ORIGINAL ARTICLE

F-16438s, Novel Binding Inhibitors of CD44 and Hyaluronic Acid

I. Establishment of an Assay Method and Biological Activity

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Abstract   In an attempt to obtain inhibitors of hyaluronic acid (HA) binding to its receptor, CD44, we established an efficient assay method to detect and quantify binding using fluorescein-labeled HA and HEK293 cells stably expressing CD44. As a result of the screening of culture broths of microorganisms, we found fungus strain *Gloeoporus dichrous* SANK 30502 produced inhibitory activity in this new assay. Five compounds, F-16438 A, B, E, F and G, were isolated from the fermentation broths, and their IC_{50} values were determined to be 10.3, 13.5, 27.3, 12.0 and 13.0 μM, respectively. F-16438 A, B, E, F and G are the first reported inhibitors of binding HA to CD44. F-16438 A, B, E and F have novel structures.

Keywords   hyaluronic acid, CD44, binding inhibitor, screening method, F-16438

Introduction

Hyaluronic acid (HA) is a high-molecular-weight, straight-chain glycosaminoglycan (GAG) composed of repeating alternating units of glucuronic acid and *N*-acetylglucosamine. Despite its structural simplicity, HA has a great number of diverse functions. It has been shown that HA participates in embryonic development and morphogenesis [1, 2], wound healing [3, 4], inflammation [5, 6], and tumor progression and invasion [7, 8]. In addition, since the molecular domain of HA encompasses a large volume of water, HA also functions as a lubricant and as a shock absorber, and acts as an important structural molecule in tissues such as the vitreous of the eye [9] and synovial joint fluid [10]. In fact, the concentration and average molecular weight of HA in the joint fluids are reduced in diseases such as osteoarthritis and rheumatoid arthritis [11, 12].

The transmembrane glycoprotein CD44 is a major HA cell surface receptor expressed abundantly on many cell types, including macrophages, lymphocytes, fibroblasts, epithelial cells, chondrocytes and several tumor cells [13~18]. Using a variety of experimental models, CD44 has been shown to mediate cell-cell and cell-ECM interactions [19~21], co-stimulate lymphocyte activation [22, 23], and promote growth and metastasis of some tumors [24, 25]. In highly invasive breast cancer cell lines, turnover of HA and expression of CD44 are upregulated [26]. By treatment with catabolic stimulators like interleukin-1 alpha and fibronectin fragment, CD44 is upregulated in chondrocytes [27]. It has also been shown that HA is taken up by these cells for degradation through the HA receptor [28~30]. Based on all those observations, we hypothesized that inhibition of HA binding to the CD44 receptor will inhibit CD44 functions and/or turnover of HA, and may therefore be of therapeutic value in

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the treatment of various diseases including cancer, osteoarthritis and rheumatoid arthritis.

In order to obtain agents having inhibitory activity of the interaction between HA and CD44, we analyzed the function of CD44 expressed exogenously in HEK293 cells and developed an efficient assay method to detect binding of HA to CD44 on HEK293 cells. In the course of a screening program, fungus strain *Gloeoporus dichrous* SANK 30502 was discovered to produce novel CD44-HA binding inhibitors, which were coded as F-16438s. In this paper, we describe the establishment of an assay method and the biological activities of these inhibitors. The fermentation, isolation and structural elucidation as well as the physico-chemical properties of these compounds are reported elsewhere [31].

Materials and Methods

Establishment of HEK293 Cells Stably Expressing Human CD44

HEK239 (293) cells were maintained in a humidified incubator at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The cDNA of human CD44 was amplified by polymerase chain reaction (PCR) using a Human Lymph Node cDNA Library (Takara) as a template, and inserted into the expression vector pRK5. HEK293 cells were co-transfected with pRK5/CD44 and pSV2neo by a calcium phosphate transfection method. A cell lineage expressing CD44 was selected using 500 μg/m of Geneticin (GIBCO) and was named CD44/293.

Western Blotting

Cells (5×10^6) were washed and collected with 1 ml of phosphate-buffered saline (PBS). After centrifugation at 3,000 rpm for 10 minutes, the supernatant was removed and 500 μl of lysis buffer (Bio-Rad) was added to the cells. The cells were lysed with sonication. The lysate was incubated at 95°C for 5 minutes and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to PVDF membranes and immunoblotted with specific antibodies for human CD44 (V6034, Biomeda), after which they were detected using an ECL Plus Western Blotting Detection System (Amersham Biosciences).

Preparation of Fluorescein-labeled Hyaluronic Acid

Fluorescein-hyaluronic acid (fl-HA) was prepared as described by de Belder and Wik [32] with a slight modification. Briefly, 50 mg of hyaluronic acid (800~1200 kDa, Seikagaku Kogyo) was dissolved in 40 ml of water, and mixed with 20 ml of DMSO. Fluorescein-amine, isotype I (25 mg, Aldrich) in DMSO (0.5 ml) containing acetaldehyde (25 μl, Fluka) and cyclohexyl isocyanide (25 μl, Fluka) was added into the mixture. The mixture was incubated at 25°C for 5 hours, and then poured into 240 ml of ethanol saturated with NaCl. The precipitated fl-HA was collected by centrifugation at 1000 g and air-dried in a chemical hood. The fl-HA was then dissolved in water and re-precipitated using NaCl-saturated ethanol 2 times. The fl-HA was further purified by exhaustive dialysis against distilled water. An aliquot of fl-HA was treated with 5 μg/ml of bovine testicular hyaluronidase (Sigma). The fl-HA with and without treatment of hyaluronidase were then characterized by Sepharose CL-2B chromatography mentioned below.

Incubation of fl-HA with CD44/293 or 293 Cells

CD44/293 or 293 cells (5×10^6) were plated in 10-cm type I collagen-coated dishes and cultured for 48 hours. The cells were then cultured in the presence of 28 μg/ml fl-HA. After 48 hours of incubation, the conditioned medium was collected and cells were washed 3 times with PBS. Then, the cells were incubated in 1 ml of 0.05% trypsin at 37°C for 5 minutes followed by 5 minutes centrifugation at 1300 g. The supernatant was defined as cell surface pool. The cell pellets were washed again 3 times with PBS and then resuspended in 50 mM Tris-HCl, pH 7.4, containing 400 μg/ml proteinase K (Sigma). The cell pellets were incubated at 37°C for 24 hours to solubilize the cells and release the total intracellular material. The solubilized fraction was termed intracellular pool.

Characterization of fl-HA Degradation Using Size-exclusion Chromatography

The fl-HA within the conditioned media, cell surface pools and intracellular pools was characterized by size-exclusion chromatography using a 1.6 cm$\times$25 cm Sepharose CL-2B column equilibrated with PBS. Fractions of 2.5 ml were collected at 0.5 ml/minute and the fluorescence (excitation, 485 nm; emission, 535 nm) was determined by an ARVO fluoroscan (PerkinElmer).

CD44-HA Binding Assay

Cells were washed and harvested with PBS, and disrupted by sonication in 50 mM Tris-HCl, pH 7.8. The suspensions of the disrupted cells were centrifuged at 400 g for 5 minutes. Then, the supernatant was subjected to centrifugation at 20,000 g for 1 hour to yield high speed pellets. The membrane fractions were resuspended and

adjusted to 1 mg/ml with 50 mM Tris-HCl, pH 7.8. A mixture of 1 ml of the membrane fractions (100 μg/ml of total protein) and 10 μl of fl-HA was incubated at room temperature for various periods of time in the presence of various concentrations of NaCl, and then centrifuged at 20,000 g for 10 minutes. After washing 3 times with 50 mM Tris-HCl, pH 7.8, containing NaCl at a concentration equal to that used for incubation, the pellet was solubilized by 200 μl of 50 mM Tris-HCl, pH 7.4, containing 400 μg/ml proteinase K at 37°C for 1 hour. The fluorescence in the lysates was measured by the ARVO fluoroscan. Non-specific binding was determined in the presence of a large excess (300 μg/ml) of unlabeled hyaluronic acid.

Organism, Fermentation and Isolation of F-16438s

Taxonomic study of *Gloeoporus dichrous* SANK30502, and fermentation and isolation of F-16438s are reported elsewhere [32].

Fig. 1 Characterization of fl-HA.

Fl-HA, treated with (○) or without (●) bovine testicular hyaluronidase was applied to a Sepharose CL-2B column and eluted with PBS. The positions of the void volume (V_o) and the total volume (V_t) are indicated.

Results and Discussion

Characterization of fl-HA

Fluorescein-amine was conjugated to high molecular mass HA and characterized by gel filtration chromatography on a Sepharose CL-2B column. As shown in Fig. 1, the majority of fl-HA was eluted in the void volume of the column and no free fluorescein or lower molecular mass contaminants were detected. When fl-HA was treated with bovine testicular hyaluronidase, no fluorescence was detected in the void volume, indicating that the fluorescein-labeled material was highly pure HA that could be a substrate for hyaluronidase.

Characterization of CD44/293 Cells

Several studies have shown that HA is endocytosed *via* hyaluronic acid receptors on the cell surface, and a significant portion of endocytosed hyaluronic acid is degraded by lysosomal hyaluronidases [28, 29]. The endocytosis and degradation of HA is inhibited by anti-CD44 antibody [28, 29], suggesting that CD44 has a critical role in HA turnover. We established that CD44/293 cells stably express human CD44 protein (Fig. 2A). The cells were cultured in the presence of fl-HA for 48 hours. After the conditioned medium (hereafter medium) was collected, the cells were washed with PBS and then treated with trypsin to remove pericellular-associated fl-HA (cell surface pool). The cell pellet was then subjected to exhaustive protease digestion to solubilize the cells and liberate intracellular fl-HA (intracellular pool). Fig. 2B shows the Sepharose CL-2B chromatographic profiles of fl-
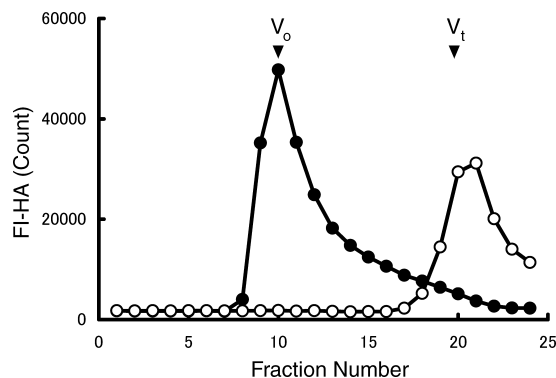
HA in these fractions isolated from CD44/293 and 293 cells. In 293 cells, most of the added fl-HA remained in the medium, and only trace amounts of fl-HA were detected in cell surface and intracellular pools. In contrast, fl-HA was incorporated into cell surface and intracellular pools of CD44/293 cells, and the amount of high molecular fl-HA in the medium was reduced to 69.7% when compared with 293 cells. In the intracellular pool of CD44/293, degraded fl-HA with smaller molecular mass was observed in addition to the intact fl-HA that was eluted in the void volume of the Sepharose CL-2B column. Considering the fact that fl-HA was not degraded in medium or the cell surface pool of either 293 or CD44/293 cells, these results indicate that CD44, exogenously expressed on the cell surface, functioned as an HA receptor, and that the endocytosed fl-HA was degraded by lysosomal hyaluronidases in CD44/293 cells as seen in other types of cells [29].

Establishment of Binding Assay Method

The whole membrane fractions of CD44/293 cells were used for the establishment of a CD44-HA binding assay. The binding was only detected when the membranes from CD44/293 cells were used (Fig. 3A), confirming that the binding was specific to CD44. Under low ionic strength conditions, HA did not bind efficiently to simian virus 40-transfected 3T3 cells [33] which are known to express CD44 abundantly [34]. For further optimization of the receptor-binding assay, the cell membranes were incubated with fl-HA and various concentrations of NaCl to determine the effect of ionic strength that was considered to be a critical factor for binding. Fig. 3B shows that 0.2 M is
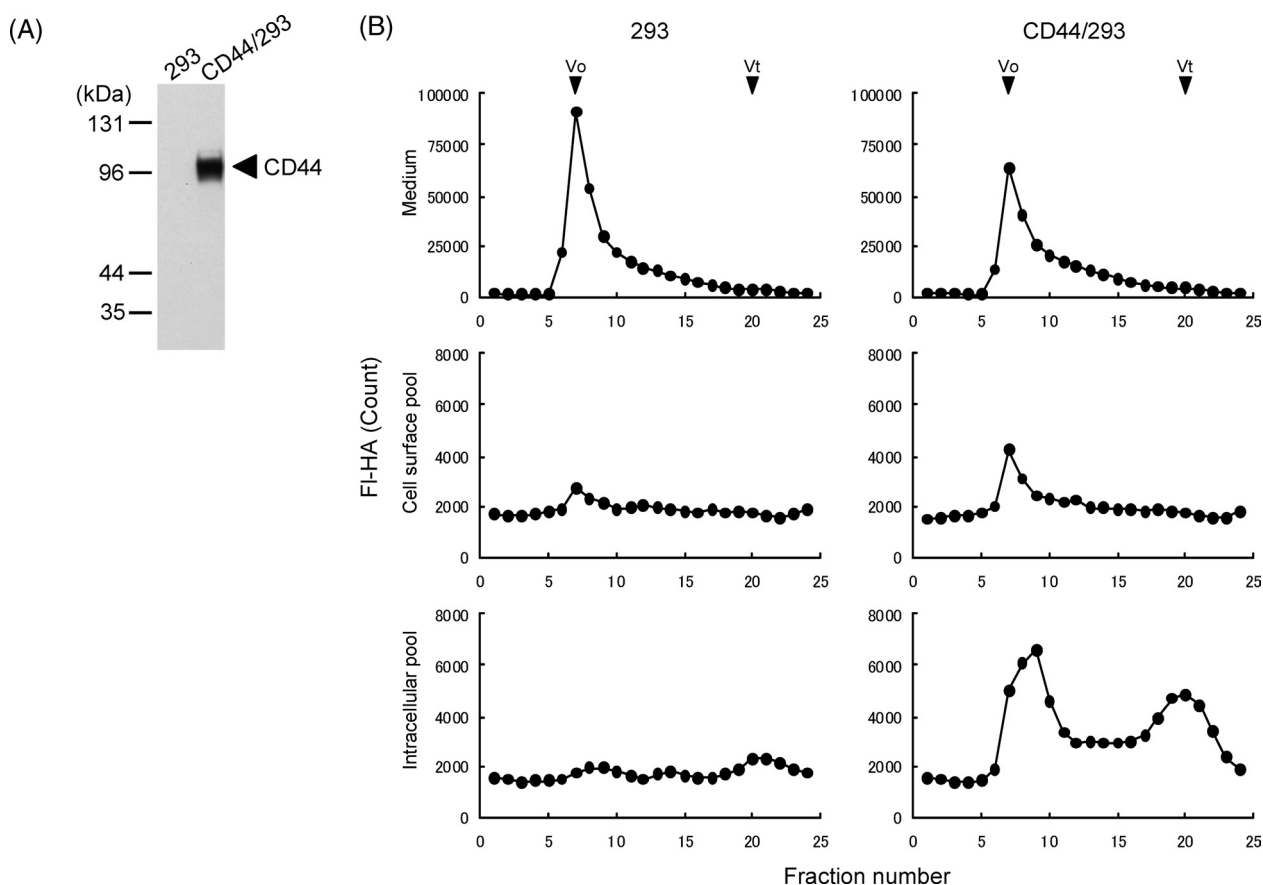
Fig. 2 Characterization of CD44/293 cells.

(A) Whole extracts of 293 and CD44/293 cells (40 μg) were analyzed by western blotting with antiCD44 antibody (V6034, Biomeda). (B) Analysis of fl-HA turnover by 293 and CD44/293 cells. Cells were incubated with fl-HA at 37°C for 48 hours. Then, media, cell surface pools and intracellular pools were applied to a Sepharose CL-2B column and eluted with PBS. The amount of fl-HA in each fraction (2.5 ml) were determined by fluorescence as described in Materials and Methods. The positions of the void volume (V_o) and the total volume (V_t) are indicated.

the optimal NaCl concentration. When the cell membranes were incubated for various periods with fl-HA, binding increased up to 1 hour (Fig. 3C) and was retained for 2 hours incubation. At longer incubation times of more than 4 hours, however, the binding decreased. Under the standard laboratory conditions, the binding did not show temperature or pH dependencies; equal binding was observed at 4°C and room temperature in the pH range from 7.0 to 7.8 (data not shown). Scatchard plot analysis under the optimized conditions (Fig. 3D) revealed that the values for Kd and Bmax were 0.35 nM and 0.50 pmol/mg protein, respectively. These values had good concordance with the values obtained from binding between HA and simian virus 40-transfected 3T3 cell membranes [35].

Search for Inhibitors from Microbial Culture Broths
Among 120,000 culture broths of microorganisms, fungus strain SANK 30502 was found to have inhibitory activities

for CD44-HA binding. The active substances were purified after consecutive column chromatographies [32]. The chemical structures of F-16348 A, B, E, F and G are shown in Fig. 4. The dose dependent inhibition of CD44-HA binding by F-16438B is shown in Fig. 5 as a representative example. The IC_{50} values of the inhibitory activity of F-16348 A, B, E, F and G were 10.3, 13.5, 27.3, 12.0 and 13.0 μM, respectively (Table 1). F-16438 A, B, E and F were found to be new found structures whereas F-16438G was a previously identified phospholipase C inhibitor [36]. The compounds did not show any cytotoxicity to HEK293 cells up to 100 μg/ml. These compounds are the first reported low-molecular weight inhibitors of CD44-HA binding; this discovery well demonstrated the utility of the new CD44-HA binding assay. It is expected that this series of compounds will serve as new chemical probes of HA-CD44 interactions and functions.
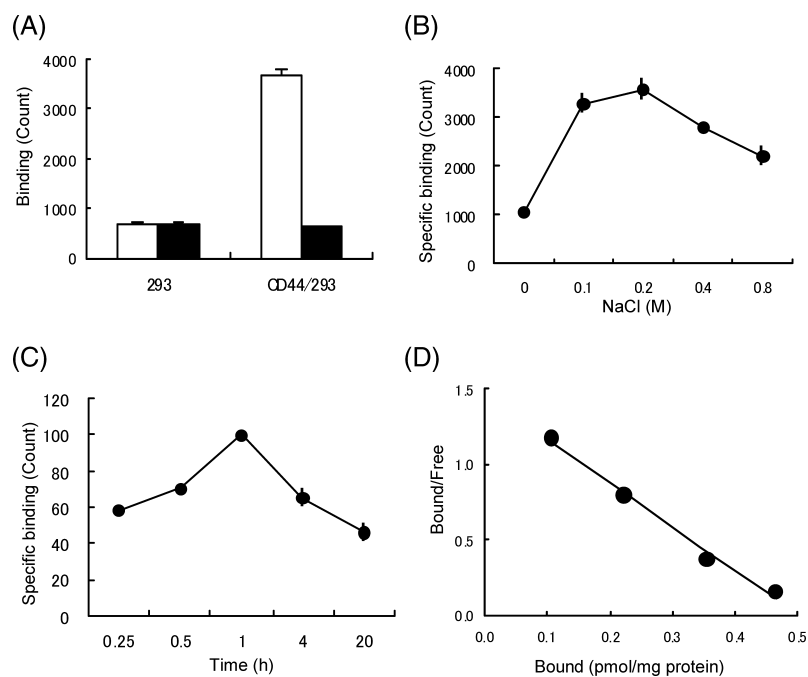
Fig. 3 Binding of fl-HA to CD44/293 cell membranes.

(A) Specificity of fl-HA binding to CD44. The cell membranes prepared from 293 or CD44/293 cells were incubated with fl-HA (3 μg/ml) at room temperature for 1 hour in the presence of 0.2 M NaCl. Following incubation, the cell membranes were washed three times and digested with proteinase K. The fluorescence in the sample was measured using an ARVO fluoroscan. Open columns and closed columns indicate total binding and nonspecific binding, respectively. Bars indicate SD. (B) Effect of NaCl concentration on binding of fl-HA to CD44. The cell membranes were incubated with fl-HA (3 μg/ml) in the presence of various concentrations of NaCl as indicated. (C) Time-dependency of fl-HA binding. The cell membranes were incubated with fl-HA (3 μg/ml) for 0.25~20 hours. (D) Scatchard plot of fl-HA binding. The cell membranes were incubated with various concentrations of fl-HA (0~10 μg/ml) under optimized conditions.

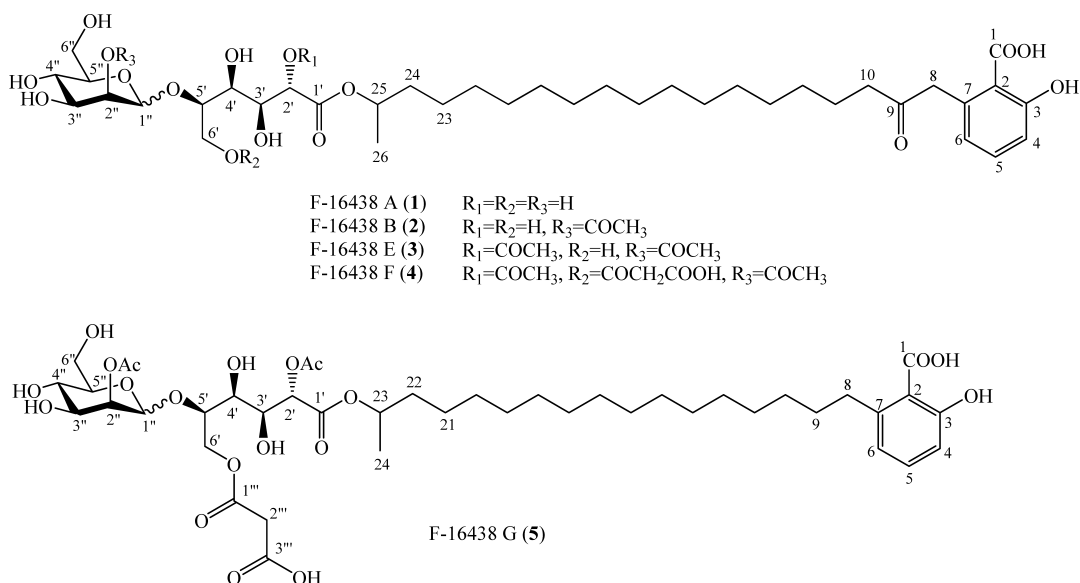|	
F-16438 A (**1**)	$R_1=R_2=R_3=H$
F-16438 B (**2**)	$R_1=R_2=H$, $R_3=COCH_3$
F-16438 E (**3**)	$R_1=COCH_3$, $R_2=H$, $R_3=COCH_3$
F-16438 F (**4**)	$R_1=COCH_3$, $R_2=COCH_2COOH$, $R_3=COCH_3$

F-16438 G (**5**)

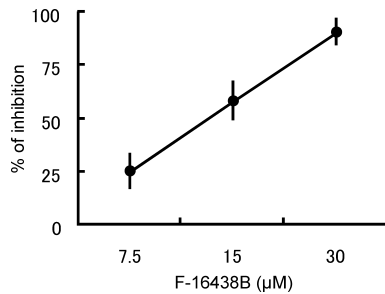**Fig. 4** Structures of F-16438 A, B, E, F and G.

Fig. 5 Dose dependent inhibition of CD44-HA binding by F-16438 B.

The cell membranes were incubated with fl-HA (3 μg/ml) under optimized conditions in the presence of various concentrations of F-16438B as indicated.

Table 1 Inhibition of binding between CD44 and HA by F-16438s

Compound	IC$_{50}$ (μM)
F-16438 A	10.3
F-16438 B	13.5
F-16438 E	27.3
F-16438 F	12.0
F-16438 G	13.0

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