Tetraspanin CD9 in Bovine Oocytes and Its Role in Fertilization

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Abstract. This study was conducted in bovine to investigate whether CD9 (a member of the tetraspanin superfamily of proteins) is present on oocytes and whether it functions in sperm-oocyte binding and fusion. First, the presence of CD9 in bovine matured oocytes was examined by immunofluorescence with the anti-CD9 monoclonal antibody (mAb) and fluorescein isothiocyanate-conjugated goat anti-mouse antibody, and the results showed that CD9 was expressed on the plasma membrane of matured oocytes. Sperm binding and fusion with oocytes was then examined by in vitro fertilization. When the zona pellucida-free matured oocytes were fertilized, both sperm binding to ooplasm and sperm penetrating into oocytes were significantly (P<0.01) reduced in anti-CD9 antibody-treated oocytes (6.3 ± 0.7 per oocyte and 41.6%, respectively) compared with untreated control oocytes (19.0 ± 0.7 per oocyte and 81.3%, respectively), indicating that the anti-CD9 mAb potentially inhibits sperm-oocyte binding and fusion. These results demonstrated that the CD9 present on bovine matured oocytes is involved in sperm-oocyte interaction during fertilization.

Key words: Bovine, CD9, In vitro fertilization, Oocyte, Sperm-oocyte fusion

CD9, a 24–27 kDa cell-surface protein with four predicted transmembrane domains, is a member of the tetraspanin family. It has been proven to participate in the regulation of sperm-oocyte interaction during fertilization in mammals [1–5].

The first indication that CD9 is functional in fertilization was obtained from experiments using an antibody against CD9 [1]. In that study, Chen et al. observed that the anti-CD9 antibody inhibited the sperm-oocyte binding and fusion. However, in subsequent studies [2–4], sperm penetration through the zona pellucida and binding to the egg plasma membrane were normal, but oocytes from CD9−/− mice showed severely impaired sperm-egg fusion. This fusion competence could be restored upon injection of mRNA from either human or mouse CD9 into CD9−/− mouse oocytes [6]. In addition, CD9 is expressed in pig oocytes during early growth and meiotic maturation and that it participates in sperm-oocyte interactions during fertilization [5].

Apart from the expression of CD9 on the plasma membrane of oocytes in the mouse [2–4, 6] and pig [5], it is also localized to the oocyte microvillar membrane [7], blastocysts in mouse [3, 8], and endometrium epithelial cells in the mouse [9], human [10] and bovine [11]. Accordingly, CD9 is required for normal microvillar shape and distribution in the mouse [7] and plays a role in inhibiting embryo implantation [8]. CD9 expression in the uterus is upregulated in an ovarian steroid hormone-dependent manner, implicating multiple roles of CD9 in regulation of embryo implantation during the peri-implantation period [9]. At the same time, CD9 is involved in bovine binucleate cell migration and fusion in the trophoblast and endometrium [11].

It is unclear whether CD9 is expressed on the plasma membrane of oocytes in bovine and what role CD9 plays in sperm-oocyte interaction. To fill in the gap in knowledge that furthers understanding of tetraspanin CD9 in bovine oocytes and its role in fertilization, in the present study, we examined (1) whether CD9 is located in bovine oocytes and (2) whether CD9 is involved in sperm-oocyte interaction during fertilization.

Materials and Methods

Reagents
All reagents were purchased from Sigma (Sigma Chemical, St. Louis, MO, USA), unless otherwise stated.

Collection of oocytes and in vitro maturation
The procedure was modified on the basis of that described in our previous report [12].

Ovaries from cows and heifers were transported from a local abattoir to the laboratory in physiological saline at 35 C. Cumulus-oocyte complexes (COCs) were aspirated using a 10H ml syringe with a 19-gauge needle. Oocytes with an intact compact cumulus and homogeneous ooplasm were collected under a stereomicroscope (Nikon SMZ645, Tokyo, Japan), washed three times in Hepes-buffered TCM199 medium and then washed twice in NaHCO3-buffered TCM-199. The COCs were transferred into 0.5 ml of maturation medium (M199 + 10 mg/ml of oFSH [Ovagen, Auckland, New Zealand] + 10 mg/ml of oLH [Ovagen] + 1 mg/ml estradiol [Ovagen] + 10% fetal bovine serum [FBS, Hyclone; Gibco BRL, Paisley, Scotland, UK] in 4-well plates (Nunc A/S, Roskilde, Denmark) and overlaid with paraffin oil. The COCs were cultured for 18–20 h at 38.5 C in a humidified atmosphere with 5% CO2.

Accepted for publication: February 18, 2009
Published online in J-STAGE: March 16, 2009
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Removal of the zona pellucida

After maturation, the COCs were freed from cumulus cells by 2 to 3 minutes of vibration in 0.1% hyaluronidase, and the mature oocytes with a first polar body (PB1) were selected. The zona pellucida (ZP) was then removed by pipetting in 5 mg/ml pronase solution for about 2 min. These ZP-free oocytes were washed 3 times in maturation medium and cultured in an incubator (humidified atmosphere of 5% \(\text{CO}_2\) at 38.5°C) for 10 min. Some of the ZP-free oocytes were then selected for immunostaining of CD9, and others with PB1 in this process were used for fertilization in vitro.

Immunostaining of CD9 in oocytes

This process was modified based on the description of Li et al. [5].

ZP-free oocytes were treated for 45 min in a culture medium containing a mouse anti-bovine CD9 mAb (IVA 50, sc-51574. Santa Cruz Biotechnology, Santa Cruz, CA 95060, USA; 1:20) in a humidified atmosphere of 5% \(\text{CO}_2\) at 38.5°C. After washing three times for 5 min each in PBS supplemented with PVA (PBS-PVA), the oocytes were fixed with 4% paraformaldehyde in PBS-PVA (pH 7.4) for 15 to 20 min at 25°C. After another three washes, the oocytes were stained with fluorescein isothiocyanate-conjugated goat anti-mouse antibody in a 100 microliter drop (1:40) for 45 min. The stained oocytes were further washed three times for 5 min each in PBS-PVA before nuclear staining with 10 \(\mu\text{g}\) propidium iodide/ml in PBS for 6 min. Finally, the oocytes were mounted on slides with antifade solution (DABCO) and observed under a phase-contrast microscopy.

In vitro fertilization (IVF)

Before in vitro fertilization, the ZP-free oocytes were treated for 45 min in the culture medium containing a mouse anti-bovine CD9 mAb (1:20) in an incubator. They were then fertilized in vitro according to our previous description [13]. In this process, the ZP-free oocytes without anti-bovine CD9 mAb treatment were used as a control.

Assessment of sperm-oocyte binding

At 8 h after insemination, the loosely binding spermatozoa were completely removed from the oocytes by pipetting. The oocytes were then stained with 5 \(\mu\text{g}\) bis-benzamide (Hoechst 33342)/ml in PBS-PVA for 5 min followed by washing 3 times for 5 min each in PBS-PVA. Finally, the oocytes were mounted on slides, and the number of sperm bound to each egg was determined by a fluorescence microscope.

Assessment of sperm penetration

Based on our previous report [14], this procedure was modified and is described briefly as follows.

At 8 h after in vitro fertilization, the ZP-free oocytes were washed 4 times in the maturation medium by pipetting to completely remove the loosely binding spermatozoa. The oocytes were then cultured for another 10 h in culture medium (M199 + 10 \(\mu\text{g}\)/ml of FSH + 10 \(\mu\text{g}\)/ml of LH + 1 \(\mu\text{g}\)/ml estradiol [Ovagen] + 10% fetal bovine serum [FBS, Hyclone; Gibco BRL, Paisley, Scotland, UK] + 100 \(\mu\text{g}\)/ml of penicillin + 10 \(\mu\text{g}\)/ml of streptomycin) to assess the sperm penetration. Subsequently, oocytes from each group were fixed in a mixture of acetic acid and ethanol at a ratio of 1:3 at 4°C for 48 h. They were then stained with 1% (w/v) orcein in 45% (v/v) acetic acid. Sperm penetration was determined under phase-contrast microscopy.

The sperm penetration was classified into (1) monospermy, as the presence of a whole sperm, a spermhead or a pronucleus with an associated sperm tail in the ooplasm; (2) polyspermy, the presence of any multiple sperm structures in the ooplasm; and (3) unpenetration, the absence of any sperm structure in the ooplasm.

Statistical analysis

All experiments were repeated at least three times. The percentage data were subjected to arcsine transformation before statistical analysis. The data were analyzed by one-way ANOVA combined with the LSD test. The level of statistical significance was set at \(P<0.05\).

Results and Discussion

CD9 distribution on the bovine matured oocyte

When the zona pellucida-free oocytes were treated with anti-CD9 mAb and fluorescein isothiocyanate-conjugated goat anti-mouse antibody, and then with PI, the CD9 (green) was observed on the plasma membrane (Fig. 1a); on the other hand the oocytes incubated with the secondary antibody alone showed no staining (Fig. 1b). The chromosomes were stained red by PI as expected (Fig. 1a and 1b).

This result is similar to the previous reports in the mouse of Chen et al. [1], who observed that CD9 was expressed on oocytes isolated from the oviducts. In addition, Chen et al. also reported that CD9 plays a role in sperm-oocyte binding and fusion in the mouse. This hints that the CD9 in bovine oocytes may also participate in the regulation of sperm-oocyte interaction. So, we conducted the following experiments.

Effect of anti-CD9 antibody on sperm-egg interaction during fertilization in vitro

After removal of the zona pellucida, oocytes were first co-cultured for 45 min with anti-CD9 antibody and then incubated for 8 h with frozen-thawed sperm; the average number of spermatozoa binding to the oocytes (6.3 ± 0.7) was significantly (\(P<0.01\)) lower than that of those binding to the controls (19.0 ± 0.7; Fig. 2). The data indicated that anti-CD9 mAb potentially inhibits sperm-oocyte binding.

As shown in Table 1, when the oocytes were incubated with CD9 antibody during in vitro fertilization, the rates of penetration were significantly (\(P<0.01\)) reduced (41.2 ± 2.2%) compared with the untreated control oocytes (81.2 ± 4.2%), showing that anti-CD9 mAb potentially inhibits sperm-oocyte fusion.

Based on the hypothesis of Chen et al. [1] that the microvillar surface of the egg may be uniquely suited for CD9-assisted tethering of integrin α6β1 to the underlying actin cytoskeleton, which may, in turn, promote binding of sperm via fertilinβ, when the
function-blocking monoclonal antibody (anti-CD9 mAb) cross-reacted with the CD9 protein, the binding of sperm and fertilinβ to the oocyte was reduced.

Taken together, the present study indicates for the first time that CD9 is present on the bovine mature oocytes and might play a critical role in regulating the sperm binding and fusion between the oocyte and sperm during fertilization.

Acknowledgements

This work was supported in part by the Research Fund for the Doctoral Program of Higher Education (No. 20060619031) of the Ministry of Education and by the National Key Technology R & D Program (No. 2006BAD14B08).

References


Table 1. Effect of anti-CD9 mAb on sperm penetration in ZP-free oocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of oocytes</th>
<th>Penetration rate (%)</th>
<th>Unpenetration rate (%)</th>
<th>Monospermy rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>137</td>
<td>41.6 ± 2.2</td>
<td>58.4 ± 2.2</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>Control</td>
<td>144</td>
<td>81.2 ± 4.2</td>
<td>18.8 ± 4.2</td>
<td>9.0 ± 1.0</td>
</tr>
</tbody>
</table>

Values with different superscripts (a and b) in the same column differ significantly (P<0.01).


