Short Communication

Generation and Characterization of Novel Monoclonal Antibodies Against Human Aurora-A

Liming Wu,¹ Tom Beito,² and Junjie Chen¹

Abstract

The mitotic kinase Aurora-A is essential for mitotic progression, including centrosome maturation, mitotic spindle formation, and faithful segregation of chromosomes to daughter cells. Several lines of evidences also suggest that the mammalian aurora kinase family proteins play a role in tumorigenesis. We have previously shown that human Aurora-A was ubiquitinated and negatively regulated by an early mitotic checkpoint protein, Chfr (checkpoint protein with FHA and RING domain). Here, we established several mouse anti-Aurora-A monoclonal antibodies (MAb). GST-tagged human Aurora-A was expressed in BL21 and used as an antigen to immunize mice. Three different hybridomas were obtained and antibodies produced by these hybridomas were analyzed. The results reveal that these antibodies specifically recognize endogenous Aurora-A in both immunoblotting and immunofluorescence experiments. They are useful tools for further analysis of human Aurora-A.

Introduction

AURORA-A BELONGS TO THE AURORA FAMILY of Ser/Thr kinases, which is essential for mitotic progression,⁽¹⁾ including centrosome maturation, mitotic spindle formation, and faithful segregation of chromosomes to daughter cells. In mammalian cells, abrogation of Aurora-A kinase activity disrupts cell cycle progression.^(2,3) Depletion of Aurora-A by RNA interference delays mitotic entry,⁽³⁾ and inhibition of Aurora-A with small molecular compounds causes chromosome alignment defect during metaphase, which lead to aneuploidy and cell death over time.^(4,5)

The mammalian aurora kinase family has been closely linked to tumorigenesis. Overexpression of Aurora-A transforms mammalian fibroblasts⁽⁶⁾ and gives rise to aneuploidy cells containing multiple centrosomes and spindles. Indeed, previous studies have shown that amplification of the AURKA locus (20q13) correlates with chromosomal instability in mammary and gastric tumors^(7,8) and poor prognosis for patients with node-negative breast cancer.⁽⁹⁾ Elevated expression of Aurora-A has also been detected in over 50% of colorectal,⁽¹⁰⁾ ovarian,⁽¹¹⁾ and gastric tumors,⁽⁸⁾ and in 94% of invasive duct adeno-

carcinomas of the breast.⁽¹²⁾ We have previously shown that an early mitotic checkpoint protein, Chfr (checkpoint protein with FHA and RING domain), ubiquitates Aurora-A and negatively regulates its expression level.⁽¹³⁾ Here we report that generation of several monoclonal antibodies specifically recognizing human Aurora-A. These antibodies are useful tools for further analysis of human Aurora-A (hAurora-A), especially for detecting Aurora-A overexpression in human cancers. We believe that these reagents can be used for diagnosis in the future.

Materials and Methods

Purification of GST-tagged Aurora-A

Human Aurora-A cDNA fragment was subcloned into pGEX4T-1 vector between EcoRI/XhoI. GST-tagged Aurora-A was expressed in *Escherichia coli* strain BL21. After 3 h induction with 0.4 mM IPTG at 37°C, cells were harvested and lysed by sonication. GST-hAurora-A protein was purified with Glutathione Sepharose-4B beads (Amersham Biosciences, Piscataway, NJ) and eluted with PBS containing 10 mM L-glutathione (Sigma, St. Louis, MO). Fractions con-

¹Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, Connecticut.

²Department of Immunology, Mayo Clinic, Rochester, Minnesota.

taining recombinant proteins were determined by SDS-PAGE and Coomassie blue staining, and then pooled, quantified, and used for mouse immunization.

Immunization and fusion

Three female BALB/c mice were immunized by subcutaneous injection of 50 μ g of antigen in complete Freund's adjuvant (Sigma) followed by biweekly subcutaneous antigen applications in incomplete Freund's adjuvant. After the fourth immunization, a mouse was boosted by intravenous injection of 50 μ g of antigen into the tail vein. Three days after the final boost, the mouse was sacrificed, and 10^8 spleen cells were used for fusion with Sp2 myeloma cells using PEG1500 (Roche Molecular Biochemicals, Mannheim, Germany). Cells were resuspended in hypoxanthine-aminopterin-thymidine (HAT) selection media containing 5 U of recombinant mouse interleukin-6 (IL-6)/mL (Roche Molecular Biochemicals). Ten days post-fusion, hybridoma culture supernatants were screened by enzyme-linked immunosorbent assay (ELISA) and then subcloned by limiting dilution.

ELISA

96-well dishes were coated with 25 ng protein/well in 100 mM sodium carbonate buffer, washed and blocked with 5% milk in PBS. 50 μ L hybridoma culture supernatant was applied for 1 h at room temperature. Plates were washed with sodium carbonate buffer and then incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). Plates were washed and incubated with 100 μ L of 1 mg/mL p-nitrophenyl phosphate disodium (PNPP) (Sigma). Positives clones were further confirmed by immunoblotting.

Cell culture and RNA interference

HeLa, Sp2 myeloma cells (a kind gift of J. Gannon), were grown in DMEM supplemented with 10% FCS, 100 U penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine at 37°C, 5% CO₂ with saturated humidity. For RNAi transfection experiments, cells of 30–50% confluency were transfected twice in 6-well plates with 200 nM siRNAs specific for hAurora-A (DAMARCON, sequence available upon request) and analyzed 36 h later by immunoblotting or immunofluorescence staining.

Immunoblotting

For immunoblotting, total cell lysates were obtained by lysing 10^6 cells in 100 μ L of 1XSDS sample buffer/10% 2-mercaptoethanol. Heat-denatured samples were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with 5% milk in TBST. Primary and HRP-conjugated goat-anti mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were diluted in PBS containing 5% milk.

Immunofluorescence staining

HeLa cells were fixed with 3% paraformaldehyde for 15 min and washed in PBS. Primary and secondary antibodies were diluted in PBS with 5% goat serum. Primary antibodies were applied for 20 min at 37°C, followed by two washes with PBS before incubation with 1:400 diluted Rhodamine-conju-

gated goat anti-mouse antibodies (Jackson ImmunoResearch) for 20 min at 37°C. Cells were washed with PBS and counterstained with DAPI for DNA. Images were taken using a Nikon microscope (100 $\times$).

Results and Discussion

In order to generate human Aurora-A (hAurora-A) specific monoclonal antibodies (MAb), we expressed the full-length hAurora-A fusion protein with N-terminal GST tag in BL21. The purified GST-hAurora-A protein was used to immunize 3 BALB/c mice. After second immunization, serum derived from these three mice was tested for recognizing overexpressed Flag-tagged hAurora-A from HEK293T cells by immunoblotting (data not shown). One mouse was selected for i.v. boosting and the spleen cells of the mouse were used for fusion with Sp2 myeloma cells. After fusion, cells were seeded in 96-well plates, and 1000 culture supernatants were screened on day 10 by ELISA. Supernatants of the positive clones were further screened by immunoblotting against Flag-tagged hAurora-A, and then those clones showing a specific single band were subcloned by limiting dilution and the culture supernatants were screened again by immunoblotting. Finally, seven positive hybridoma clones were expanded and clones 1A3, 2E1, and 7B5 were selected for further analysis.

As shown in Figure 1, all three MAbs—1A3, 2E1, 7B5—recognized a single band of 45 kDa, which was reduced in the

FIG. 1. Aurora-A MAb specifically recognizes endogenous Aurora-A protein in the cell. HeLa cells were mock treated or transfected with Aurora-A-specific siRNAs. Cells were collected and immunoblotting was performed using all three anti-Aurora-A MAbs. Immunoblotting with Actin was included as a load control.

cell extract prepared from four different Aurora-A siRNA treated cells, indicating that these MABs recognized endogenous hAurora-A. Aurora-A has been shown to localize at centrosome and mitotic spindle during mitosis.^(6,14) To test whether these three MABs were also suitable for immuno-

fluorescence staining, untreated as well as #2 hAurora-A siRNA treated HeLa cells were incubated with MABs 1A3, 2E1, and 7B5, respectively, followed by Rhodamine-conjugated goat anti-mouse secondary antibody. As shown in Figure 2A, MAB 1A3 recognizes hAurora-A, which accumulates

FIG. 2. Specificity of anti-Aurora-A MABs was assayed by immunofluorescence. HeLa cells were transfected with control or Aurora-A-specific siRNAs. Cells were fixed and immunostained with anti-Aurora-A MABs 1A3 (**A**), 2E1 (**B**), and 7B5 (**C**). The specific localization of Aurora-A at different mitotic phases was presented.

FIG. 2. (Continued).

at the centrosome in prophase and the centrosome and mitotic spindle during mitosis, while the staining pattern was almost completely abolished in hAurora-A siRNA treated cells. Similar results were also observed for MAbs 2E1 and 7B5 (Fig. 2B and C).

In summary, we have generated three MAbs specifically recognizing the human mitotic kinase Aurora-A. These an-

tibodies can be used to detect endogenous Aurora-A in both immunoblotting and immunofluorescence-staining experiments. In addition, these three MAbs most likely recognize different epitopes of hAurora-A by IgG isotype determination (data not shown). Thus, these specific anti-Aurora-A MAbs are useful reagents not only for cell-based studies, but also have potential use as an immunohistochemistry tool for

FIG. 2. (Continued).

the detection of tumors with Aurora-A overexpression. Since therapeutic agents that specifically inhibit Aurora-A kinase activity are being developed as potential anti-cancer agents, we believe that these Aurora-A-specific antibodies can be

used in the future to identify the subset of patients with Aurora-A overexpression. It is possible that these patients will likely benefit from treatment using these Aurora-A-specific inhibitors.

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Address reprint requests to:

Junjie Chen
Department of Therapeutic Radiology
Yale University School of Medicine
333 Cedar Street
PO Box 208040
New Haven, CT 06520

E-mail: Junjie.Chen@yale.edu

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