Arch Pharm Res Vol 24, No 6, 514-517, 2001

Archives of Pharmacal Research
http://apr.psk.or.kr

Virus-cell Fusion Inhibitory Activity for the Polysaccharides from Various Korean Edible Clams

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(Received February 2001)

In order to find potent virus-cell fusion inhibitory components from Korean edible clams, thirteen prepared polysaccharides were introduced to syncytia formation inhibition assay, which is based on the interaction between the HIV-1 envelope protein gp120/41 and the cellular membrane protein CD4 of T lymphocytes. Among them, *Meretrix petechialis* showed a potent virus-cell fusion inhibitory activity. Fusion index (FI) and percent (%) fusion inhibition of the polysaccharide of this clam were 0.21 ± 0.02, and 67.52 ± 4.09 at 100 μg/ml, respectively. It exhibited almost equivalent virus-cell fusion inhibitory activity to that of dextran sulfate which was used as a standard control.

Key words: Anti-HIV, gp120/41, Syncytia, Fusion, Clams

INTRODUCTION

The vast majority of anti-human immunodeficiency virus-1 (anti-HIV-1) therapeutic agents have been limited to reverse transcriptase and protease inhibitors (Langtry *et al.*, 1989; Faulds *et al.*, 1992; Whittington *et al.*, 1992). In addition, many efforts were carried out searching for HIV-1 integrase inhibitors (Kim *et al.*, 1998). The adverse side effects of nucleoside inhibitors and the advent of resistant strains to many of the inhibitors demand the new drugs based on another mode of action (De Clercq, 1994). The envelope glycoproteins of HIV-1 consist of two noncovalently associated subunits gp 120 and gp 41 which are derived from the gp 160 precursor. Gp 120 initiates binding between virions and CD4 molecules of T lymphocytes and triggers fusion between the viral envelope and the host cell membranes, allowing infection to proceed (Hart *et al.*, 1991; Lin *et al.*, 1996). Therefore, gp120-CD4 interaction, gp120-gp 41 interaction, membrane fusion, and so forth, could provide an additional means of treating viral infection (Ryu *et al.*, 1998). Polyanionic compounds, such as polysulfates, polysulfonates, polycarboxylates have been shown to interfere with virus cell binding and syncytia formation between infected and uninfected cells (De Clercq, 1987). However, the biological activities of these compounds do not appear to be specific for HIV-1. Several recent reports showed that synthetic peptides derived from the HIV-1 gp 41 sequence and triterpene derivatives displayed potent virus-cell fusion inhibition (Mayaux *et al.*, 1994; Pereira *et al.*, 1995; Ryu *et al.*, 1999; Nakashima *et al.*, 1996). But, clinically useful compounds effective at blocking viral entry or virus-cell fusion inhibition have not thus been developed so far. In order to reduce the risk of any infection from HIV-1, a recombinant vaccinia virus, carrying HIV-1 *env gene* was introduced for our assay system (Earl *et al.*, 1990). The gp 120-CD4 interaction, and gp120-gp41 interaction which result in syncytia formation can be quantitatively expressed by the fusion index (FI) (Cheserbo *et al.*, 1988). Thirteen polysaccharides prepared from Korean edible clams were evaluated.

In this paper, we describe the virus-cell fusion inhibitory activity of the polysaccharides prepared from Korean edible clams and the systematic assay involving vPE 16 and HeLa/CD4$^+$ cells which carry HIV *env gene* and CD4 molecules of its surface, respectively.

MATERIALS AND METHODS

Ham's F12, new born calf serum (NBCS), trypsin (1:250 diluted), penicillin G/streptomycin solution and Na_2EDTA

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were purchased from GIBCO BRL (New York, U.S.A.). Dextran sulfate (average M.W. 10,000), trypan blue, neutral red, and cetylpyridinium chloride were purchased from Sigma (St. Louis, U.S.A.). DMSO was purchased from Merck (Darmstadt, Germany). Alcalase (alkaline protease mixture) was from Novo Korea (Seoul, Korea).

Preparation of clam polysaccharides

The polysaccharides were prepared according to the previously published method (Kim *et al.*, 1996). In brief, the shell of each clam was removed and the whole soft body was de-fatted using three times of 24 h extractions with acetone. The fat-free dried tissue was cut into a fine powder using a razor blade. Approximately 4 g of dried, de-fatted, pulverized powder was suspended in 40 ml of 0.05 M sodium carbonate buffer (pH 9.2). The suspension was shaken for 48 h at 2,000 rpm at 60°C after adding 2 ml of Alcalase (0.6 AU/g). The digestion mixture was cooled to 4°C, and trichloroacetic acid was added to a final concentration of 5%. The sample was mixed and allowed to stand for 10 min, then centrifuged for 20 min at 8,000 *g*. The supernatant was recovered by decanting. Three volumes of 5% potassium acetate in ethanol was added to one volume of supernatant. After mixing, the suspension was stored overnight at 4°C, then centrifuged for 30 min at 8,000 *g*. The supernatant was discarded, and the precipitate was washed with absolute alcohol. The precipitate (1 g) was dissolved in 40 ml of 0.2 M NaCl and centrifuged for 30 min at 8,000 *g* and insoluble material was discarded. To the supernatant, 0.5 ml of cetylpyridinium chloride (5%) was added and the precipitate was collected by centrifugation. The precipitate was dissolved in 10 ml of 2.5 M NaCl and five volumes of ethanol were added and the precipitate was centrifuged for 30 min at 10,000 *g*. The precipitate was dissolved in water, dialyzed against 100 volumes of water and the dialyzate was freeze-dried. Weighed samples of 3 mg each were dissolved in 3 ml of the culture medium. They were filtered with a microfilter (Sterivex 0.22 μm), and then used for the syncytia formation inhibition assay.

Analysis of clam polysaccharides

The homogeneity of each polysaccharide was demonstrated on 1% agarose gel-electrophoresis as previously described (Wu *et al.*, 1998). In brief, it was performed in a 1% gel poured in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0). Approximately, 100 μg of purified polysaccharides were loaded on the gel and constant voltage (100 V) was applied for 1 hr at room temperature. The gel was visualized with 0.5% Azure A in 1% acetic acid and destained in water.

Cells and viruses

CD4 positive HeLa cells carrying a CD4 protein on its surface were cultured with Ham's F12 medium supplemented with 10% (v/v) heat-inactivated newborn calf serum (NBCS), 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate and $NaHCO_3$ (1.176 g/l). Syncytia formation activity of the $CD4^+$ HeLa cells with vPE 16 was constant throughout the experiments. The cells were maintained at 37°C in a humidified atmosphere with 5 % CO_2. The cells were subcultured twice a week at 5×10^5 cells/ml. Recombinant vPE 16 was prepared from a vaccinia virus which expresses HIV-1 envelope protein gp 120 and gp 41 on its surface. The stock of vPE 16 was prepared from culture supernatant of vPE 16 infected vero cells. The virus titer of the supernatant was determined using a plaque assay. The virus stock was stored as aliquots at -80°C until used. Both the $CD4^+$ HeLa cells and recombinant vPE 16 were kindly provided by Dr. M. Nishijima at NIH in Japan.

Syncytia formation inhibition assay

The assay using $CD4^+$ HeLa cells and vPE 16 was carried out as described by Tochikura *et al.* with some modifications (Woo *et al.*, 1997). $CD4^+$ HeLa cells (2×10^5 cells/ml) in log-phase were seeded in 12-well culture plates (3 ml cell suspension/well). Three days later, the plates were washed with fresh medium to remove unadhered cells then 0.2 ml of culture medium including test compounds or various concentrations of dextran sulfate were added. The culture plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 30 min. Then 10 μl of diluted vPE 16 (7.3×10^2 PFU) was added and incubated for an additional 30 min. After the incubation, 0.8 ml of culture medium was added and again incubated. After 16-20 h of incubation, syncytia formation was observed under the microscope. The fusion index (FI) and the percentage of fusion inhibition are calculated as follows :

$$FI = \frac{\text{Total number of nuclei}}{\text{Total number of cells}} - 1$$

$$\% \text{ fusion inhibition} = \left(1 - \frac{FI_1}{FI_2}\right) \times 100$$

where FI_1 is the fusion index of the test sample and FI_2 is the fusion index of the control sample.

RESULTS AND DISCUSSION

The intervention of the HIV replicative cycle could function as targets for anti-HIV drugs. The compounds that are presently available as anti-HIV drugs are targeted at either

reverse transcriptase or viral protease. However, developing strategies aimed at inhibiting other steps of the virus replicative cycle, such as gp 120/41-CD4 interaction, and membrane fusion could provide an additional means of treating the viral infection.

Virus-cell fusion can be considered as an attractive target for anti-HIV chemotheraphy since the compounds that interfere this process may be expected to block viral spread through both virus-to-cell and cell-to-cell contact. Peptides derived from gp 41, i.e. C51, and C27, effectively block the gp120/41 mediated virus-cell fusion (Ryu *et al.*, 1999). Moreover, the betulinic acid derivatives represent the first low-molecular weight compound to be recognized as fusion inhibitors and are prime candidates to be pursed for HIV-1 infections (Mayaux *et al.*, 1994).

In our previous report, various plant extracts were evaluated for the virus-cell fusion inhibitory activity, and the methanolic extract of *Eugenia caryophyllata* afforded several active compounds (Woo *et al.*, 1997; Kim *et al.*, 2001). For a continuation of our search for anti-HIV compounds from marine natural products, thirteen polysaccharides prepared from Korean edible clams were tested for the syncytia formation inhibition assay. Several sulfated polysaccharides, such as dextran sulfate, β-cyclodextrin sulfate, and prunellin isolated from *Prunella vulgaris* showed potent anti-HIV activity based on the virus-cell fusion inhibitory activity (Yao *et al.*, 1992). The virus-cell fusion inhibitory activity of dextran sulfate and its congeners appear to be mediated by a specific interaction with the V3 loop of gp 120. In addition, sulfated polysaccharides may also directly interfere with the binding of HIV particles to the cell. They are not known to lead to the development of virus-drug resistance, and they should be effective against HIV mutants that are resistant to AZT or other RT inhibitors (De Clercq, 1995). The migration of each polysaccharide on agarose gel was shown in Fig. 1. Seven of them including *Batillus crnutus, Scapharca sbcrenata, Scapharca broughtonii, Ruditapes philippinarum, Meretrix petechialis, Sinonovacula constricta,* and *Halocynthia roretzi* showed major spots at the same location as dextran sulfate (M_r 10,000). These results suggest that their homogeneity is relatively good, although the extraction and precipitation steps were only taken for the purification of polysaccharides. In general, the structure of clam polysaccharide is composed of neutral sugars containing a substitution of sulfate groups (Amornrut *et al.*, 1999). Among thirteen polysaccharides, a polysaccharide prepared from *Meretrix petechialis* exhibited almost equivalent virus-cell fusion inhibitory activity to that of dextran sulfate (Table I). *Meretrix petechialis* mainly distributed at the west coast of the Korean peninsula is a good natural source for the preparation of active polysaccharide. In addition, they can be prepared easily and made available in large quantities at a reasonable cost. In order to overcome the low bioavailability of polysaccharides, the study for the structure-activity relationship and chemical modification of the purified polysaccharide from *Meretrix petechialis* is currently under way.

Table I. Inhibition of vPE 16-induced syncytia formation in CD4 HeLa cells by thirteen polysaccharides prepared from Korean edible clams.

Sample	Fusion Index (FI)	% Fusion inhibition
Batillus crnutus	0.60 ± 0.02	6.70 ± 2.36
Cyclophorus herkloisi	0.64 ± 0.05	1.03 ± 8.18
Halocynthia roretzi	0.53 ± 0.08	18.04 ± 12.66
Meretrix petechialis	0.21 ± 0.03	67.53 ± 4.09
Meretrix lusoria	0.65 ± 0.04	0
Mytilus coruscus	0.48 ± 0.06	25.26 ± 9.06
Octopus minor	0.59 ± 0.04	8.76 ± 5.58
Ruditapes philippinarum	0.39±0.05	40.21±7.31
Scapharca broughtonii	0.69 ± 0.09	0
Scapharca sbcrenata	0.52 ± 0.05	19.07 ± 7.93
Sinonovacula constricta	0.37 ± 0.05	42.78 ± 7.09
Stichopus japonicus	0.56 ± 0.05	13.92 ± 7.94
Styela clava	0.54 ± 0.06	15.98 ± 9.32
Control	0.65 ± 0.05	0
Dextran sulfate	0.23 ± 0.04	63.92 ± 5.43

CD4 positive HeLa cells were incubated for 3 days and 100 μg/ml of polysaccharides, dextran sulfate and vPE 16 were added. After 16-20 h incubation, syncytia formation was observed. The fusion index (FI) and percent (%) fusion inhibition were calculated as described in the text. Each value is the mean ± S.E. of three experiments.

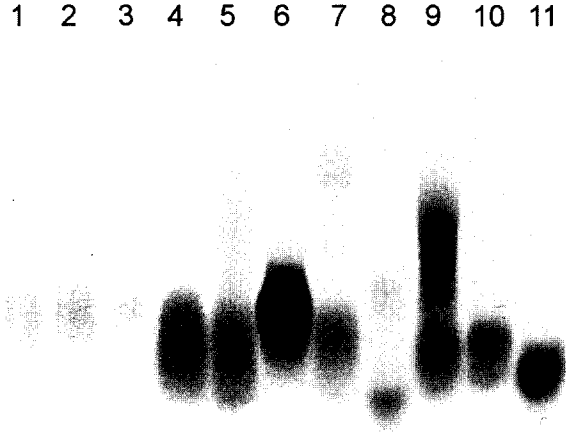

Fig. 1. Agarose gel-electrophoresis of clam polysaccharides. After running the electrophoresis, the gel was stained in 0.5% Azure A in 1% acetic acid and destained in water. Lane 1. *Batillus crnutus*, Lane 2. *Scapharca sbcrenata*, Lane 3. *Scapharca broughtonii*, Lane 4. *Mytilus coruscus*, Lane 5. *Ruditapes philippinarum*, Lane 6. *Meretrix petechialis*, Lane 7. *Sinonovacula constricta*, Lane 8. *Cyclophorus herkloisi*, Lane 9. *Styela clava* Lane 10. *Halocynthia roretzi*, Lane 11. *Dextran sulfate (MW: 10,000)*

ACKNOWLEDGEMENTS

This work was supported by the Korea Health 21 R & D Project, Ministry of Health & Welfare, Republic of Korea (HMP-00-B-21600-0126).

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