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Characterization of monoclonal antibodies against MHC class II-associated p41 invariant chain fragment

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Mouse monoclonal antibodies directed against human MHC class II-associated p41 invariant chain fragment have been generated. Mice were immunized with human recombinant Ii-isoform p26. For hybridoma production mouse splenocytes and myeloma cells were fused. Hybridoma cells were screened using ELISA and immunoblotting. Three cell lines (42B10, 42G11 and 43C8) were used for production of specific antibodies, which reacted with p41 fragment and did not bind to cathepsins L or S or their proenyzmes. As primary antibody for immunofluorescence staining of lymph node tissue sections clone 2C12 MAb was selected. Specific localization of p41 fragment in certain cells in lymph nodes was observed.

Introduction

MHC class II molecules display antigenic peptides on cell surface of APC (dendritic cells, B cells, macrophages, thymic epithelial cells) for recognition by $CD4^+$ T lymphocytes.[1,2,3] The MHC class II-associated Ii is a transmembrane protein that complexes with newly synthesized MHC class II heterodimers and directs their trafficking through the endosomal compartments of APC. The luminal domain of Ii organizes MHC class II dimers into nonomeric complexes and prevents premature association of MHC class II molecules with endogenous polypeptides. Within endosomal/lysosomal compartments, Ii undergoes stepwise proteolytic degradation to yield progressively smaller fragments that remain associated with the peptide-binding groove of MHC class II dimmers.[4] Dissociation of CLIP (a set of 3 kDa peptides) from the peptide-binding groove allows loading and subsequent surface expression of MHC class II molecules with antigenic peptides generated from endocytosed or phagocytosed protein. The key enzymes that degrade Ii are the cysteine proteases (cathepsins S[5], L[6] and/or V[7]). In human Ii exists in two alternatively spliced forms, p31 in p41[3], the latter containing an additional 64-amino acid sequence at the C-terminal end (hereafter called the p41 fragment). The discovery of p41 fragment-cathepsin L complex isolated from human liver[8] and its known crystal structure[9], led to the suggestion that the p41 fragment (Ii respectively) may enhance antigen presentation by providing a mechanism to inhibit otherwise destructive cathepsin L but not cathepsin S activity.[10] The aim of the present study was to generate specific mouse monoclonal antibodies directed against MHC class II-associated p41 invariant chain. Furthermore these antibodies were used in immunohistochemical studies of p41 fragment in lymph-node tissue.

Materials and methods

Preparing and purification of antibodies

Human recombinant Ii-isoform p26, comprising only the luminal domain[11] (together with p41 fragment) was purified by Ni-chelate chromatography (a gift from Dr. Klaus Dornmair, Max Planck Institute, Martinsried) and was subsequently used as an antigen for immunization of mice. BALB/c mice were injected subcutaneously with p26 (50 μg/mouse) emulsified in complete Freund's adjuvant, followed by intraperitoneal injections of the same amount of antigen in incomplete Freund's adjuvant. Test bleeds were taken and titer of specific antibodies determined using antigen immobilized ELISA in which recombinant p26 and p41 fragment-cathepsin L complex, respectively, were used as antigens. The mouse with the highest titer was boosted intraperitoneally with p26 (50 μg/mouse). For hybridoma production splenocytes and myeloma cells (NS1/1-Ag4-1) were fused by a modification of the method of Kohler and Milstein.[12] The screening of the positive wells was performed by antigen immobilised ELISA as described above for test bleeds. Hybridomas producing antibodies against p41 fragment were cloned twice by means of the limiting dilution method[13] and expanded into large volumes. The cell culture supernatants were concentrated by ultrafiltration and MAbs purified by affinity chromatography on Protein A-Sepharose (Pharmacia, Sweden). Antibody containing fractions were pooled and dialysed against PBS, pH 7.2. Small aliquots of purified antibodies were stored at – 20 °C.

Immunoblotting

Samples were first separated by SDS-PAGE on 8 – 25 % polyacrylamide gels using Phast-System (Pharmacia, Sweden). After the electrophoresis the proteins were transferred onto PVDF membrane (Millipore, USA) by passive diffusion accelerated with higher temperature. Non-specific binding sites were blocked with 0.4 % Tween 20 in PBS, pH 7.2. After this and all subsequent steps the membrane was washed with PBS, pH 7.2 containing 0.5 % Tween. Primary anti-p41 fragment antibodies were incubated with the membrane, followed by secondary goat anti-mouse IgG conjugated to HRP (Dianova, Germany). Detection was performed using 0.05 % DAB (Sigma, USA) and 0.01 % H_2O_2 in 50 mM Tris-HCl buffer, pH 7.5.

Immunofluorescence tissue staining

Sections from formalin fixed, paraffin embedded lymph nodes were used for IHA. Tissue sections on micro cover glasses were deparaffinised in xylene and rehydrated through ethanol series. They were placed in 10 mM sodium citrate buffer, pH 6.0 and put into a microwave oven (5 min, 400 W) for antigen retrieval. Non-specific binding sites were blocked with 3 % BSA in PBS, pH 7.4. After this and all subsequent steps tissue sections were rinsed in PBS, pH 7.4. Primary anti-p41 fragment antibody was added (clone 2C12, 25 μg/ml, for 2 h at 37 °C), followed by Alexa Fluor™ 488-labeled goat anti-mouse IgG secondary antibody. Molecular Probes, USA). Tissue sections were mounted on slides with ProLong™ AntiFade Kit (Molecular Probes, USA). Fluorescence microscopy and optical slicing were performed by confocal laser scanning microscope LSM 510 (Carl Zeiss Inc., USA).

Results

Purified monoclonal antibodies were tested for specificity using immunoblotting. As shown in Figure 1, 2C12 MAb (derived from clone 42B10) reacted with p41 fragment in complex with cathepsin L (32 kDa, lane 5),

with p41 fragment detached from the complex (14 kDa, lane 1 and 2), as well as with recombinant p26 (lane 3). There was no cross-reactivity observed with recombinant procathepsin L (lane 4), cathepsin L (31 kDa, lane 1) nor its heavy chain (25 kDa, lane 1) and light chain (below 14 kDa, lane 1), respectively.

Also, antibody specificity and cross-reactvity towards different cathepsins was tested by ELISA. Recombinant (pro)cathepsins L and S were added to the wells instead of p26 or p41 fragment-cathepsin L complex. With all three selected cell lines negligible reactivity was observed. For immunohistochemical localization of p41 fragment in lymph node tissue sections 2C12 MAb was selected. Results are shown in Figure 2.

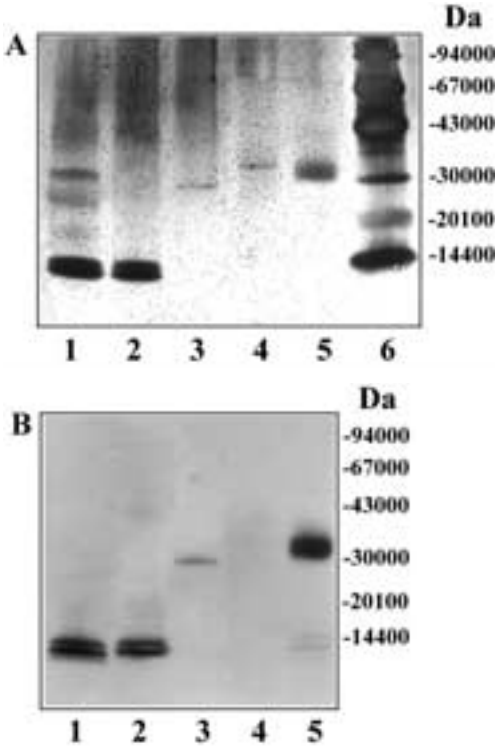

Figure 1. Demonstration of the specificity of anti-p41 fragment Mab. (A) SDS-PAGE silver staining. (B) Immunoblot of the equivalent gel stained with 2C12 MAb. Samples: (lane 1) p41 fragment-cathepsin L complex reduced (with 5% 2-mercaptoethanol) and exposed to 100 °C in the presence of SDS for 5 minutes; (lane 2) p41 fragment detached from the native complex by HPLC; (lane 3) p26 reduced (with 5% 2-mercaptoethanol) and exposed to 100 °C in the presence of SDS for 5 minutes; (lane 4) nonreduced recombinant procathepsin L; (lane 5) nonreduced p41 fragment-cathepsin L complex; (lane 6) LMW standards.

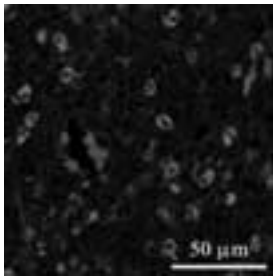

Figure 2. Immunohistochemical staining of lymph-node tissue sections for p41 fragment.

Conclusions

Specific monoclonal antibodies recognizing MHC class II-associated p41 fragment were successfully produced. They do not cross react with cathepsins L nor S or their proenzymes. We have shown specific localization of Ii fragments in certain cells in lymph nodes. These antibodies provide new tools for investigating subcellular colocalization of Ii together with cathepsins S and L.

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