Doxorubicin-Conjugated Anti-Midkine Monoclonal Antibody as a Potential Anti-Tumor Drug

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Background: Midkine is a heparin-binding growth factor preferentially expressed in tumor cells. The present study was performed to utilize anti-midkine antibody for tumor therapy.

Methods: A monoclonal antibody to midkine was raised by immunizing mice deficient in the midkine gene. The binding site of the antibody was studied by using N-terminal half and C-terminal half of midkine, both of which were chemically synthesized. Doxorubicin (DOX)-conjugate of the antibody was produced by chemical conjugation. The effects of the antibody and the conjugate on cell growth were examined using a midkine-secreting tumor cell, i.e. human hepatocellular carcinoma cell (HepG2).

Results: The monoclonal antibody bound to the N-terminal half of midkine. The antibody did not inhibit the growth of HepG2 cells probably because the active domain of midkine is in the C-terminal half. We produced the antibody conjugated with DOX with the hope that the conjugate would be internalized accompanied with midkine. Indeed, the antibody-DOX conjugate significantly inhibited the growth of HepG2 cells compared with DOX-conjugated control IgG.

Conclusion: The result raises the possibility of using anti-midkine antibody conjugated with DOX for cancer therapy.

Key words: antibody – monoclonal – cancer – growth substances – immunotoxins

INTRODUCTION

Growth factors and the downstream signaling system are often overexpressed in tumors, and become the target of their treatment (1). Midkine (MK) is a heparin-binding growth factor with a molecular weight of 13 kDa (2–4), which has approximately 45% sequence identity with pleiotrophin (PTN) (5,6), but is not related with other growth factors. MK promotes the growth, survival, differentiation and migration of various target cells (2,7–11). It also enhances the fibrinolytic activity of endothelial cells (12) and exhibits angiogenic activity through cellular interactions (13,14).

MK is frequently overexpressed in human carcinomas (15–23). MK is expected to increase the growth, survival, migration and angiogenic activity of tumor cells, thereby, contributing to the aberrant growth of these cells. In support of this view, the strong expression of MK in tumors is correlated with a poor prognosis among patients with neuroblastoma (16), urinary bladder carcinoma (22) and glioblastoma (23). Furthermore, anti-MK antibody inhibits the growth of Wilm’s tumor cells in vitro (24), and antisense oligo DNA to MK inhibits the growth of colon carcinoma cells in vivo (25), making MK a suitable molecular target of tumor therapy. Here, we explored the possibility of utilizing anti-MK antibody conjugated with an anti-tumor drug, doxorubicin (DOX). DOX is a potent anti-cancer drug and has been used frequently to produce antibody-toxin conjugates (26–31).

MATERIALS AND METHODS

CELL CULTURE

Human hepatocellular carcinoma cells (HepG2) (Riken Cell Bank, Ibaraki, Japan) were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum, in a humidified atmosphere with 5% CO2 at 37°C. Routinely, the cells that were adherent were split when a subconfluent monolayer was formed.

MONOCLONAL ANTIBODY (mAb) TO MK

The mAb to MK was produced by an established procedure (32) except that female mice deficient in the MK gene (33)
were immunized with MK. This animal experiment was performed according to the National Research Council’s criteria (NIH publication No. 86-23). The first immunization was made subcutaneously with MK in incomplete Freund adjuvant. The second and third immunizations were given intraperitoneally with MK in complete Freund adjuvant. The final immunization was with MK through subocular venulae. The dose of MK in each injection was 10 µg, and the immunization period was 3 weeks. Among more than 10 hybridoma clones obtained, clone MM964 was used for the present study. Anti-MK mAb (MM964) was produced by growing the hybridoma in the peritoneum of nude mice. The mAb was purified by chromatography on Protein A Sepharose CL-4B (Amersham Pharmacia Biotech AB, Sweden). The bound mAb was eluted at 4°C with 0.1 M glycine–HCl buffer, pH 2.7, and the eluate was immediately neutralized with 1 M Tris–HCl buffer, pH 9.0.

CONJUGATION OF mAb WITH DOX

6-Maleimidocaproyl hydrazide of DOX (MC-DOXHZN) was synthesized from DOX hydrochloride (ALEXIS Biochemicals, Lausen, Switzerland) and 6-maleimidocaproylhydrazide trifluoracetate salt (Toronto Research Chemicals Inc., North York, Canada) as described previously (34). The mAb (26.5 mg) in 4.0 ml of phosphate-buffered saline (PBS) was reduced with dithiothreitol and reacted with MC-DOXHZN (1.12 mg in 0.22 ml of H2O) as described previously (35). The reaction mixture was passed through a column of 8.0 g of Bio-Beads (SM-2) (Bio-Rad Laboratories, Richmond, CA, USA) equilibrated with PBS. The solution was filtered again through a sheet of cellulose acetate membrane. The amount of protein in the DOX conjugate was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The amount of DOX was determined by absorbance at 495 nm. Mouse IgG was conjugated with DOX in a similar manner.

CELL SURVIVAL RATE ASSAY

HepG2 cells suspended in 90 µl of the culture medium were plated in a 96-well microtiter plate (Falcon 3072, Becton Dickinson, Franklin Lakes, NJ) at a density of 0.32 × 10^4 cells/well. After 8 h of culture, various dilutions of the conjugated or non-conjugated mAb, or mouse IgG in 10 µl of culture medium were added. Then culture was continued in the CO2-incubator at 37°C for 48 and 96 h. In the assay with 96 h incubation, the culture medium was changed at 48 h. The WST-1 assay using a Cell Counting Kit (Wako, Osaka, Japan) was carried out according to the manufacturer’s instructions. Briefly, 10 µl of the Cell Counting Kit solution was applied to each well. After incubation for 1 h at 37°C, absorbance was measured at 450 nm using a microplate reader (Lab systems Multi-scan MS; Dainippon Seiyaku, Osaka, Japan) with a reference wavelength of 620 nm. The survival rate was calculated according to the following equation:

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\text{survival rate} = \frac{\text{[absorbance of treated cells]}}{\text{[absorbance of cells treated with PBS alone]}} \times 100
\]

WESTERN BLOTTING

SDS-PAGE and immunoblotting were carried out under standard conditions. Samples were boiled for 3 min in the sample buffer and subjected to SDS-PAGE using a 13% gel under reducing conditions. The proteins were transferred onto nitrocellulose membrane at 40 V for 2 h at 4°C. After being blocked for 1.5 h with 5% skim milk in PBS (w/v), the nitrocellulose sheet was incubated with the mAb overnight at 4°C. After being washed with PBS containing 0.1% Tween-20 for 1 h, the membrane was incubated with rabbit anti-mouse IgG coupled to horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA, USA) in 5% skim milk in PBS at a dilution of 1 : 2500 for 1 h. Finally, the signal was visualized with an ECL kit (Amersham Biosciences, Piscataway, NJ).

MK AND THE DERIVATIVE

Human MK, its N-terminal half and C-terminal half, and human PTN were synthesized as described previously (36,37).

ELISA ASSAY

One hundred nanograms of sample (full-length MK, N-half or C-half of MK, full-length PTN) in PBS was added to the well of a 96-well plate (Falcon 3915, Becton Dickinson, Franklin Lakes, State) and was kept at room temperature for 2 h. The solution was removed and the wells were washed three times with PBS. After blocking with 3% BSA in PBS for 1 h, 100 µl of mAb solution was added to each well, and the plate was incubated at room temperature. After 2.5 h, the antibody solution was removed, the well was washed three times with PBS, and rabbit anti-mouse IgG coupled to horseradish peroxidase at a dilution of 1 : 5000 was added to each well and incubated for 30 min at room temperature. Washing was done in the manner described above, and visualization was performed with o-phenylenediamine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at room temperature for 30 min. The optical density was measured at 450 nm with an ImmunoMINI NJ-2300 (InterMed, Tokyo, Japan).

IMMUNOCYTOCHEMISTRY

Staining with anti-MK mAb was performed as described previously (24). Briefly, cells were grown on chamber slides, then washed three times with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min, and finally blocked with 1% goat serum in PBS at room temperature for 20 min. The treated cells were incubated overnight at 4°C with the mAb at a concentration of 10 µg/ml. After three washes with PBS, fluorescein isothiocyanate-conjugated goat anti-mouse IgG
(Sigma-Aldrich Chemie Gmbh) was used as the secondary antibody at a dilution of 1:150, and the cells were incubated at room temperature for 30 min. Finally they were washed again as above and examined with a confocal microscopic system (MRC 1024 system, Bio-Rad Laboratories).

RESULTS

LOCALIZATION OF THE ANTIGEN-BINDING SITE IN MK

We coated whole MK or its N-terminal or C-terminal half on ELISA plates, and assayed the binding of mAb MM964, which specifically reacted with MK but not PTN (Fig. 1). We found that it reacted with the N-terminal, but not the C-terminal half of MK (Fig. 1). Other anti-MK mAbs also reacted with the N-terminal half (data not shown).

CYTOTOXIC ACTION OF DOX-CONJUGATED ANTI-MK ANTIBODY AGAINST HEPG2 CELLS

As tumor cells with MK expression, we selected HepG2 cells. The cells synthesized and secreted into the medium large amounts of MK as shown by western blot analysis (Fig. 2A) and staining with anti-MK antibody (Fig. 2B). However, the mAb had no effect on the growth of Hep-G2 cells at a concentration of up to 400 μg/ml for 96 h. We considered that the inability to suppress the growth of the tumor cells is owing to the fact that the mAb is not directed at the active site of the MK molecule.

We then employed a different approach. Since MK is actively internalized by the cells and eventually transported to the nucleus (38), we considered that mAb bound to MK is also internalized. Therefore, the mAb conjugated with a toxin was expected to be toxic to tumor cells expressing MK. To test this possibility, we produced DOX-conjugated anti-MK mAb (mAb-DOX) using MC-DOXHEZN. The molar ratio of DOX to the mAb was 6.59. Mouse IgG was similarly conjugated with DOX (IgG-DOX), and the molar ratio was 5.84.

The binding activity of the mAb-DOX to MK was scarcely different from that of the unconjugated mAb (Fig. 3).

We tested the possible growth inhibitory effects of the mAb-DOX on HepG2 cells. Forty-eight hours after its addition, the mAb-DOX showed slight effects at 400 μg/ml, but no effect at 100 μg/ml (Figure 4A). At 96 h, this conjugate inhibited the growth of HepG2 cells 73% relative to the control at a dose of 100 μg/ml (Fig. 4B). IgG-DOX showed no significant effect. At 400 μg/ml, the mAb-DOX inhibited growth to 37%, while the control IgG–DOX conjugate inhibited growth to 72% of the control, the difference being significant (Fig. 4B). We also examined the effects of free-DOX. DOX at the concentration of 24 μM, which corresponds to the amount of DOX in 400 μg of mAb-DOX, inhibited the growth to 52% of the control in 48 h and 26% in 96 h.
DISCUSSION

An obvious approach to using MK as a molecular target in tumor therapy is the use of antibody. Indeed we found that affinity-purified anti-MK inhibited the growth of Wilms’ tumor cells (24). However, the monoclonal anti-MK antibody did not inhibit the growth of Hep-G2 cells (this study) or that of Wilms’ tumor cells (unpublished results). We considered that this is because the antibody is directed at the N-terminal half of MK, which is not the region with the major active site: MK activity is principally carried by the C-terminal half (38). Our repeated attempts to obtain antibody directed towards the functional domain remain unsuccessful.

Exogenously added MK is internalized after binding to low-density lipoprotein receptor-related protein (38), which also serves as an MK receptor (40), and eventually reaches the nucleus by binding either with laminin-binding protein precursor or nucleolin (39,41). Thus, we expected mAb-DOX to be selectively taken up by tumor cells secreting MK. Indeed, mAb-DOX showed significant growth inhibitory effects at doses of 100–400 μg/ml, and prolonged incubation was required to exert the effects. One problem of such an approach is that the clone is eventually degraded; and the DOX released will be non-specifically incorporated. However, IgG-DOX formed as a control exhibited lesser effects than the mAb-DOX. The involvement of MK in tumor growth (24,25) implies that a clone resistant to the antibody-conjugate will be difficult to develop. Free DOX exhibited slightly stronger effects than mAb-DOX. Thus, the merit of mAb-DOX is in expected selective action to MK-secreting cells.

MK is overexpressed in a variety of human carcinomas, but its expression in normal adult tissue is restricted (2). Tumors in which overexpression of MK is observed include esophageal, gastric, colon, hepatocellular, lung, breast, urinary bladder, thyroid and prostate carcinoma and neuroblastoma, glioblastoma and Wilms’ tumor (15–23). Among the above tumors, approximately 80% of those examined overexpressed MK. Furthermore, low-dose lipoprotein receptor-related protein, which is important for the internalization of MK, is broadly distributed (42). This is also the case for shuttle proteins involved in the nuclear transportation of internalized MK, namely laminin-binding protein precursor and nucleolin. The broad expression in tumor, restricted expression in normal tissue and internalization using a broadly distributed system are all beneficial in the application of the mAb-DOX to tumor therapy.

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References


