bal adhesion[2]. Therefore, prevention of *C. trachomatis* transmission during sexual intercourse is most important. Currently, the only effective technique to prevent genital *C. trachomatis* infection is the proper use of a condom. If used correctly, it can reduce the risk of *C. trachomatis* transmission. However even under these circumstances, development of a new preventive measure is considered worthwhile.

Infection of the organism to the host epithelial cells has been reported to be mediated by the chlamydial attachment to heparan sulfate proteoglycans on the host extra-cellular matrix. We previously described that heparan sulfate analogues, heparin and its 2-O-desulfated derivative, significantly inhibited *C. trachomatis* serovar L2 infection[3]. However, *C. trachomatis* serovar L2 is rare. Therefore, we needed to examine if their analogues are also effective on the inhibition *C. trachomatis* serovar D which accounts for more than 50% of genital C. *trachomatis* infection.

Using serovar D in this study, we have examined whether heparin, its 2-O-desulfated derivative, and 6-O-desulfated derivative (Fig. 1) reduce the infectivity of *C. trachomatis* serovar D to genital epithelial cells. We also determined whether these polysaccharides are potentially beneficial as antichlamydial microbicides.

MATERIALS AND METHODS

Heparin and its derivatives

The periodate-oxidized heparin, its chemically 2-O-desulfated derivatives (2-ODS heparin), and its 6-O-desulfated derivatives (6-ODS heparin) were obtained from Seikagaku Corp. (Tokyo, Japan). Periodate-treated heparin and these O-sulfated derivatives have been shown to have no anti-coagulation activities[4]. Without exception, heparin and the derivatives used in this study are all periodate-treated.

Assessment of inhibition of C. trachomatis infectivity

HeLa 229 cells were plated in 96-well tissue

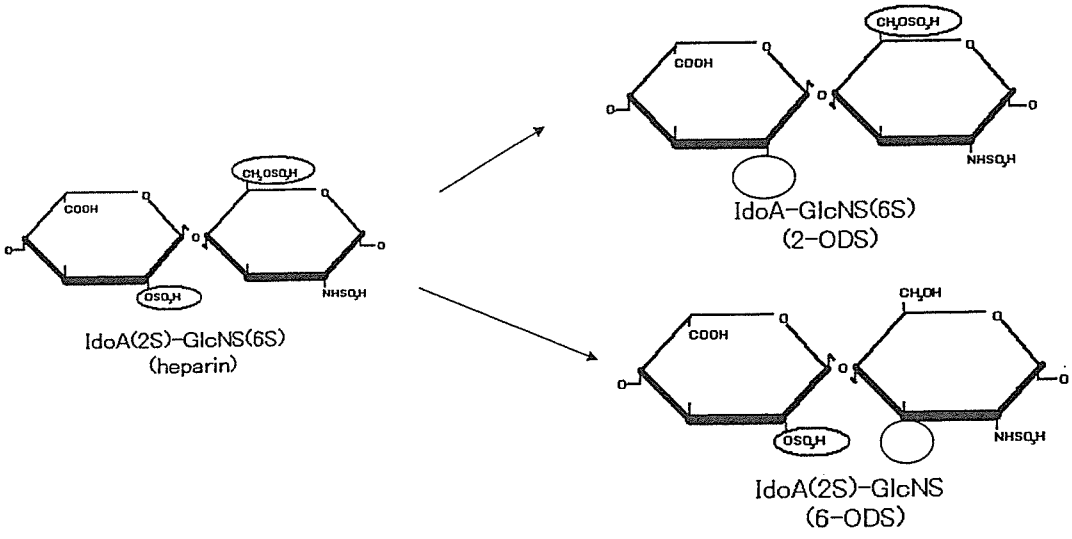

Fig. 1. Structure of heparin isomer unit
The periodate-oxidized heparin, its chemically 2-O-desulfated derivatives (2-ODS heparin), and its 6-O-desulfated derivatives (6-ODS heparin) is shown. Periodate-treated heparin and these O-sulfated derivatives have been shown to have no anti-coagulation.

culture plastic plates and maintained at 37℃ in 5% CO_2 for 24 hr in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum. After the HeLa 229 cells formed confluent monolayers, the medium was removed. *C. trachomatis* server D/UW-3/Cx was diluted with a sucrose-phosphate glutamic acid (SPG) medium to a concentration of 4×10^4 IFU/ml, and the monolayered HeLa 229 cells were then inoculated with 250 μl of *C. trachomatis*. The infected monolayers were centrifuged for 1 hr at 600 ×g at room temperature, and the supernatants containing *C. trachomatis* were discarded. Each well was washed with a fresh culture medium once and incubated with 500 μl of the culture medium supplemented with cyclohexamide (1 μg/ml) at 37℃ in 5% CO_2 for 72 hr. After incubation, infected monolayers containing HeLa 229 cells were fixed with ethanol. *C. trachomatis* inclusions produced in the cytoplasm of HeLa 229 cells were then stained by means of ABC technique using monoclonal antibody of mouse anti-human *C. trachomatis* (Biomeda) as a primary antibody solution. Inhibition of infectivity by various reagents was determined by counting the average number of inclusions in four microscopic fields containing infected cells stained with hematoxylin and eosin.

Inhibition of C. trachomatis serovar D infectivity by heparin

HeLa 229 cells were inoculated with *C. trachomatis* serover D and then incubated with heparin or its derivatives to assess inhibition of *C. trachomatis* serovar D infectivity. Furthermore, we investigated if an inhibitory effect of heparin on the intracellular chlamydial growth either before or after *C. trachomatis* is attached to the host cells. In this experiment, reagents were added either to SPG, designated SPG (+) culture fluid (−), or to culture fluid, designated SPG (−) culture fluid (+). Heparin or the derivatives at concentrations of 0.1 μg/ml, 1.0 μg/ml, 10 μg /ml, and 100 μg/ml were added to either SPG or culture fluid, and the mixed fluids remained for 30 min at room temperature, followed by centrifugation.

Inhibition of C. trachomatis serovar D infectivity after receiving heparin derivatives

Heparin and its derivatives were examined for the inhibition activity of *C. trachomatis* infectivity by the methods described above. To determine whether inhibition by heparin or its derivatives involves *C. trachomatis* before adhesion to host cells or HeLa 229 cells, 4×10^4 IFU of *C. trachomatis* was adsorbed for 30 min to SPG-diluted heparin or its derivatives at concentrations of 0.1 μg/ml, 1.0 μg/ml, 10 μg/ml, and 100 μg/ml. The pretreated *C. trachomatis* (500 μl) was added to the top of 500 μl of 30% sucrose in a 1.5 ml tube and centrifuged for 1 hr at 8000 ×*g*, by which *C. trachomatis* was separated from heparin or the heparin derivatives. A yield of 50 μl of sediment containing *C. trachomatis* was obtained from the bottom of the tube and then mixed with 100 μl of SPG. The mixture was inoculated to each of 96-well plastic plates containing confluent monolayered HeLa 229 cells, and inhibition of inclusion formation was examined. After *C. trachomatis* and heparin at various concentrations of 0.1 μg/ml, 1.0 μg/ml, 10 μg/ml, and 100 μg/ml were added to SPG, the mixture stood at room temperature for incubation. After 30 min, the number of inclusions was measured before and after contact with HeLa 229 cells.

Each assay was performed in duplicate, and in each figure bars indicate standard devia-

tion. We used t-test in each assay.

RESULTS

Inhibition of C. trachomatis serovar D infectivity by heparin

Inhibition of *C. trachomatis* infection was assessed by the addition of heparin before and after attachment of the organism to HeLa 229 cells. The group receiving reagents before attachment of *C. trachomatis* was defined as SPG (+) culture fluid (−), and the other group receiving reagents after attachment of *C. trachomatis* was defined as SPG (−) culture fluid (+). The SPG (+) culture fluid (−) showed a dose-dependent inhibition of infectivity with a reagent concentration of 1 μg/ml or larger, whereas SPG (−) culture fluid (+) did not (Fig. 2).

Inhibition of C. trachomatis serovar D infectivity by heparin derivatives

Two different heparin derivatives, 2-ODS and 6-ODS, were tested for effects on *C. trachomatis* infection of HeLa 229 cells. Addition of 2-ODS before attachment to the host cells revealed a significant dose-dependent reduction of infectivity when its concentration was more than 0.1 μl/ml. In contrast, 6-ODS did not show any obvious inhibition (Fig. 3).

Further experiments were done to determine whether effective reduction of *C. trachomatis* infectivity by heparin and 2-ODS results from their influence on the organism before attachment to host cells, or their direct influence on the host cells. *C. trachomatis*, mixed with SPG supplemented with heparin or 2-ODS, was centrifuged to remove reagents, and was inoculated to host cells. Although heparin or 2-ODS heparin was absent at time of attachment of *C. trachomatis* to host cells, inhibition of infectivity occurred in a dose, dependent manner with concentrations more than 0.1 μg/ml (Fig. 4).

DISCUSSION

In this in vitro experimental study we have examined whether heparin and its derivatives as well as heparin, inhibit infectivity and growth of *C. trachomatis* serovar D in cultured HeLa 229 cells derived from human cervical carcinoma. The effect of heparin on *C. trachomatis* serovar L2 had already been

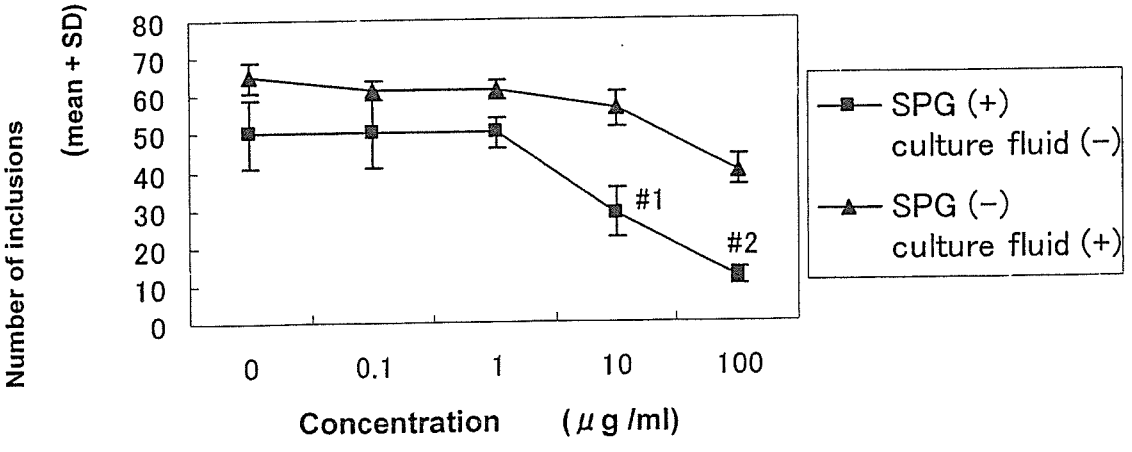

Fig. 2. Inhibition of *C. trachomatis* infection by heperin
The SPG (+) culture fluid (−) showed a dose-dependent inhibition of infectivity with a reagent concentration of 1 μg/ml or larger, whereas SPG (−) culture fluid (+) did not.
#1, #2 $p<0.001$ vs 0 μg/ml (SPG+culture fluid−)

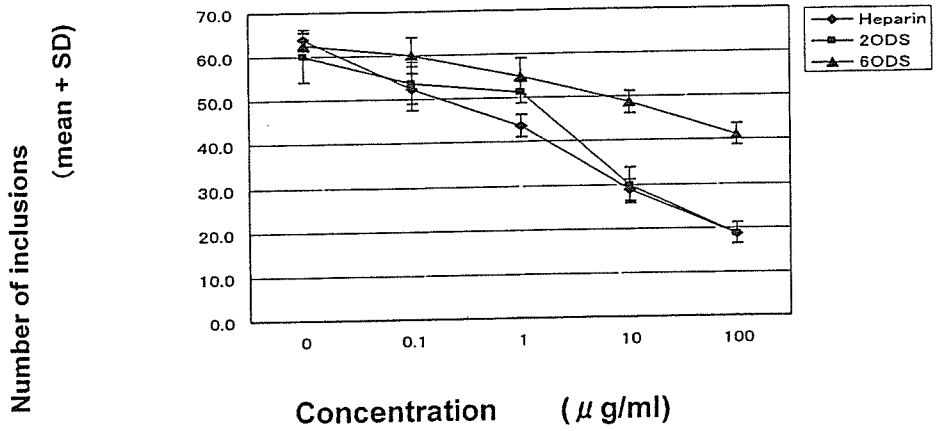

Fig. 3. Effect of heparin, 2-ODS and 6-ODS on inhibition *C. trachomatis* infection
Addition of 2-ODS before attachment to the host cells revealed a significant dose-dependent reduction of infectivity when its concentration was more than 1 μg/ml. In contrast, 6-ODS did not show any obvious inhibition.
n.s. heparin vs 2-ODS (1 μg/ml~100 μg/ml)
$p<0.01$ heparin vs 6-ODS (1 μg/ml~100 μg/ml)

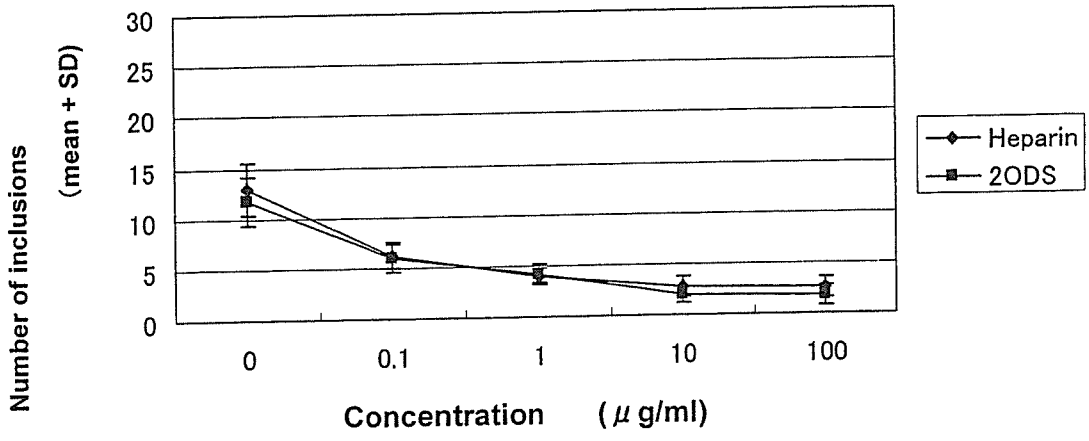

Fig. 4. Inhibition of infection of after removing excess heparin and heparin derivatives
After removing excess heparin and heparin derivatives, heparin and 2-ODS showed a dose-dependent inhibition of infectivity.

examined in our previous study[3].

Heparan sulfate proteoglycans are a group of cell surface components usually anchored to the plasma membranes via transmembrane core proteins or glycosylphosphatidylinositol[4]. These proteoglycans bind to a large spectrum of cellular ligands, including matrix components, growth factor, lipolytic enzymes, protease inhibitors, and transcriptional regulators[5]. In addition, heparan sulfate proteoglycans have been shown to function as receptors for a variety of infectious agents[6,7].

Our results demonstrated that heparin is able to lead to a significant reduction in intracytoplasmic inclusions of *C. trachomatis* serovar D when its concentration is 10 μm/ml or more. Furthermore, we showed that formation of intracytoplasmic inclusions due to *C. trachomatis* serovar D is markedly inhibited by addition of heparin when administered before attachment of *C. trachomatis* serovar D to host cells. Therefore, it is likely

that heparin also affects the attachment of *C. trachomatis* serovar D to the host cell cytoplasm.

Results of our in vitro experiments provide evidence that both 2-ODS heparin and heparin, of which had been lost completely, by periodate oxidization anti-coagulant activities are also able to reduce infectivity, as revealed by a decrease in the number of intracytoplasmic inclusions by *C. trachomatis* serovar D. These results suggest that inhibition appears to depend on the heparan sulfate structure and that administration of heparin before *C. trachomatis* serovar D attachment to the genital epithelial cells may produce a potential chemotherapeutic interaction for the prevention of *C. trachomatis* serovar D. The results from this study on *C. trachomatis* serovar D are the same as those of our previous study on serovar L2. Since inhibition of *C. trachomatis* infectivity was shown by both serovar groups, the result supports the effectiveness of heparin and 2-ODS heparin, and the idea that they could be used in practice. Collectively, 2-ODS heparin as well as heparin interacts with *C. trachomatis* prior to adhesion to host cells. Antigen detection PCR is available for diagnosis of *C. trachomatis*-caused diseases with a high degree of certainty and a course of macrolide and new quinoline antibiotics is the treatment of choice for *C. trachomatis*-caused genital diseases. Aside from diagnosis and therapy, few measures are available for the prevention of *C. trachomatis* infection. Of the heparin and its derivative evaluated in this in vitro study, 2-ODS heparin without anticoagulation action can be used for the prevention of *C. trachomatis* infection. Further clinical studies are needed in this issue.

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