Glycosaminoglycans Prevent Induction of Fragmentation of Porcine Oocytes Stimulated by Dibutylrlyl Cyclic Adenosine 3',5'-Monophosphate in Culture

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ABSTRACT. The *in vitro* effects of derivatives of cyclic adenosine 3',5'-monophosphate (cAMP) and glycosaminoglycans (GAGs) on the spontaneous induction of fragmentation of cultured porcine oocytes were examined. Oocytes cultured for 72 h or longer undergo spontaneous fragmentation, and the percent of fragmented oocytes increased thereafter. The fragmented oocytes consisted of several "blastomeres" showing uneven distribution of DNA among the "blastomeres". Cytoplasmic bodies were also identified on the surface of fragmented oocytes and in the space among fragmented "blastomeres". Dibutylrlyl cyclic adenosine 3',5'-monophosphate (dbcAMP) at concentrations exceeding 50 μ M markedly increased the induction of fragmentation. 8-bromoadenosine 3',5'-cyclic monophosphate, butyrate, cAMP and GAGs isolated from porcine follicular fluid (pFF) did not stimulate the induction of fragmentation. pFF-GAGs added to the suspending medium at concentrations of 10 μ g/ml or greater prevented the occurrence of dbcAMP-stimulated fragmentation of isolated porcine oocytes in a dose-dependent manner. Preparations of hyaluronic acid and chondroitin sulfate from a commercial source prevented the occurrence of fragmentation stimulated by dbcAMP. The present findings suggest that cAMP may involve the induction of fragmentation of porcine oocytes and GAGs prevent the activation of cAMP-dependent fragmentation.

Porcine oocytes isolated from antral follicles and cultured *in vitro* will resume meiosis and undergo maturation spontaneously and become arrested at the second metaphase (13, 14). These *in vitro* matured oocytes are fertilizable (8, 12, 15). Also these cultured oocytes undergo spontaneous activation and degeneration characterized by formation of nucleus-like structure and fragmentation (18). A similar type of fragmentation was observed in aged or degenerating oocytes situated within the ovary (2), or in the Fallopian tube (23). In the course of our study of the mechanism of spontaneous activation and degeneration of oocytes, we observed that a significant number of oocytes underwent fragmentation and cytolysis when cultured for 3 or more days (18). The observation that the occurrence of fragmentation and cytolysis is higher with cumulus-free oocytes than cumulus-enclosed oocytes (18) suggests that the cumulus cells may produce component(s) that would maintain viability and delay or prevent the onset of the degenerative process. We have demonstrated that the porcine follicular fluid (pFF) contains glycosaminoglycans (GAGs) which suppress the cytolysis of oocytes in cul-

ture (21). Other studies, conducted with bovine oocytes, have revealed that dibutylrlyl cyclic adenosine 3',5'-monophosphate (dbcAMP) can stimulate the spontaneous induction of fragmentation observed *in vitro* (19).

In the present study, we examined the effects of derivatives of cyclic adenosine 3',5'-monophosphate (cAMP) and ovarian GAGs on the spontaneous induction of fragmentation of cultured porcine oocytes. Particular emphasis has been placed on the morphological characteristics of dbcAMP-induced fragmentation and the ability of ovarian GAGs to overcome the cAMP-induced fragmentation of porcine oocytes so that an evaluation can be made of the possible physiological role of GAGs and cAMP in the intrafollicular regulation of oocyte fragmentation.

MATERIALS AND METHODS

Chemicals. Hyaluronic acid and chondroitin sulfate from bovine trachea, heparin from porcine intestinal mucosa, and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Purity of GAGs used in the present study was determined by high performance liquid chromatography (TSK gel, DEAE-2SW) and found to be 71.3, 68.2, and

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41.4% for hyaluronic acid, chondroitin sulfate and heparin respectively.

In vitro culture of porcine oocytes. Ovaries were excised from non-cycling, prepuberal gilts of approximately 100 kg body weight at a local slaughterhouse and transported to the laboratory in physiological saline maintained at 32–35°C within 1 hr. Oocytes were collected by puncturing follicles (diameters ranging from 3 to 5 mm). The oocytes with adhering cumulus cells and germinal vesicles (GV) were washed twice with modified Krebs Ringer bicarbonate solution (modified KRB) containing 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄, 25.07 mM NaHCO₃, 21.58 mM sodium lactate, 0.5 mM sodium pyruvate, 5.56 mM glucose, 1 mg/ml crystalline bovine serum albumin, 50 µg/ml streptomycin sulfate and 75 µg/ml potassium penicillin (22). Groups of 12–17 oocytes with cumulus cells suspended in 0.2 ml of modified KRB solution were cultured for 120 hr at 37.0 ± 0.1°C under an atmosphere of 5% CO₂ in air, and scored for fragmentation of oocytes by light microscopy. The mean percentages of oocytes showing fragmentation were calculated for each control and experimental group. Significant differences between the means were determined by chi-squared analysis. The values presented in each figure are means of three replicate experiments. To determine whether the fragmented "blastomeres" contained DNA or nuclear fragments the specimens were stained with Hoechst dye (33342) (24) for 15 min at a final concentration of 10 µg/ml, and examined under an inverted fluorescence microscope.

Electron microscopy. Oocytes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 2 hr at 4°C.

Fig. 1. Effect of dbcAMP (100 µM) on the time course of spontaneous fragmentation of porcine oocytes cultured *in vitro*. (○—○) Control, (●—●) dbcAMP. Values are mean ± S.D. (n=3). *P<0.05, **P<0.01.

After an initial fixation, the oocytes were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.3) for 2 hr at 4°C, and subsequently rinsed in the same buffer. They were dehydrated in a graded series of ethanol, and embedded in Luveak 812 (Nacalai tesque Co., Japan). Ultrathin sections were cut with a Sorvall Porter-Blum MT-1 ultramicrotome and mounted on copper grids. They were stained with uranyl acetate and lead citrate and examined with a Hitachi H-300 transmission electron microscope at 73 kV.

Preparation of pFF-GAGs. Fresh porcine immature ova-

Table I. EFFECTS OF OF DIBUTYRYL CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE (dbcAMP), 8-BROMOADENOSINE 3',5'-CYCLIC MONOPHOSPHATE (8-Br-cAMP), BUTYRATE, CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE (cAMP) AND PORCINE FOLLICULAR FLUID-GLYCOSAMINOGLYCANS (pFF-GAGs) ON THE INDUCTION OF FRAGMENTATION OF PORCINE OOCYTES IN CULTURE.

	Dose	Oocytes examined (n)	Fragmented oocytes		
			(n)	%	SD
Control	—	48	4	8.5	4.2
DbcAMP (µM)	20	64	2	2.3	3.9
	50	48	17	35.4	4.6**
	100	54	23	41.6	8.5**
8-Br-cAMP (µM)	20	47	3	6.5	3.9
	50	31	3	9.0	5.5
	100	40	6	14.8	4.3
Butyrate (µM)	20	56	4	5.4	3.6
	50	52	3	5.3	3.2
	100	52	4	7.0	4.8
cAMP (µM)	20	20	2	8.5	6.1
	50	24	1	4.2	7.2
	100	41	3	6.5	4.1
pFF-GAGs (µg/ml)	10	42	3	3.4	3.4
	50	64	1	1.7	2.9
	100	54	0	0	—

Duration of incubation; 96 hr

**, P<0.01 (Chi-square analysis)

ries were obtained from a local abattoir, placed in physiological saline (0.154 M NaCl), packed in ice, and transported to the laboratory within 3 hr. The contents of the ovarian follicles ranging in diameter from 3 to 5 mm were aspirated within 5 hr after the slaughter and centrifuged at $10,000 \times g$ for 20 min at 4°C . The supernatant of pFF was collected and lyophilized. GAGs were prepared from the pFF according to the method described by Schiller *et al.* (22), with slight modifications and pFF-GAGs which suppress the cytolysis of oocytes in culture were isolated using Dowex 1-x 2 and TSK gel DEAE-2SW columns according to the method described in the previous paper (21). Uronic acid was determined with a modified method of Dische's carbazole reaction in the presence of borate, as described by Bitter and Muir (3). The isolated materials were dissolved in culture medium and assayed.

RESULTS

Porcine oocytes were incubated in modified KRB solution and examined at 24 hr intervals for fragmentation. Fragmentation occurred after 72 hr and the percentage of fragmented oocytes increased thereafter (Fig.

1). At 96 hr after incubation, about 8% of oocytes induced fragmentation (Fig. 1). The addition of dbcAMP to the medium at concentrations exceeding $50 \mu\text{M}$ increased significantly the incidence of fragmentation (Table I, Fig. 1). However, 8-bromoadenosine 3',5'-cyclic monophosphate (8-br-cAMP), butyrate, cyclic adenosine 3',5'-monophosphate (cAMP), and pFF-GAGs did not stimulate spontaneous induction of fragmentation of porcine oocytes in culture (Table I).

The fragmented oocytes consisted of several "blastomeres" of varying sizes which were retained within the zona pellucida (Fig. 2A, C). The fragmented oocytes were stained for DNA with Hoechst dye (Fig. 2B, D). In all specimens one of the "blastomeres" stained intensely while the others showed minimal or no staining. Cytoplasmic bodies were identified on the surface of fragmented oocytes (Fig. 3B-D) and filled in the space among fragmented "blastomeres" (Fig. 3A). Asymmetrical distribution of DNA among the "blastomeres" and cytoplasmic bodies on the surface of fragmented oocytes might suggest that fragmentation is a degenerative process rather than mitotic or meiotic division.

Fig. 2. Photomicrograph of fragmented porcine oocytes cultured with dbcAMP ($100 \mu\text{M}$) for 96 hr. (A, C) Oocytes undergoing fragmentation to form "blastomeres". $\times 200$. (B, D) Fragmented oocytes stained with Hoechst dye and photographed under a fluorescent microscope. Note uneven fluorescence of each blastomere. $\times 200$.

Fig. 3. A, B

Fig. 3. C, D

Fig. 4. (A) Effect of ovarian GAGs on the time course of fragmentation of porcine oocytes stimulated with dbcAMP (100 μ M) in culture. (●●) dbcAMP (control), (○○) dbcAMP+GAGs (100 μ g/ml). Values are mean $\pm$ S.D. (n=3). *P<0.05, **P<0.01. (B) Effect of ovarian GAGs on the fragmentation of porcine oocytes stimulated with dbcAMP (100 μ M) in culture for 96 hr. Values are mean $\pm$ S.D. (n=3). *P<0.05, **P<0.01.

Porcine FF-GAGs at a concentration of 10 μ g/ml or greater added to the cultured medium markedly delayed the onset of fragmentation in oocytes incubated with dbcAMP at a concentration of 100 μ M (Fig. 4, A; B). The percentage of fragmented oocytes decreased with increasing concentration of pFF-GAGs in the medium (Fig. 4B). Partially purified samples of hyaluronic acid, chondroitin sulfate and heparin were obtained from a commercial source and tested for suppressive activity. Hyaluronic acid and chondroitin sulfate decreased the incidence of dbcAMP-stimulated fragmentation (Table II). Hyaluronic acid was more effective and its suppressive activity was concentration-dependent. In contrast, heparin had minimal suppressive activity even at a concentration of 1 mg/ml.

DISCUSSION

Porcine nonatretic cumulus-enclosed oocytes collected from follicles of 3–5 mm diameter possess GV. When cultured in vitro in modified KRB solution, progression from GV stage to the first metaphase takes place at 14 hr of incubation and the majority of oocytes progress to second metaphase after 30 hr in incubation, although considerable variation is found in the time required for the progression of oocytes to the second metaphase. Spontaneous "pronucleus" formation was observed at 57–60 hr after incubation (18). As shown in Fig. 1, a small percentage of fragmented oocytes was ob-

served after 72 h of incubation for cumulus-enclosed oocytes. These observations may suggest that fragmentation occurs mainly at the oocytes progressed to second metaphase and formed "pronucleus".

Ovaries contain a large number of oocytes at birth (for example, about 700,000 in human) (25). The maximum number of oocytes ovulated represents less than 0.1% of the total present at birth. Other 99.9% are lost by atresia of ovarian follicles (25). In most atretic follicles, meiosis-like changes such as germinal vesicle breakdown (GVBD), chromosomes at metaphase, or expulsion of polar body are observed (25). These maturation-like changes in the oocytes are followed by degeneration of oocytes including fragmentation and disappearance of chromosomes and then finally degenerated oocytes are removed from follicles by phagocytic activity of granulosa cells (2, 10, 25). Upon ovulation, oocytes at the second metaphase passing through the oviduct and remaining unfertilized will undergo degeneration and death within a short period (1, 5, 23). The similarities in the morphological changes of degeneration observed with cultured oocytes and those in atretic follicles and in oviducts suggest that the process of cell death in these situations may be related and might be due to a deficiency or loss of essential ingredients in microenvironments of oocytes for oocyte viability. Our present results suggest that cAMP and GAGs may have significant roles for oocyte viability.

In higher organisms, cell death can be classified into

Fig. 3. Electron microphotograph of fragmented porcine oocytes cultured with dbcAMP (100 μ M) for 96 h *in vitro*. (A) Numerous cytoplasmic bodies filled in the space among fragmented "blastomeres" with vacuolated cytoplasm ($\times 21,000$). (B) Cytoplasmic bodies filled in perivitelline space. Z, zona pellucida ($\times 30,000$). (C, D) Cytoplasmic bodies on the surface of fragmented oocytes. Note the large vacuities (V) containing fragmenting cytoplasm and broken membranes (C, $\times 40,000$; D, $\times 60,000$).

Table II. EFFECTS OF HYALURONIC ACID, CHONDROITIN SULFATE, AND HEPARIN ON FRAGMENTATION OF PORCINE OOCYTES STIMULATED WITH dbcAMP (100 μ M) IN CULTURE.

	Dose (mg/ml)	Oocytes examined (n)	Fragmented oocytes		
			(n)	%	SD
Control	—	63	27	41.7	8.4
Hyaluronic acid	0.5	54	11	19.5	6.6*
	1.0	56	5	8.7	6.0**
Chondroitin sulfate	0.5	54	14	26.4	6.2
	1.0	58	13	22.3	5.6*
Heparin	0.5	57	20	37.6	8.2
	1.0	62	24	37.8	7.1

Duration of incubation; 96 hr

*, $P < 0.05$; **, $P < 0.01$ (Chi-square analysis)

two different categories: necrosis, which occurs as a result of massive tissue damage; and apoptosis, which is a process of active cellular self destruction that requires the expression of a number of genes (4). Apoptotic cell may be fragmented into a number of smaller "apoptotic bodies", some of which contain portions of the fragmented nucleus and an array of intact organelles such as mitochondria and lysosomes (4). Fragmented porcine oocytes showed asymmetrical distribution of DNA among the "blastomeres" (Fig. 2). As shown in Fig. 3, cytoplasmic bodies were identified on the surface of fragmented oocytes and filled in the intercellular space of fragmented "blastomeres". These morphological profiles of fragmentation might be recognized as a step of apoptosis.

Cyclic AMP or its dibutyryl derivative causes regression of some primary carcinogen-induced animal tumours, several transplantable tumours and many cell lines in vitro (27). In some instances the regression is known to be mediated by cell death with the morphology of apoptosis. DbcAMP also causes premature deletion of embryonic palatal shelf tissue (17), where the morphology is that of apoptosis. In the present study, the increased incidence of fragmentation of cultured porcine oocytes was also observed in the presence of dbcAMP.

Jensen and Zachariae (9) prepared GAGs from FF by trypsin digestion, TCA solubilization, and ethanol precipitation and showed that the content of GAGs in FF was about 0.2 to 0.3% (w/v). The major constituent of the GAGs was identified as chondroitin sulfate, based on the finding that chondroitinase A, B, and C hydrolyzed 90% of the GAGs produced by the granulosa cells (28). Heparin-like substances, dermatan sulfate, and hyaluronic acid was also identified as components of GAGs prepared from ovarian and follicular tissues (6). In the previous study, a substance, having a retention time on TSK gel DEAE-2SW column nearly coincident with that of hyaluronic acid, possessed the highest activity to prevent cytolysis of oocytes (21). In this study, the

same fraction separated from pFF possessed the fragmentation-preventing activity as well as cytolysis-preventing activity.

GAGs of the extracellular matrix have generally been observed to exert their influence on cell behavior via an effect on cell adhesion and cytoskeletal organization, the latter presumed to be mediated by physical linkages between the cell surface binding sites of GAGs and cytoskeleton (7). It was reported that testicular hyaluronidase promoted DNA synthesis of chondrocytes in vitro when applied at concentrations high enough to digest cell-associated proteoglycans (16). Such an effect of hyaluronidase on cell growth might indicate that hyaluronic acid influences the cells to be quiescent by acting on the cell surface. This evidence, shown in chondrocytes, suggests that one possible function of isolated pFF-GAGs and hyaluronic acid for cumulus-oocyte complexes is to make oocytes quiescent. Hyaluronic acid stimulates protein kinase activity in intact cells (26) and induces local changes in membrane properties by influencing ion or lipid distribution (11). Such evidence indicates that hyaluronic acid may influence cell behavior and organization by signal transduction via protein kinase activity as well as by direct action to cell membrane. The detailed molecular mechanisms of isolated pFF-GAGs and hyaluronic acid to suppress the fragmentation of porcine oocytes incubated with dbcAMP remains to be determined.

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