PD4 on Cell Proliferation Cycle of MGC-803 Cells

Flow cytometry analysis showed that only 8.3% of MGC-803 cells treated with PD4 were in the G2 phase of the cell cycle as compared with 20.5% of control cells (Table 1). Treatment with McAb PD4 yielded 59.1% of cells in the G1 phase, where as 46.0% of control cells were in the G1 phase (P<0.05). Thus, McAb PD4 arrested the cell cycle between the S and G2 phase, allowing cells in the G1 phase accumulate.

Analysis of DNA Fragmentation in MGC-803 Cells Induced by McAb PD4

The breaking of DNA into 180-200 bp fragments by endonucleases is a typical feature of apoptosis. Treatment of MGC-803 cells with McAb PD4 resulted in a ladder pattern of DNA on electrophoresis that is typical of apoptosis (Figure 1).

Detection of Apoptosis by TdT Assay

Terminal deoxynucleotidyl transferase (TdT) can add fluorescein-labeled dUTP to the free 3'-OH termini of DNA fragments, allowing for the detection of apoptotic cells under fluorescence microscopy. Few control MGC-803 cells treated with mormal mouse IgG were labeled with fluorescein (Figure 2A), whereas most MGC-803 cells treated with McAb PD4 were labeled with fluorescein (Figure 2B). Under higher magnification, we found that the nuclear material was broken into knoblike pieces and showed some morphological changes of apoptosis (Figure 2C).

Experiments with the immunohistochemical cell death kit also confirmed McAb PD4- induced cell apoptosis (data not shown).

Table 1. The effect of McAb PD4 on cell cycle of gastric carcinoma cell MGC-803

Treatment of MGC-803 cells	G1 phase	G2 phase	S phase
Untreated group	46.0%	33.5%	20.5%
McAb PD4 treated group	59.1%	32.6%	8.3%
Normal mouse IgG treated group	49.1%	31.4%	19.5%

MGC-803 cells were analyzed by flow cytometry 24 h after they were treated with McAb PD4 or normal mouse IgG as described in materials and methods.

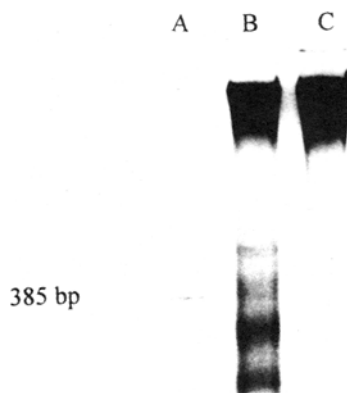

Fig. 1. DNA cleavage after McAb PD4 treatment of gastric carcinoma cell MGC-803

A: 385bp DNA marker

B: DNA from MGC-803 treated for 48 h with McAb PD4 (50 μg/ml)

C: DNA from MGC-803 treated for 48 h with normal mouse IgG (50 μg/ml) carcinoma cell MGC-803

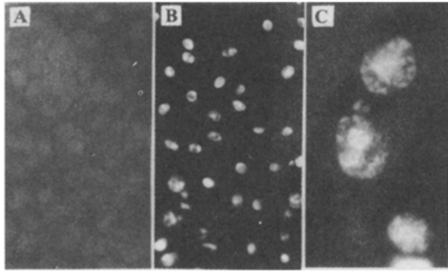

Fig. 2. Apoptosis detected by TdT assay with in situ death detection kit under fluorescence microscope

A: MGC-803 cells treated with normal mouse IgG (50μg/ml) (×200)

B: MGC-803 cells treated with McAb PD4 (50 μg/ml) (×200)

C: Higher magnification of MGC-803 cells in B (×800)

Measurement of Fas Expression on MGC-803 Cell Surface

We designed experiments to determine if P40, the target antigen for McAb PD4, was the same molecule as the Fas antigen. Competitive inhibition experiments showed that anti-Fas antibody does not inhibit the binding of McAb PD4 to P40. Furthermore, McAb PD4 alone had strong binding ability to MGC-803 cells; anti-Fas antibody alone did not bind to MGC-803 cells.

Effect of McAb PD4 on Tumorigenicity in Nude Mice

Each of the eight points of control group developed tumor by day 15 after injection. Treatment with McAb PD4 significantly reduced tumor growth and size (mean value of eitht points) in nude mice ($P<0.01$). The data was shown in Figure 3.

DISCUSSION

Our present studies showed that McAb PD4 can block tumor cell proliferation cycle and trigger cell deletion by inducing apoptosis.

McAb PD4 was prepared from mice immunized with the human gastric carcinoma cell line MGC-803. McAb PD4 reacted strongly with 3 of 4 gastric cancer cell lines and weakly with 4 of 4 lung cancer cell lines, but it did not react with lymphocytes, erythrocytes or myeloplasts (Dong et al., 1985). McAb PD4 recognized a membrane protein with a molecular weight of 40KD.

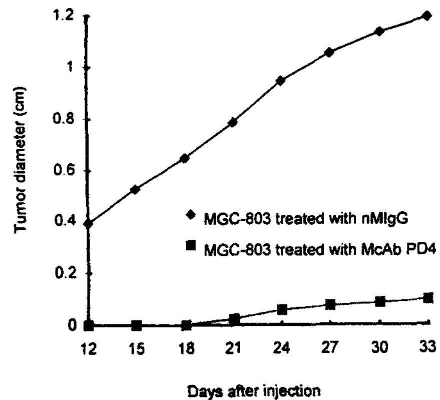

Fig. 3: Inhibition of tumor growth in nude mice treated with McAb PD4

Apoptosis, a common form of eukaryotic cell death during embryonic development, metamorphosis, tissue atrophy and tumor regression, eliminates unwanted or dangerous cells and helps maintain homeostasis. Many factors can induce apoptosis. Fas/APO-1, a membrane protein with a molecular weight of 38 to 43 KD, has been studied as an important factor in apoptosis. The Fas antigen is expressed at high levels on lymphocyte and low levels on some kinds of tumor cells (Owen-Schaub et al., 1992; Owen-Schaub et al., 1994). Binding of the Fas antigen by anti-Fas antibody or Fas ligand activates an acid sphingomyelin enzyme. The enzyme can hydrolyze sphingomyelin to produce ceramide which as a second messenger activates a series of kinase and phosphatase. Finally, the concentration of Ca^{2+} in the cytoplasm increases and apoptosis occurs (Cahill et al., 1996).

The pathway and mechanism whereby McAb PD4 induces apoptosis are unknown. Our findings show that the anti-Fas antibody does not bind to MGC-803 cells, indicating that Fas is not expressed on MGC-803 cells. Therefore, we believe that McAb PD4 target antigen P40 is not the Fas/APO-1 protein. It is possible that the binding of McAb PD4 or a ligand-like antibody to P40, a molecule similar to the Fas protein, induces apoptosis directly. Alternatively, P40 may be involved in stimulating cell proliferation and inhibiting

apoptosis. An autocrine factor from tumor cells may react with P40, thereby keeping tumor cells activated. The binding of McAb PD4 to P40 may block the function of the autocrine factor and relieve the inhibition of apoptosis. Undoubtedly, molecular cloning of P40 is essential for clarifying the pathway and mechanism of apoptosis induced by McAb PD4. This work is in progress. Since McAb PD4 reduces tumor growth in vivo, induction of apoptosis by human monoclonal antibody may prove to be a useful therapeutic approach in the treatment of cancer.

Acknowledgments

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