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Further characterization of sulfated homopolysaccharides as anti-HIV agents

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Summary. Fucoidan and dextran sulfate showed anti-HIV activities against mononuclear cells from AIDS patients, and they abrogated HIV reverse transcriptase (RT) activity by interacting with the HIV envelope in the membranes of target cells. Furthermore, they showed a synergistic effect with azidothymidine (AZT).
Key words. Dextran sulfate; fucoidan; anti-HIV agents.

It is important and urgent to find a potent anti-HIV agent that can be utilized safely under clinical conditions. We have previously reported that sulfated homopolysaccharides with immunomodulating activities are more potent anti-HIV agents than sulfated heteropolysaccharides[1]. This study evaluates the actions of dextran sulfate and fucoidan, two typical sulfated homopolysaccharides, against peripheral mononuclear cells (MNC) from two AIDS patients in vitro, and attempts to elucidate the mechanism of action of these chemicals.

Materials and methods

Peripheral MNC from two hemophiliacs with AIDS (cases 1 and 2) were obtained by centrifugation over Ficoll-Paque cushions at 2000 rpm for 20 min[2]. The MNC were co-cultured for 10 days with lymphoblasts activated with PHA (10 μg/ml) for three days in the presence of dextran sulfate or DEAE dextran as a negative control. Reverse transcriptase (RT) activities in the culture supernatants were measured according to the method of Daniel et al.[3]. Briefly, Molt-4. clone no. 8 cells were cultured with the supernatant from HIV-infected TALL-1 cells. The cell suspension was centrifuged at 1300 rpm for 10 min. The supernatant (1.5 ml) was centrifuged at 25,000 rpm for 90 min to pellet viruses. The virus pellet was resuspended in 10 μl of dissociation buffer. To this was added 90 μl of a solution containing 0.04 M Tris-HCl, 0.1 M $MgCl_2$, 0.045 M KCl, poly (rA): oligo (dT)/ml, and 0.004 M dithiothreitol, and then 2.5 μl of [methyl-^{3}H]thymidine 5'-triphosphate (30 Ci/nmol) was added to the reaction mixture. After shaking in a water bath at 37 °C for 60 min, each sample (100 μl) was spotted onto a glass fiber filter, washed, and rinsed with ethanol. The filter was counted in a liquid scintillation counter. The background count (HIV-free) was usually less than 2500 cpm.

Next, the following experiments were carried out to elucidate the mechanism of action of dextran sulfate and fucoidan. Molt-4. clone no. 8 cells (10^6/ml) as a target[4] were cultured overnight at 37 °C in a humidified incubator with 5% CO_2 in air in the presence of fucoidan (100 μg/ml) or dextran sulfate (100 μg/ml). Thereafter, the target cells were carefully washed three times and were cultured for 10 days with HIV-containing supernatants whose RT activity was more than 5×10^5 cpm/ml. The effect of fucoidan or dextran sulfate upon HIV was evaluated in terms of RT activity and cytopathic affect (CPE) score[5].

Furthermore, in order to clarify the mechanism of action in more detail, Molt-4. clone no. 8 cells were cultured in the presence of HIV for four different time intervals (2, 6, 12, and 24 h). Thereafter, the cells were carefully washed three times so that the remaining HIV were removed completely. The HIV-treated cells were cultured in the presence of fucoidan and dextran sulfate for 10 days and RT activities in the culture supernatants, as well as the CPE score, were determined as described above. A

ten-day culture was most suitable for measurement of RT activity.

We also carried out experiments to see whether fucoidan and dextran sulfate react with HIV in a target cell-free system or not. Fucoidan or dextran sulfate was reacted with intact HIV-containing supernatants for 30 or 60 min. After centrifugation at 15,000 rpm for 90 min, various supernatants were subjected to RT assay as stated previously.

Fucoidan and azidothymidine (AZT) were added to microculture wells in the presence of Molt-4. clone no. 8 (1×10^6/ml) and HIV and co-cultured for 10 days. Thereafter, CPE score and RT activities were measured[3, 5].

Results and discussion

There was little or no RT activity in the dextran sulfate (100 µg/ml)-treated group, whereas RT activity was present in the no-treatment group and the DEAE dextran-treated group, which suggests that dextran sulfate has an anti-HIV effect on MNC from patients with AIDS (table 1). HIV could survive in the Molt-4. clone no. 8 cells pretreated with fucoidan and dextran sulfate, which indicates that fucoidan was not taken up into the cytoplasm of Molt-4. clone no. 8 cells, but dextran sulfate was slightly taken up into the cytoplasm (table 2). No statistically significant difference was found in RT activity between the fucoidan-pretreated group and the no-pretreatment group.

Table 3 summarizes the results of the RT assay and CPE scores. In both fucoidan and dextran sulfate-treated groups, there was almost no RT activity in the supernatants of cells pretreated with HIV for 2 to 12 h, suggesting that HIV invade the cytoplasm of target cells slowly and that fucoidan and dextran sulfate can still react with HIV in the cell membranes. RT activities were significantly decreased when HIV were cultured with fucoidan or dextran sulfate; indeed, fucoidan almost completely abrogated RT activity (table 4). This suggests that these compounds bind to *env* protein of HIV. Fucoidan synergized with AZT at much lower concentrations (table 5). For example, a combination of 0.1 µg/ml fucoidan and 0.014 µM AZT might be used clinically. If fucoidan, dextran sulfate and AZT are given clinically at the total doses of 300 mg im, 300 mg im and 1200 mg per os, respectively, it is possible to maintain concentrations of more than 0.1 µg/ml fucoidan and dextran sulfate and

Table 1. Effect of dextran sulfate on co-culture of mononuclear cells from AIDS patients with PHA-stimulated lymphocytes from healthy volunteers*.

Case no.	Chemical used	RT activity (cpm) at day 10
1	Dextran sulfate (10 µg/ml)	81,414
	Dextran sulfate (100 µl/ml)	28,210
	–**	92,417
2	DEAE dextran (100 µg/ml)	9,781
	Dextran sulfate (100 µg/ml)	617
	–**	13,703

*Mononuclear cells from AIDS patients (cases 1 and 2) were co-cultured with 3-day cultured PHA-stimulated (10 µg/ml) lymphocytes from healthy volunteers for 10 days in the presence of dextran sulfate or DEAE dextran. RT activities in the culture supernatants were determined according to the method of Daniel et al.[3]. **no addition of a chemical.

Table 2. Effect of one-day pretreatment with fucoidan or dextran sulfate on HIV infection against Molt-4. clone no. 8 cells pretreated with fucoidan or dextran sulfate*.

Chemical	RT activity (cpm)	CPE score
Fucoidan (100 µg/ml)	339,738	4
Dextran sulfate (100 µg/ml)	93,482	2
–**	1,694	-
HIV alone	308,177	3

*RT activities in the culture supernatants were determined by the method of Daniel et al.[3]. CPE was judged based on the criteria proposed by Lifson et al.[5]. **no addition of a chemical.

Table 3. Effect of fucoidan or dextran sulfate, added at different time intervals, on HIV-infected Molt-4. clone no. 8 cells*.

Chemical	Preincubation time (hour) 2	6	12	24
Fucoidan (100 µg/ml)	2,220(–)	3,439(–)	4,181(–)	12,429(–)
Dextran sulfate (100 µg/ml)	2,225(–)	3,385(–)	4,020(1)	14,501(2)
–**	1,179(–)	1,086(–)	1,004(–)	1,482(–)
HIV alone	61,311(3)	151,910(4)	276,461(4)	820,799(4)

*RT activities (cpm) determined by the method of Daniel et al.[3]; CPE score evaluated by the method of Lifson et al.[5]. **no addition of a chemical.

Table 4. Effect of fucoidan or dextran sulfate on Molt-4. clone no. 8 cell-free HIV-containing supernatant*.

Chemical used (µg/ml)	Incubation time of a chemical with HIV 30 min	60 min
–**	537,623 (cpm)	655,231 (cpm)
Fucoidan (100)	79,020	71,397
Dextran sulfate (100)	105,895	158,946

*The figure represents RT activity (cpm) measured by the method of Daniel et al.[3]. **no addition of a chemical.

Table 5. Synergistic anti-HIV activity induced by fucoidan and AZT.

Fucoidan (µg/ml)	AZT (µM) 0.375	0.125	0.042	0.014	0.005	–+
10	1330* (1)**	921 (1)	1977 (1)	2660 (1)	6584 (1)	12,100 (2)
1	5116 (1)	4610 (1)	25,413 (1)	8655 (2)	30,191 (3)	159,157 (3)
0.1	9708 (1)	3716 (1)	8054 (2)	9177 (2)	14,861 (4)	46,153 (4)
–+	9608 (3)	3186 (3)	7742 (3)	8162 (3)	13,315 (4)	90,704 (4)

*RT activity (cpm); **CPE score; +no addition of a chemical.

more than 0.014 μM AZT in sera (Personal communication with Dr S. Kimura). These doses may be used for the treatment of hemophiliacs with AIDS.

It is well known that sulfated polysaccharides act as anticoagulants as well as mitogens[6-12]. Our data suggest that these sulfated homopolysaccharides could be used in combination with AZT for the treatment of AIDS which would be advantageous because the combination significantly lowers occurrence of side effects.

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The ear-shell (*Sulculus diversicolor aquatilis*) myoglobin is composed of an unusual 39 kDA polypeptide chain

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Summary. An unusual myoglobin was isolated from the buccal mass of the ear-shell *Sulculus diversicolor aquatilis*. The myoglobin consists of a 39 kDa polypeptide chain which is about double the size of the usual myoglobin subunit, contains one heme per molecule, and has an unusual spectral property in the oxy-form. On the basis of these properties and partial amino acid sequencing, we propose that *Sulculus* myoglobin has a didomain structure, and that one of the two domains does not function as an oxygen-binding domain. So far, a myoglobin of this type has not been described in molluscs.
Key words. Myoglobin; didomain structure; *Sulculus*.

A number of molluscs have remarkable red muscles in the buccal mass and triturative stomach[1,2]. Myoglobin is abundant in such red muscle, and the subunit structure of the molluscan pigment is either a monomer or dimer consisting of a molecular weight (M_r) 15,000–18,000 polypeptide chain in all species investigated so far.

Recently, an unusual didomain structure was found in the hemoglobins of the clams *Barbatia reeveana*[3] and *Barbatia lima*[4]. Interestingly, a closely related species, *Barbatia virescens*, has no hemoglobin with such a structure[4]. Thus, molluscan globins show remarkable diversity in subunit structure and constituent chain[2], and therefore they would be an excellent source for the elucidation of the molecular evolution of globins.

We isolated myoglobin from the ear-shell *Sulculus diversicolor aquatilis* and found that the myoglobin has unique characteristics in spectral property, autoxidation and subunit structure, compared with other molluscan myoglobins.

Materials and methods

About 25 g of buccal mass of *Sulculus* was used in one preparation. All procedures were carried out at low temperature (2–4 °C) as far as possible. The water extract was fractionated with ammonium sulfate between 55 and 90% saturation at pH 7.2. The crude myoglobin fraction was dissolved in a minimum volume of 50 mM phosphate buffer (pH 7.2) and was passed through a Ultrogel AcA 44 column (3 × 110 cm) equilibrated with 50 mM phosphate buffer (pH 7.2). The myoglobin fraction was pooled and ammonium sulfate was added up to 55% saturation. This solution was then applied to a Butyl-Toyopearl 650 M column (1.6 × 13 cm) equilibrated with 50 mM phosphate buffer (pH 7.2) containing 55% ammonium sulfate, and eluted with a linear gradient of the same solution (300 ml) to 50 mM phosphate buffer (pH 7.2) (300 ml). The myoglobin thus obtained was dialyzed against 20 mM Tris-HCl buffer (pH 8), and was kept at 2 °C until used. The heme concentration of myoglobin