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Full Length Research Paper

Preparation and identification of monoclonal antibodies against humanin

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To generate and characterize a monoclonal antibody (mAb) against humanin (HN), BALB/c mice were immunized with the purified pet-44a-HN in adjuvant and their splenic lymphocytes were fused with myeloma SP2/0 cells. The hybridoma cell lines were screened for HN-specific antibodies by indirect enzyme-linked immunosorbent assay (ELISA), and anti-HN mAb-producing hybridoma clones were obtained using a limiting dilution assay. The specificity and affinity of the antibodies were characterized by western blot assays and indirect ELISA. Following fusion, screening and cloning, four hybridoma clones were obtained, and the clone 5A8H3 was demonstrated to stably produce anti-HN IgG2a. Further characterization of 5A8H3 revealed that this mAb specifically recognized HN, the fusion proteins of pet-44a-HN protein and pGEMEX-1-HN, but not control (*Escherichia coli* proteins). This mAb interacted with HN at an affinity constant (Ka) of 2.0×10^8 M^{-1}. The HN-specific IgG2a mAb was successfully generated. It interacted with HN specifically and sensitively, providing a valuable tool for further study of the biological functions of HN.

Key words: Humanin, monoclonal antibodies, characterization.

INTRODUCTION

Humanin (HN) is a 24-amino acid peptide that was originnally found in the occipital lobe of a patient with Alzheimer's disease (AD) in 2001 (Hashimoto et al., 2001a). It can bind to its specific receptor on the cell membrane and activate the JAK/STAT3 pathway (Hashimoto et al., 2005). HN has been found to protect neurons from AD-relevant neurotoxicity (Hashimoto et al., 2001b, 2001c). It can also exhibit neuroprotective activity against toxicity by familial amyotrophic lateral sclerosis (ALS)-related mutant superoxide dismutase (Matsuoka et al., 2006). Furthermore, HN can protect lymphocytes and undifferentiated PC12 cells from serum-deprivation-promoted apoptosis (Kariya et al., 2002, 2003) and inhibit prion-peptide-induced apoptosis of cortical neurons *in vitro* (Sponne et al., 2004). However, little is known about the pattern of HN expression in rodents and humans due to the lack of specific antibody against it.

Currently, polyclonal antibodies against HN (P04) are commercially available (Tajima et al., 2002). However, because of the heterogeneous nature, polyclonal antibodies usually have low specificity. In contrast, an antigen-specific monoclonal antibody (mAb) usually has high specificity and affinity and is able to detect the antigen specifically and sensitively. Here, we report the generation and characterization of a mAb against HN. One HN-specific mAb, 5A8H3, showed high specificity and affinity. The successful generation of anti-HN mAb provides a useful tool for further investigation of the expression and function of HN.

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Abbreviations: HN, Humanin; **mAb,** monoclonal antibody; **ELISA,** enzyme-linked immunosorbent assay; **BSA,** bovine serum albumin; **IgG,** immunoglobulin G; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Materials

The recombination plasmids for pet-44a-HN and pGEMEX-1-HN were constructed in our laboratory as previously described (Luo et al., 2004). Freund's complete adjuvant (FCA) and Freund's incomeplete adjuvant (FIA) were purchased from Sigma (St. Louis, MO, USA). The HN peptide (Met-Ala-Pro-arg-G1y-Phe-Ser-Cys-Leu-Leu-Leu-Leu-Thr-Ser-Glu-Ile-Asp-Leu-Pro-Val-Lys-Arg-Arg-Ala) was synthesized by the Chinese Peptide Company (CPC, Hangzhou, China). Horseradish peroxidase-conjugated goat anti-mouse IgG (HRP-IgG) was obtained from Zhongshan Goldbridge Biotechnology (Beijing, China). RPMI-1640 medium was purchased from Hyclone (Logan, UT, USA) and fetal bovine serum (FBS) was obtained from Gibco (Grand Island, NY, USA). Polyethylene glycol 4000 (PEG4000) was purchased from Haotian Biotechnology Co. (Hangzhou, China), and the Iso-2 Mouse Monoclonal Antibody Isotyping Kit was obtained from Roche (Indianapolis, IN, USA). Mouse myeloma cell line SP2/0 was routinely maintained in our laboratory.

Mice and immunization

Female BALB/c mice (8 weeks of age) weighing about 20 g were obtained from the Laboratory Animal Center, Zhejiang University School of Medicine (Qualification No. SYXK, Zhejiang, 02004-0052). Individual mice were housed in the specific pathogen-free facility of our campus. The experimental protocols were evaluated and approved by the Animal Research Protection Committee of Zhejiang University.

Blood samples (40 µl) were collected from the ophthalmic venous plexus of individual mice prior to immunization, and the sera were used as negative controls for later experiments. Individual mice were immunized intradermally with 200 µl of the purified pet-44a-HN fusion protein (100 µg) in 50% CFA and boosted intraperitoneally with the same amount of purified pet-44a-HN fusion protein in 50% IFA twice with an interval of 10 days. Three days after the third immunization, their blood samples were collected for evaluation of antigen-specific antibody responses using indirect ELISA. After confirming the antigen-specific antibody responses, the mice were challenged intravenously with 100 µg of soluble protein antigen.

Generation of hybridoma clones

Three days after antigen challenge, splenic mononuclear cells were isolated from individual mice for generation of hybridoma clones by fusing splenic mononuclear cells with the mouse SP2/0 myeloma cells at a ratio of 10:1 in 50% (v/v) PEG 4000. The hybridoma cells were selected in HAT medium for 7 days and then maintained in HT medium for 9 – 10 days. Subsequently, their supernatants were screened for the presence of anti-HN antibodies by indirect ELISA using the HN peptide (2 µg/ml) as the coating antigen. Selected hybridoma cells were subcloned using a limiting dilution assay to obtain hybridoma clones producing antibody against HN.

Production of ascites and purification of the anti-HN MAb

Individual BALB/c mice were intraperitoneally injected with 0.5 ml mineral oil. Two weeks later, the mice were intraperitoneally injected with 10^6 hybridoma cells (0.5 ml). Ten days after injection, the generated ascites were collected by syringe and the anti-HN IgG was purified using a 1 ml protein A-agarose column (Sigma).

Characterization of the anti-HN mAb

The subclasses of anti-HN mAbs were determined using the Iso-2 Mouse Monoclonal Antibody Isotyping Kit according to the manufacturer's protocol. The affinity of anti-HN IgG was determined by indirect ELISA using different concentrations of anti-HN IgG (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} mol/L) and coating antigen (2, 4 and 8 µg/m1). The affinity constant (Ka) was calculated as previously described (Beatty et al., 1987). The titer of anti-HN IgG was detected with indirect ELISA using 2µg/ml HN peptide and HRP-IgG (diluted 1:5000) as the coating antigen and the detecting antibodies, repectively. The plate wells that had been coated with bovine serum albumin (BSA) or coated with HN peptide and probed with mouse IgG from unmanipulated BALB/c mice, were used as negative controls.

The specificity of anti-HN IgG was analyzed by western blot assays. In brief, the purified pet-44a-HN and pGEMEX-1-HN fusion protein, the synthesized HN peptide, the cell lysate of *Escherichia coli* BL21 (DE3) colonies that had been transformed with the plasmid of pet-44a-HN, pGEMEX-1-HN, or negative control pGEMEX-1, and BSA were separated by SDS-PAGE. After transferring and blocking, the HN proteins on the membranes were probed with anti-HN IgG and then detected with HRP-IgG, followed by visualization via enhanced chemiluminescence (Thermo Fisher Scientific Inc, Rockford, IL, USA) and exposure to x-ray film. The same isotype of mouse IgG was used as a negative control.

RESULTS AND DISCUSSION

To generate a mAb against HN, mice (n = 4) were immunized with the purified pet-44a-HN fusion protein in CFA and boosted with antigen in IFA, followed by challenge with soluble antigen. Their blood was collected and the anti-HN antibodies contained in the sera were identified by indirect ELISA using the synthesized HN peptide as the coating antigen. The serum titers of anti-HN IgG reached $1:10^4$ to $1:10^5$. Subsequently, their splenic mononuclear cells were isolated and fused with SP2/0 myeloma cells at a ratio of 10:1. Following selection, 25 hybridoma lines (a fusion rate of 88%) were obtained and their supernatants were screened for anti-HN IgG using indirect ELISA. Following limited dilution three times, a total of four hybridoma clones that produced HN-specific IgG were identified. Of the four hybridoma clones, the 5A8H3 clone was found to secrete the highest concentration of anti-HN IgG (30 µg/ml) in the supernatant. Importantly, after being frozen in liquid nitrogen for 6 months, this hybridoma clone continually produced high concentration of anti-HN IgG. Thus, this clone was further characterized for its antigen specificity and affinity.

Analysis of the IgG subclass of this anti-HN mAb revealed that it was IgG2a, and the titer of ascitic mAb reached $1:10^6$ (as determined by indirect ELISA). Importantly, this mAb specifically recognized the 63 KD of pet-44a-HN fusion protein and the 31 KD of pGEMEX-1-HN fusion protein, but not any of the *E. coli* proteins (no band developed in the cell lysates of *E. coli* that had not been transformed with the HN-containing plasmid) (Figure 1A). In addition, this anti-HN IgG reacted with the synthesized HN peptide (Figure 1B) but not with control BSA, suggesting that it specifically recognized HN, at

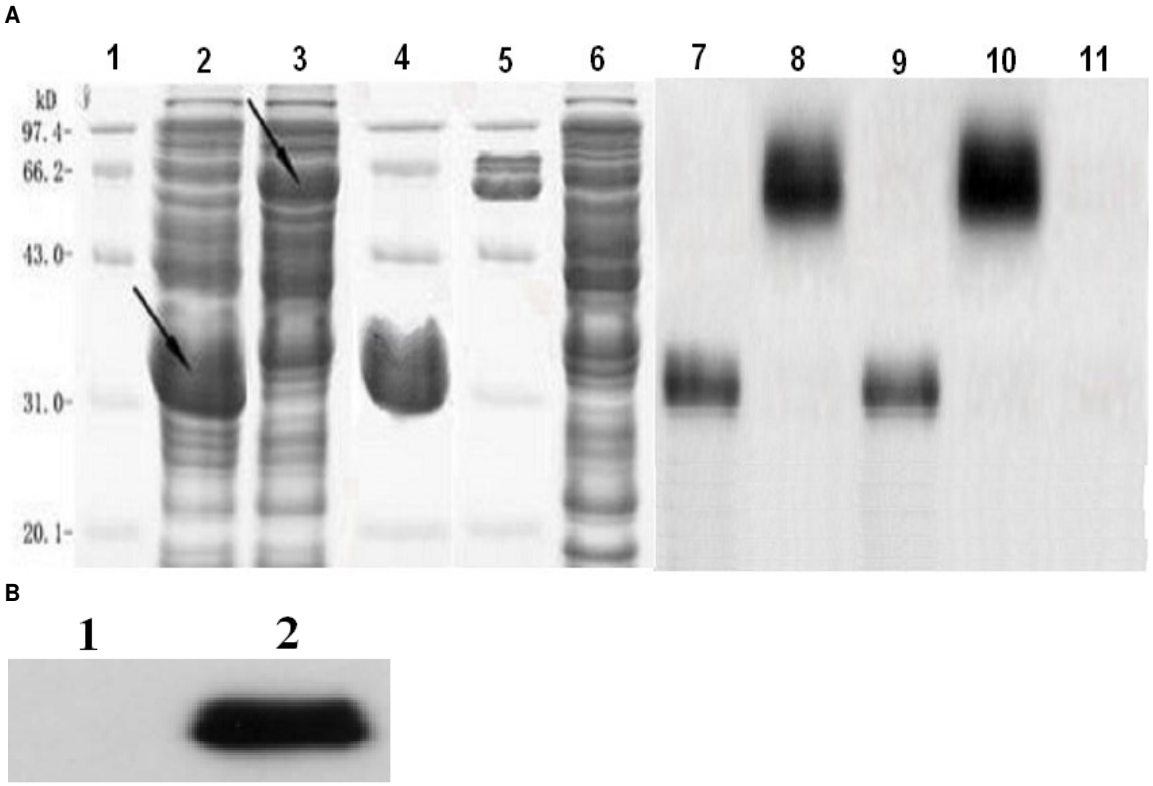

Figure 1. Western blot analysis of anti-HN IgG2a. (A): The protein markers (lane 1), the cell lysate of *E. coli* BL21 (DE3) that had been transformed with the pGEMEX-1-HN plasmid (lane 2), the cell lysate of *E. coli* BL21 (DE3) transformed with the pet44a-HN plasmid (lane 3), the purified pGEMEX-1-HN proteins (lane 4), the pet-44a-HN proteins (lane 5) and the cell lysate of *E. coli* BL21 (DE3) transformed with control plasmid of pGEMEX-1 (lane 6) were separated by 15% SDS-PAGE. The target fusion proteins are indicated by arrows. Following transfer, the proteins were probed with 2μg/ml of anti-HN IgG2a and subsequently detected with HRP-IgG, followed by visualization with enhanced chemiluminescence. Lanes 7 – 11 represent the same proteins in the lanes 2 – 6. The specificity and recognition of anti-HN IgG2a were determined by western blot assay using the synthesized peptide of HN (lane 2) and control BSA (lane 1, the panel B). Data shown are representatives of images from three independent experiments.

least in Western blot assay. Further analysis revealed that the anti-HN IgG interacted with the synthesized peptide in a dose-dependent manner and its Ka reached 2.0×10^8 M^{-1} (Figure 2). Therefore, the mAb of anti-HN IgG generated in this study specifically recognized HN antigen and interacted with the antigen with a high affinity.

HN has been found to regulate the pathogenesis of Alzheimer's disease (AD) and the apoptotic process of many types of cells. HN can interact with Bax, insulin-like growth factor binding protein 3 (IGFBP-3), and formyl peptide receptor-like 1 (FPRL1) to regulate cell growth, migration, and apoptosis (Guo et al., 2003, Ikonen et al., 2003, Ying et al., 2004). However, due to the lack of a specific mAb that recognizes HN, the distribution of HN in rodent and human subjects remains unknown. In this study, we immunized BALB/c mice with the purified fusion protein of pet-44a-HN and generated a hybridoma cell clone that stably secreted high levels of anti-HN IgG2a. Analysis of specificity and affinity revealed that this mAb specifically recognized HN and the fusion proteins of pet-44a-HN and pGEMEX-1-HN, but not *E. coli* proteins and control BSA. Furthermore, this mAb interacted with HN with a Ka of 2.0×10^8 M^{-1}. These unique features of this mAb make it suitable for detection of HN specifically and sensitively. Using this mAb, we might be able to determine the pattern of HN expression in rodents and humans. If this mAb can neutralize the interaction of HN with Bax, IGFBP-3, and FPRL1, it may be used as a therapeutic regiment for promoting tumor cell apoptosis.

HN is a small peptide with low immunogenicity, which makes it difficult to induce a strong immune response. In this study, we immunized BALB/c mice with the purified fusion protein of pet-44a-HN and successfully generated hybridoma clones that stably produced HN-specific mAb. This experimental strategy was easily performed and avoided the complex procedure of peptide conjugation with a carrier protein and subsequent purification, which is a procedure commonly used for generating high immunogenicity of peptide antigens for immunization. In

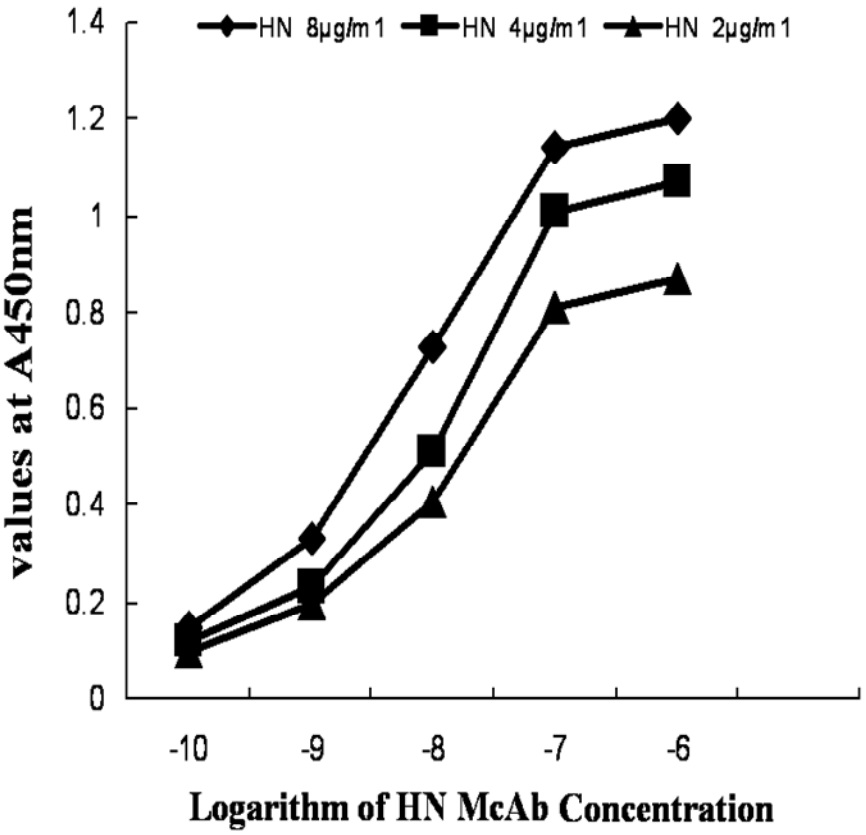

Figure 2. ELISA analysis of anti-HN IgG2a. The recognition of anti-HN IgG2a was determined by indirect ELISA using the indicated concentrations of HN peptide as the coating antigen and anti-HN for antibody detection. The plate wells coated with BSA or coated with HN peptide and probed with isotype control IgG2a were used as negative controls. The optical density values of control wells were around 0.05 – 0.1. Data shown are the mean values from three separate experiments and the intra-group variation was less than 10% of the mean value.

addition, we used the synthesized peptide as the coated antigen for ELISA, which led to specific detection of anti-HN hybridoma clones. Therefore, application of the purified fusion protein for immunization and the synthesized peptide as the antigen for *in vitro* screening of hybridoma clones is feasible and effective for generation and characterization of anti-peptide antigen-specific mAbs. The mAbs we prepared in the current study have been successfully used in ELISA and western blot analysis. However, whether it could be used for immunohistochemical staining and flow cytometry analysis and have neutralization activity remains to be further determined.

In summary, we successfully generated hybridoma clones that stably produced HN-specific mAbs. The mAb 5A8H3 was an IgG2a that interacted with HN specifically and sensitively. To the best of our knowledge, this was the first mAb against HN reported in the world. Conceivably, this mAb can be a useful tool for further investigation of the expression and function of HN in rodents and humans.

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