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Characterization of antibody clones that bind exclusively to insoluble fibrin

Hirobumi Fuchigami[a] and Yasuhiro Matsumura[a,b,c]

Previously, we established an antibody, termed 102-10, which recognizes insoluble fibrin exclusively, unlike the previously established anti-insoluble fibrin antibodies that also cross-reacted with fibrinogen. We established that the epitope of this antibody is on the β chain that lines an indented structure that becomes exposed only when insoluble fibrin is formed. The amino acid sequence of the epitope is completely conserved from mouse to humans. This study attempted to determine the most suitable insoluble fibrin clone for future diagnostic and therapeutic development. Binding kinetics and properties of antibodies were evaluated by the surface plasmon resonance analysis (SPR) and ELISA among 1101, 99, 443, and 102-10. Immunohistochemical staining for mouse and human pancreatic cancer tissues were also performed. For frozen sections, visually appropriate staining results were observed at an antibody concentration of 1–10 μg/ml, while for paraffin sections, 10 μg/ml was required. From immunohistochemistry and ELISA analyses, clone 99 and clone 1101 showed almost no nonspecific binding in normal pancreatic tissues. Hybridoma production for 1101 yielded more antibodies than that of 99 and demonstrated good long-term stability. It was, therefore, concluded that clone 1101 would be useful for future clinical development as well as basic research. *Blood Coagul Fibrinolysis* 34:20–27

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[a]Division of Developmental Therapeutics, Exploratory Oncology Research & Clinical Trial Center, National Cancer Center, Chiba, [b]Department of Immune Medicine, National Cancer Center Research Institute, National Cancer Center and [c]Research Division, RIN Institute Inc., Tokyo, Japan

Correspondence to Yasuhiro Matsumura, MD, PhD, Department of Immune Medicine, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
E-mail: yhmatsum@east.ncc.go.jp

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Introduction

Cancer-induced hypercoagulability was first reported by Trousseau, and is currently being studied by many researchers, including us, both fundamentally and clinically [1,2]. We found that cancer stroma formation associated with increased blood coagulation in solid cancers impedes the permeability of high-molecular-weight antibodies and formulations categorized by drug delivery systems and diminishes their therapeutic effect [3,4]. First, we considered establishing antibodies against collagen that forms cancer stroma and then delivering anticancer drugs with those antibodies [5]. However, collagen also plays an important role in normal tissues and is abundant therein. Next, we considered an antibody against insoluble fibrin. Although insoluble fibrin is not cancer-specific, it is at least specific to the lesion site, including in solid cancer tissues. We were able to establish an anti-insoluble fibrin antibody clone called 102-10 [6]. Unlike conventional anti-insoluble fibrin antibodies, 102-10 was found to bind insoluble fibrin but not related soluble substances, including fibrinogen, fibrin monomer, and fibrin degradation products (FDP) [7]. The epitope of the 102-10 antibody was found to be part of the insoluble fibrin β chain that lines the pit structure, which becomes exposed only when insoluble fibrin is formed. In a soluble state, the epitope forms a strong hydrophobic bond with the hydrophobic portion of the γ chain. These properties result in specific and exclusive binding of 102-10 to insoluble fibrin. Importantly, the amino acid sequence of this epitope is completely conserved from fish to humans and, of course, conserved in mouse. Previously, we reported that radiation-labeled 102-10 pet probes selectively accumulate in stromal-rich and insoluble fibrin-rich chemically induced mouse tumor tissues [7]. Following this, we succeeded in producing an antibody drug conjugate (ADC) in which the anticancer drug monomethyl auristatin E (MMAE) was bound to 102-10 with a valine–leucine–lysine linker specifically cleavable by plasmin. The ADC was intravenously injected into mice bearing insoluble fibrin-rich pancreatic tumors, where it accumulated in solid tumors via the Enhanced Permeability and Retention effect and bound to insoluble fibrin in the stroma, followed by specific release of MMAE from the linker with plasmin activated only on insoluble fibrin. This ADC exerted a remarkable anti-tumor effect on tumors with the fibrin-rich stroma. As plasmin is completely inhibited outside the insoluble fibrin by the innate inhibitor α_2-plasmin inhibitor, the release of MMAE occurs only on the insoluble fibrin, resulting in little toxicity outside the tumor. As the anticancer drug MMAE is a small molecule,

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it was concluded that the significant anti-tumor effect was because of complete penetration of MMAE throughout the entire solid tumor mass [8].

The purpose of the present study was to produce several anti-insoluble fibrin monoclonal antibodies (anti-IF mAbs) by immunizing mice with the identified epitope peptide and to identify a suitable mAb for future clinical development.

Methods and materials

Production of new anti-insoluble fibrin monoclonal antibodies

To produce new anti-insoluble fibrin mAbs, 6-week-old BALB/cAnNCrlCrlj mice (Charles River Japan, Yokohama, Japan) or rats were immunized intraperitoneally with an emulsion of Freund's complete adjuvant (DIFCO, New Jersey, USA) and a saline solution containing 50 μg of the epitope peptide from the fibrinogen β chain that we previously discovered [7]. Three to five successive booster injections were administered intraperitoneally at 2-week intervals using the same amount of antigen in an adjuvant system (Merck, Hessen, Germany). A final boost was provided by administering the same amount of antigen intravenously. Animals were sacrificed under anesthesia, and the iliac lymph node from rats or spleen from mice was extracted and cells were fused to the P3X63-Ag8.653 myeloma cell line. Then, primary hybridoma cells were selected with HAT medium and cloned by limited dilution. Hybridoma cells were acclimated to appropriate serum-free media for large scale culture. Supernatant titer was confirmed using the Human IgG ELISA Kit (Bethyl Laboratories, Massachusetts, USA) and mAbs were obtained by affinity purification and size exclusion chromatography. The isotypes of the mAbs were determined using IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche, Basel-Stadt, Switzerland).

Surface plasmon resonance analysis

Experiments were performed using a Biacore T200 instrument, CM5 biosensor chips, and amine coupling according to the manufacturer's instructions (GE Healthcare, Illinois, USA). Kinetic evaluations were performed using an immobilized Glutathione S-Transferase (GST)-tagged peptide (CNIPVVSGKECEEIIR), which contains the insoluble fibrin mAb epitope (~90 RU) or GST tag alone as a control (~90 RU) in 10 mM sodium acetate at pH 5.0. The insoluble fibrin antibody (46.8–3000 nM), was injected using multicycle kinetics. Injections were administered at 30 μ/min at 25 °C. An HBS-N buffer at pH 7.4 (GE Healthcare) was used as a running buffer, and 10 mmol/l glycine–HCl at pH 1.5 was used as a regeneration buffer. Binding analyses were performed using the 1 : 1 binding model of the Biacore T200 evaluation software, version 1.0. The resonance unit against the GST-tagged epitope was subtracted from that against GST-tag alone in order to obtain the real K_D value against the epitope.

ELISA

One microgram of fibrinogen (Merck, Darmstadt, Germany) was immobilized onto a 96-well plate for 12 h. The fibrinogen-immobilized plates were then treated with a thrombin solution at 37 °C for 1 h to prepare fibrin clot plates. The wells were then blocked using N102 (Nichiyu, Tokyo, Japan) for 2 days. insoluble fibrin mAb and control mAb were conjugated to horseradish peroxidase (HRP) using Peroxidase Labeling Kit–NH_2 (Dojindo Molecular Technologies, Kumamoto, Japan) and diluted with PBS containing 1% Block Ace (KAC Hyougo, Japan) at 0.25–1 μg/ml. Subsequently, the antigen-coated plates were incubated in diluted HRP-labeled mAbs for 1 h and the wells were washed with Tris-buffered saline (TBS) containing 0.05% Tween 20. Finally, the mAbs bound to the wells were visualized using a 1-Step Slow TMB-ELISA (Thermo, Massachusetts, USA) as a substrate for 5 min.

Animal models

Conditional LSL-$Trp53^{R172H/+}$ (National Cancer Institute, Frederick, Maryland, US), LSL-$Kras^{G12D/+}$ (a gift from Y. Kawaguchi, C. Wright, and D. Tuveson), and Ptf1a-Cre46 (a gift from Y. Kawaguchi, C. Wright, and D. Tuveson) strains were interbred to obtain LSL-$Kras^{G12D/+}$; LSL-$Trp53^{R172H/+}$; Ptf1a-Cre (KPC) mice with a mixed 129R1/C57BL/6 background. These KPC mice spontaneously developed pancreatic ductal adenocarcinoma (PDAC) 8 weeks after birth. Commercially obtained C57BL/6JJms (Japan SLC, Shizuoka, Japan) was used as a normal mouse control. All animals were sacrificed under anesthesia and tumor or normal pancreatic tissue was resected for immunohistochemistry (IHC) analysis. The study was approved by the Committee for Animal Experimentation of the National Cancer Center, Tokyo, Japan. All animal procedures were performed in accordance with the Guidelines for the Care and Use of Experimental Animals established by the Committee. These guidelines meet the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan.

Immunohistochemistry

Insoluble fibrin mAb and control mAb were conjugated with horseradish peroxidase using the Peroxidase Labeling Kit–NH_2 and diluted with TBS at 1–10 μg/ml. A frozen human PDAC tissue section (OriGene Technologies, Maryland, USA) and a normal human pancreatic tissue section (BioChain, California, USA) were purchased commercially. Resected pancreatic cancer tissues were embedded in an optimal-cutting-temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan) and frozen at −80 °C. For frozen sections, samples were fixed for 10 min using chilled acetone (Wako Pure

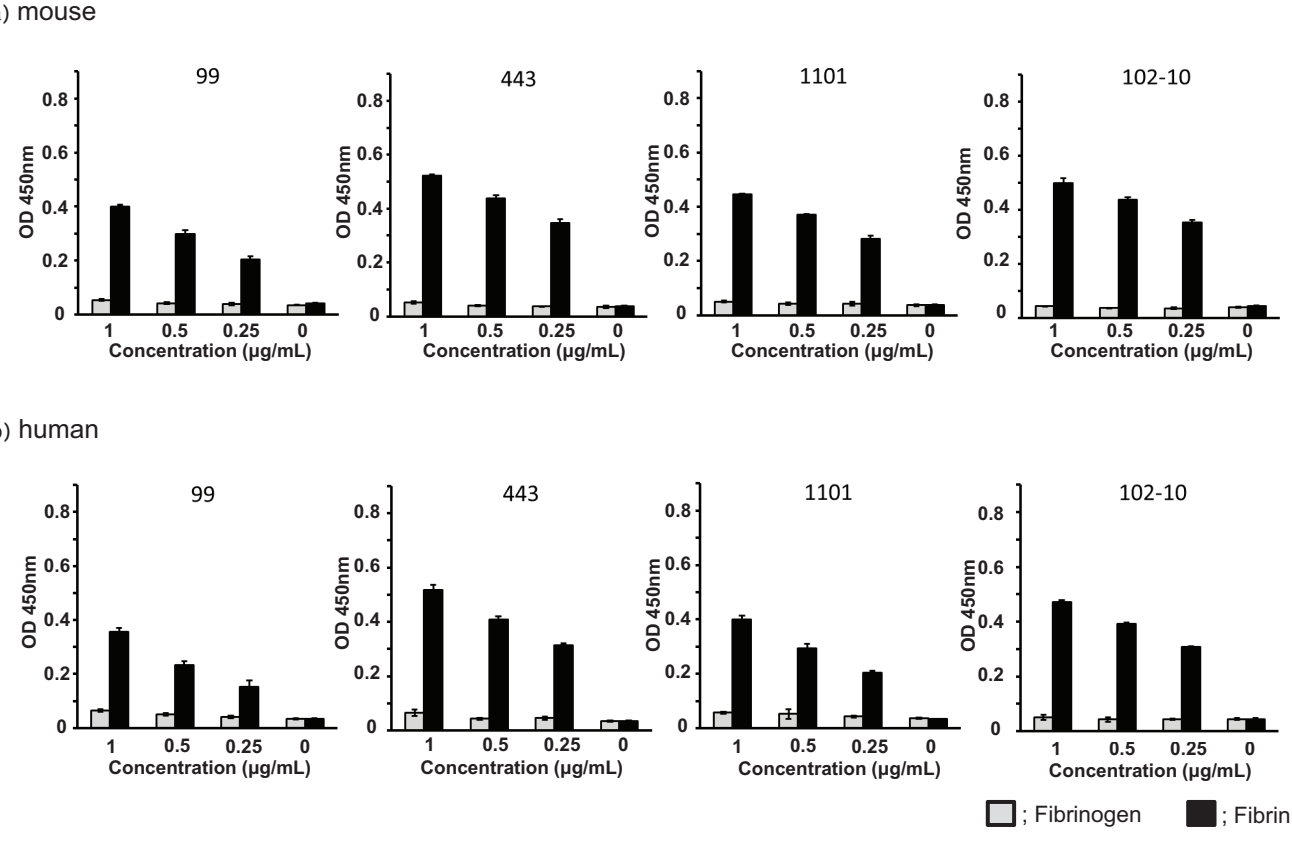
Table 1 Biochemical characteristics of anti-fibrin antibodies

Clone No.	Host species	Immunization antigen	K_D (mol/l)
99	Mouse	Epitope peptides of 102-10 (β chain)	8.762×10^{-8}
1101	Mouse	Epitope peptides of 102-10 (β chain)	6.525×10^{-8}
443	Rat	Epitope peptides of 102-10 (β chain)	3.561×10^{-7}
102-10	Chinese hamster (mouse/human chimera)	Insoluble fibrin	2.440×10^{-7}

KD, equilibrium dissociation constant.

Chemical, Osaka, Japan). Then, endogenous peroxidase activity was inhibited with 0.3% hydrogen peroxide for 20 min and the specimens were blocked with 5% skimmed milk (Becton Dickinson, New Jersey, USA) at room temperature for 30 min. Sections were then incubated with 0.25–1 μg/ml of HRP-conjugated insoluble fibrin mAb or control mAb overnight at 4 °C. Immunostaining was performed on paraffin-embedded tissue from human PDAC, normal pancreas, and a human thrombus sample (generously provided by Dr Genichiro Ishii, Pathology Division of the National Cancer Center Hospital East). In brief, sections 6 μmol/l thick were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol in water. Inhibition of endogenous peroxidases was performed by incubating the slides in 3% hydrogen peroxide (Wako, Osaka, Japan) for 20 min. Antigen retrieval was achieved by microwaving the slides for 10 min in hot (98 °C) Tris–HCl buffer at pH 9.0, followed by 30 min of cooling to room temperature. The sections were then washed with PBS (Wako, Osaka, Japan) for 10 min. After blocking with 5% skimmed milk (Becton Dickinson, New Jersey, USA) at room temperature for 30 min, the sections were incubated overnight at 4 °C in a humidified chamber with 10 μg/ml of HRP-conjugated 1101 IgG antibody. In the case of human thrombus, the sections were stained similarly with or without a 100-fold higher concentration of nonlabelled 1101 IgG in order to verify the specificity of the anti-insoluble fibrin mAb. After washing with PBS for 5 min, color development was achieved by applying diaminobenzidine tetrahydrochloride (DAB) staining reagent (Agilent, California, USA) for 2 min. The sections

Fig. 1



Examination of antibody binding affinity and specificity. ELISA assays were performed to detect antibody dose-response binding affinity and fibrin clot specificity. (a) Plates were coated with mouse fibrinogen and fibrin. (b) Plates were coated with human fibrinogen and fibrin.

were then counterstained with hematoxylin (Muto Pure Chemicals, Tokyo, Japan), dehydrated via ethanol and xylene, and coverslipped using Mount-Quick mounting medium (Daido, Saitama, Japan).

Results

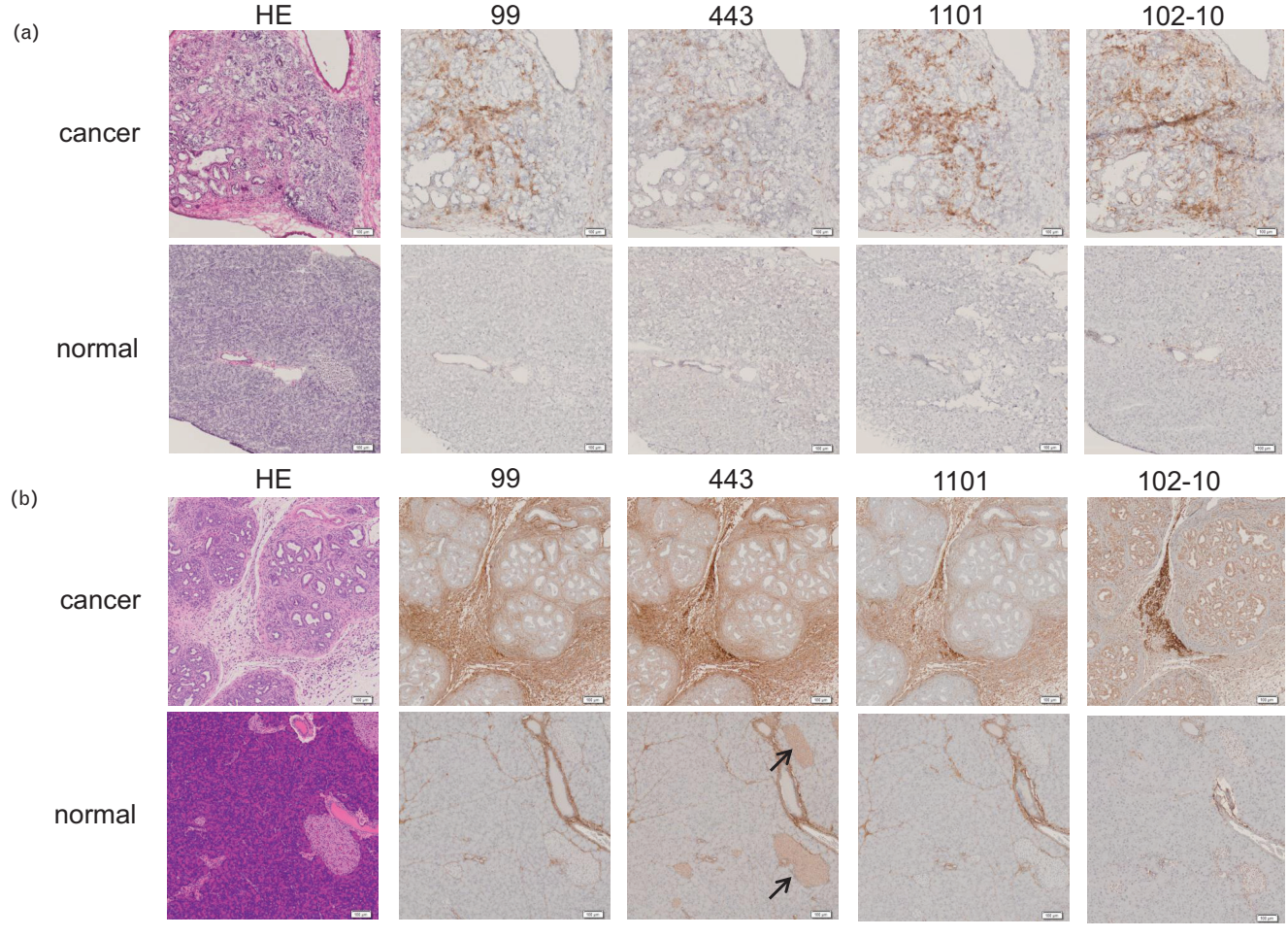
Character and surface plasmon resonance analysis analysis of insoluble fibrin monoclonal antibodies

Mice and rats were immunized with insoluble fibrin-epitope peptide and two mouse mAb clones (99 and 1101) as well as one rat antibody clone (443) were obtained. The isotypes of clones 99, 1101, and 443 were IgG1, IgG1, and IgG2a, respectively. Clone 102-10 mouse/human chimeric antibody, which we obtained previously, was used to compare reactivity, affinity, and dyeability. As it is difficult to analyze affinity to insoluble proteins such as insoluble fibrin, affinity of the mAbs to the epitope peptide was assessed using SPR analysis. Clones 99 and 443 had a higher affinity to the epitope peptide than 102-10 (8.762×10^{-8} and 6.525×10^{-8}, respectively, vs. 2.440×10^{-7}, Table 1), while 1101 had a slightly lower affinity to the epitope peptide than 102-10 (3.561×10^{-7} vs. 2.440×10^{-7}, Table 1). Although 1101 had the lowest affinity of all three clones, its K_D suggested both fast attachment to and fast dissociation from the antigen (Supplementary Table 1, http://links.lww.com/BCF/A139).

Specificity and cross-reactivity to mouse/human insoluble fibrin

The reactivity of anti-insoluble fibrin mAbs with mouse/human insoluble fibrin or fibrinogen were assessed with ELISA. All anti-insoluble fibrin mAb clones reacted with both human/mouse insoluble fibrin in a concentration-dependent manner, and did not react with mouse/human fibrinogen (Fig. 1), indicating both specific and cross-reactivity with mouse/human insoluble fibrin.

Fig. 2



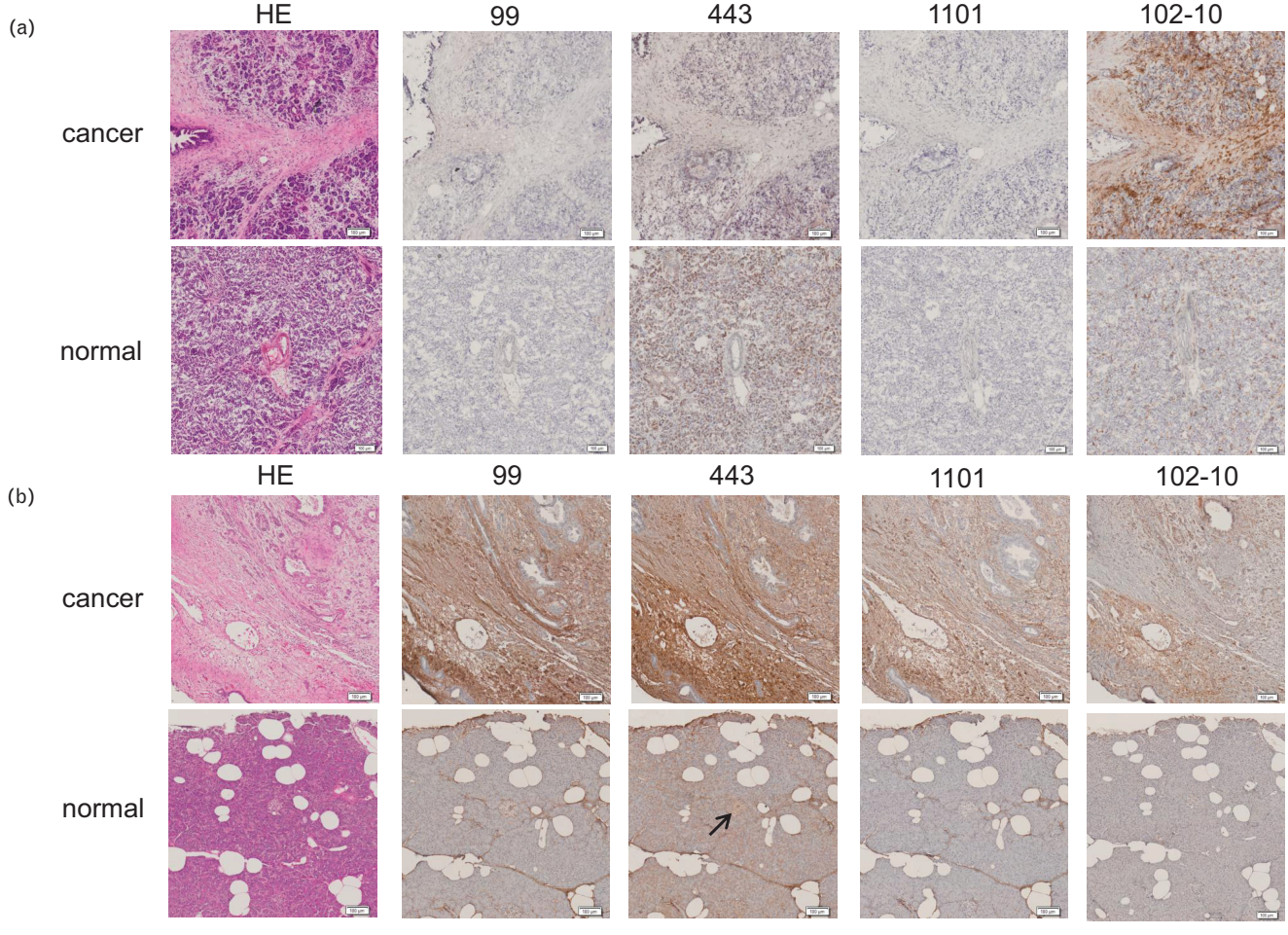
Fibrin deposition stained with anti-fibrin antibodies in mouse pancreatic tissue samples. (a) Frozen tissue samples were incubated with 1 μg/ml of HRP-conjugated anti-fibrin antibodies. (b) FFPE tissue samples were incubated with 10 μg/ml of HRP-conjugated anti-fibrin antibodies. The arrows indicate islets of Langerhans. HE staining is shown in the leftmost column. Scale bar, 100 μm. HRP, horseradish peroxidase.

Dyeability and insoluble fibrin specificity of anti-insoluble fibrin monoclonal antibodies

Although the previously established chimeric antibody (102-10) showed good dyeability in the staining of frozen tissue specimens, only a few nonspecific staining was observed in formalin-fixed paraffin-embedded (FFPE) (Fig. 2b and 3b). For this reason, the three anti-insoluble fibrin mAb clones (99, 1101, and 443) were established, and dyeability and specificity were examined in frozen tissue samples or FFPE tissue samples by direct methods. To evaluate staining, mouse and human PDAC tissues were used because of their rich deposition of insoluble fibrin. Normal tissues from mouse and human were used as a control. Results showed that in frozen mouse tissues, similar insoluble fibrin staining was observed with all three clones, while no or only slight insoluble fibrin staining was seen in normal tissues (Fig. 2a). In mouse FFPE tissues, strong insoluble fibrin staining was seen with 99, 1101, and 443, and while 102-10 also stained insoluble fibrin, nonspecific staining of the lumen structure was also observed with this clone. With clone 443, the islets of Langerhans were stained nonspecifically in normal mouse FFPE specimens (Fig. 2b). In frozen human tissues, very weak insoluble fibrin staining was observed with clones 99 and 1101, while strong staining was observed with 102-10. With clone 443, moderate staining was observed, but nonspecific background was seen in normal tissue (Fig. 2b). In human FFPE sections, a similar tendency as with mouse tissues was observed (Fig. 3b). Although 99 and 1101 exhibited weak staining in frozen human cancerous tissues at a concentration of 1 μg/ml, they markedly improved at a concentration of 10 μg/ml, with no enhancement of nonspecific staining noted (Fig. 4).

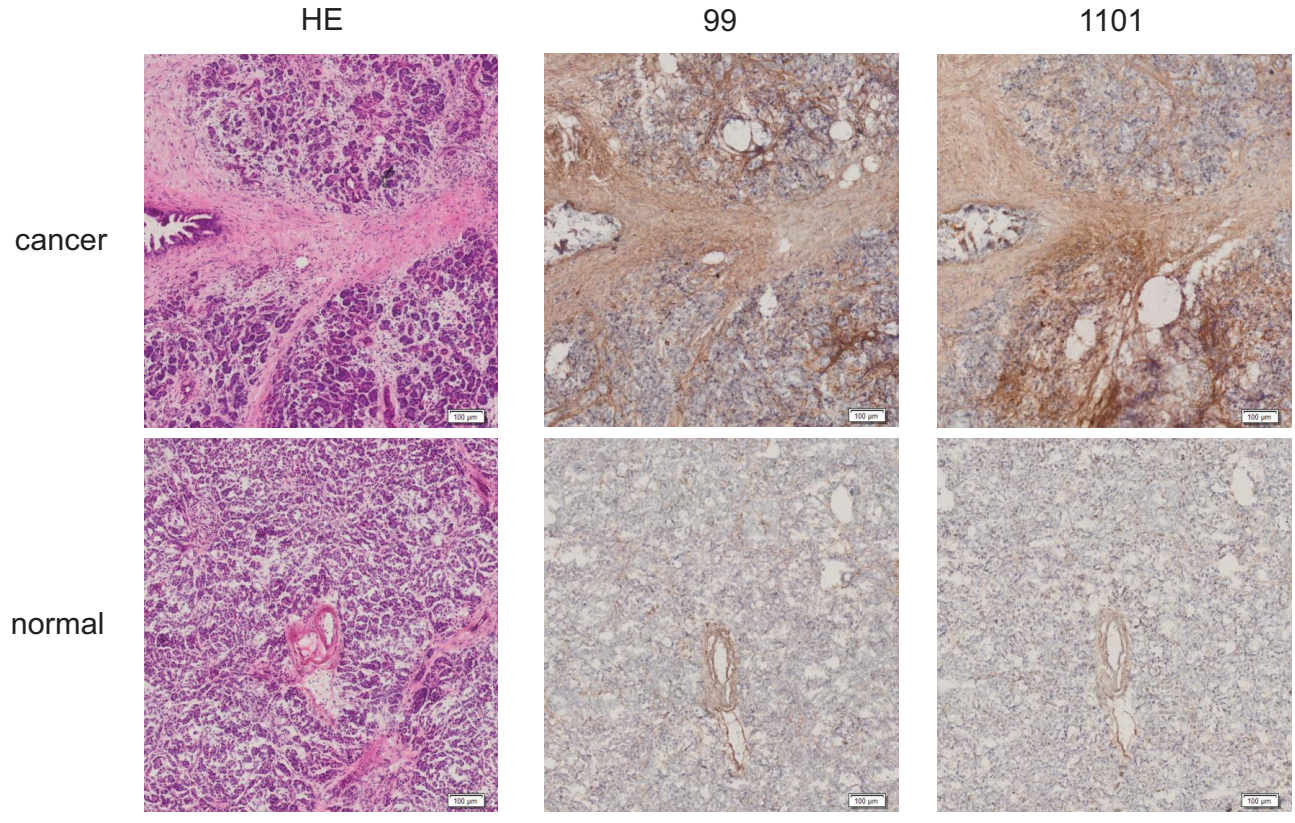
The results of immunostaining of human thrombotic tissue with clone 1101 clearly showed suppression of

Fig. 3



Fibrin deposition stained with anti-fibrin antibodies in human pancreatic tissue samples. (a) Frozen tissue samples were incubated with 1 μg/ml of HRP-conjugated anti-fibrin antibodies. (b) FFPE tissue samples were incubated with 10 μg/ml of HRP-conjugated anti-fibrin antibodies. The arrow indicates islets of Langerhans. HE staining is shown in the leftmost column. Scale bar, 100 μm. HRP, horseradish peroxidase.

Fig. 4



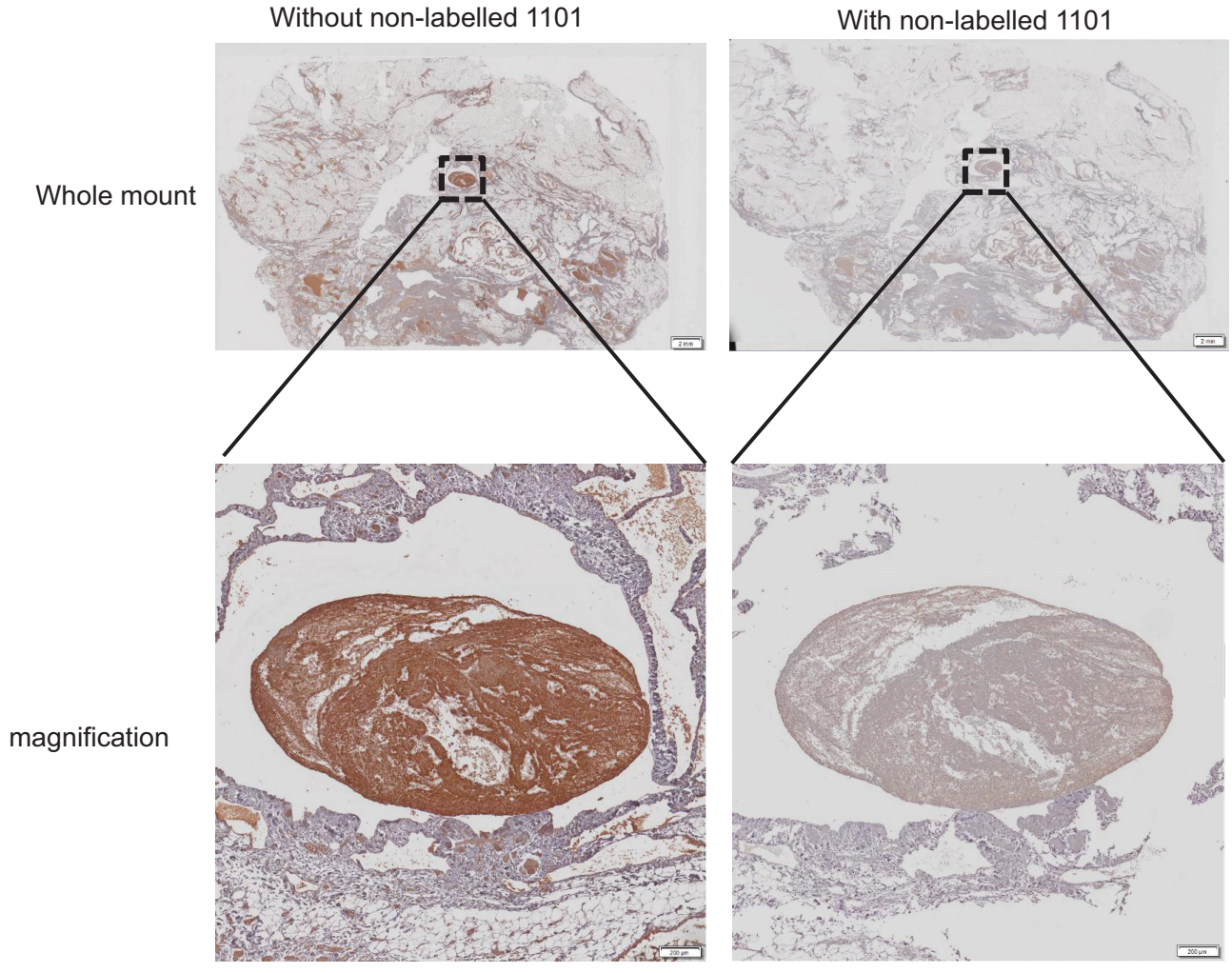
Improvement of human pancreatic tissue staining with HRP-conjugated 99 and 1101 clones. Frozen tissue samples were incubated with 10 μg/ml of HRP-conjugated 99 or 1101 antibody. Scale bar, 100 μm. HRP, horseradish peroxidase.

staining in the presence of unlabeled 1101, demonstrating the specificity of this antibody (Fig. 5).

Discussion

When the original 102-10 clone anti-insoluble fibrin antibody was established, its epitope was determined to localize to the β chain, and in an aqueous environment, it is completely covered by a hydrophobic bond with the neighboring γ chain. Therefore, our anti-insoluble fibrin mAbs do not have access to the epitope in soluble fibrinogen, fibrin monomers, and FDP molecules. They can access to the epitope only when insoluble fibrin is formed [7]. Immunizing mice with this epitope yielded many anti-insoluble fibrin antibody-producing hybridomas. Among them, clones 99, 1101, and 443 were selected for further studies following ELISA screening and analyses on binding avidity and specificity to insoluble fibrin. We established the optimal conditions for immunostaining with the four mAbs. In consideration of simplifying the IHC method, each antibody was directly labeled with HRP, with no use of secondary antibody. Antibody concentration was 1 μg/ml in mouse frozen sections, and visually appropriate staining was observed in frozen human sections, with no increase in nonspecific staining observed at 10 μg/ml. This suggests that for the IHC method for frozen sections, a concentration range of 1–10 μg/ml is desirable. On the other hand, regarding the IHC method for FFPE sections, a visually appropriate staining reaction only occurred at an antibody concentration of 10 μg/ml for both mouse and human sections. Both clone 99 and 1101 yielded similar clear staining results for frozen and FFPE sections, with almost no nonspecific reaction seen in normal tissues. Clone 443, like clones 99 and 1101, showed similar staining of the tumor stroma from mouse and frozen human sections as well as FFPE sections, but nonspecific staining was also observed in normal tissues. Although antibody clone 102-10 showed a significant reaction with insoluble fibrin in the tumor stroma, nonspecific staining was also seen in cell nuclei from both mouse and human sections. From ELISA results, all four clones were deemed highly active against mouse and human insoluble fibrin, and the reaction was concentration-dependent. Moreover, almost no reaction was seen with mouse and human fibrinogen, suggesting that for all clones, activity is specific to insoluble fibrin. Regarding the amount of antibody produced from hybridomas, 1101 yielded a stable amount of antibody. The specificity of 1101 was also clarified in the results of immunostaining of human

Fig. 5



Immunohistochemistry on a blood clot found near excised lung cancer tissue was performed using HRP-conjugated-1101 with or without nonlabeled 1101. Upper left: without nonlabeled 1101, upper right: with nonlabeled 1101. The lower row is an enlarged view of each. Scale bar, 2 mm (upper panels) and 200 μm (lower panels).

thrombotic tissue. Altogether, the present studies suggest that clone 1101 is a promising candidate for future use in basic research and for the production of humanized antibodies for clinical application. We believe that this particular anti-insoluble fibrin antibody will contribute significantly to the study of blood coagulation. In addition, we are aiming to develop a new ADC with this antibody via linkage to an anticancer drug [8], as well as an efficient thrombolytic agent via fusion to urokinase [9].

Conclusion

It was therefore concluded that clone 1101 would be useful for future clinical development as well as basic research.

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Conflicts of interest

Y.M. is a co-founder, shareholder, and Board Member of RIN Institute Inc., which owns the anti-insoluble fibrin antibody discussed herein.

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