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The pseudoproteoglycan probes synthesized by conjugating unsulfated dextran with poly-L-lysine exhibit anti-HIV-1 activity

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ABSTRACT

Proteoglycans (PGs), which are present at animal cell surfaces and in the extracellular matrices, are involved in various biological events. PG consists of 1 or more sulfated glycosaminoglycan chains linked with a core protein, and a heparan sulfate-PG, syndecan is involved in the initial step of HIV-1 infection to the CD4+ lymphocytes and macrophages. Based on the structures of natural PGs, we synthesized new conjugated compounds that simulate macromolecular structure of PG, by combination of various types of unsulfated glycans and a core polypeptide, and named them as "Pseudoproteoglycans" (pseudoPGs). A pseudoPG synthesized by coupling 10 kDa poly-L-lysine (PLL) with 10 kDa dextran (Dex) exhibited remarkable anti-HIV-1 activity, which is more effective against R5 virus than sulfated polysaccharides, although neither PLL nor Dex has such an activity. Here we show the concept of the pseudoPG and the biological data focusing on the antiviral effects of the related compounds, to discuss the structure-activity relationship of the antiviral conjugates.

KEYWORDS: unsulfated glycan, dextran, α-poly L-lysine, HIV-1 suppression, pseudoproteoglycan (pseudoPG).

INTRODUCTION

The growth rate of the number of AIDS patients worldwide has declined in the last decade, while the number of those in the developing countries is still increasing, as reported by WHO media center (http://www.who.int/mediacentre/factsheets/fs360/en/). Because long-term administration of a combination therapy using various HIV-1 inhibitors increases the selective pressure on viruses to induce multiple drug-resistancy of the virus variants, an urgent need remains for novel antiretroviral drugs to HIV-1. Through a heparin-binding V3 loop in the envelop region, HIV-1 has been reported to utilize the cell surface heparan sulfate PG, syndecan, as a *cis* receptor to infect macrophages and as a *trans* receptor to infect T lymphocytes. Accordingly, use of the sulfated polysaccharides as competing agents has been

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reported to reduce HIV-1 attachment and entry into the macrophages and T-cells [1].

PGs are natural glycoconjugates which are composed of a core protein substituted with acidic polysaccharide chains, for example, sulfated glycosaminoglycan chains such as heparan sulfates, chondroitin sulfates, keratan sulfates, etc., and are present on the cell surfaces and in the extracellular matrices of various animals. Simulating the natural PG structure, we synthesized a group of neoglycoconjugates by conjugating various polysaccharide(s) with the linear amino acyl polymer, poly L-lysine (PLL) of various sizes (see Fig. 1). The whole structures of the neoglycoconjugates resemble those of the natural PGs present in animals, and therefore, they were designated as "pseudoPGs". Simulating the higher-order structure of PGs (Fig. 1), the new compounds were referred to as "pseudoPGs (pseudoPGs)" [2]. The conjugates contained on average 8 mol of Dex chains (10 kDa) per mole of PLL chain (10 kDa), based on the average molecular mass of each monomer, which was measured by SEC-MALLS [3].

The utility of a pseudoPG synthesized using dextran and PLL (Dex-PLL) as anti-HIV-1 reagent was expected from its binding activity to cyclophilin A which was reported to associate with the HIV-1 capsid protein and play important roles in viral assembly and disassembly [1]. In fact, the pseudoPGs were found to have inhibitory activity to the binding and entry of HIV-1 toward both the lymphocytes and macrophages [3]. In this study, we introduce a conception of the pseudoPGs and the basic data which demonstrate the utility of the pseudoPGs as probes to investigation and treatment of HIV-1.

MATERIALS AND METHODS

Materials

α-PLL hydrobromide (PLL, Molecular Weight (Mw) of 10 kDa), dextran sulfates (Mw of 50 k and 8 k), and azidothymidine (AZT), were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dextran T10 (Dex, Mw of 10 kDa) was purchased from GE Healthcare UK Ltd. (Buckinghamshire, England).

Synthesis of Dex-PLLs

Dex and PLL were conjugated by reductive amination as described previously [3]. Briefly, Dex (343.0 mg) and PLL (21.3 mg) were mixed in 600 µl of 0.25 M borate buffer (pH 8.1) in a round-bottomed flask, and then 400 µl of 2 M NaCl was added to the mixture. After pre-incubation at 45 °C for 2 hrs, 33.9 mg of $NaCNBH_3$ as a reducing agent was added to the mixture and incubated with continuous shaking at 45 °C for 14 days. The synthesized Dex-PLL was purified by gel filtration using TOYOPEARL® HW-50F column (25 mm i.d. x 800 mm, Tosoh Corporation,

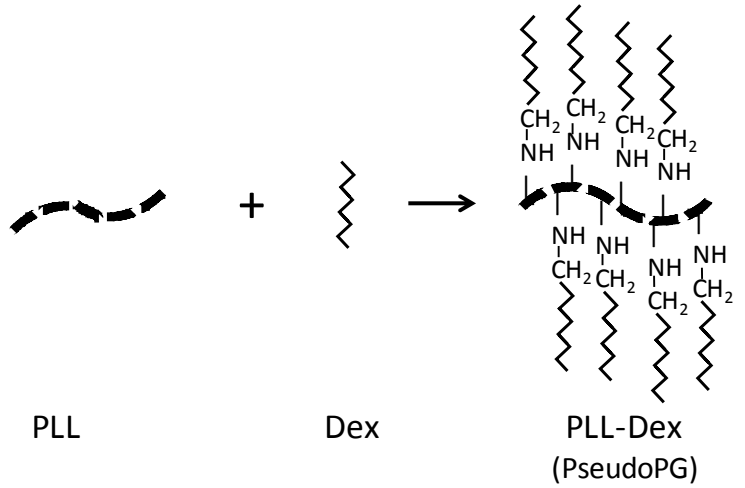

Fig. 1. Synthesis of PseudoPG. PLL was conjugated with Dex by reductive amination to produce pseudoPG [2] which simulates the natural PG structure.

Tokyo, Japan) with 0.067 M phosphate buffer (pH 7.4) as a running buffer at a flow rate of 0.9 mL/min and monitored at 220 nm. The first eluted, high-molecular-weight peak fractions which corresponded to the Dex-PLL were dialyzed against 0.1 M sodium acetate, and then desalted and concentrated by ultrafiltration using the Ultrafiltration membrane (NMWL: 1,000; Millipore Corporation, MA, USA). The purified Dex-PLL was lyophilized and stored at -25 °C until use. The concentrations of PLL and Dex were measured by BCA protein assay kit (Pierce Biotechnology, IL, USA) and by the phenol- sulfuric acid method, using PLL and glucose as standards, respectively. The concentration of the complex, Dex-PLL is expressed in mass concentration, which is equivalent to the sum of concentration of each raw material.

O-Sulfation of Dex-PLL

O-Sulfation was performed by chemical sulfation using chlorosulfuric acid after conjugation of Dex-PLL in a draft chamber. Briefly, Dex or Dex-PLL (about 0.6 mg each) was dissolved in 3 mL of formamide, and chlorosulfonic acid (200 µl) was slowly added on ice. By attaching a reflux condenser, the mixture was reacted at 5 °C for 5 hrs, and then dialyzed against water. For lower-sulfation of Dex or Dex-PLL, the mixture was reacted for 1 hr. After the reaction, the sulfated Dex-PLL was dialyzed against water. The sulfation was confirmed by proton nuclear magnetic resonance (^{1}H-NMR) or carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy.

Anti-HIV-1 activity and cellular toxicity

Anti-HIV-1 activity and toxicity assays were performed using N4R5/iGFP or N4X4/iGFP cells which are NP-2/CD4/CCR5 or NP-2/CD4/CXCR4 cells with HIV-1 CCR5-tropic Ba-L or CXCR4-tropic IIIB virus strain, respectively, and express GFP in nuclei when infected with the virus as described previously [3, 4]. Briefly, N4R5/iGFP or N4X4/iGFP cells were seeded at 5 x 10^3 cells/100 µl each on wells of 96 well plates using Eagle's minimum essential medium containing 10% (v/v) fetal calf serum and cultured overnight under 95% air and 5% CO_2 atmosphere at 37 °C. The N4R5/iGFP or N4X4/iGFP cells were incubated with 10 µl of various pseudoPGs dissolved in the medium (10 µl) at 37 °C for 1 hr, and then were infected by 100 µl of HIV-1, CCR5-tropic Ba-L or CXCR4-tropic IIIB virus solution, respectively. After 2 days of culture, IC_{50} and CC_{50} of each experiment were measured. IC_{50} of the pseudoPGs against HIV-1 was calculated from the numbers of GFP-positive cells compared to those of control. CC_{50} of the pseudoPGs was calculated from the cell number counted using the counting kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

RESULTS

The anti-HIV-1 activity of the pseudoPG, Dex-PLL

Dex-PLL was shown to have remarkable anti-HIV-1 activity in spite of the fact that both the materials, Dex and PLL do not have such an anti-HIV-1 activity. As shown in Fig. 2, using the system of the fluorescent-reporter cells developed by Islam *et al*. [4], Dex-PLL was found to have distinct anti-HIV-1 activity, i.e., suppression of proliferation of HIV-1 in human glioma NP-2 cells which had been prepared to express GFP as a signal of HIV-1 infection [3]. The activity was exhibited against both the macrophage-tropic R5 virus, Ba-L strain (Fig. 2, *left*) and T-cell-tropic X4 virus, IIIB strain (Fig. 2, *right*) as well as AZT, when compared to that of control (without drug), although the material compounds, neither PLL nor Dex, has such an activity.

Characterization of structural variation of pseudoPG

Table 1 summarizes the results of anti-HIV 1 activity (IC_{50}) and cellular toxicity (CC_{50}) of the synthesized pseudoPGs with or without sulfation in comparison with the materials, Dex and PLL and other related compounds. Though both the materials do not have anti-HIV-1 activity, the pseudoPG, Dex-PLL inhibited the infection of both Ba-L and IIIB viruses with low cytotoxicity. The inhibition was a little more effective to IIIB than to Ba-L virus. However, the IC_{50} of Dex-PLL was 1.8 times lower than that of dextran sulfate (50 k) against Ba-L while the IC_{50} of Dex-PLL was 5.7 times higher than that of dextran sulfate (50 k) against IIIB virus, indicating that Dex-PLL was more effective against R5 virus than sulfated polysaccharides. Dex-PLL suppressed a clinically isolated R5 virus from primary peripheral blood

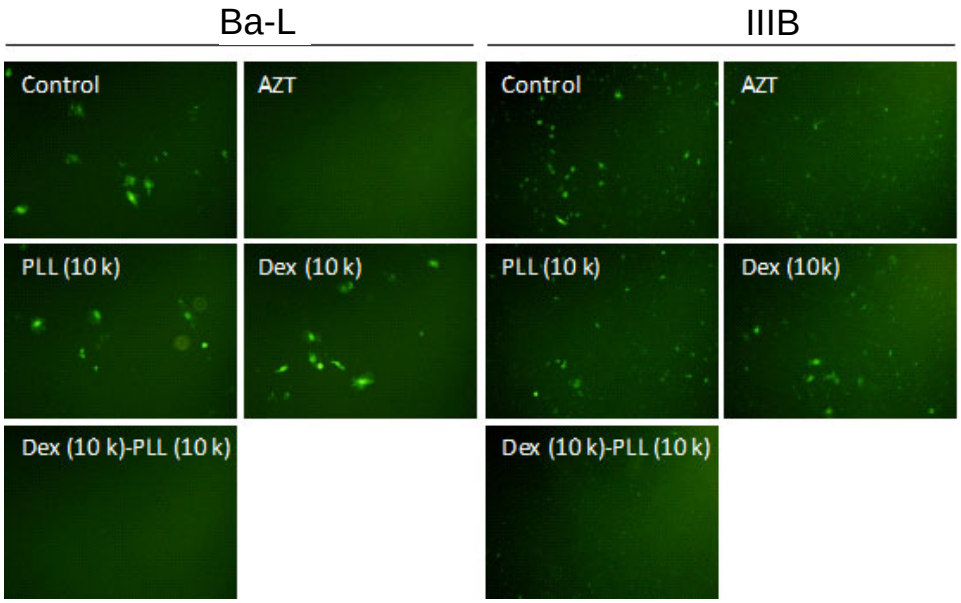

Fig. 2. Anti-HIV-1 activities of Dex-PLL, the components of Dex-PLL, and AZT. The anti-HIV-1 activities of PLL, Dex, PLL-Dex, and AZT against viral strain Ba-L were measured using N4R5/iGFP cells (*left*), and against viral strain IIIB using N4X4/iGFP cells (*right*). AZT (10 μg/ml), a known anti-HIV-1 compound, was used as the positive control. Concentrations of PLL, Dex, and PLL-Dex were 1, 100, and 100 μg/ml, respectively. The green fluorescence indicates the HIV-1-infected cell.

Table 1. Anti-HIV-I activity and cellular toxicity of pseudoPGs, their components and the related compounds.

PseudoPG	IC50 (μg/ml)		CC50 (μg/ml)	Ref.
	N4R5/iGFP	N4X4/iGFP	N4R5/iGFP	
	Ba-L	IIIB		
Dex	>100	>100	>100	[3]
PLL	>1	>1	25	[3]
Dex-PLL	3.8	1.2	>100	[3]
High-sulfated Dex	>100	5.3	>100	
Low-sulfated Dex	>100	10	>100	
High-sulfated Dex-PLL	>100	>100	>100	
Low-sulfated Dex-PLL	>100	33	>100	
Sulfated PLL	>100	>100	10-100	
Dextran sulfate (50 k)	6.7	0.21	>100	[3]
Dextran sulfate (8 k)	0.14	0.02	>100	
AZT	0.02	0.02	>100	[3]

mononuclear cells. Sulfation of Dex-PLL markedly lowered its antiviral activity, suggesting that the anti-HIV-1 mechanism of Dex-PLL is different from that of dextran sulfate. The anti-HIV-1 activity of Dex-PLL also showed the dependency of the chain lengths in the three kinds of assay systems including a human $CD4^+$ T-cell line [3].

DISCUSSION

Our findings indicate that the pseudoPG synthesized by conjugating PLL with various unsulfated glycans acquire the anti-HIV-1 activity upon conjugation, which open new insights into both structural and functional aspects of the new synthetic pseudoPG probes to anti-HIV-1 reagents.

As a treatment of AIDS, multiple drugs against HIV-1 have been simultaneously administered to the patients, which may often cause the tendency of the virus to drug resistancy. We found that Dex-PLL shows a remarkable anti-HIV-1 activity, although neither material had such an activity. Dex-PLL also suppressed a clinically isolated R5 virus from primary peripheral blood mononuclear cells [3]. Because the variability of the structure of pseudoPG conjugate has a wide potential to generate different conjugates by only changing the glycan moieties, it is expected to rapidly produce new therapeutic medicines having different structures for the patients whose virus attained resistency against existing drugs.

CONCLUSION

The preventive mechanisms of the pseudoPG, Dex-PLL against HIV-1 have been studied to some extent [3], and the results suggested that Dex-PLL may have unique multiple preventive mechanisms against HIV-1 at not only adsorption and entry to the cells, but at the multiple stages including intracellular steps (our unpublished results). In contrast to the hitherto reported anti-HIV-1 compound based on sulfated polysaccharide origin, the pseudoPGs showed a contrasting anti-HIV-1 efficacy by suppressing the sulfated glycan-resistant R5 virus as well as X4 virus (Table 1). However, the detailed elucidation and verification of the anti-HIV-1 mechanism of pseudoPG remains to be clear, because our preliminary study suggested that the pseudoPGs may act on the viruses at multiple stages of infection. The elucidation of anti-HIV-1 mechanism of pseudoPG will open new insights into the development of a potential anti-HIV-1 agent acting *via* a novel prevention mechanism.

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CONFLICT OF INTEREST STATEMENT

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