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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