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Direct cytocidal effect of galectin-9 localized on collagen matrices on human immune cell lines

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ABSTRACT

Background: There is a continuous demand for new immunosuppressive agents for organ transplantation. Galectin-9, a member of the galactoside-binding animal lectin family, has been shown to suppress pathogenic T-cell responses in autoimmune disease models and experimental allograft transplantation. In this study, an attempt has been made to develop new collagen matrices, which can cause local, contact-dependent immune suppression, using galectin-9 and collagen-binding galectin-9 fusion proteins as active ingredients.
Methods: Galectin-9 and galectin-9 fusion proteins having collagen-binding domains (CBDs) derived from bacterial collagenases and a collagen-binding peptide (CBP) were tested for their ability to bind to collagen matrices, and to induce Jurkat cell death in solution and in the collagen-bound state.
Results: Galectin-9-CBD fusion proteins exhibited collagen-binding activity comparable to or lower than that of the respective CBDs, while their cytocidal activity toward Jurkat cells in solution was 80 ~ 10% that of galectin-9. Galectin-9 itself exhibited oligosaccharide-dependent collagen-binding activity. The growth of Jurkat cells cultured on collagen membranes treated with galectin-9 was inhibited by ~90%. The effect was dependent on direct cell-to-membrane contact. Galectin-9-CBD/CBP fusion proteins bound to collagen membranes via CBD/CBP moieties showed a low or negligible effect on Jurkat cell growth.
Conclusions: Among the proteins tested, galectin-9 exhibited the highest cytocidal effect on Jurkat cells in the collagen-bound state. The effect was not due to galectin-9 released into the culture medium but was dependent on direct cell-to-membrane contact.
General significance: The study demonstrates the possible use of galectin-9-modified collagen matrices for local, contact-dependent immune suppression in transplantation.

1. Introduction

Galectins comprise a family of soluble calcium-independent animal lectins that are defined based on their affinity for ß-galactosides and conserved amino acid sequences in the carbohydrate recognition domain (CRD) [1]. There are currently ten members of the human galectin family, which can be classified into three subtypes based on their structures. The proto-type (galectin-1, -2, -7, -10 and -13) and chimera-type (galectin-3) galectins have a single CRD, while the tandem-repeat-type galectins (galectin-4, -8, -9, and -12) have two CRDs joined by a linker region [2]. Human galectin-9 was first reported as a tumor antigen in patients with Hodgkin's disease [3]. Recent studies suggest that galectin-9 is a novel type of modulator of immune functions and that it acts mainly through regulation of T-cell development and homeostasis [4]. Galectin-9 promotes and represses the differentiation of naive T-cells into regulatory T-cells (Tregs) and T-helper 17 cells (Th17), respectively [5]. In addition, galectin-9 induced the death of differentiated T-helper1 (Th1) and Th17 cells [6,7]. Several receptors/binding partners for galectin-9 have been reported to date, including glucose transporter 2 [8], T-cell immunoglobulin mucin-3 (Tim-3) [9], CD44 [10], protein disulfide isomerase [11], Forssman glycosphingolipid [12], and IgE [13]. Among them Tim-3 is postulated to be the functional receptor for galectin-9 in Th1 and Th17 cells, although contradictory evidence exists [11,14].

Could be expected from its regulatory function in specific T-cell subpopulations, therapeutic effects of galectin-9 in autoimmune and inflammatory disease models and experimental organ/tissue transplantation have been reported. The administration of recombinant galectin-9 resulted in beneficial effects in mice with rheumatoid arthritis [5,15], experimental autoimmune encephalomyelitis [9], and type I diabetes mellitus [16,17]. Chen and his colleagues have demonstrated that treatment with recombinant galectin-9 significantly prolonged the

Abbreviations: CBD, collagen-binding domain; CBP, collagen-binding peptide; CRD, carbohydrate recognition domain; G9Null, protease resistant form of galectin-9; ssG9, highly stable and soluble form of galectin-9; Tim-3, T-cell immunoglobulin mucin-3

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survival of allogeneic skin grafts [18] and fully mismatched cardiac allograft [19] in mice. Although the use of galectin-9 alone failed to induce tolerance, galectin-9 in combination with rapamycin induced tolerance in the cardiac allograft transplantation model [20]. The systemic administration of chemical immunosuppressants is usually associated with side effects and complications due to non-specific suppression of the immune system. In the case of galectin-9, harmful side effects have not been reported, indicating a low risk of unfavorable pan-immunosuppression associated with galectin-9 treatment. However, one cannot exclude the possibility that systemic administration of galectin-9 would prevent proper functioning of the immune system in some manner. It is desirable, in this context, to avoid systemic administration of galectin-9 in cases where only local immunosuppression is required, i.e., organ/tissue (especially skin) transplantation.

Skin autografting is the ideal choice for burn wound coverage, but a lack of a patient's own unburned skin (donor site) and/or unsuitability of the wound for autografting may require at least temporary use of wound dressings or skin substitutes to accelerate the healing process, reduce the infection risk, etc. [21]. These alternatives include skin allografts (human living or deceased donor skin), xenografts, cultured autologous or allogeneic epithelial cells, and bioengineered skin substitutes. The use of these alternatives is associated with more or less risk of graft rejection. In the present study, we performed *in vitro* experiments in order to develop a collagen-based local, contact-dependent immunosuppressant using galectin-9 and collagen-binding galectin-9 fusion proteins as active ingredients. The modified collagen matrix may be used for tissue engineering including the treatment of burns. The collagen-binding domains (CBDs) derived from Clostridial collagenases have been successfully used to localize bioactive agents on collagen fibrils *in vitro* and *in vivo* [22,23]. Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) fused to the N-terminal of the CBD of *Clostridium histolyticum* class II collagenase (ColH) were retained for more than one week after being injected subcutaneously into mice [22]. The latter protein (collagen-binding bFGF) strongly stimulated the DNA synthesis in stromal cells at the site of injection. CBD was expected to tightly anchor galectin-9, which itself exhibits oligosaccharide-dependent collagen-binding activity, on collagen matrices in the present study. In addition to galectin-9-CBD fusion proteins, galectin-9 having a short collagen-binding peptide (TKKTLRT) [24] at the C-terminal was also produced. This peptide has been used to produce a wide variety of collagen-binding growth factors [25].

2. Materials and methods

2.1. Construction of expression vectors

The PCR primers used for construction of the expression vectors are listed in Table 1. Two mutant forms of galectin-9, protease-resistant galectin-9 (G9Null) [26], and a highly stable and soluble form of galectin-9 (ssG9) [27], and four types of CBDs derived from *C. histolyticum* collagenases (CBD302, CBD305, CBD112 and CBD115) [28,29] were used in the present study (Fig. 1). The CBD302 DNA contains an *Nde*I site. To efficiently construct expression vectors, a single nucleotide substitution (CATATG → CCTATG) was introduced into CBD302 DNA without an amino acid substitution. The modified CBD302 DNA was used as a target for construction of expression vectors. To construct a collagen-binding galectin-9 having CBD302 at the C-terminal of G9Null and ssG9 (G9Null-302 and ssG9-302), CBD302 DNA was amplified by PCR using forward (CBD302-F) and reverse (CBD302-R) primers tagged with *Bam*HI and *Bgl*II sequences, respectively, and then digested with *Bam*HI and *Bgl*II. The digested DNA was inserted into the *Bam*HI site of pET-11a (Stratagene, La Jolla, CA, USA) to construct pET-CBD302. G9Null and ssG9 cDNAs were amplified using forward (G9-F) and reverse (G9-R) primers tagged with *Nde*I and *Bam*HI sequences, respectively, and then digested with *Nde*I and *Bam*HI. The digested cDNAs were inserted into the *Nde*I–*Bam*HI sites of pET-CBD302, which yielded expression vectors for G9Null-302 and ssG9-302 (pET-G9Null-302 and pET-ssG9-302). pET-305, pET-112 and pET-115 were constructed as described for pET-CBD302 using the primer pairs of (CBD305-F + CBD302-R), (CBD112-F + CBD112-R), and (CBD115-F + CBD112-R), respectively. The digested cDNAs for G9Null and ssG9 were inserted into the *Nde*I–*Bam*HI sites of pET-CBD305/112/115 as described above to construct pET-G9Null-305–pET-ssG9-115. To construct a collagen-binding galectin-9 having CBP at the C-terminal of G9Null and ssG9 (G9Null-CBP and ssG9-CBP), G9Null and ssG9 cDNAs were amplified using forward (G9-F) and reverse (CBP-R) primers tagged with *Nde*I and *Bam*HI sequences, respectively, digested, and then inserted into the *Nde*I–*Bam*HI sites of pET-11a, which yielded expression vectors for G9Null-CBP and ssG9-CBP. The DNA sequences of all the expression vectors were confirmed by automated sequencing. The nucleotide and amino acid sequences of all the recombinant proteins used in the present study are summarized in Supplementary Fig. 1.

2.2. Expression and purification of recombinant proteins

Expression of the recombinant proteins in *Escherichia coli* (*E. coli*) BL21(DE3) cells was carried out as described previously [30] except that *E. coli* was cultured for 16 h at 20 °C after the addition of isopropyl-ß-D-thiogalactopyranoside. To compare the solubility and yields of galectin-9 and galectin-9 fusion proteins, expression and purification were carried out under the same conditions for all the proteins: recombinant proteins in the *E. coli* cell extract derived from 400-ml of culture were recovered by batch-wise absorption on 1.5 ml of lactose-agarose gel (J-Oil Mills, Inc., Tokyo, Japan). The gel was packed into a column and then washed with 15 ml of 20 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 0.03% 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonic acid (TBS, 0.03% CHAPS). Recombinant proteins were eluted with 3 ml of TBS, 0.2 M lactose and then dialyzed against PBS. The dialysate was centrifuged at 25,000 ×*g* for 20 min. The resulting supernatant was sterilized by filtration and then used as the purified preparation. The purified preparation was stored at 4 °C. The recombinant proteins used for the collagen-binding assay (see below) were dialyzed against TBS after elution from lactose–agarose. Protein concentrations were determined using BCA protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL, USA) and bovine serum albumin (BSA) as a standard.

2.3. Cell proliferation assay

The Jurkat T lymphocyte, MOLT-4T lymphoblast and THP-1 monocyte cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 unit/ml penicillin and 100 μg/ml streptomycin at 37 °C under a 5% CO_2–95% atmosphere. The antiproliferative effect of recombinant proteins on Jurkat cells in solution (i.e., in the case that the proteins were added to the culture medium of Jurkat cells in the absence of collagen matrices) was determined by means of the WST-8 assay as described previously [31]. Jurkat cells (5×10^4 cells in 90 μl of medium/well) were plated on 96-well plates and then cultured for 2 h. Test samples (10 μl/well) were added at various concentrations, and then the cultures were continued for 24 h. WST-8 reagent (Cell Counting Kit-8; Dojin Laboratories, Kumamoto, Japan) was added to the culture medium (10 μl/well), followed by incubation for 2 h. Using a plate reader, the viable cell number was determined by measuring the difference between the absorbance at 450 and that at 620 nm. Each assay was performed in triplicate. The half inhibition concentration of each recombinant protein was determined from the dose–response curve on the assumption that there is a linear negative relationship between the cell number and molar concentration (in logarithmic scale) of the test sample in the inhibition range. G9Null was always used in the assay as a standard, and the antiproliferative

activity of galectin-9 fusion proteins was calculated taking the activity of G9Null as 100%.

The antiproliferative effect of recombinant proteins localized on collagen membranes was determined using 33-mm-diameter collagen vitrigel membranes (bovine skin type I collagen) attached to the bottoms of 35-mm dishes (AGC Techno Glass Co., Ltd., Shizuoka, Japan). Each collagen vitrigel membrane was rehydrated with 2 ml of TBS for 10 min before use. Galectin-9 (G9Null and ssG9), galectin-9-CBP (G9Null-CBP and ssG9-CBP), and galectin-9 fusion proteins having CBDs were dissolved in TBS, TBS/40 mM lactose and TBS/5 mM $CaCl_2$/40 mM lactose, respectively. Each protein solution was added to a collagen vitrigel membrane in a 35-mm dish (1 ml/dish) and then incubated for 1 h. After removal of the solution, the dish was washed three times with 2 ml of 20 mM HEPES-NaOH (pH 7.5), 0.15 M NaCl (10 min × 3). The dish was further washed with 2 ml of the culture medium for 30 min at 37 °C. In some experiments, galectin-9 (G9Null) was recovered from the membrane by treatment with TBS, 0.2 M lactose to determine the amount of adhered G9Null by enzyme-linked immunosorbent assay (see below). Jurkat cells (1×10^6 cells in 2 ml of medium/dish) were inoculated into the dish, and then cultured for 24 h. In some experiments, MOLT-4 and THP-1 cells were used instead of Jurkat cells. At the end of the culture, three 100-μl aliquots of cell suspension were withdrawn from a single dish and then inoculated into a 96-well plate. Cells attached to a collagen vitrigel membrane (see Results) were released by gentle pipetting. The cell numbers were determined using WST-8 reagent as described above. Each assay was performed in triplicate (three collagen vitrigel membranes/assay). The viability of cells attached to a collagen vitrigel membrane was determined by propidium iodide (PI) staining. Jurkat cells attached to the membrane were washed with PBS, followed by incubation with a PI solution (2 μg/ml in PBS) for 20 min at 37 °C in dark. After washing with PBS, the stained cells on the membrane were observed by confocal laser scanning microscopy. Jurkat cells cultured on a control collagen vitrigel membrane, which did not become attached to the membrane, were collected by centrifugation before staining with PI.

In some cell proliferation assays, insoluble type I collagen fibrils (bovine achilles tendon type I collagen; Sigma–Aldrich, St. Louis, MO, USA) were used. Collagen fibrils placed in the inner filter cup of a Nanosep MF centrifugal device (Pall Corporation, Port Washington, NY, USA) (10 mg/cup) were sterilized by overnight incubation in 70% ethanol (0.3 ml of 70% ethanol in the cup). After removal of the ethanol solution by centrifugation, the collagen fibrils were washed three times with 0.3 ml of TBS (10 min × 3). Galectin-9 and galectin-9 fusion protein solutions (see above) were added to the cup containing collagen fibrils (240 μl/cup), followed by incubation for 1 h. After removal of the solution, the collagen fibrils were washed three times with 0.3 ml of 20 mM HEPES–NaOH (pH 7.5), 0.15 M NaCl (10 min × 3) and then further washed with 0.3 ml of the culture medium for 30 min at 37 °C. The washed collagen fibrils were transferred to a 1.5-ml tube. Jurkat cells (5×10^5 cells in 0.4 ml of medium/tube) were inoculated into the tube and then cultured for 24 h. WST-8 reagent was added to the culture medium (40 μl/tube), followed by incubation for 1 h. The tube was centrifuged at 10,000 rpm for 5 min. Two 100-μl aliquots of the resulting supernatant were withdrawn from a single tube and then transferred to a 96-well plate. Using a plate reader, the cell numbers were determined as described above.

Table 1
Nucleotide sequences of PCR primers.

CBD302-F: 5′-CGTCCTGGATCCGAAATAAAGGATCTTTCAGAA-3′
CBD302-R: 5′-CGACCGAGATCTTTATCTTCCTACTGAACCTTC-3′
CBD305-F: 5′-CGTCCTGGATCCGTATATCCAATAGGCACTGAA-3′
CBD112-F: 5′-CGTCCTGGATCCACAACAACACCTATAACTAAA-3′
CBD112-R: 5′-CGACCGAGATCTTTATTTATTTACCCTTAACTC-3′
CBD115-F: 5′-CGTCCTGGATCCAACGAGAAATTGAAGGAAAAA-3′
CBP-R: 5′-CGACCGGGATCCCTAAGTACGCAGAGTTTTTTTAGTT GTCTGCACATGGGTCAGCTG-3′
G9-F: 5′-CGTCCTCATATGGCCTTCAGCGGTTCCCAGGCT-3′
G9-R: 5′-CGACCGGGATCCTGTCTGCACATGGGTCAGCTG-3′

2.4. Collagen binding assay

Collagen-binding activity was assayed using insoluble type I collagen fibrils (bovine Achilles tendon type I collagen) as described previously [22,28] with modifications. Collagen fibrils placed in the inner filter cup of a Nanosep MF centrifugal device (10 mg/cup) were washed twice with 0.3 ml of TBS (10 min × 2) before use. Galectin-9 and galectin-9 fusion protein solutions (see Cell proliferation assay) and CBDs dissolved in TBS, 5 mM $CaCl_2$ were added to the cup containing collagen fibrils (240 μl/cup) and then incubated for 1 h. Protein solutions of at least five different concentrations (2.5, 5.0, 7.5, 10.0, and 12.5 μM) were used for each protein. The highest concentration (12.5 μM) was selected because the highest concentration of G9Null achievable in TBS/PBS is typically lower than 13 μM. After incubation, the device was centrifuged to obtain a filtrate containing the unbound protein. The filtrate and the original protein solution were subjected to SDS-PAGE together with a series of different amounts of the protein as the quantitation standard. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. The stained gels were then scanned and quantitated using ImageJ software [32]. Typical results of SDS-PAGE analysis are shown in Supplementary Fig. 2.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The concentration of galectin-9 in the culture medium was quantified by ELISA as described previously [15]. Ninety-six-well plates

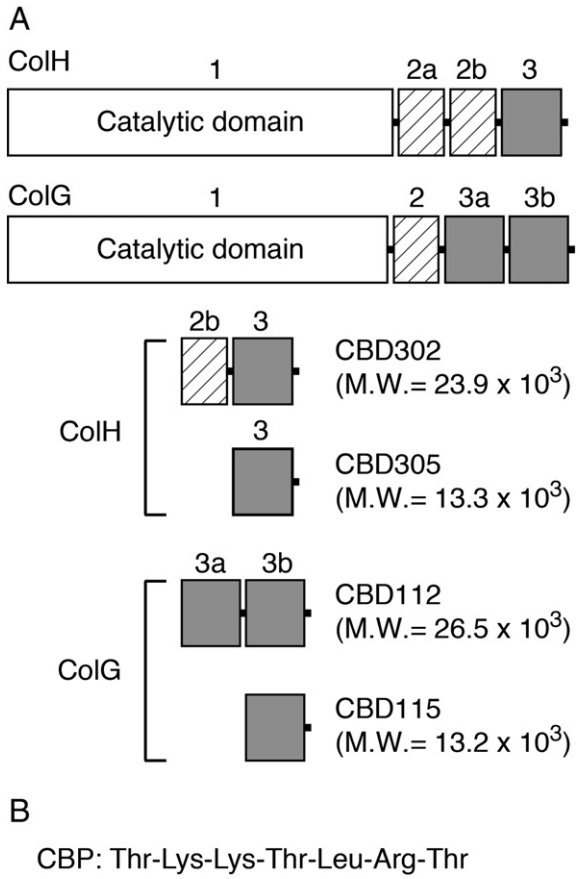

Fig. 1. Schematic representation of collagen-binding domains (CBDs) derived from Clostridial collagenases (ColH and ColG) and the amino acid sequence of a collagen-binding peptide (CBP).

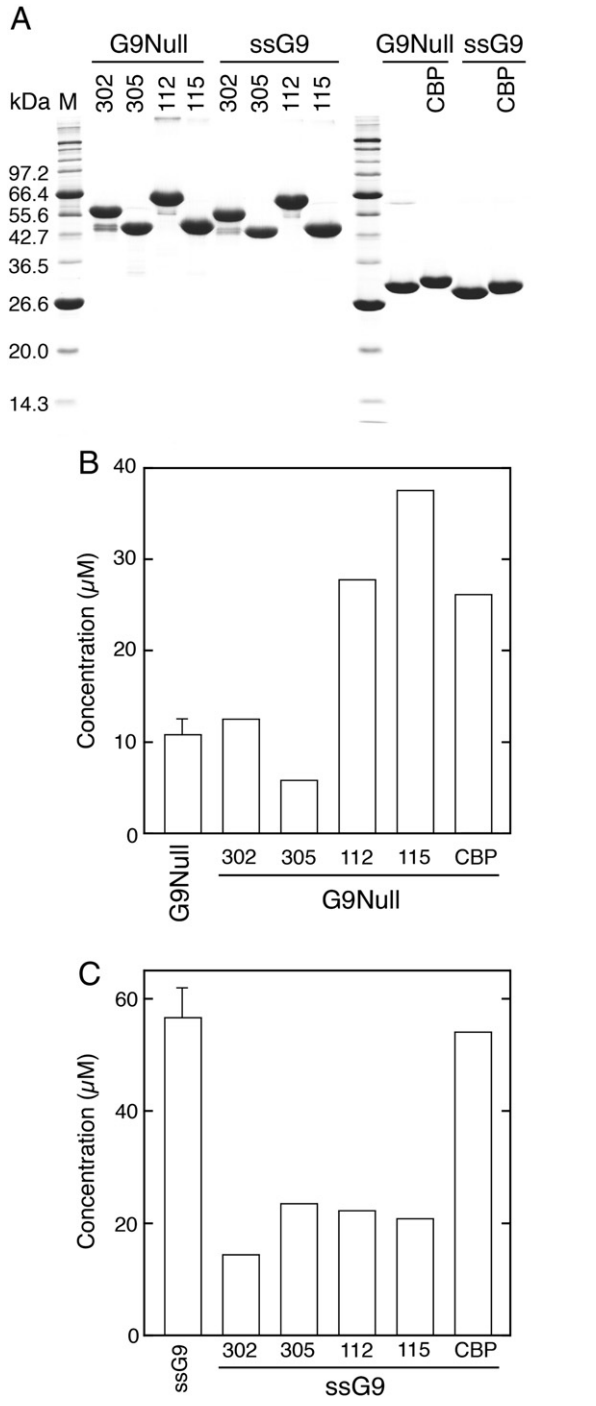

Fig. 2. Purity and solubility of galectin-9 and galectin-9 fusion proteins. (A) Purified recombinant proteins (3 μg/lane) were electrophoretically separated in SDS/12.6% polyacrylamide gels under reducing conditions. The gels were stained with Coomassie brilliant blue R-250. M, molecular weight marker proteins. (B & C) The protein concentration of the purified preparation of G9Null, ssG9 and galectin-9 fusion proteins. To compare the solubility and yields, expression and purification were carried out under the same conditions for all the proteins as described under Materials and methods. The results represent the means ± SD for twelve experiments and five experiments for G9Null and ssG9, respectively, and the means for two experiments for galectin-9 fusion proteins.

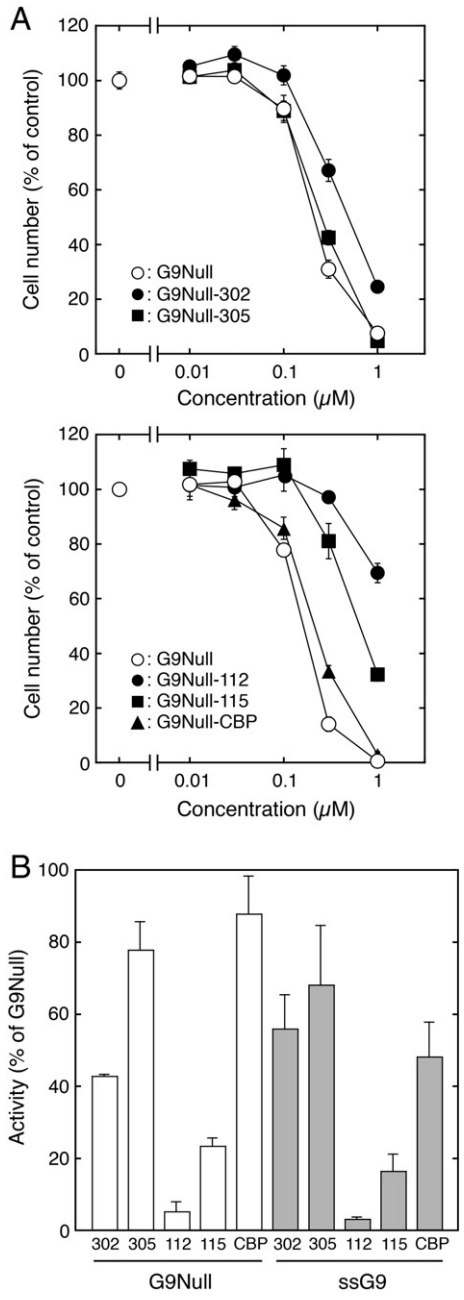

Fig. 3. The antiproliferative activity of galectin-9 and galectinn-9 fusion proteins toward Jurkat cells added to the culture medium. (A) Typical dose–response curves on WST-8 assaying for G9Null and G9Null fusion proteins. (B) The quantitative antiproliferative activity of galectin-9 fusion proteins. The half inhibition concentration of each recombinant protein was determined from the dose–response curve as described under Materials and methods. The activity was expressed taking that of G9Null as 100%. The data represent the means ± SD for triplicate experiments.

(Nunc, Naperville, IL, USA) were coated with an anti-human galectin-9 monoclonal antibody (3 μg/ml, 50 μl/well) overnight at 4 °C. The wells were washed five times with PBS-T (PBS containing 0.05% Tween-20) and then blocked with 3% fetal bovine serum containing 0.05% Tween 20 in PBS for 1 h at 37 °C. The wells were washed with PBS-T, followed by incubation with samples and standards (50 μl/well) for 1 h at 37 °C. After washing of the wells with PBS-T, biotinylated polyclonal anti-human galectin-9 antibody (1 μg/ml, 50 μl/well) was added to each well, followed by incubation for 30 min at 37 °C. The wells were then washed with PBS-T, followed by incubation with streptavidin–horseradish peroxidase (1:10,000 dilution, 50 μl/well; Invitrogen, Carlsbad, Ca, USA) for 30 min at 37 °C. The wells were washed with PBS-T, and then a 3,3′-5,5′-tetramethylbenzidine solution (100 μl/well; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA) was added to each well, followed by incubation at room temperature for 20 min to allow sufficient blue product to accumulate. Reactions were stopped by adding 1 M phosphoric acid (100 μl/well). Colorimetric analysis was carried out at 450 nm. A standard curve, ranging from

0.00128 to 20 ng/ml of recombinant galectin-9, was generated to calculate the galectin-9 concentrations in the samples.

2.6. Western blot analysis

Samples and standards (10 μl/lane) were electrophoretically separated in SDS/12.6% polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA, USA), followed by immunodetection with affinity-purified rabbit anti-galectin-9 antibody and horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Kirkegaard and Perry Laboratories, Inc.). G9Null was visualized using an ECL detection system (GE Healthcare BioSciences, Piscataway, NJ, USA).

3. Results

3.1. Design and production of galectin-9 fusion proteins

We designed and produced ten novel galectin-9 fusion proteins comprising different combinations of two galectin-9 mutant proteins (G9Null and ssG9), and four types of CBDs and a CBP (Fig. 1). CBD and CBP were fused to the C-terminal of galectin-9, because the addition of proteins and peptides to the N-terminal of galectin-9 frequently results in a drastic decrease in the yield of a recombinant protein. Galectin-9 and galectin-9 fusion proteins were expressed in *E. coli* and then purified by single step lactose-affinity chromatography (Fig. 2A). The solubility of G9Null (and wild-type galectin-9) is low: the maximum concentration of G9Null in PBS is typically 0.3–0.4 mg/ml (10–13 μM). The addition of CBDs derived from ColH had a negligible (CBD302) or negative (CBD305) effect on the solubility of G9Null (Fig. 2B). On the other hand, the solubility of G9Null fusion proteins having CBDs derived from ColG and CBP was 2–3.5 times higher than that of G9Null on a molar basis. ssG9 was recently developed to overcome the low solubility of G9Null [27] and is about five times more soluble than G9Null. The solubility of ssG9 was greatly impaired by the addition of CBDs, while the effect of CBP was minimal (Fig. 2C). The yield of a recombinant protein paralleled the protein concentration in a purified preparation, because the preparation volume was almost constant for all the recombinant proteins.

3.2. Antiproliferative effect of galectin-9 and galectin-9 fusion proteins on Jurkat cells in solution

The antiproliferative activity of galectin-9 fusion proteins was measured using G9Null as a standard to assess the effects of CBD and CBP moieties on the activity under the standard assay conditions, that is, they were not in contact with collagen matrices. Typical dose–response curves used to calculate the antiproliferative activity of G9Null and G9Null fusion proteins are shown in Fig. 3A. The antiproliferative activity of all the fusion proteins was lower than that of G9Null. The addition of CBD112 and CBD115 markedly reduced the activity of both G9Null and ssG9 to less than 10% and about 20% of that of G9Null, respectively (Fig. 3B). CBD302, CBD305 and CBP showed a moderate to negligible effect on the activity under the assay conditions used. As the activity of ssG9 itself was 133 ± 5.7% of G9Null, the negative effect of collagen-binding moieties was more prominent on ssG9 than on G9Null.

3.3. Collagen-binding activity of galectin-9 and galectin-9 fusion proteins

The collagen-binding activity of galectin-9 and galectin-9 fusion proteins was determined using insoluble collagen fibrils, which have been used to characterize collagen-binding properties of CBDs [22,28]. The assay was performed in the presence of 40 mM lactose to eliminate the interaction between galectin-9 moiety and oligosaccharide chain(s) of collagen in the case of fusion proteins (Figs. 4A, B). Initially, we attempted to analyze the binding data by means of Scatchard plots. However, a typical Scatchard plot was not obtained for most of the recombinant proteins under the experimental conditions used. Therefore, the binding data were plotted as "Bound vs. Free", not "Bound/Free vs. Free". The data indicate that the binding affinity of the fusion proteins for insoluble collagen fibrils is in the following order regardless of the galectin-9 moiety: 112 > 115 ≃ 302 > 305 ≃ CBP, although the *Kd* values are not available. The collagen-binding activity of the fusion proteins was comparable to or lower than that of corresponding CBDs (Supplementary Fig. 3). In the case of CBD305, the activity was markedly reduced by the addition of galectin-9 moiety. The affinity of G9Null and ssG9 for collagen fibrils, which was assayed in the absence of

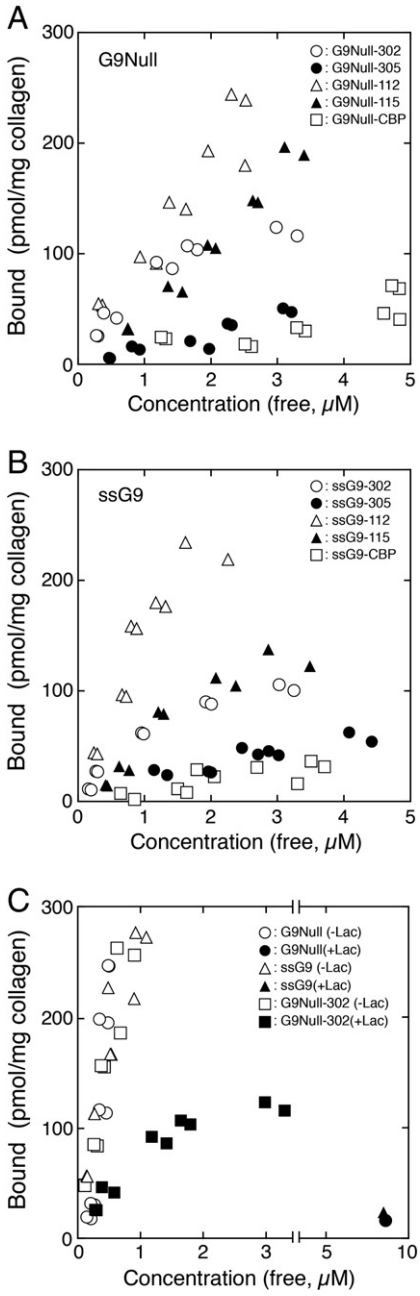

Fig. 4. Collagen-binding activities of galectin-9 and galectin-9 fusion proteins. (A & B) The collagen-binding activities of galectin-9 fusion proteins were determined using insoluble type I collagen fibrils in the presence of lactose. (C) The activities of G9Null, ssG9 and G9Null-CBD302 were determined in the absence (−Lac) or presence (+Lac) of lactose. The binding data for G9Null-CBD302 determined in the presence of lactose were taken from panel A.

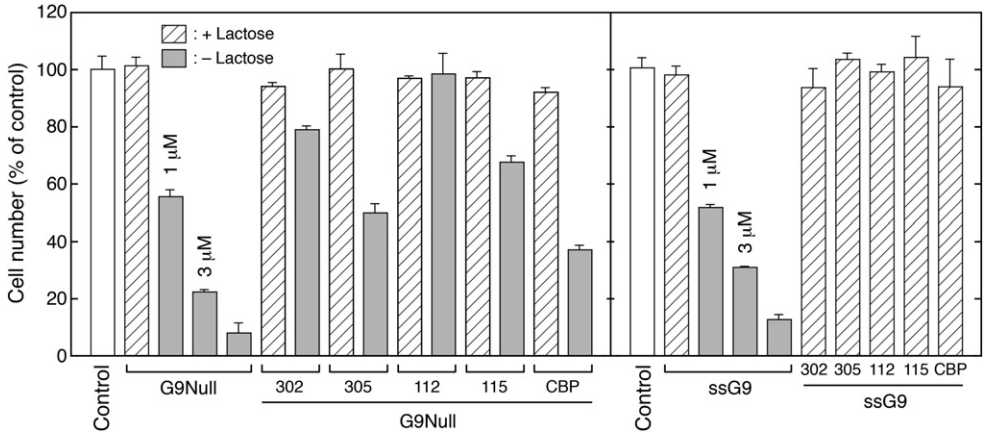

Fig. 5. The antiproliferative effect of vitrigel membranes treated with galectin-9 and galectin-9 fusion proteins on Jurkat cells. Collagen vitrigel membranes treated with galectin-9 and galectin-9 fusion protein solutions (10 μM unless otherwise indicated) in the presence (hatched columns) or absence (gray columns) of lactose were used as a culture substratum. The number of Jurkat cells cultured on an untreated collagen vitrigel membrane was taken as 100%. The data represent the means ± SD for triplicate experiments.

lactose, was apparently higher than that of G9Null-302 and the other fusion proteins (Fig. 4C). In the absence of lactose, however, the binding profile of G9Null-302 (and other G9Null fusion proteins, data not shown) became indistinguishable from those of G9Null and ssG9 (Fig. 4C). The binding of G9Null and ssG9 to insoluble collagen was almost completely inhibited by lactose (Fig. 4C). The binding capacity of collagen for galectin-9 was comparable to or higher than that for the fusion proteins.

Calcium chloride was included in the assay buffer for galectin-9-CBD fusion proteins as calcium ion is known to induce a conformational change in CBDs and increases their affinity for collagen [33]. Contrary to in the case of the CBD fusion proteins, the binding of CBP fusion proteins (G9Null-CBP and ssG9-CBP) to collagen fibrils was inhibited about 60% in the presence of 1 mM or higher concentrations of $CaCl_2$, while EDTA showed no effect on the binding (data not shown). Hence, the binding data for G9Null-CBP and ssG9-CBP were obtained using the assay buffer without $CaCl_2$.

3.4. Antiproliferative effect of galectin-9 and galectin-9 fusion proteins localized on collagen matrices

The ability of the recombinant proteins to induce Jurkat cell death under the conditions when the proteins were bound to collagen was determined using collagen vitrigel membranes as a culture substratum. A collagen vitrigel membrane attached to the bottom of a 35-mm dish was treated with a galectin-9/galectin-9 fusion protein and then washed to remove unbound protein. Jurkat cells inoculated onto collagen vitrigel membranes treated with G9Null and ssG9 became attached to the membranes: About 93% and 99% of Jurkat cells inoculated onto membranes treated with 1 μM and 10 μM G9Null, respectively, became attached, while the cells did not attach to a control membrane. The growth of Jurkat cells cultured on membranes treated with 1 μM, 3 μM, and 10 μM G9Null was inhibited by about 45%, 80%, and 90%, respectively (Fig. 5). In addition to that of Jurkat cells, the growth of two other immune cell lines, MOLT-4 (a human lymphoblast cell line) and THP-1 (a human monocyte cell line) cells, inoculated onto membranes treated with 10 μM G9Null was inhibited by about 90% (Fig. 6A). Determination of cell viability by propidium iodide staining indicated that the observed effect on cell growth was not cytostatic in nature but was due to a cytocidal effect of galectin-9 (Fig. 7), as in the case when galectin-9 was added to the culture medium of Jurkat cells [34]. The membranes treated with ssG9 exhibited a growth-inhibiting effect similar to that of G9Null (Fig. 5).

Jurkat cells inoculated onto membranes treated with galectin-9 fusion proteins in the presence of lactose did not become attached to the membrane at all. In addition, the membranes did not affect the growth of Jurkat cells regardless of the fusion partner (Fig. 5). We examined whether or not galectin-9 fusion proteins could exert a growth-inhibiting effect when they were loaded onto the membranes in the absence of lactose, i.e., under the conditions when they could interact with collagen through both the CBD/CBP moiety and the galectin-9 moiety. The membranes treated with G9Null fusion proteins, except for G9Null-112, inhibited cell growth by about 20%–60% (Fig. 5). A variable degree of cell adhesion was observed with the membranes. The activity of ssG9 fusion proteins was not determined under these conditions.

In addition to collagen vitrigel membranes, we tested insoluble collagen fibrils in the assay. Insoluble collagen fibrils were sterilized by incubation with 70% ethanol before use. The collagen-binding assay revealed that the treatment did not affect the binding characteristics of the fibrils for both CBD302 and G9Null-302 (data not shown). The growth of Jurkat cells was inhibited by about 73% in the presence of insoluble collagen fibrils treated with 10 μM G9Null, while the fibrils treated with 10 μM G9Null-302 (and other G9Null fusion proteins, data not shown) in the presence of lactose showed no effect (Fig. 6B).

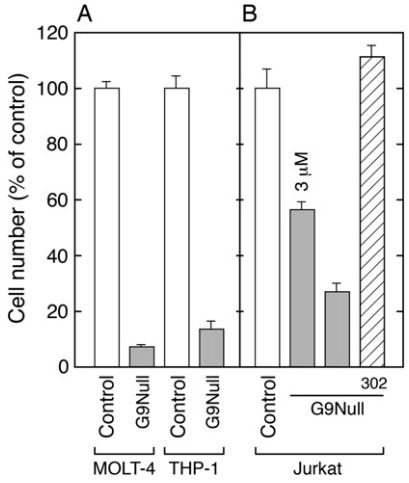

Fig. 6. The antiproliferative effect of vitrigel membranes and insoluble collagen fibrils treated with galectin-9 and a galectin-9 fusion protein on human immune cell lines. (A) Collagen vitrigel membranes treated with galectin-9 solution (10 μM) in the absence of lactose were used as a culture substratum. The number of MOLT-4/THP-1 cells cultured on an untreated collagen vitrigel membrane was taken as 100%. (B) Insoluble collagen fibrils treated with galectin-9 and galectin-9 fusion protein solutions (10 μM unless otherwise indicated) in the presence (hatched columns) or absence (gray columns) of lactose were used as a culture substratum. The number of Jurkat cells cultured on untreated insoluble collagen fibrils was taken as 100%. The data represent the means ± SD for triplicate experiments.

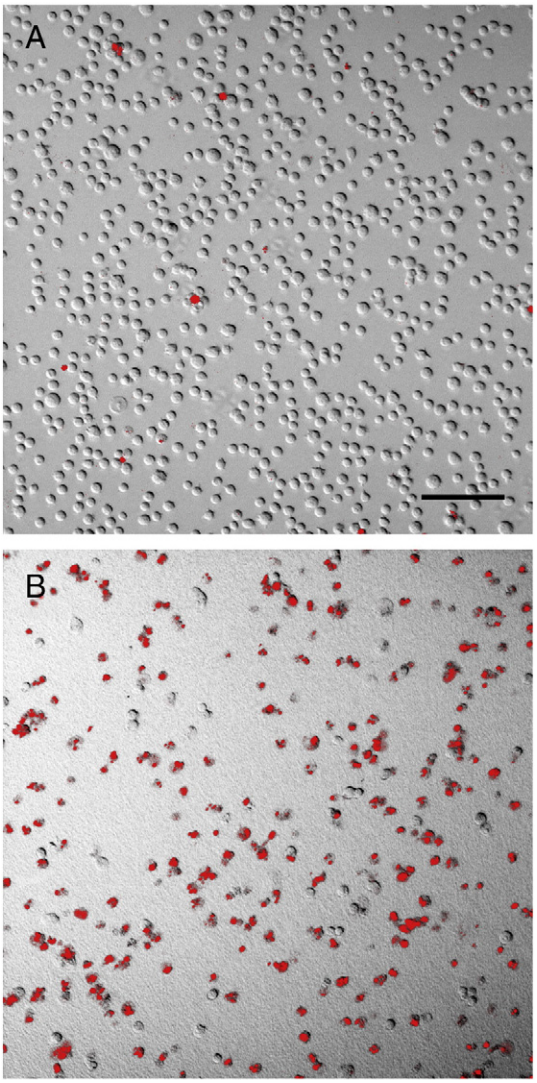

Fig. 7. Detection of dead cells by propidium iodide (PI) staining. (A) Jurkat cells cultured on a control collagen vitrigel membrane were collected by centrifugation, followed by staining with PI. After washing with PBS, the stained cells were placed on a glass slide and observed by confocal microscopy. (B) Jurkat cells cultured on a membrane treated with 10 μM G9Null in the absence of lactose became attached to the membrane. The cells were stained with PI on the membrane and then observed by confocal microscopy. Merged images of the PI fluorescence image and differential interference contrast (DIC) image in the same field are shown. Scale bar, 100 μm.

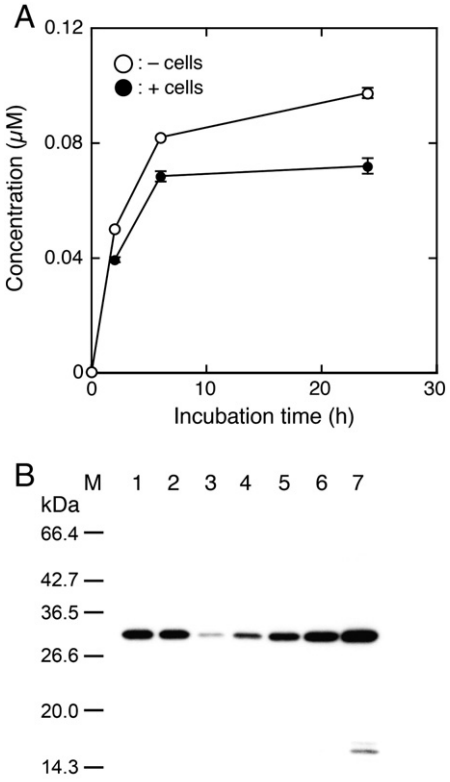

Fig. 8. Quantitation of G9Null released from collagen vitrigel membranes by ELISA and Western blot analysis. (A) A collagen vitrigel membrane treated with 10 μM G9Null in the absence of lactose was incubated in the culture medium in the absence and presence of Jurkat cells. An aliquot of the culture medium was withdrawn after 2, 6 and 24 h of incubation. The content of G9Null was determined by ELISA. The data represent the means ± SD for triplicate experiments. (B) The conditioned medium (24-h culture in the presence of Jurkat cells) was analyzed by semi-quantitative Western blotting using anti-galectin-9 polyclonal antibody as the first antibody. M, molecular weight marker proteins, Lanes 1 and 2, conditioned medium derived from two independent experiments (×1/20 dilution); lanes 3–7, standard G9Null (0.2, 0.5, 1, 2 and 4 ng/lane).

3.5. Release of galectin-9 from collagen matrices

The above-mentioned results suggest that collagen matrices loaded with galectin-9 (G9Null and ssG9) exerted a cytocidal effect through direct contact with Jurkat cells. However, the possibility that free galectin-9 released from the collagen matrices played some role, major or minor, in the growth-inhibiting effect cannot be excluded. The concentration of G9Null in the culture medium upon incubation of collagen vitrigel membranes treated with G9Null was determined in the absence and presence of Jurkat cells by ELISA and semi-quantitative Western blot analysis. The concentration determined by ELISA nearly reached the maximum level after 6 h incubation in both the absence and presence of Jurkat cells (Fig. 8A). The concentrations of G9Null in the culture medium after 24 h incubation were 0.097 ± 0.002 μM (3.2 ± 0.06 μg/ml, 6.4 ± 0.12 μg/dish; in the absence of cells) and 0.072 ± 0.003 μM (2.4 ± 0.09 μg/ml, 4.8 ± 0.18 μg/dish; in the presence of cells). The concentration determined by semi-quantitative Western blot analysis was 0.075 ± 0.004 μM after 24 h incubation in the presence of the cells, which was closely similar to that obtained on ELISA. Degradation products of G9Null were not detected in the culture medium on Western blot analysis (Fig. 8B). The amount of G9Null bound to collagen vitrigel membrane at the beginning of the experiment was 33.8 ± 1.6 μg/dish (ELISA). Therefore, about 20% of G9Null bound to the membrane was released during 24-h incubation in the absence of cells.

4. Discussion

We have demonstrated that the C-terminal region of *C. histolyticum* collagenases (ColH and ColG) serves as a substrate recognition/binding site, which forms CBD structurally independent of a metalloproteinase domain of the enzymes [28,35]. CBD can act as an anchoring domain for collagen even when fused to heterologous proteins [22]. We chose CBDs derived from ColH and ColG as promising candidates for the anchoring domain for localizing galectin-9 on collagen matrices. In addition to CBDs, we also tested CBP, because CBP has been used to produce at least eight collagen-binding growth factors [25]. Galectin-9 is composed of two CRDs, N-CRD and C-CRD, both of which exhibit relatively high affinity for N-linked oligosaccharides and glycolipid-type glycans [36]. The intrinsic sugar-binding activity of galectin-9 makes it possible to bind to collagen, a glycoprotein, without the assistance of CBD/CBP. Type IV collagen in basement membrane and fibrillar type V collagen are known to be highly glycosylated [37,38]. However, type I collagen in skin and tendons, of which all most all commercially available collagen matrices are made, exhibited low levels of glycosylation [39,40]. Therefore, it was expected that CBDs and CBP facilitate the localization of galectin-9 on collagen matrices made of type I collagen.

Two types of mutant galectin-9 (G9Null and ssG9) were used in the present study. G9Null, a protease resistant form of galectin-9, has been used in most *in vivo* experiments to demonstrate the therapeutic potential of galectin-9. ssG9 was recently developed to improve the solubility of G9Null through optimization of the structure of the linker region. The solubility of G9Null fusion proteins was comparable to or higher than that of G9Null except for that of G9Null-305 (Fig. 2). The two- to three-fold increases in the solubility of fusion proteins containing CBD112, CBD115 and CBP may reflect the high solubility of the collagen-binding moieties (CBP contains three amino acid residues with a positive charge). In the case of ssG9, the fusion proteins exhibit lower but acceptable solubility compared to that of ssG9. Currently, the reason for the negative effect of CBDs on the solubility of ssG9 is not clear.

The formation of fusion proteins with CBDs more or less abolished growth-inhibiting (apoptosis-inducing) activity of galectin-9 on Jurkat cells (Fig. 3). This negative effect may in part be due to the interference with the interaction between cell surface receptor(s) and one or both CRDs of fusion proteins by the collagen-binding moieties through simple steric hindrance: The negative effects of CBD302 (M.W. = 23.9×10^3) and CBD112 (M.W. = 26.5×10^3) were higher than those of CBD305 (M.W. = 13.3×10^3) and CBD115 (M.W. = 13.2×10^3), respectively. In addition, attachment of CBP (a heptapeptide) to G9Null had a negligible effect on the activity. However, in the case of CBD112 and CBD115, the presence of an interaction between CBD and galectin-9 CRD(s), which leads to masking of the sugar-binding site of CRD(s), cannot be ruled out, because the negative effects of CBD112 and CBD115 were significantly higher than those of CBD302 and CBD305, respectively. Although the growth-inhibiting activities of G9Null-112/115 and ssG9-112/115 were very low (less than about 20% of that of G9Null), these fusion proteins were not excluded from further characterization, as it is possible that the interdomain interactions in the proteins change upon binding to collagen.

Insoluble type I collagen from bovine Achilles tendon is so far the best substrate commercially available for elucidating the collagen-binding properties of CBDs, though it is highly heterogeneous in nature. Insoluble collagen was also used in the present study to elucidate the collagen-binding properties of galectin-9 fusion proteins and galectin-9. Scatchard analysis is one of the most popular procedures for binding data analysis in biochemical experiments. Although we tried to apply Scatchard analysis to data obtained on collagen-binding assaying, a typical Scatchard plot was not obtained for most of the fusion proteins (Fig. 4). This was probably due to the presence of multiple binding/interaction sites in both insoluble collagen fibrils and galectin-9/galectin-9 fusion proteins. In addition to the fusion proteins, a typical Scatchard plot was not obtained for CBDs including CBD302 and CBD305 (Supplementary Fig. 3). Currently, we cannot adequately explain the discrepancy between our previous results [28] and those of the present study. The data expressed as “Bound vs. Free”, however, clearly show the order of affinity for collagen of the fusion proteins. The collagen-binding characteristics of G9Null/ssG9-112/115 were closely similar to those of the corresponding CBDs (Supplementary Fig. 3C & D). On the other hand, the collagen-binding activity of CBDs derived from ColH, especially CBD305, was decreased upon formation of the fusion proteins (Supplementary Fig. 3A & B). This is contrary to the situation in the antiproliferative activity of fusion proteins: the activity of G9Null was only slightly affected by the addition of CBD305 moiety (Fig. 3). These results suggest that it is difficult to predict the effect of inter-domain interactions in the fusion proteins on the activity of each functional domain.

G9Null and ssG9 showed significant affinity for insoluble collagen fibrils (Fig. 4C). The binding of galectin-9 to collagen was almost completely inhibited by lactose, indicating the oligosaccharide-dependent nature of the interaction. The finding was unexpected because type I collagen in skin and tendons was reported to exhibit low levels of glycosylation. Mature type I collagen does not contain potential N-glycosylation sites, and only the presence of glucosylgalactosylhydroxylysine and galactosylhydroxylysine residues has been reported [39,40]. Although we cannot completely exclude the possibility that galectin-9 binds to contaminating substance(s) in the collagen preparation, it is possible that galectin-9 has relatively high affinity for these O-linked saccharides and that the number of glycosylated hydroxylysine residues per collagen molecule is higher than the number of the binding site(s) for CBDs. Comprehensive analysis of the oligosaccharide moieties of collagens with regard to their affinity for members of the galectin family is needed not only for therapeutic application, but also for understanding of the physiological role of collagen as an extracellular reservoir for galectins.

CBP (a heptapeptide, TKKTLRT) has been employed to construct a variety of collagen-binding growth factors including EGF, bFGF and vascular endothelial growth factor. The fusion proteins were reported to have beneficial effects compared to the parental growth factors *in vivo* [41–43]. Galectin-9 fusion proteins having CBP exhibited the lowest affinity for collagen among the fusion proteins tested. In previous studies, CBP was fused to the N-terminal of the growth factors, while it was placed at the C-terminal of galectin-9 in the present study. It is possible that the affinity of the CBP moiety for collagen depends on its position in the fusion proteins. CBP is said to be derived from a mammalian collagenase [24]. However, more correctly, the sequence was found using “the principle of complementary hydropathy” [44], and it is not a part of collagenase sequences. Together with the finding that the binding of galectin-9-CBP to collagen was greatly reduced in the presence of a physiological concentration of calcium, the nature of CBP as a binding peptide specific for collagen should be carefully reexamined.

In order to determine the ability of recombinant proteins immobilized on collagen matrices to induce Jurkat cell death, collagen vitrigel membranes were used as a culture substratum. A collagen vitrigel membrane is a thin, transparent collagen membrane with enhanced strength, which is prepared via three processes, i.e., gelation, vitrification (removal of free and bound water by evaporation), and rehydration [45]. We selected collagen vitrigel membranes based on their advantages for forthcoming *in vivo*/therapeutic studies. All the galectin-9 fusion proteins could not induce Jurkat cell death when they were loaded on the membranes in the presence of lactose, that is, under the conditions when the fusion proteins can interact with collagen only through their collagen-binding moieties (Fig. 5). This was not due to the difference in the binding capacity for CBDs/CBP between insoluble collagen fibrils and collagen vitrigel membranes, because insoluble collagen fibrils treated with G9Null-CBD302 in the presence of lactose had no effect on growth of cells. On the other hand, the membranes treated with the G9Null fusion proteins in the absence of lactose significantly suppressed the growth of Jurkat cells, except for G9Null-112. The growth-inhibiting effect of the membranes roughly corresponded to the activities of the loaded proteins in solution. These results suggest that the growth-inhibiting effect of collagen vitrigel membranes treated with G9Null fusion proteins depends on both the amount (distribution density) of the immobilized protein and the activity of the protein in solution, and that the distribution density of G9Null fusion proteins immobilized through only CBDs/CBP is not high enough to induce cell death. In accordance with the results, the membranes treated with G9Null and ssG9 in the absence of lactose exerted the highest growth-inhibiting activity among the recombinant proteins tested. This effect seems to depend on direct interaction between immobilized galectin-9 and cell surface receptor(s): Jurkat cells became attached to the membrane treated with galectin-9 soon after inoculation. The cells remained attached throughout the culture period and died on the membranes (Fig. 7). In addition, the concentrations of galectin-9 in the culture medium (galectin-9 released from the membranes) after 24-h culture were about 0.1 μM and 0.07 μM in the absence and presence of Jurkat cells, respectively. G9Null at 0.1 μM induced only a 10 to 20% reduction in the Jurkat cell growth when added to the culture medium at the beginning of the culture (Fig. 3A). Because more than 80% of G9Null in the medium was released within 6 h of incubation, optimization of the washing conditions for the membranes after incubation with a galectin-9 solution

may reduce the unfavorable release of the protein *in vitro* and *in vivo*. It is also suggested that the distribution density needed to induce cell death is higher than that needed to support cell attachment, because more than 90% of Jurkat cells became attached when inoculated on membranes treated with 1 μM G9Null, while cell growth was inhibited by about 45%. The observation that galectin-9 bound to collagen can induce cell death via direct interaction is important not only from the therapeutic point of view but also for understanding the physiological functions of galectin-9. Galectin-9 localized in the extracellular matrix (ECM) and even on the cell surface of galectin-9-resistant cells may exert its function through cell–ECM and cell–cell interactions.

We created ten novel galectin-9 fusion proteins having CBD/CBP, which was expected to tightly anchor galectin-9 on collagen matrices. However, unexpectedly, galectin-9 itself exhibited apparent affinity for collagen. The lactose-sensitive nature of the interaction suggests that galectin-9 binds to the oligosaccharide chain(s) of collagen molecules. Induction of Jurkat cell death requires at least 0.1 μM or higher concentrations of galectin-9 in solution; on the other hand, most growth factors including EGF and bFGF exert their maximum activity at concentrations lower than 1 nM. Therefore, it is probable that the requirement of high concentrations of galectin-9 (high distribution density on the membranes) is the major reason for the inability of galectin-9 fusion proteins, which are immobilized on collagen via only CBD/CBP, to induce cell death. Nevertheless, the present study indicates that collagen vitrigel membranes and other collagen matrices loaded with G9Null or ssG9 can exert a contact-dependent cytocidal effect on human immune cells and that the collagen matrices may be used for tissue engineering where local immunosuppression is needed.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.01.019.

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