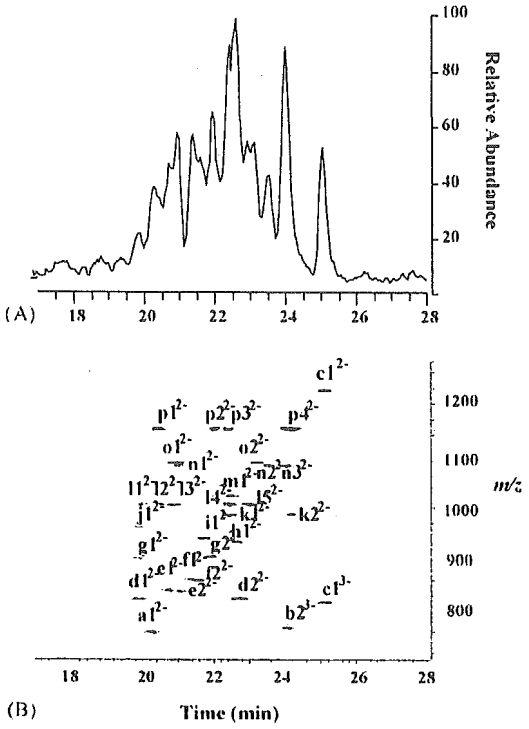

Fig. 6. TIC of a mixture of equal amount of d_0-PA N-linked oligosaccharides from rhCG and d_4-PA N-linked oligosaccharides from hCG (A), and its 2D display (B). Oligosaccharides (from 2 μg rhCG and hCG) were analyzed by GCC-LC/MS in the negative ion mode.

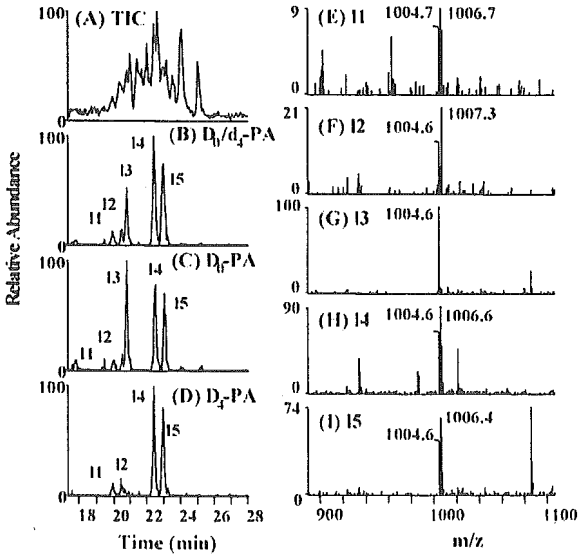

Fig. 7. TIC of a mixture of equal amount of d_0-PA N-linked oligosaccharides from rhCG and d_4-PA N-linked oligosaccharides from hCG (A). Extracted ion chromatograms of d_0-, and d_4-PA monosialylated biantennary (set *m/z* values, 1004–1007) (B), d_0-PA monosialylated biantennary (set *m/z* values, 1004–1005) (C), and d_4-PA monosialylated biantennary oligosaccharides (set *m/z* values, 1006–1007) (D). Mass spectra of peak I1–I5 (E–I).

Fourteen oligosaccharides were detected only in hCG, and most of them were fucosylated complex type. These results show the differences in glycosylation between rhCG and hCG and suggest that many hybrid type oligosaccharides linked to rhCG, while fucosylated oligosaccharides attach to hCG.

4. Discussion

Alteration of glycosylation is known to cause many changes in the biological activity as well as the physical properties of proteins. Several procedures of oligosaccharide profiling have been reported for the assessment of alteration of glycosylation, however, most of them can be used for only either qualitative or quantitative analysis. Although mass spectrometric oligosaccharide profiling is useful for the qualitative analysis, it has a problem on precision, and some isomers are still indistinguishable if their retention times are closed to others. In this study, we demonstrated that the use of isotope-tagged internal standards and GCC-LC/MS made it possible to do both quantitative and qualitative carbohydrate analysis.

First, we demonstrated the monosaccharide composition analysis using the isotope tag method. The use of internal standards that were heated under the same hydrolysis condition as an analyte glycoprotein resulted in good precision and accuracy in the monosaccharide composition analysis. Several HPLC methods for determination of monosaccharides have been reported. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been widely used for monosaccharide composition analysis [20,22–25]. Although HPAEC-PAD gives high resolution of all common monosaccharides and has the advantage of not requiring derivatization, this method is also known to have a disadvantage of limited selectivity [26]. The isotope tag method with SIM mode is equal to the HPAEC-PAD in sensitivity and is better than it in selectivity.

Next, we demonstrated the potentiality of the isotope tag method for quantitative oligosaccharide profiling using rhCG and hCG as model glycoproteins. hCG consists of an α subunit (MW 14.7 kDa) and a β subunit (MW 23.0 kDa), and oligosaccharides link to Asn52, and 78 in the α subunit and Asn13 and 30 in the β subunit. It has been reported that the majority of N-linked oligosaccharides in rhCG and hCG are fucosylated or non-fucosylated di-, tri-, and tetra-antennary forms with a various level of sialylation [27–30]. We prepared d_0-PA oligosaccharides and d_4-PA oligosaccharides from rhCG and hCG, respectively, and an equal part of d_0-PA and d_4-PA oligosaccharides was injected into LC/MS. We demonstrated that the oligosaccharides existing in one side protein were detected as single ions, whereas common oligosaccharides were detected as paired ions. We could easily realize that monosialo-, and disialobiantennary oligosaccharides linked to both hCG and rhCG, while fucosylated oligosaccharides and some hybrid type oligosaccharides linked to only hCG and rhCG, respectively. In addition, we demonstrated the pos-

sibility of the quantitative comparison the oligosaccharides between two quite similar glycoproteins. This quantitative oligosaccharide profiling is expected to be a powerful tool in various stages, including quality control and comparability assessment of glycoprotein products, and elucidation of glycan alteration in some diseases.

Acknowledgements

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Thrombomodulin Enhances the Invasive Activity of Mouse Mammary Tumor Cells

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Thrombomodulin (TM) is a thrombin receptor on the surface of endothelial cells that converts thrombin from a procoagulant to an anticoagulant. Thrombin promotes invasion by various tumor cells, and positive or negative correlations are found between the expression of TM and tumorigenesis in some patients. In this study, we used an invasion assay to investigate the effect of TM on the invasive activity of a mouse mammary tumor cell line, MMT cells, and the effects of TM were compared with those of thrombin as a positive control. In the presence of 1% fetal calf serum (FCS), TM significantly stimulated MMT cell invasion in a dose-dependent manner, resulting in an approximately 3-fold increase at 1–10 pg/ml over the untreated control. Thrombin also caused a similar degree of stimulation at 50 ng/ml. Since thrombin activity was detected in the components of the assay system, an invasion assay was also performed in a thrombin-activity-depleted assay system constructed to eliminate the effect of thrombin activity; TM (10 pg/ml) plus thrombin (1 pg/ml) stimulated invasion by approximately 3.5-fold in this assay system. Hirudin, a specific thrombin inhibitor, inhibited stimulation by TM as well as by thrombin in both the presence and absence of 1% FCS. Investigations of the effects of TM on proliferation, adhesion and chemotaxis to clarify the mechanism of stimulation by TM revealed that TM does not affect proliferation or adhesion in the presence of 1% FCS, but stimulates chemotaxis by approximately 2.3-fold. Similar results were obtained in experiments using thrombin. TM (10 pg/ml) plus thrombin (1 pg/ml), on the other hand, stimulated chemotaxis by approximately 2.3-fold in the thrombin-activity-depleted assay system. Binding studies using [^{125}I]-thrombin revealed that the cells have specific saturable binding sites for thrombin. These results show that TM stimulates the invasive activity of MMT cells, probably by acting as a cofactor for the thrombin-stimulated invasion of the cells *via* its receptor and lowering the effective concentration of thrombin. The findings also indicate that the stimulation of invasive activity in the presence of 1% FCS and in the thrombin-activity-depleted assay system may mainly be mediated by the stimulation of chemotaxis.

Key words: invasion, thrombin, thrombomodulin.

Abbreviations: TM, thrombomodulin; MEM, modified Eagle's medium; CS, calf serum; FCS, fetal calf serum; MMP, matrix metalloprotease; ECM, extracellular matrix; Boc-Asp(Obzl)-pro-Arg-MCA, Boc-β-benzyl-Asp-Pro-Arg-4-methyl-coumaryl-7-amide; PBS, phosphate-buffered saline.

Thrombomodulin (TM) is a thrombin receptor on the surface of endothelial cells (*1*) that was first discovered as a cofactor for the thrombin-catalyzed activation of the anticoagulant protein C (*2*). Biologically active soluble forms of TM, which probably represent the products of limited proteolytic cleavage of cell-surface TM, were later detected in human plasma (*3*), suggesting a possible role of the soluble forms *in vivo*. TM also positively or negatively regulates various functions of thrombin as described below. TM stimulates the inactivation of pro-urokinase-type plasminogen activator (*4*), the activation of TAF I (*5*), and the activation of progelatinase A (*6*). TM inhibits the activation of platelets (*7*), the activation of factor X (*8*) and human endothelial cells (*9*), the stimulation of fibrin formation (*8*), and the proliferation of arterial smooth muscle cells (*10*) and human umbilical vein endothelial cells (*11*).

On the other hand, there are several direct and indirect lines of evidence indicating that thrombin stimulates invasion and/or metastasis by tumor cells (*12–18*), and it has recently been reported that the expression of TM is increased or decreased in some carcinomas. The expression of TM increases in squamous carcinomas of the lung (*19*), colorectal carcinomas (*20*), and some transitional

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carcinomas (*21–22*), and its expression level is negatively correlated with the malignancy of carcinoma of the esophagus (*23*), hepatocellular carcinoma (*24*), and ovarian tumors (*25*). There is also evidence of increased serum levels of TM in some tumors, including pancreatic cancer (*26*), digestive tract carcinoma (*27*), and glioblastoma (*28*). Based on this evidence, it is likely that TM plays some role in the regulation of tumor metastasis.

In this study, we investigated the effects of TM on the invasive activity of a mouse mammary tumor cell line, MMT, by an *in vitro* invasion assay, because tumor cell invasion through the basement membrane is a critical step in the process of metastasis (*29–30*). We also compared the effects of TM with those of thrombin as a positive control.

MATERIALS AND METHODS

Materials—TM was a kind gift of Asahi Kasei Pharma, Japan. The TM was prepared as described by Gomi *et al.* (*31*). Plasmids containing the cDNA encoding TM (residues 1–498) were transfected into COS-1 cells, and the recombinant TM was purified from serum-free COS-1-cell-conditioned medium. The purified TM yielded a single band at 90 kDa in SDS-PAGE under reducing conditions. The recombinant TM was confirmed to be thrombin-free by a protein C activating assay developed in our laboratory (*32*). Thrombin (1,140 units/mg protein) was a kind gift of Mochida Pharmaceutical Co., Ltd., Japan. Hirudin was purchased from Wako (Osaka, Japan). A fluorogenic substrate, Boc-β-benzyl-Asp-Pro-Arg-4-methyl-coumaryl-7-amide (Boc-Asp(Obzl)-pro-Arg-MCA), was purchased from Peptide Institute, Inc. (Osaka, Japan).

Cell Culture—MMT mouse mammary tumor cells were obtained from the Japanese Health Science Research Resource Bank and cultured in modified Eagle's medium (MEM) supplemented with 10% calf serum (CS) on 60-mm diameter culture dishes, 4×10^5 cells per dish. After 7 d, the subconfluent MMT cells were detached from the culture dishes with 0.25% trypsin/EDTA, treated with MEM containing 10% CS, and collected by centrifugation. The cells were then washed with MEM and used in experiments. In some experiments, the thrombin activity associated with cells was depleted as described below, and the resultant cells were used for various experiments in which MEM containing 0.1% BSA was used as the basal medium. We refer to this assay system as the thrombin-activity-depleted assay system below. To deplete thrombin activity, the cell suspension (1.5×10^5 cells in 10 ml of MEM) was incubated in a non-adherent form on 100-mm diameter non-treated culture dishes pre-coated with BSA (10 mg/ml) for 2 h in a humidified chamber at 37°C under 5% CO_2, and then washed with MEM.

In Vitro Invasion Assay—In vitro invasion by MMT cells was measured in a Matrigel invasion chamber (Collaborative Biomedical Products, Bedford, MA, USA). The chamber (upper compartment) was placed in a 24-well culture plate (lower compartment), and the cell suspension (1.6×10^5 cells in 500 μl) and the basal medium (750 μl) containing various factors were added to the upper and lower compartments, respectively. MEM containing 1% fetal calf serum (FCS) or 0.1% BSA was used as the basal medium. Matrigel invasion chambers were precoated with fibronectin as described below before use in the thrombin-activity-depleted assay system. Human plasma fibronectin solution (IWAKI, Japan) was diluted to a final concentration of 5 μg/ml with phosphate-buffered saline (PBS), and a 300 μl aliquot was added to the chamber and a 750 μl aliquot to the 24-well culture plate. The chamber and 24-well plate were allowed to stand at 37°C for 2 h and were then washed with PBS. After incubating the cells for 18 h, the filters were fixed with methanol and stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping with cotton swabs, and the number of cells that had migrated to the lower surface of the filters was counted under a microscope.

Measurement of Thrombin Activity—Thrombin activity in FCS, CS, and on cells was measured by the method of Kawabata *et al.* (*33*). A 10 μl volume of 10% FCS or CS was mixed with 90 μl of reaction buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10 mM $CaCl_2$, with or without hirudin (0.5 unit/ml). Packed cells (1×10^6 cells) were suspended in 100 μl of reaction buffer with or without hirudin (0.5 unit/ml). After adding 1 μl of 10 mM substrate, Boc-Asp(Obzl)-pro-Arg-MCA solution to the cell suspension, the mixture was incubated for 20 min at 37°C, and the reaction was stopped by adding 600 μl of 0.6 M acetic acid. The fluorescence of the aminomethylcoumarine released was measured with a fluorospectrophotometer at an excitation wavelength of 380 nm and an emission of 460 nm. A blank solution was prepared by adding 1 μl of substrate solution to the reaction buffer mixed with 600 μl of 0.6 M acetic acid. Thrombin activity was calculated using 1, 2.5, 5 and 10 ng/ml thrombin solutions as standards and subtracting the fluorescence obtained in the presence of hirudin from that in the absence of hirudin. A linear dose-response curve was obtained between 0.5–5 ng/ml of thrombin, and its activity was inhibited by more than 98% by hirudin (0.5 unit/ml). The fluorescence of each sample was within the linear range.

Proliferation Assay—The cell suspension (1×10^5 cells in 4 ml) was seeded on 60-mm diameter culture dishes and incubated with each factor for 18 h. MEM containing 1% FCS was used as the basal medium. The cells were then detached from the culture dishes with 0.25% trypsin/EDTA, treated with MEM containing 10% CS, collected by centrifugation, and counted with a hemocytometer.

Adhesion Assay—Adhesion assays were performed by a modification of the method of Deryugina *et al.* (*34*). A 300 μl aliquot of fibronectin (5 μg/ml), prepared as described above, was added to each well of 24-well plates (IWAKI, Japan). The plates were allowed to stand overnight at 4°C, washed with PBS, blocked with 1% BSA in PBS for 1 h at 37°C, and finally washed in PBS. MMT cells (55×10^4 cells) were exposed to each factor in 2 ml of MEM containing 1% FCS for 30 min at 37°C. After washing with 2 ml of MEM, the cell suspensions (1×10^5 cells in 0.38 ml of MEM) were seeded on each well. After incubation for 30 min at 37°C, non-adherent cells were removed by washing with PBS, and the adherent cells were fixed and stained with 0.2% crystal violet in 10% ethanol for 10 min. After three washes with 2 ml of PBS,

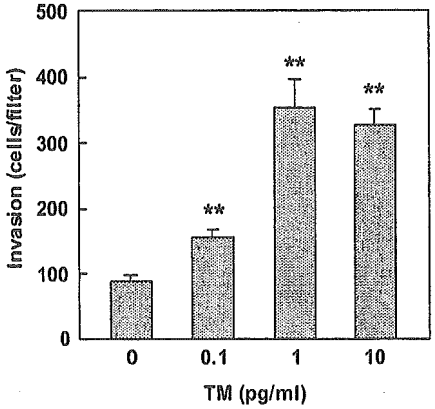

Fig. 1. **Dose dependency of the effect of TM on invasiveness.** MEM containing 1% FCS was used as the basal medium. The concentrations of TM indicated are the concentrations in the lower compartment. The data shown are means ± SD of the data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). The deviation in each experiment was less than 10%.

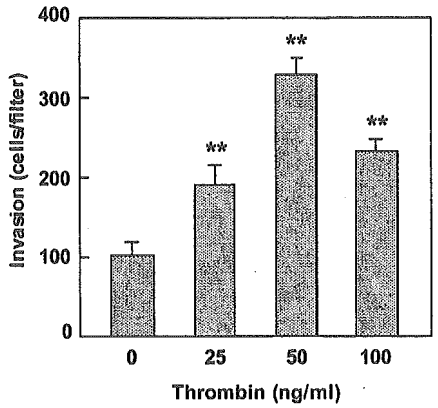

Fig. 2. **Dose dependency of the effect of thrombin on invasiveness.** MEM containing 1% FCS was used as the basal medium. The concentrations of thrombin indicated are the concentrations in the lower compartment. The data shown are means ± SD of data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). The deviation in each experiment was less than 10%.

the dye was extracted in an end-over-end mixer with 600 μl of 50% ethanol in 50 mM sodium phosphate (pH 4.5) for 10 min, and absorbance was measured at 540 nm. The correlation between absorbance and cell number was confirmed in a preliminary experiment.

Chemotaxis Assay—Chemotaxis assays were performed with control inserts (Collaborative Biomedical Products, Bedford, MA, USA) in a similar manner to the invasion assay described above. The control inserts were not coated with Matrigel. MEM containing 1% FCS was used as the basal medium. The control inserts were pre-coated with fibronectin (5 μg/ml) as described for the pre-coating of the Matrigel invasion chamber in the thrombin-activity-depleted assay system.

Iodination of Thrombin and Determination of Binding—Thrombin was iodinated to a specific activity of 19.1 × 10^7 cpm/μg by the chloramine T method as described previously (*35–36*). After precoating 24-well plates with fibronectin (5 μg/ml) as described above, the cell suspensions (1.22 × 10^5 cells in 0.45 ml of MEM) were seeded into each well and incubated in a humidified chamber at 37°C under 5% CO_2 for 2 h. The cells were then washed with 0.4 ml of MEM containing 15 mM Hepes (pH 7.2) and 0.1% BSA and incubated for 1.5 h at 37°C in the same buffer with various concentrations of [^{125}I]-thrombin in the presence or absence of a 100-fold excess amount of unlabeled thrombin. After washing the cells four times with the same ice-cold buffer, the cells were solubilized with 0.4 ml of 1 N NaOH for 1 h at 37°C. Specific binding was calculated as the difference between total binding and nonspecific binding.

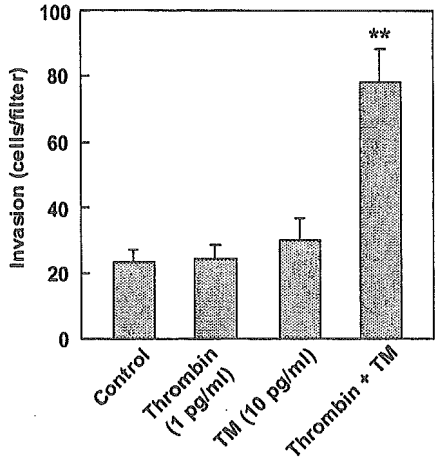

Fig. 3. **Effect of TM and thrombin on invasiveness in the thrombin-activity-depleted assay system.** Cells on which thrombin activity was depleted were used in the experiment. MEM containing 0.1% BSA was used as the basal medium. The data shown are means ± SD of data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). The deviation in each experiment was less than 10%.

RESULTS

Effect of TM on Invasiveness—Figure 1 shows the effects of TM on the invasive activity of MMT cells in the presence of 1% FCS. TM significantly stimulated invasive activity in a dose-dependent manner, resulting in an approximately 3-fold stimulation at 1–10 pg/ml. Figure 2 shows the effects of thrombin used as a positive control. Thrombin also stimulated invasive activity in a dose-dependent manner.

On the basis of these findings, we investigated the possibility that the stimulation of invasion by TM might be dependent on thrombin that may have been introduced into the assay system as described below. First, thrombin activity in the assay system was measured. The thrombin concentrations in freshly prepared 10% FCS and CS measured by the thrombin activity assay were 200 pg/ml and 2.8 ng/ml, respectively. The amount of thrombin on the cells measured in a similar manner was 35 pg/10^6 cells. Based on these values, the thrombin concentrations in the assay system with or without 1% FCS were estimated to be 24.48 and 4.48 pg/ml, respectively. Second, the action of TM was examined in the thrombin-activity-depleted assay system described in "MATERIALS AND METHODS," and depletion of thrombin activity in the

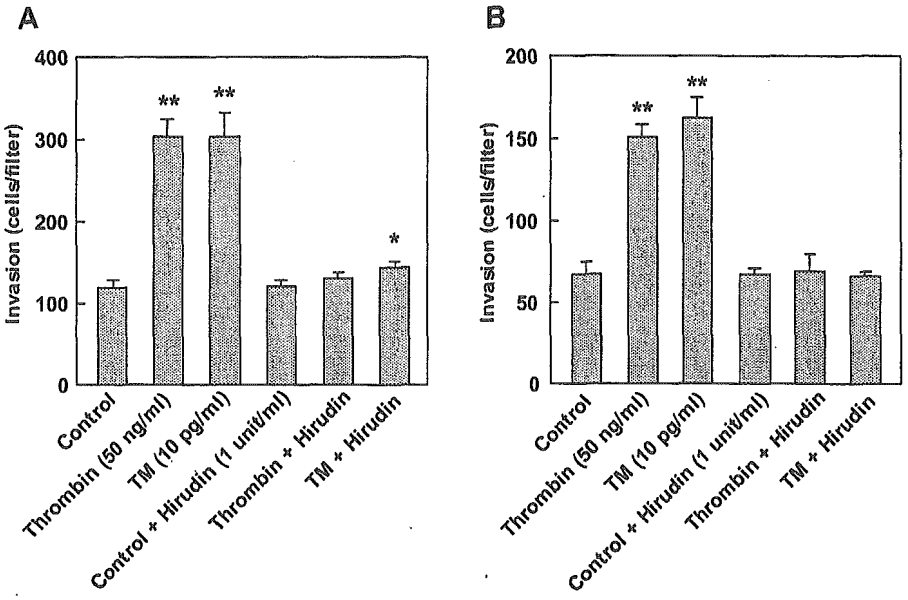

Fig. 4. **Effect of hirudin on the stimulation of invasion by TM.** (A) MEM containing 1% FCS was used as the basal medium. The indicated concentration of each factor is that in the lower compartment. The data shown are means ± SD of data obtained in duplicate wells in three experiments (*$p < 0.05$ *vs.* control; **$p < 0.01$ *vs.* control) (B) MEM containing 0.1% BSA was used as the basal medium. The data shown are means ± SD of data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). The deviation in each experiment was less than 10%.

assay system was confirmed by the absence of any detectable thrombin activity on the cells. Figure 3 shows the effects of thrombin (1 pg/ml) and TM (10 pg/ml) on the invasive activity of cells in the thrombin-activity-depleted assay system. While neither thrombin or TM had any effect on invasion, TM plus thrombin stimulated invasion by approximately 3-fold.

Effect of Hirudin on the Stimulation of Invasion by TM—The action of TM was also examined in the presence of the specific thrombin inhibitor hirudin to investigate the possibility described above. Fig. 4, A and B, shows the effects of hirudin on the invasion-stimulating activity of TM in the presence and absence of 1% FCS. We used a 1 unit/ml concentration of hirudin in this experiment, because 50 ng/ml thrombin corresponds to 0.057 unit/ml, and so 1 unit/ml hirudin seemed adequate to inhibit this concentration of thrombin. As expected, hirudin (1 unit/ml) not only inhibited the stimulation by thrombin to control levels, but the stimulation by TM as well.

Effect of TM on Proliferation—Since tumor cell invasion consists of a series of events, including adhesion to the extracellular matrix (ECM) and chemotaxis, we investigated the effects of TM on these two events to clarify the molecular mechanism of the stimulation of invasive activity by TM. Before investigating the effect of TM on these processes, we investigated its effects on cell proliferation to confirm that the stimulation of invasive activity by TM is not an artifact of the enhancement of cell proliferation.

Figure 5 shows the effects of TM on MMT cell proliferation in the presence of 1% FCS. The numbers of cells in the presence of TM or thrombin did not differ from the numbers in the control cultures.

Effect of TM on Adhesion to Fibronectin—Figure 6 shows the effects of TM on adhesion to fibronectin, a basal lam-

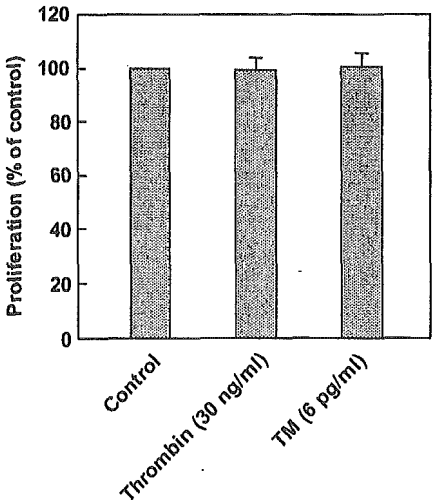

Fig. 5. **Effect of TM on proliferation.** MEM containing 1% FCS was used as the basal medium. The data shown are means ± SD of data obtained in duplicate dishes in three experiments. The deviation in each experiment was less than 10%.

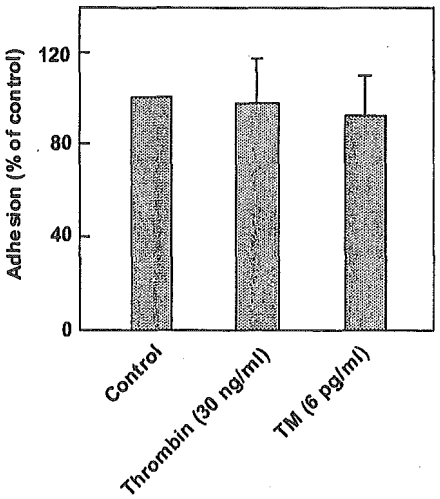

Fig. 6. **Effect of TM on adhesion to fibronectin.** MEM containing 1% FCS was used as the basal medium. The data shown are means ± SD of data obtained in duplicate wells in three experiments. The deviation in each experiment was less than 10%.

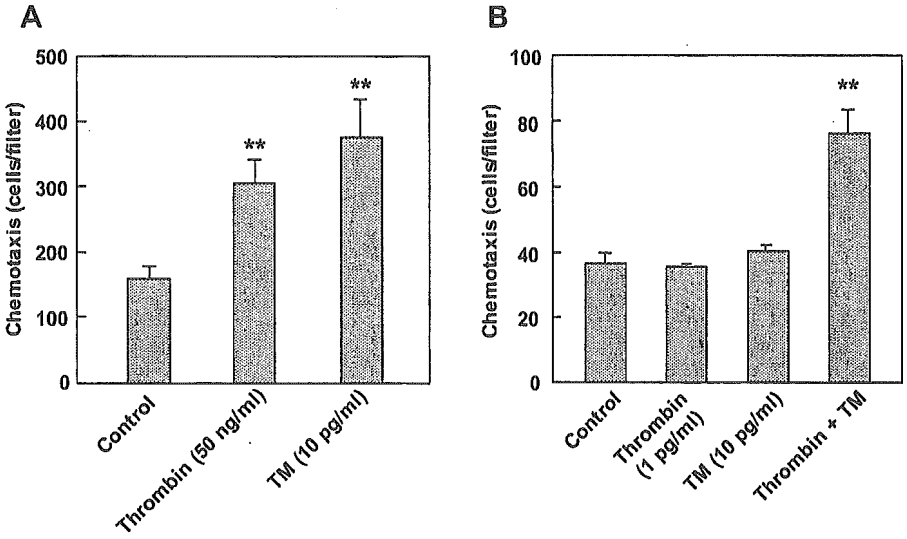

Fig. 7. **Effect of TM on chemotaxis.** (A) MEM containing 1% FCS was used as the basal medium. The data shown are means ± SD of data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). (B) Cells on which thrombin activity was depleted were used in the experiment. MEM containing 0.1% BSA was used as the basal medium. The data shown are means ± SD of data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). The deviation in each experiment was less than 10%.

ina component. Neither TM nor thrombin affected adhesion to fibronectin.

Effect of TM on Chemotaxis—Figure 7 (A and B), shows the effects of TM on chemotaxis by MMT cells in the presence of 1% FCS and in the thrombin-activity-depleted assay system, respectively. Both TM (10 pg/ml) and thrombin (50 ng/ml) significantly stimulated chemotaxis by MMT cells by approximately 1.9–2.3-fold in the former system, but neither TM (10 pg/ml) nor thrombin (1 pg/ml) affected chemotaxis in the latter system; TM plus thrombin, on the other hand, stimulated chemotaxis by approximately 2-fold.

Binding of Thrombin—Figure 8 shows the binding curves for specific [^{125}I]-thrombin binding sites on cells in the presence and absence of TM (10 pg/ml). These binding curves show the specific [^{125}I]-thrombin binding to be saturable at approximately 40 ng/ml, and that TM has no effect on the specific binding of [^{125}I]-thrombin. A similar binding experiment was performed in the [^{125}I]-thrombin concentration range of 1–10 pg/ml, but no specific binding was detected independent of the presence or absence of TM, probably because the absolute amount of radioactivity used was too low to be detected as specific binding.

DISCUSSION

In the present study, we show that, at its maximum effective dose, TM stimulates the invasive activity of MMT cells *in vitro* by approximately 3-fold. As far as we know, this is the first time that TM has been shown to stimulate the invasive activity of tumor cells *in vitro*. Similarly, exogenous thrombin causes maximal stimulation of invasion at 50 ng/ml, which is a concentration more than 1,000-fold higher than the maximum effective dose of TM.

Since TM acts as a cofactor for the thrombin-catalyzed activation of protein C and increases the rate of the reaction by >1,000-fold (*8*), the stimulation by TM may have been due to TM interacting with thrombin, which had been introduced into the assay system, and acting as a cofactor for thrombin-stimulated invasion of MMT cells, thus lowering the effective concentration of thrombin. This possibility seems to be supported by the detection of thrombin activity in the assay systems, the requirement for thrombin for stimulation by TM in the thrombin-activity-depleted assay system, and the inhibition of stimulation by hirudin. It is noteworthy that the thrombin concentration required for stimulation by TM in the thrombin-activity-depleted assay system is more than 20,000-fold less than the concentration required for thrombin to stimulate invasion. On the other hand, the control level was not inhibited by hirudin in the presence of 1% FCS, probably due to the lower thrombin concentration in the assay system compared with the effective concentration of exogenous thrombin.

There have been two studies examining the effect of TM on the invasive activity of tumor cells. Matsushita *et al.* showed that a subcloned human esophageal squamous cell carcinoma line with low TM expression is more invasive than a high TM-expressing clone (*37*). In their study, the action of TM does not seem to be due to an acceleration of its thrombin cofactor activity, because the difference between the cell lines with low and high TM expression with respect to their cofactor activity for protein C activation by thrombin was less than 13% and significantly lower than their TM levels and invasive activities. Hosaka *et al.* showed that TM (10–100 ng/ml)

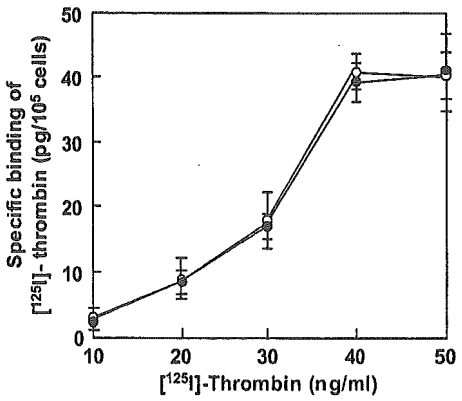

Fig. 8. **Binding of [^{125}I]-thrombin.** Specific binding of [^{125}I]-thrombin to cells in the absence (solid circles) and presence of TM (10 pg/ml) (open circles) was measured. The data shown are representative specific binding curves for [^{125}I]-thrombin binding sites and are means ± SD of data obtained in triplicate wells.

inhibits the invasive activity of mouse melanoma cells *in vitro* (*38*), and TM has also been found to decrease the proliferation of tumor cell lines subcloned from patients with malignant melanomas (*39*). None of these inhibitory effects were inhibited by hirudin.

In contrast to these studies, the present study shows that TM enhances the invasive activity of MMT cells and indicates that the action of TM is entirely dependent on thrombin as described above. Therefore, the mode and mechanism of action of TM in MMT cells seems to be different from its mode and mechanism of action in the squamous cell carcinoma line and melanoma cells.

It is useful to speculate on the role of TM in tumorigenesis based on the findings of our study, because tumor cell invasion through the basement membrane is a critical step in the process of metastasis (*29–30*). Several studies have shown that TM levels in serum increase in patients with certain tumors (*26–28*), as described in the Introduction. Thus, the results of this study suggest that the soluble form of TM may play a positive role in the malignancy of some kinds of tumors, probably by enhancing the metastatic potential of thrombin. On the other hand, TM on the cell surface may act as a negative regulator to thrombin, because thrombin is degraded as the thrombin-TM complex by its internalization after binding to TM on the cell surface (*40*). This possibility may be supported by the findings that the expression level of TM is negatively correlated with the malignancy of some carcinomas (*23–25*), as described in the Introduction.

Tumor cell invasion is a complex process that involves adhesion to ECM, degradation of ECM, and chemotaxis (*41*). Chemotaxis is the essential step in invasion as reviewed by Wells (*42*). The results of the present study show that both TM and thrombin stimulate chemotaxis in the presence of 1% FCS, and that TM plus thrombin stimulate chemotaxis in the thrombin-activity-depleted assay system. Both of these findings are consistent with previous reports that thrombin stimulates chemotaxis (*17*, *18*), and the presence of specific binding sites for thrombin on cells indicates that these actions are mediated by thrombin receptors.

However, other actions of thrombin may also be involved in the stimulation of tumor cell invasion, because the enhancement of chemotaxis alone is insufficient to account for the increase in tumor cell invasion. One such other possible action of thrombin is the stimulation of the matrix metalloprotease (MMP)–mediated degradation of a variety of ECM proteins, including collagens type IV, V, VII, and X, fibronectin, laminin (*43–47*), elastin (*48–49*), proteoglycans (*49–51*), and entactin (*52*). Several reports have indicated that thrombin increases the active forms of MMP-2 and MMP-9 (*53–59*), and plays important roles in the enhancement of tumor cell invasion and metastasis (*34*, *60–68*). There are also reports that thrombin stimulates the release of MMP-2 (*69*), the expression of MMP-1 and MMP-3 (*70*), and the expression of MMP-9 mRNA (*71*). Another possible action is the stimulation of MMP-independent degradation of ECM. This possibility appears to be supported by the finding that thrombin stimulates the expression of urokinase-type plasminogen activator, a factor involved in the degradation of ECM protein (*72*), and stimulates the heparinase-mediated release of heparan sulfate from ECM (*73*).

In conclusion, the results of this study show that TM stimulates the invasive activity of MMT cells, probably by acting as a cofactor for the thrombin-stimulated invasion of cells mediated by thrombin receptors, and by lowering the effective concentration of thrombin. Further, the results indicate that the stimulation is mainly caused by an enhancement of chemotaxis.

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抗体医薬の現状と展望

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State and Perspective of Antibody Therapeutics

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Toru KAWANISHI* and Takao HAYAKAWA**

はじめに

抗体医薬の始まりは19世紀終わりのエミール・ベーリングと北里柴三郎による血清療法にさかのぼる．彼等は加熱変性させたジフテリア菌毒素をウサギに注射することにより，ジフテリアに対する抵抗性を獲得させた．更に，そのウサギの血清を他のウサギに注射すると，ジフテリアに対する抵抗性をワクチンの接種されていないウサギに移せることを発見し，これをヒトに応用した[1]．その後，ヒト血液から精製したガンマーグロブリン製剤が開発され老人や術後の患者の日和見感染症，川崎病の自己免疫病に多用されている．

1975年にモノクローナル抗体作成技術がケラーとミルシュタインにより[2]開発されてから，対象となるターゲットに対して高親和性と特異性の高いマウスモノクローナル抗体については基礎研究だけでなく治療薬を目指した膨大な研究が行われてきた．実際，モノクローナル抗体治療薬は①分子化合物に比較して基本的に細胞毒性が低いかもしくは無い，②比較的長期にわたり血中濃度の維持が容易である，③結合対象となるリガンド選択性・特異性に優れている，④抗原の捕捉だけでなく生体内からの排除が可能であるといったメリットがある．しかしながら，マウスモノクローナル抗体の治療薬としての利用はヒトへの免疫原性により繰り返し投与時の効果の減弱，アナフィラキシーショックの危険性のために，非常に限られたものであった．そこで今日までに，免疫原性，アナフィラキシーショックの危険性を低減し，繰り返し投与を可能にするヒト型モノクローナル抗体を作成する様々な技術が生み出された．このようにして作成されたヒト型モノクローナル抗体の一部は医薬品として承認され臨床で用いられており，現在臨床応用を目指して開発中のものも多い(Table 1, Table 2)．そこで本稿においては抗体医薬の基礎と臨床応用，問題点等について概説する．

1. 抗体医薬の作成

1.1 キメラ抗体，ヒト化抗体

キメラ抗体は遺伝子組換え技術を用いてマウスモノクローナル抗体の定常 constant (C) 領域をヒト抗体のC領域に置き換えたものである[3] (Fig. 1)．更にヒト化抗体は抗体タンパク質の三次元構造をもとに，抗原が実際に結合する相補性決定領域 complementarity determining region (CDR) の1から3を残して，それ以外の部分であるフレーム領域

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Table 1 認可された抗体医薬

商品名 (抗体名)	企業	日本での 販売元	タイプ (抗原)	適応症	認可 (年)
Repro® (*Abciximab*)	Centocor社/ Eli Lilly社	全薬工業（株）	キメラ (gp II b III a)	PTCA後再狭窄	1994
Rituxan® (*Rituximab*)	IDEC社/Roche 社/Genentech社		キメラ (CD20)	ノンホジキン リンホーマ	1997
Zenepax® (*Daclizumab*)	Roche社		ヒト化 (IL-2R)	腎移植	1997
Remicade® (*Avakine*)	Centocor社		キメラ (TNF-α)	クローン病 慢性関節リウマチ	1998
Synagis® (*Palibizumab*)	MedImmune社/ Abott社		ヒト化 (RSV)	RSV小児感染	1998
Simulect® (*Basilicimab*)	Novartis社		ヒト化 (IL-2)	腎移植	1998
Herceptin® (*Trastuzumab*)	Genentech社/ Roche社	日本ロシュ（株）	ヒト化 (HER2)	乳癌	1998
Mylotarg® (*Gemtuzumab*)	Cellutech社/ AHP社		抗癌剤－コンジュゲート (CD33)	急性骨髄性白血病	2000
Zevalin® (*Iritumomab*)	IDEC社/ Schering AG社		^{90}Y-コンジュゲート (CD20)	ノンホジキン リンホーマ	2002
Bexxar® (*Tositumomab*)	Coulter社/ SKB社		^{131}I-コンジュゲート (CD20)	ノンホジキン リンホーマ	2002

（文献122より許可を得て転載）

Table 2 開発中の抗体医薬

段階	数					
	合計	キメラ抗体	ヒト化抗体	完全ヒト抗体	マウス抗体	その他
発売中	11	4	5	0	2	0
申請中	3	0	1	1	1	0
フェーズIII	20	1	9	2	8	0
フェーズII	60	7	25	15	4	9
フェーズI	35	5	13	6	6	5
合計	129	17	53	24	21	14

（2003年1月時点）（文献123より許可を得て転載）

frame region (FR) をすべてヒト抗体に置き換えたものである[4] (Fig. 1)．以下に，マウスハイブリドーマ細胞から，遺伝子としてcDNAを用い遺伝子組換え法によるキメラ抗体，ヒト化抗体の作製法を紹介する．

第一のステップは，マウス抗体産生ハイブリドーマからのマウス抗体をコードする遺伝子（以下マウス抗体遺伝子）のクローニングである．ハイブリドーマ細胞よりRNAを抽出し，①cDNAを作製後，プラークハイブリダイゼーション法あるいはPCR法により抗体遺伝子をクローニングするか，②RNAより直接PCR法により抗体遺伝子をクローニングする方法が用いられている．ハイブリドーマ細胞は，目的の抗体遺伝子以外に，融合パートナーのミエローマ由来の抗体遺伝子，タンパク質に翻訳されない偽抗体遺伝子が含まれていることもある．

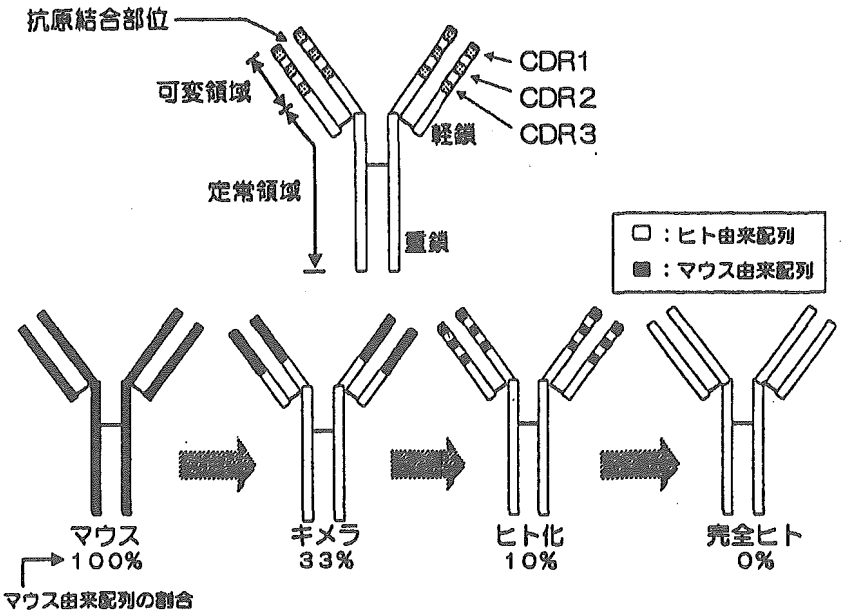

Fig. 1　抗体の構造（上段）及びマウスモノクローナール抗体のヒトに対する抗原性低減技術の進展（下段）
（文献 124 より許可を得て転載）

したがって，精製したモノクローナル抗体 V 領域のアミノ酸配列を一部決定し，クローニングした抗体遺伝子と一致しているか確認することが重要である．

キメラ抗体は，クローニングしたマウス抗体の variable（V）領域遺伝子にヒトの C 領域遺伝子を連結し，適当な発現ベクターに挿入して培養細胞で生産する．また，抗体産生ハイブリドーマのマウスイムノグロブリン（Ig）C 領域をヒト Ig C 領域に組換える相同組換え法やトランスジェニックマウスによっても作成される．

ヒト化抗体遺伝子の作製は以下の複雑なステップからなる．ヒト化抗体作製の第一ステップでは，クローニングしたマウス抗体可変（V）領域における抗原との結合に寄与する超可変領域（CDR）配列とヒト抗体 V 領域におけるアイソタイプ固有のアミノ酸配列をもつフレームワーク領域（FR）からなる V 領域をコードする遺伝子を構築する．ヒト化抗体作製における最も重要な点は，CDR を移植するヒト FR 領域のデザインである．マウス抗体の CDR を単純にヒト FR へ移植した抗体では，結合活性の低下，消失がみられる．これはマウス FR 領域中のいくつかのアミノ酸が CDR の高次構造維持に大きな影響を与えており，それらのアミノ酸残基を CDR とともに移植しなければいけないことを示している．CDR の高次構造に影響を与えるアミノ酸残基がいくつか同定されているが[5]，その法則は確立されておらず，コンピューターモデリングなどを組み合わせて個々の抗体で試行錯誤しているのが現状である[6]．また，この方策として目的のマウス抗体 V 領域と最も高いホモロジーを示すヒト抗体 V 領域を選択し，その FR 領域を用いている場合もある．

最終的に，構築された抗体 heavy（H）鎖及び light（L）鎖遺伝子が挿入された発現ベクターを動物細胞に導入し，遺伝子組換え抗体を発現する．現在，上市されている抗体の製造細胞で実績があるのは，チャイニーズハムスター卵巣由来の CHO 細胞，マウスミエローマ由来の NS0 細胞及び SP2/0 細胞である[7]．動物細胞が産生に用いられるのは以下の理由による．まず，抗体は H 鎖及び L 鎖各 2 本が複数の S-S 結合を介して結合しており，正確な立体構造の構築には動物細胞での発現が最適である．また，抗体の Fc 領域には N 型糖鎖が結合しており，糖鎖は C_H2 ドメインの立体構造の維持，後述する複数のエフェクター活性に必須である．したがって，動物細胞で発現しないと糖鎖が付加されないため，抗体のエフェクター活性が損なわれてしまうからで

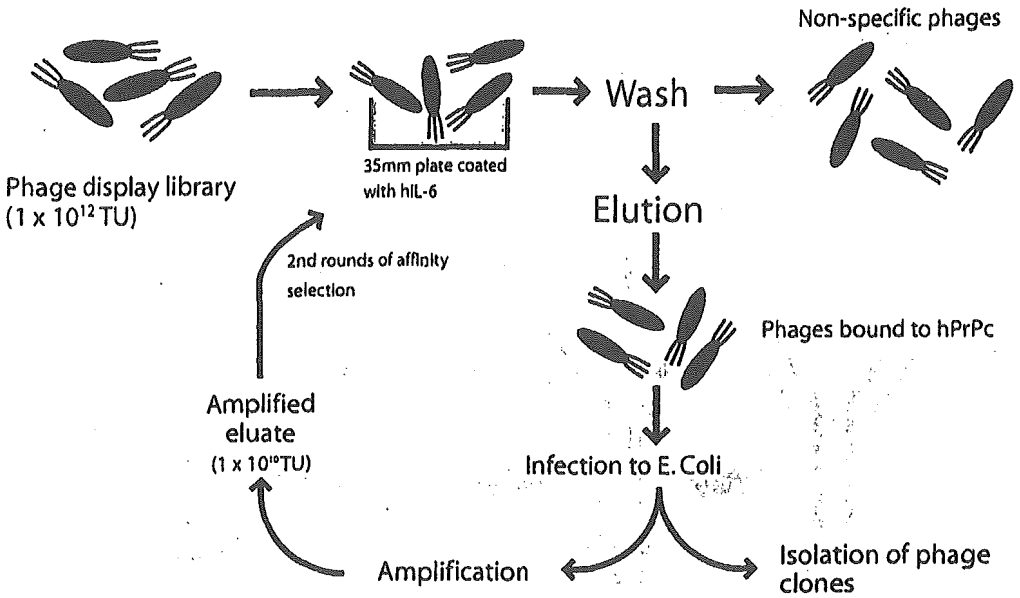

Fig. 2 ファージディスプレイライブラリーを用いたパンニング方法
（文献125より許可を得て転載）

ある．

1.2 ファージディスプレイヒト抗体

ファージディスプレイ法は大腸菌ウイルスの一つであるM13やT7などの繊維状ファージのコートタンパク質（g3pやg10pなど）のN末端側にファージの感染性を失わないよう外来遺伝子を融合タンパク質として発現させるシステムである[8]．一度に10^7種類以上の多種類の分子種を呈示したライブラリーを構築でき，また粒子ごとに目的の機能や性質をもった分子種を選択できる．その技術を用いて外来遺伝子として抗体の結合部位である2つのポリペプチド鎖V_HとV_Lを短いリンカーで直列につないだ一本鎖Fv single-chain Fv (scFv) をファージにディスプレイさせたものが抗体ファージライブラリーである[19,10]．そしてファージライブラリーからパンニングと呼ばれる固相化された抗原分子上で特異的抗体ファージを濃縮する操作を繰り返して特異的抗体ファージをスクリーニングする[11] (Fig. 2)．それを最終的にファージから切り離したものがファージディスプレイ抗体である．

1.2.1 抗体ファージライブラリーの種類

現在，世界中で様々な抗体ライブラリーが作製・報告されているが，その抗体遺伝子ソースの性質の違いに応じて，以下の3つに分類される (Fig. 3)．

1.2.1.1 免疫ライブラリー

感染症回復者や対象抗原をワクチン接種して血中抗体価を上昇させたヒト，自己抗体を保有する患者，担癌患者などのリンパ球を出発材料としてRT-PCRにより増幅したV遺伝子より構築したライブラリーである．中和抗体を有する各種感染症に対する抗体の創出に有効と考えられる．

最初から目的抗体遺伝子がライブラリー中に多く含まれていることから，比較的小さなサイズのライブラリーからでもかなり高い確率で特異性，親和性の高いヒト抗体を単離することが可能である．一方，対象抗原によっては，リンパ球ソースの入手方法の点など倫理的な面で問題となることもある．更に対象抗原ごとにあるいは患者ごとにライブラリーを構築しなければならないため，手間がかかるという欠点がある．本法により，ヒト血液凝固第Ⅷ因子 (FⅧ) に対するインヒビター抗体を有する患者よりFⅧに高い親和性 ($Kd=10^{-11}M$) を有し，FⅧの活性を阻害するscFvクローンが得られている[12]．

1.2.1.2 ナイーブ/非免疫ライブラリー

正常なヒトが保有するV_H，V_L遺伝子をRT-PCRにより分離し，ランダムに組み合わせた抗体可変領域ドメインを提示したライブラリーである．ヒトがもともと生体内に有し，産生している抗体可変領域を組み合わせて作製するため，ヒト抗原に対する治療用のヒト抗体作製に，最もよく利用されて

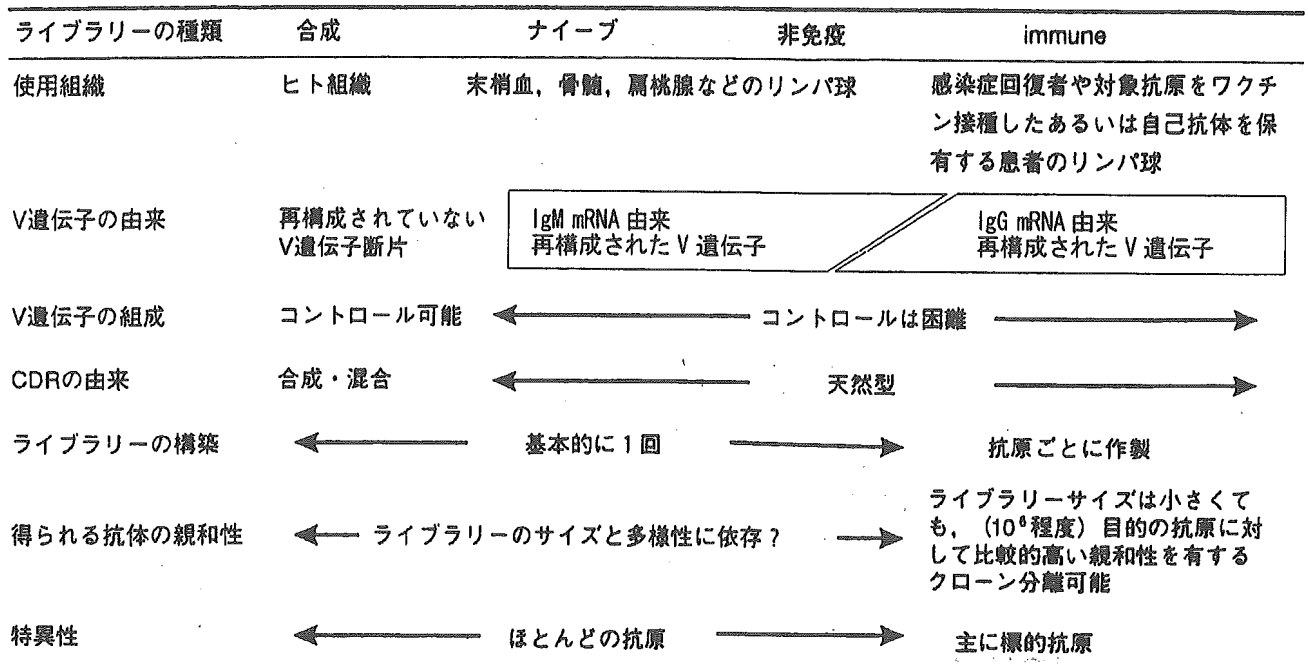

Fig. 3　合成，ナイーブ，非免疫，immune 各ライブラリーの比較：ファージディスプレイヒト抗体ライブラリーの作成法による分類
（文献 126 より許可を得て転載）

いる．通常，正常人の末梢血，骨髄，扁桃腺などのリンパ球を出発材料とし，複数のドナーを用いることでより多くの多様性を有するライブラリーを構築する．このライブラリーでは用いるドナーがどのような疾病歴，抗体価，遺伝系などのバックグラウンドを有するかなどの選択が難しく，V 遺伝子の組成や由来のコントロールに課題がある．厳密にいえば抗原感作によるクラススイッチが起こっていないという観点から，IgM クラスの mRNA のみを V_H 遺伝子の増幅に用いているものが特にナイーブライブラリーと呼ばれる[13]．以下にヒト抗体ライブラリーの構築（B 細胞を出発材料としたナイーブ/非免疫ライブラリーの場合）法の概略を示す[14]（Fig. 4）．

ヒトリンパ球由来 mRNA から，イムノグロブリンの γ，μ，κ，λ 鎖の C 領域に特異的なプライマーを用いて，V 遺伝子の cDNA を作製する．次に，各遺伝子（γ，μ 鎖由来の V_H 並びに κ，λ 由来の V_L）を V 遺伝子ファミリーに特異的な DNA プライマーのセットを用いて V 遺伝子の cDNA を作製する．次に，各遺伝子（γ，μ 鎖由来の V_H 並びに κ，λ 由来の V_L）を V 遺伝子ファミリーに特異的な DNA プライマーのセットを用いて合成し，それらリンカー DNA を用いて PCR により連結し，scFv 遺伝子を作製する．それをファージタンパク質 g3p の N 末端に融合タンパク質遺伝子としてファージミドベクター上で連結させ（Fig. 4），大腸菌に形質転換後，ヘルパーファージを用いて，ファージディスプレイ抗体ライブラリーを調製する．

1.2.1.3　合成ライブラリー

ヒト B 細胞内で実際に抗体産生に用いられている遺伝子を選び，V 遺伝子断片と CDR 3 領域に相当する適当な長さのランダムなアミノ酸配列をもつ合成 DNA を用いて抗体可変領域遺伝子を構築したライブラリーである[15,16]．最初から機能的な scFv を産生する V_H と V_L 遺伝子の組み合わせでライブラリーを構築することができるため，得られる抗体の発現効率や安定性が高いとされる．人工的なランダムオリゴ DNA を用いているため，ゲノム中の抗体遺伝子のみを利用した抗体ライブラリーより高い多様性が得られる．逆に特定の CDR 領域のみの多様性であるため，他の CDR 領域が多様性に寄与するような抗体は得られない．

1.3　トランスジェニックヒト抗体

完全なヒトモノクローナル抗体取得のもう一つの戦略は，ヒト抗体を産生するトランスジェニック動物の利用である．内因性 Ig をノックアウト（KO）

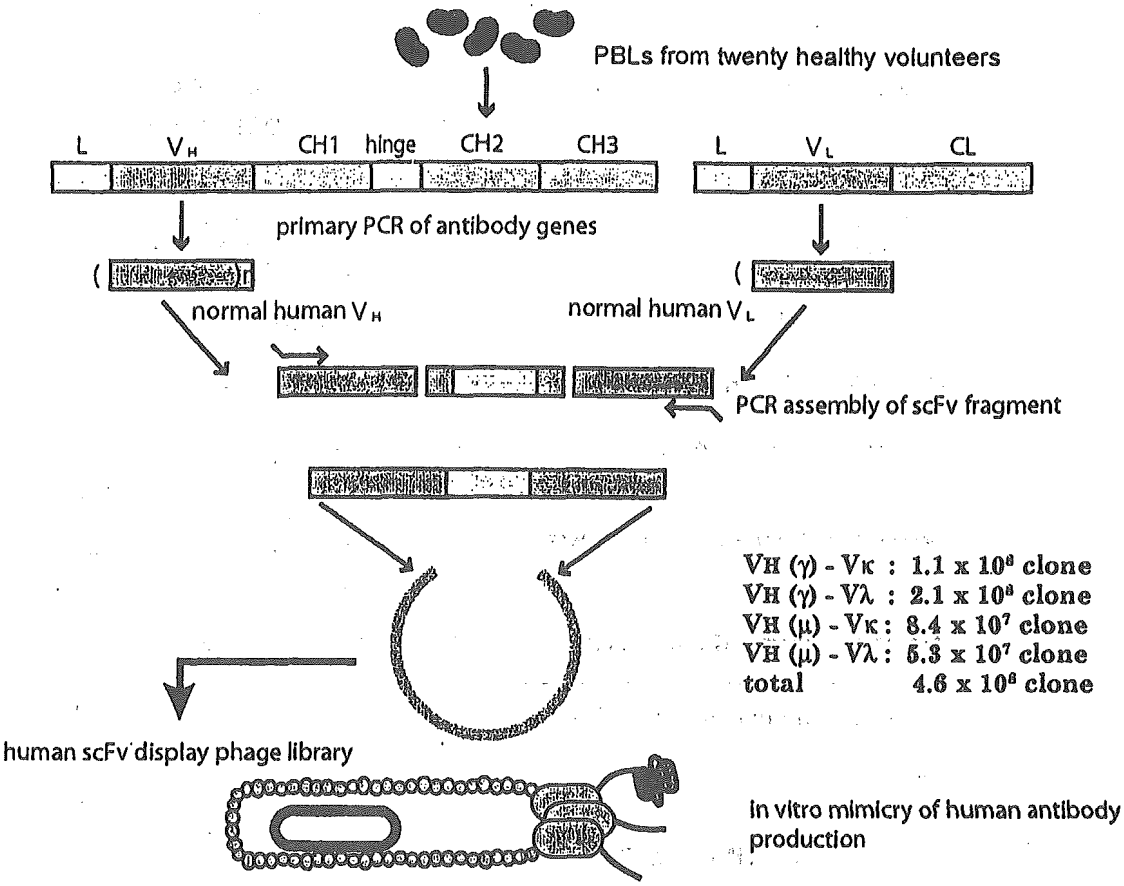

Fig. 4 ナイーブヒト抗体ファージライブラリー構築の概略
(文献 125 より許可を得て転載)

したマウスに機能的なヒトのIg遺伝子を導入すれば，マウス抗体の代わりに多様な抗原結合能を持つヒト抗体が産生されると考えられる．更に，このマウスを免疫すればヒトモノクローナル抗体を従来のハイブリドーマ法で容易に得ることが可能と考えられる．

1.3.1 ヒト抗体重鎖，軽鎖ミニ遺伝子を導入したヒト抗体産生マウス

ヒト抗体H鎖，L鎖ミニ遺伝子を導入したトランスジェニックマウスとマウス内在性抗体遺伝子を破壊したノックアウトマウスを掛け合わせてヒト抗体産生マウスを作成する[17,18] (Fig. 5)．抗原を接種

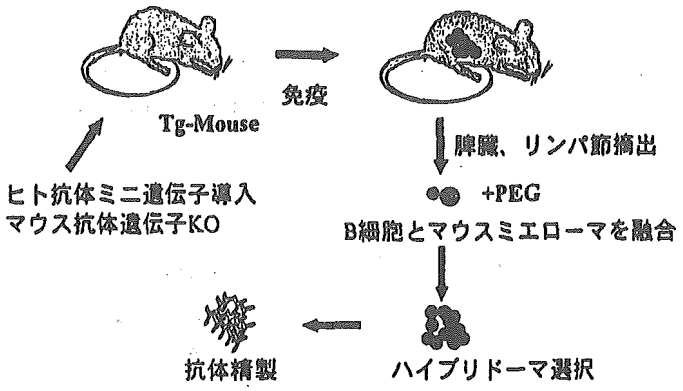

Fig. 5 トランスジェニックヒト抗体技術
(文献 127 より許可を得て転載)

したヒト抗体産生マウスから通常のハイブリドーマ法によりヒトモノクローナル抗体を作成する．また，抗原を接種したヒト抗体産生マウスの抗体産生細胞を胸腺から分離し，抽出した遺伝子をCHO細胞に導入しヒト抗体を産生させる技術も開発されている(Fig. 6)．本法によりIL-8, EGFR, TNF-α, CD4などに対するヒト抗体が得られている．しかしながら，本法では用いるベクターにクローン可能なDNA長は通常数kbから数百kbであり，ヒト抗体遺伝子の全長（H鎖1.5 Mb，L鎖κ 2 Mb，L鎖λ 1 Mb）を入れることはできない．また，ヒト抗体の一部（IgGのC領域）はコスミド，BAC（バクテリアの人工染色体），YAC（酵母の人工染色体）にはクローン化できない．

一方，ヒトIg遺伝子において例えば，H鎖の場合，14番染色体上の約1Mbにわたってクラスターを形成している約80種のV断片，約30種のD断片，6種のJ断片が様々に組み合わされたVDJエクソンが抗原結合部位をコードするが，この過程（VDJ組換え）が抗体の多様性に大きな役割を果たしている（Fig. 7）．L鎖κ（2番染色体，約2 Mb），L鎖λ（22番染色体，約1 Mb）遺伝子についても同様である．したがって，本法ではヒトで観察されるものと同様に多様な抗体レパートリーをマウスで再現するには限界があった．

1.3.2　KMマウス

このような問題を解決するためキリンビール社は以下のようにしてヒト抗体重鎖及び軽鎖κ遺伝子の全種類を導入したヒト抗体産生マウスを作製，ヒト抗体を産生することに成功した[19]（Fig. 8）．ま

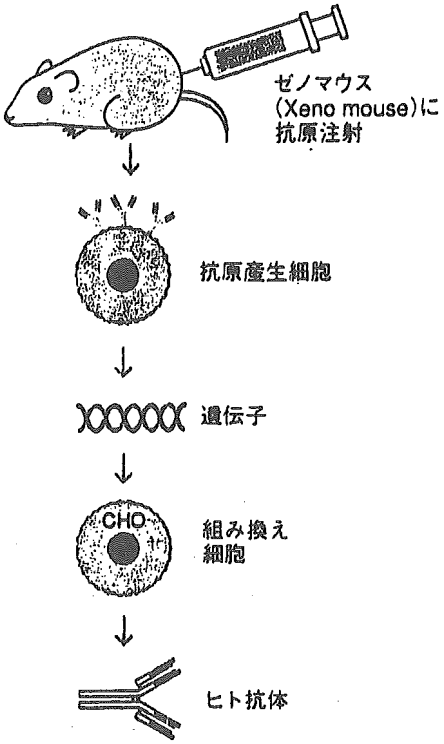

Fig. 6　アブジェニックス社のヒト抗体作製技術
（文献123より許可を得て転載）

ず，ヒト-マウスハイブリッド細胞の独立クローンからなるライブラリーをスクリーニングし，Ig遺伝子を含むヒト染色体自然断片（human chromosome fragment; hCF）のなかでH鎖：14番染色体由来とL鎖κ：2番染色体由来を選抜する．選択細胞を48時間程度コルセミド処理することにより1～数本の染色体が取り込まれた核膜構造体であるミクロセルを形成させた．ミクロセルにサイトカラシンBを加えて遠心分離して脱核させ，染色体が1つ1つ

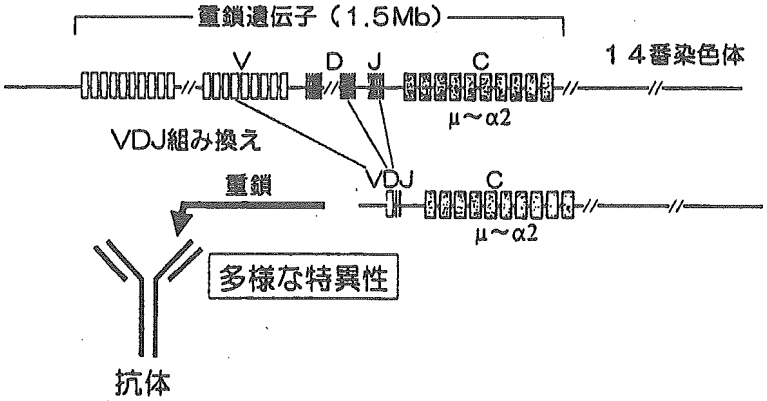

Fig. 7　ヒト抗体重鎖遺伝子の構造とVDJ組換えによる多様性生成
（文献124より許可を得て転載）

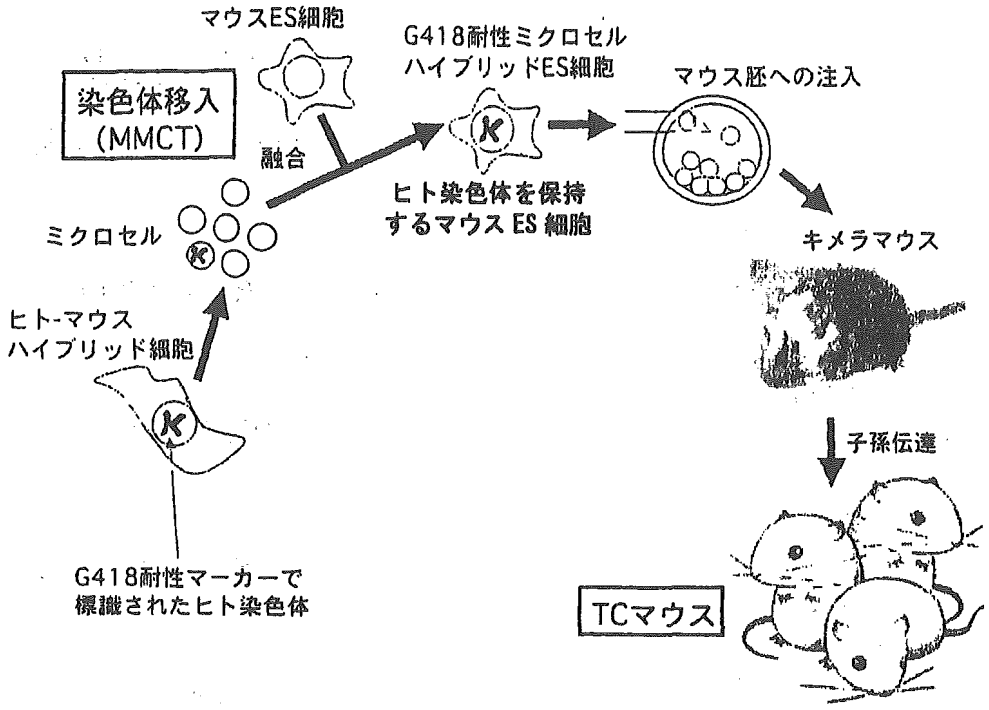

Fig. 8 トランスクロモマウス作成法の概略
(文献 124 より許可を得て転載)

核膜，細胞膜に包まれたミクロセルを分離する．単離したミクロセルと染色体受容細胞（マウス ES 細胞）をポリエチレングリコールで融合する．このような一連の操作はミクロセル融合法と呼ばれる．融合細胞を 8 細胞期受精卵に注入して，擬似妊娠雌マウスの子宮へ移植する．得られたキメラマウスは ES 細胞由来の体細胞においてヒト染色体断片を保持し，導入したヒト染色体上のヒト遺伝子を組織特異的に発現させることができる．このようにヒト染色体をもつトランスジェニックマウスはトランスクロモマウス（TC マウス）と呼ばれる．更に，ヒト抗体 H 鎖遺伝子を含む 14 番染色体断片を保持する TC マウス，ヒト抗体 L 鎖 κ 遺伝子を含む 2 番染色体断片を保持する TC マウス，内在性マウス抗体重鎖遺伝子を破壊したマウス（KO マウス），内在性マウス抗体軽鎖 κKO マウスを交配することにより，4 つの形質をすべて保持するヒト抗体を作るダブル TC/KO マウスが作成された[20]（Fig. 9）．このヒト抗体産生マウスは，ヒト抗原を免疫することにより抗原特異的なヒト抗体（IgG）力価が上昇し，その脾臓から抗原特異的なヒト IgG を産生するハイブリドーマクローンが取得された．しかし，そのハイブリドーマ取得率は，正常マウスの 10%程度であり，その原因はヒト第 2 染色体の保持率が ES 細胞及び体細胞において低いことによる（Table 3）．

一方，Medarex 社のヒト抗体産生マウス（HuMab）はヒト Igκ 鎖遺伝子の 50%を含むが，重鎖遺伝子は 10%程度しか含まないため，抗原に対する応答性が必ずしもよくないという問題点があった[21]（Table 3）．ヒト Igκ 鎖遺伝子については，一種の可変領域クラスターが倍化した構造のため，一方のクラスター（50%）を含む HuMab マウスにおいても，100%含む場合と比較して遜色のない κ 鎖の多様性が生み出されていると考えられる．更に，HuMab マウスにおいては，Igκ 鎖を含む酵母染色体ベクター（Igκ-YAC）がマウス染色体 DNA に挿入されているため，安定に保持される．そこで，ダブル TC/KO マウスの不安定な hCF2 の代わりに Igκ-YAC を導入するという改良がなされた[22,23]．実際には，ダブル TC/KO マウスをヒト抗体 L 鎖 κ 領域全域の 50%を含んでいる Medarex 社の HuMAb マウスと交配させ，ヒト 14 番染色体断片とヒト軽鎖 κ トランスジーンとを保持する KM マウス（Kirin-Medarex マウス，KM マウス™）が作成された（Fig. 10）．作成された KM マウスを用いたハイブリドーマの取得率，得られた抗体の特異性

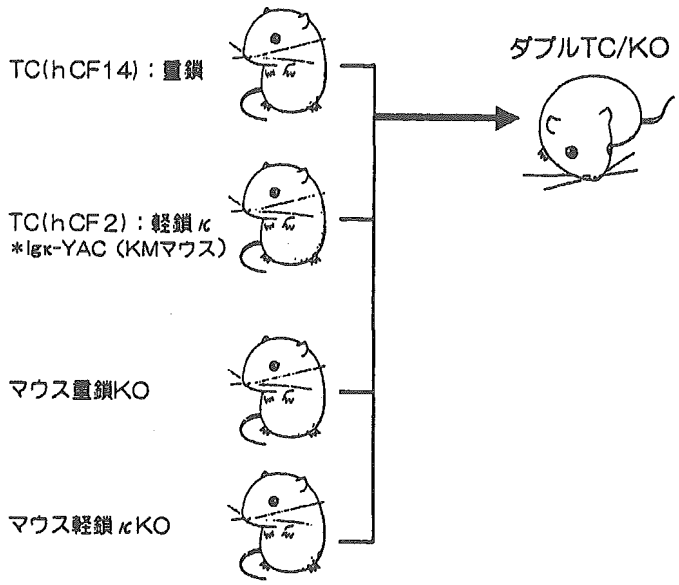

Fig. 9　ヒト抗体産生マウス（ダブルTC/KO）の作製
（文献124より許可を得て転載）

Table 3　ヒト抗体マウスの改良

	KMマウス (Kirin/Medarex社)	TCマウス (Kirin)	HuMabマウス (Medarex社)	通常マウス
重鎖遺伝子	ヒト V_H (81) 全種類	ヒト V_H (81) 全種類	ヒト V_H (4)	マウス V_H 全種類
軽鎖κ遺伝子	ヒト Vκ (38) 全種類	ヒト Vκ (76) 全種類×2	ヒト Vκ (38) 全種類	マウス Vκ 全種類
定常領域サブクラス	IgM, D, G1～G4, A1～A2, Eすべて	IgM, D, G1～G4, A1～A2, Eすべて	IgM, D, G1	マウス定常領域すべて
安定性	OK	軽鎖2番染色体断片が不安定	OK	OK
ハイブリドーマ取得効率	よい	ハイブリドーマが不安定なため，取得効率低下	V_H が少数のため，抗原への反応性弱い	よい

（文献24より許可を得て転載）

は，通常のマウスを用いた場合と比較して遜色ない結果が得られている（Table 3）．

1.3.3　KMマウスの品種改良

1.3.3.1　KM（FcγRIIb-KO）マウス

免疫するヒト抗原によってはアミノ酸配列あるいは立体構造上，マウスのものと非常に近いか，同一である場合，抗体が得られにくいことがある．そのような場合に対処するため，自己抗体を産生するFcγRIIb-KOマウスの形質を入れたKM（FcγRIIb-KO）マウスも作成されている[24,25]．

IgGのFc領域と結合するFc受容体であるFcγRIIbはimmuno tyrosine inhibitory motif（ITIM）を細胞質に持つ膜タンパク質である．過剰な抗原・抗体複合体のIgG Fc領域がB細胞上の活性化シグナルを入れるFc受容体（FcγRI）と同時に抑制性シグナルを入れるFcγRIIbと結合すると，B細胞への活性化シグナルは遮断され，B細胞にアポトーシスが誘導され，過剰な抗体産生が抑制される（Fig. 11）．そこで，寛容が打破されたFcγRIIb-KOマウスの形質をKMマウスへ導入するため，交配を行い，KM（FcγRIIb-KO）マウスが作製された．このマウスをウシコラーゲンタイプIVで免疫し，ウシとマウスのコラーゲンに共通のエピトープ（抗原決定部位）に反応するヒト抗体の有無が調べられた．通常のKMマウスではマウスコラーゲンに反応するヒトIgG抗体は血清中に観察されな

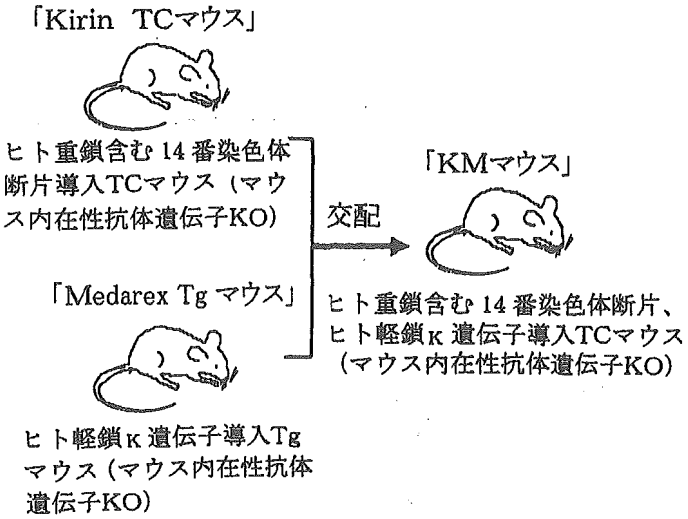

Fig. 10 KMマウスの作製
（文献128より許可を得て転載）

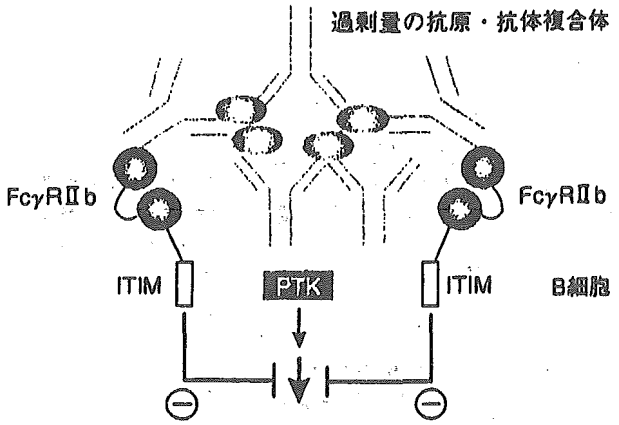

Fig. 11 FcγRIIbの機能
（文献24より許可を得て転載）

かった．一方，KM（FcγRIIb-KO）マウスではマウスコラーゲンに結合する抗体を生産するハイブリドーマが得られた．

1.3.3.2 H-2D導入KMマウス

細胞表面の膜タンパク質を認識するモノクローナル抗体を取得する場合に，ヒト組換えマウス細胞を免疫する方法が用いられる．その際マウスに主要組織適合抗原複合体が異なるマウス細胞を注入すると，強い免疫反応が引き起こされる．このような場合には，免疫原となる組換えマウス細胞とKMマウスの遺伝的背景（バックグラウンド）を一致させることが望ましい．通常のKMマウスは，H-2k（C3H）とH-2b（C57BL/6）のミックスバックグラウンドであるが，場合によってはH-2d（Balb/c）のバックグラウンドの入ったKMマウスも望まれる．そこで，Balb/cマウスとの交配により，H-2d形質の入った雑種マウスであるKM（H-2d）も作製されている[25]．

1.3.4 HACマウス

ヒト2番染色体の保持率低下を克服する方法として他のアプローチも試みられている．それは軽鎖遺伝子を安定な14番染色体断片上に組み込み，ヒト人工染色体human artificial chromosome（HAC）を作成する方法である．そこで，テロメア配列を挿入することでヒト染色体を任意の部位で切断する方法を確立し[26]，更には染色体上にloxP配列を組み込み，Creリコンビナーゼを作用させることでヒト14番染色体上にヒト22番染色体断片を転座させ，ヒト重鎖遺伝子とヒトλ鎖遺伝子を1つの染色体にもつHACが作成された[27,28]（Fig. 12）．実際には，ヒト14番染色体由来hCF20を保持するDT40細胞において，相同組換えによりloxP配列がSC20上のRNR2遺伝子座に導入された（DT40/SC20）．このloxP部位は様々なヒト染色体を転座させるための，いわばクローニングサイトといえる．クローニングするヒト染色体領域としては，ヒト22番染色体上のIgλ鎖遺伝子周辺10Mbが選ばれた．インタクトな22番染色体を保持するDT40細胞において，ヒトテロメアリピート配列を相同組換えによりLIF遺伝子座に挿入すると，この部位に新たな