Inhibition of in Vitro Fertilization of Mouse Gametes by Sulfated Sialic Acid Polymers

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The effect of sialic acid (N-acetyl neuraminic acid), sialic acid dimer, sialic acid polymers (colominic acid) and sulfated colominic acid on the activity of hyaluronidase, on the dispersion of cumulus cells by mouse sperm and on in vitro mouse fertilization (sperm penetration of zona pellucida) were evaluated. Bovine testicular hyaluronidase activity was significantly inhibited by colominic acid and sulfated colominic acid, but not by sialic acid and its dimer. The dispersion of cumulus cells from eggs by mouse sperm was also inhibited by colominic acid and sulfated colominic acid. In vitro fertilization of mouse gametes was inhibited by sulfated colominic acid. The IC₅₀ value of sulfated colominic acid-induced inhibition of fertilization was 0.3 mg/ml (ca. 0.9 ms). The value changed from 0.9 ms for cumulus-surrounded egg to 1.5 ms for cumulus free-egg. On the other hand, colominic acid showed little or no inhibitory effect on mouse in vitro fertilization at 0.5 mg/ml (ca. 1.6 ms). This antifertility activity by sulfated colominic acid did not appear to be due to an effect on sperm motility or on the oocytes. These results suggest that (1) the cumulus cells surrounding the eggs were dispersed by sperm hyaluronidase, (2) hyaluronidase was inhibited by colominic acid and by sulfated colominic acid, (3) sulfated colominic acid inhibits sperm penetration of zona pellucida by the inhibition of hyaluronidase and/or some enzymes required for mouse gametes fertilization.

Key words sulfated sialic acid polymers; fertilization; hyaluronidase

The head of the mammalian spermatozoon is occupied mostly by the nucleus and acrosome. The acrosome originates from the Golgi complex in the spermatic tubules and contains enzymes (such as acrosin, hyaluronidase, neuraminidase and arylsulfatase) necessary for the sperm to penetrate and/or fuse with the plasma membrane of the egg to achieve fertilization. In those enzymes, acrosin and hyaluronidase are unique to the sperm acrosome and are major constituents of the acrosome. 1) Hyaluronidase, a sperm acrosomal enzyme involved in fertilization, 2,3) is a bifunctional protein with putative roles in the dispersion of cumulus oophorus cells as well as zona adhesion. 4,5) The enzyme is found in acrosomes in a soluble form, but also exists as a membrane-bound form. 5,6) The cell surface hyaluronidase was reported to be inhibited by gossypol or fully sulfated glycosaminoglycans. 7,8) Recently, Moreno et al. reported that sulfated polysaccharide bind to proacrosin and acrosin and inhibit the sperm from binding to the zona pellucida. 9) Baba et al. reported that acrosin is not essential for sperm penetration 10) but is involved in the dispersal of the acrosomal matrix after acrosome reaction. 11) Therefore, the inhibition of hyaluronidase and/or acrosin may prevent the sperm-egg interaction. The role of the other acrosomal enzymes is unclear.

The newly ovulated mammalian egg is surrounded by a mass of cumulus oophorus and zona pellucida. Rabbit testicular arylsulfatase alone disperses the cumulus oophorus of rabbit ova and in combination with hyaluronidase accelerates the rate of dispersion. 12) As sialic acid is an important component of mouse zona pellucida, sperm-associated sialyltransferase activity may affect sperm-zona interaction. 13)

Recently, we prepared sulfated sialic acid polymers. 14) In this study, we measured the hyaluronidase inhibitory activity (i.e. inhibition of the dispersion of cumulus cells) of sialic acid polymers and assayed the effect of sialic acid polymers on in vitro mouse fertilization.

MATERIALS AND METHODS

Materials Apigenin, hyaluronic acid (from human umbilical cord), bovine testicular hyaluronidase (Type 1-S), N-acetyl neuraminic acid and bovine serum albumin (BSA, Fr V) were purchased from Sigma Chemical Co.(St. Louis, U.S.A.). Hoechst 333258 was from Calbiochem (La Jolla, U.S.A.). Colominic acid (Escherichia coli), of which the mean Mr is 17 kDa, was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were from Wako Pure Chemical Industries (Osaka, Japan) and Nacalai Tesque. Sulfated colominic acid (Col-S) and N-acetyl neuraminic acid dimer (DP-2) were prepared as previously described. 15) Sulfated colominic acid with a sulfur content of 5.3% and a mean Mr of 17 kDa was used as sulfated colominic acid.

Hyaluronidase Activity To prepare the inhibitory solutions, sialic acid derivatives were dissolved in 20 mM phosphate buffer (pH 6.8) at a concentration of 3.2 mg/ml. Apigenin was initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mM. These stock solutions were subsequently diluted with the same phosphate buffer. Hyaluronidase activity was assayed as follows. Three hundred micro liter of bovine testicular hyaluronidase solution (10 units/ml in the same phosphate buffer) and 0.1 ml of inhibitor solution in the same buffer were preincubated for 5 min at 37°C. The enzyme reaction was started by the addition of 0.3 ml of substrate solution (hyaluronic acid, 0.5 mg/ml in 0.3 M phosphate buffer, pH 5.32) and incubated at 37°C. At 20 min, 3 ml of acid albumin solution (0.1% BSA in acetate buffer, composed of 0.326 g sodium acetate/0.456 ml acetic acid in 100 ml distilled water) was added to the solution. After exactly 5 min at room temperature, the turbidity of the
solution was analyzed at 600 nm by spectrophotometer (Shi-
madzu UV 120-02). To evaluate the inhibitory effect of sialic acid derivatives on hyaluronidase activity, apigenin was used as a standard.

**Preparation of Sperm Suspension** Sperm were collected from ICR male mice (35–40 g) as previously described. The cauda epididymis from mature male mice was removed. The end of the epididymal ducts and the epidi-
didymis were cut into several pieces and gently placed in 3 ml of modified Kreb's Ringer bicarbonate buffer containing 4 mg/ml of BSA (m-KRB) and incubated at 37°C. After 10 min, approximately 40 µl of the upper part of the medium, containing 4 × 10⁴ motile sperm, was used as sperm suspension.

**Collection of Eggs and Preparation of Cumulus-Free Eggs** Eggs were collected from ICR female mice (25–30 g) as previously described. Mice were injected intraperi-
toneally with 10 i.u. PMSG (Teikokuiz, Tokyo, Japan) fol-
lowed 48 h later by 10 i.u. hCG (Teikokuiz, Tokyo, Japan). The mice were killed 14 h after receiving hCG. The oviducts were excised and teased in m-KRB. The oocytes in cumulus masses were transferred to m-KRB. The eggs were incubated in m-KRB gassed with 5% CO₂–95% air at 37°C until use. Cumulus-free eggs were prepared as follows. The cumulus-
surrounded eggs were transferred into 0.1 units/ml of hyaluronidase solution in m-KRB and incubated in a CO₂ in-
cubator for 5 min. When the eggs were freed from follicle cells, they were washed twice with m-KRB and incubated in m-KRB gassed with 5% CO₂–95% air at 37°C until use.

**Dispersion of Cumulus Cells and Fertilization in Vitro** The sperm (4 × 10⁶) were incubated in 0.4 ml of m-KRB with eggs for various periods at 37°C in the presence (1.6 mM) or absence of sialic acid derivatives. The dispersion of cumulus cells was evaluated under a phase contrast microscope (Type IMT, Olympus). Sperm motility and sperm penetration of zona pellucida was assessed by phase contrast microscopy at 120 min. For sperm-egg fusion assay, sperm and eggs were incubated in 0.4 ml of m-KRB in the presence or absence of sialic acid derivatives for 6 h. Hoechst 33258 was added to the medium at a final concentration of 2 µM and the gametes were further incubated for 30 min. The formations of male and female pronuclei were observed under a fluorescence micro-
scope (Type VANOX-T, Olympus). At 24 h after insemination, the percentages of 2-cell eggs were determined using a phase contrast microscope.

**RESULTS**

The inhibitory effects of sialic acid derivatives on hy-
aluronidase activity, i.e., hydrolysis of hyaluronic acid, are depicted in Fig. 1. All assays were performed in triplicate. Sulfated sialic acid polymer (Col-S) strongly inhibits bovine testicular hyaluronidase in a concentration-dependent fashion. The 50% inhibitory concentration (IC₅₀) was 70 µg/ml (ca. 71 µM) for Col-S, 300 µg/ml (ca. 310 µM) for colominic acid, 230 µg/ml for apigenin. NeuNAC and DP-2 did not inhibit hyaluronidase activity up to the concentration of 450 µg/ml (ca. 460 µM). The concentrations of colominic acid and sulfated colominic acid were calculated on the basis of the con-
tents of NeuNAC.

Cumulus-surrounded eggs were incubated with sperm in the presence or absence of sialic acid derivatives. The dispersion of cumulus-cells from eggs by sperm hyaluronidase was time-dependent. After 90 min, almost all cumulus cells had dispersed from the eggs. In the presence of 0.5 mg/ml (1.6 mm) of colominic acid or Col-S, about half of the cells were remained, whereas almost all were dispersed in the presence of the same concentrations of NeuNAC or DP-2 (Fig. 2).

The effect of sialic acid derivatives on sperm motility, sperm penetration of zona pellucida, sperm fusion with the cumulus-surrounded eggs or cumulus-free eggs and the development of fertilized eggs to the 2-cell stage were assessed. About twenty cumulus-surrounded eggs were incubated with sperm in the presence or absence of sialic acid derivatives. The pronuclear formation and the development of eggs to the 2-cell stage were observed as described in the Materials and Methods. As shown in Fig. 3, the development of fertilized eggs to the 2-cell stage was inhibited by Col-S. On the other hand, the addition of colominic acid, sialic acid and DP-2 did not significantly inhibit gametes fertilization, even at the concentration of Col-S that showed about 70% inhibition of sperm egg fertilization. In these conditions, none of the inhibitors reduced sperm motility (data not shown).

Furthermore, we investigated the effect of sialic acid derivatives on male and/or female pronuclei formation as shown in Fig. 4. In the case of cumulus-surrounded eggs, the formation of male pronucleus was inhibited by Col-S (90% inhibition), NeuNAC (55% inhibition) and DP-2 (33% inhibition) but was not inhibited by colominic acid at 0.5 mg/ml. In the case of cumulus-free eggs, the male pronuclear formation was inhibited by Col-S (45% inhibition) but not the other sialic acid derivatives. In the presence of the same concentration of various inhibitors (0.5 mg/ml), the levels of inhibition by all the sialic acid derivatives were lower in the case of cu-

Figure 5 shows that the male pronuclear formation was in-
hibited by Col-S in a dose-dependent manner. When Col-S was added to the fertilization medium, the IC₅₀ value for cu-
mulus-surrounded eggs was 0.28 mg/ml (ca. 0.9 mm) and for cumulus-free eggs was 0.46 mg/ml (ca. 1.5 mm).
Sulfated colomonic acid inhibited fertilization in vitro. However, the apparent IC₅₀ value of was quite high because the concentrations of colomonic acid and sulfated colomonic acid were calculated based on the NeuNAc concentration. Sulfated colomonic acid used in this experiment had a sulfur content of 5.3% and a mean Mr of 17 kDa. One molecule of sulfated colomonic acid is composed of NeuNAc with an average of about 30 molecules. In other words, the molar concentration of sulfated colomonic acid becomes about 1/30 of the value shown on the x-axis of each graph.

DISCUSSION

We previously reported that sulfated colomonic acid inhibited the cytotoxic action of bee and snake venom.¹⁵ We found that synthetic sulfated colomonic acids remarkably inhibited the cytotoxicity of bee and snake venom toward mouse fibroblast cells, but colomonic acids did not show any inhibition themselves, indicating the important role of sulfate groups in the inhibitory activity of sulfated colomonic acid. The sulfated colomonic acid also exhibited potent inhibition of melittin, a highly basic peptide, which is a major cytotoxic component of bee venom. This suggests that the inhibition of bee and snake venom by sulfated colomonic acid is due to inhibition of melittin and cardiotoxin, which is a cytolytic peptide in snake venom. By inhibiting melittin, sulfated colomonic acid seems to be a simple ionic interaction between sulfated colomonic acid and melittin. It also seems to be also important to the conformational structure of sulfated colomonic acid. Melittin, a basic peptide of 26 amino acid
residues, is a major component comprising about 50 weight % of dried venom. Melittin interacts with the negatively-charged groups of lipids and disturbs lipid bilayers in cell membranes, thereby causing cytolsis.\(^{16,17}\) The positively-charged region near the C-terminal of the melittin molecule, which is composed of two arginine and two lysine residues, plays an important role in the binding process.\(^{18,19}\) For the binding of sulfated colominic acid to melittin leading to loss of its cytotoxic activity, the sulfate groups of sulfated colominic acid must be properly arranged to interact with lysine and arginine residues of melittin molecules, which play an important role in the cytolytic activity.

In this study, both sulfated colominic acid and colominic acid inhibited bovine testicular hyaluronidase activity (i.e., hydrolysis of hyaluronic acid) and mouse sperm hyaluronidase activity (i.e., dispersion of cumulus cells of eggs). Sulfated colominic acid inhibited fertilization in vitro whereas colominic acid did not. Moreno et al. reported that sulfated polysaccharides (i.e., fucoidan) bind to proacrosin and acrosin and inhibit sperm binding to the zona pellucida.\(^{20,21}\) In addition, sperm from acrosin gene-knockout mice could still penetrate the zona pellucida but the acrosin-deficient sperm exhibited a delay in penetrating the zona pellucida.\(^{20,21}\) These results suggest that sulfated colominic acid inhibits some enzymes (such as acrosin) required to penetrate the zona pellucida as well as hyaluronidase, while colominic acid inhibits hyaluronidase but not other enzymes. Therefore, we speculated that sulfated colominic acid inhibits sperm penetration of zona pellucida by binding to acrosin as well as hyaluronidase. The binding of sulfated colominic acid to hyaluronidase may lead to the loss of its dispersing activity of cumulus-cells, while the binding to acrosin may lead to the delay in sperm penetration (the dispersion of acrosomal enzymes from the acrosome). The delay of the dispersion of cumulus cells surrounding eggs may cause the inhibition of sperm penetration of zona pellucida. However, it is well known that the mass of cumulus cells around the zona pellucida disperses spontaneously even when the eggs are incubated in m-KRB without sperm. This fact may mean that colominic acid inhibits the dispersion of cumulus cells from eggs but does not lead the inhibition of the fertilization (Figs. 2, 3 and 4). These results suggested that unsulfated colominic acid did not bind to acrosin while sulfated colominic acid bound to acrosin by its negative charge. To clarify the inhibitory mechanism of sulfated colominic acid, further research on the sperm acrosomal enzyme is necessary.

**REFERENCES**

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