Immunohistochemical localization and expression of the hyaluronan receptor CD44 in the epithelium of the pig oviduct during oestrus

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Hyaluronan is related to essential reproductive processes in pigs. Hyaluronan produced by cumulus cells builds, via specific cell surface receptors, an extracellular matrix responsible for cumulus cell cloud expansion during final oocyte maturation, a preparatory event for ovulation and fertilization. In addition, hyaluronan that has been localized in the pig oviduct both in the intraluminal fluid and on the surface of the lining epithelium of the pre-ovulatory sperm reservoir, has proven beneficial during *in vitro* fertilization and embryo culture, thus indicating that it has a role *in vivo*. This study monitored the immunolocalization, protein determination and gene expression of the major cell surface hyaluronan receptor CD44 in the epithelial lining of the pig oviduct during selected stages of standing oestrus, in relation to spontaneous ovulation. The CD44 immunostaining in the lining epithelium was localized to the surface membrane and the supranuclear domain of mainly the secretory cells, particularly in the sperm reservoir of both treatment (inseminated) and control (non-inseminated) specimens.

Up to four hyaluronan-binding protein (HABP) bands (60, 90, 100 and 200 kDa) were detected in the tubal epithelium, and the 200 kDa band was determined as CD44 by immunoblotting. The expression of CD44 mRNA was higher before than after ovulation ($P < 0.05$), most conspicuously in the uterotubal junction (UTJ). In addition, CD44 expression in the preovulatory UTJ and the ampullary–isthmic junction (AIJ) of control animals was higher than in those that were inseminated ($P < 0.05$ and $P < 0.01$ for UTJ and AIJ, respectively). The results demonstrate for the first time that the specific hyaluronan receptor CD44 is expressed by the oviduct epithelial cells during spontaneous oestrus, and is particularly abundant in the sperm reservoir before ovulation. Presence of spermatozoa in this segment seemed to downregulate the receptor. The variation in the expression of CD44 in relation to spontaneous ovulation and the presence of spermatozoa indicate that the hyaluronan CD44-signalling pathway may play a role in oviduct function during sperm storage and fertilization in pigs.

Introduction

As one step of sperm transport in female pigs, a sperm reservoir is established in the uterotubal junction (UTJ) and adjacent caudal isthmus of the oviduct (Viring *et al.*, 1980; Hunter, 1981). There, spermatozoa are immersed in the intraluminal fluid or are in contact with the lining epithelium, maintaining viability and potential fertilizing ability during oestrus (Smith and Yanagimachi, 1990; Pollard *et al.*, 1991). *In vivo*, the sperm reservoir appears to prevent the process of sperm capacitation (Rodriguez-Martinez *et al.*, 2001). In addition, the sperm reservoir controls the progression of suitable spermatozoa, in very

restricted numbers, to the site of fertilization of the newly ovulated oocytes (Rodriguez-Martinez, 2001).

Hyaluronan (hyaluronic acid), one of the most abundant glycosaminoglycans, is present on the surface of the lining epithelium, the intraluminal fluid and the extracellular matrix of the lamina propria of the pig oviduct (Tienthai *et al.*, 2000). The epithelial localization is conspicuous in the deep furrows of the sperm reservoir, where clusters of spermatozoa can be seen near the hyaluronan-positive epithelium (Rodriguez-Martinez *et al.*, 1998). *In vitro*, hyaluronan has been shown to influence boar spermatozoa. For example, hyaluronan exposure before and during IVF seems to induce capacitation-like changes (as detected by chlortetracycline-mediated fluorescence, the so-called CTC technique) without eliciting the acrosome reaction

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(Rodriguez-Martinez *et al.*, 1997; Suzuki *et al.*, 2002), to diminish polyspermy of homologous oocytes after IVF (Suzuki *et al.*, 2000, 2002) and to support the development of *in vitro* matured and fertilized oocytes to the blastocyst stage in pigs (Kano *et al.*, 1998).

Hyaluronan appears to exert its biological effects through interactions with specific cell-associated receptors, such as CD44 (Underhill, 1992) or the receptor for hyaluronan-mediated motility (RHAMM, Turley *et al.*, 1991). CD44 is a membrane glycoprotein that is present in a variety of isoforms and is considered to be the major cell-surface receptor for hyaluronan (Ponta *et al.*, 1998). This CD44 family belongs to a larger group of hyaluronan-binding proteins (HABPs), termed hyaladherins, and it has been implicated in a wide variety of hyaluronan-mediated cellular events, that is cell–cell and cell–extracellular matrix functions (for review, see Toole, 1990). The various isoforms of CD44 have been detected in the cervical epithelium (Woerner *et al.*, 1995) and human endometrium (Behzad *et al.*, 1994; Saegusa *et al.*, 1998). Moreover, cumulus granulosa cells in humans and pigs clearly express CD44, encompassing abundant production of hyaluronan during cumulus expansion in humans (Ohta *et al.*, 1999) and pigs, both *in vitro* (Kimura *et al.*, 2002a) and *in vivo* (Yokoo *et al.*, 2002). In addition, HABP is present on the spermatozoa of different mammalian species (Ranganathan *et al.*, 1994). Taken together, hyaluronan, CD44 and other HABPs appear involved in the function of gametes and the female genital tract in terms of cell proliferation, adhesion, migration, maturation, protein phosphorylation and development. The physiological roles of hyaluronan in the pig oviduct have not been reported, but the conspicuous localization of hyaluronan in the sperm reservoir indicates a role during sperm capacitation and preparatory interactions for fertilization. The disclosure of the presence and location of eventual hyaluronan receptors is required to ascertain a possible autocrine mediation for this glycosaminoglycan.

The present study attempted to determine whether CD44 and CD44 mRNA are expressed in the oviduct epithelium of the pig during selected periods of oestrus in relation to spontaneous ovulation, using immunohistochemistry, ligand and western blot analyses and RT–PCR. The results indicate that the specific hyaluronan receptor CD44 is expressed by the oviduct epithelial cells during spontaneous oestrus, and is particularly abundant in the sperm reservoir before ovulation. The presence of spermatozoa in this segment seems to downregulate the receptor.

Materials and Methods

Animals and general management

Multiparous crossbred (Swedish Yorkshire × Swedish Landrace) sows ($n = 52$, parity 2–5) were recruited for

these experiments from a commercial farm on the day of weaning and individually penned at the Department of Obstetrics and Gynaecology, SLU, Uppsala. The sows received water *ad libitum* and standard ration, according to the Swedish breeding stock standard for dry sows (Simonsson, 1994). A fertile boar was always penned in the vicinity. Experienced personnel checked the sows two or three times per day for behavioural oestrus. A group of sows (hereby called treatment, $n = 29$) was inseminated with undiluted semen collected from, or mated by, either one of two fertile boars 12 h after observed onset of oestrus, whereas the other sows ($n = 23$) were not inseminated or mated (hereby called control). The experimental design has previously been reviewed and approved by the local Ethical Committee for Experimentation with Animals.

Oviductal tissue collection

The ovaries were periodically subjected to transrectal ultrasonography to detect the presence of preovulatory follicles, as described by Mburu *et al.* (1995). The animals were slaughtered at well-defined stages during standing oestrus, namely before ovulation (approximately 8 h before expected ovulation, $n = 19$), or after ovulation (approximately 4–8 h after spontaneous ovulation, $n = 33$). The oviducts were retrieved and kept at approximately 39°C during transportation to a nearby laboratory.

For immunohistochemistry, tubal segments containing the UTJ, distal isthmus, ampullary–isthmic junction (AIJ) and ampulla, were dissected out and immersion-fixed overnight or longer in 1% (v/v) paraformaldehyde in 0.15 mmol phosphate-buffered saline l^{-1} (PBS, pH 7.35). Tissues were retrieved from a total of 17 control (before ovulation = 5, after ovulation = 12) and 23 treatment (before ovulation = 8, after ovulation = 15) sows.

Oviducts ($n = 12$) were also retrieved for RT–PCR, ligand and western blot analyses, from non-inseminated ($n = 6$) or inseminated ($n = 6$) sows in standing oestrus, either before ovulation ($n = 6$) or after ovulation ($n = 6$), immediately post mortem, allotting three tissue specimens per treatment and timing. All segments were promptly retrieved and deep-frozen in liquid nitrogen until isolation of total RNAs or proteins.

Isolation of total RNA from oviductal epithelium

The lining epithelium of the collected UTJ, isthmus, AIJ and ampulla segments was scraped using the blunt side of a scalpel blade. Representative samples of scraped epithelial cells and the tubal segment after scraping were fixed in 2.5% (v/v) glutaraldehyde solution in 0.067 mmol sodium cacodylate buffer l^{-1} (pH 7.2–7.4) and after routine preparation, examined by scanning electron microscope as shown (Fig. 1a,b). Total RNAs were isolated from these epithelial cells according

Fig. 1. Representative scanning electron micrographs showing (a) a conglomerate of pig oviductal epithelial cells scraped from an isthmus segment and (b) surface of the isthmus segment. Note the area where the surgical blade removed part of the epithelial lining (*). Scale bars represent 10 μm .

to the instructions supplied with the RNeasy mini kit (QIAGEN GmbH, Hilden, Germany). In brief, the samples were digested and homogenized in the presence of a highly denaturing guanidinium isothiocyanate-containing buffer, and adjusted with ethanol. This solution was applied to a spin column contained in

the RNeasy mini kit (QIAGEN) in which the total RNA binds to the membrane by centrifugation. The total RNA was treated with 83 U of DNAase I on the spin column at room temperature for 15 min and eluted in diethyl pyrocarbonate-treated distilled water after washing eventual contaminants.

Preparation of oviductal epithelium for ligand and western blot analyses

Oviductal epithelial cells were removed from the pig oviduct as described above. Proteins were extracted from the scraped epithelial layer by vortexing and stirring in 600 μ l lysis buffer that consisted of 50 mmol Tris I^{-1} (pH 7.5), 1 mmol phenylmethylsulphonyl fluoride (PMSF) I^{-1} , 0.1 mol 6-amino-*n*-caproic acid I^{-1} , 5 mmol benzamidine HCl I^{-1} and 1% (v/v) 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulphonate (CHAPS) (all chemicals from Sigma, St Louis, MO) at 4°C for 1 h. The samples were centrifuged at 10 000 *g* for 30 min and the supernatants removed for blotting.

CD44 immunohistochemistry

Immunohistochemical staining was performed using the Vectastain avidin-biotin complex technique (Vectastain-Elite ABC Kit, Vector Laboratories, Burlingame, CA). In brief, paraffin wax-embedded blocks of oviduct segments were transversally sliced into 4 μ m sections and mounted on poly-L-lysine-coated microscope slides. After incubating at 40°C for 4 h, the sections were de-paraffinized and rehydrated. The sections were rinsed with Tris-HCl buffer, covered with avidin blocking (Vector Laboratories) for 15 min, rinsed for 5 min with Tris-HCl, then covered with biotin blocking (Vector Laboratories) for 15 min and rinsed again with Tris-HCl for 5 min. Endogenous peroxide activity was eliminated by incubation with 3% (v/v) H_2O_2 in methanol for 20 min. All sections were rinsed in Tris-HCl and incubated in the blocking serum provided in the kit (normal horse serum, Vector Laboratories) at a dilution of 1:10 for 30 min, at room temperature. After this step, and without rinsing, the sections were incubated with the anti-porcine CD44 monoclonal antibody (1 mg ml^{-1} , PORC24A, VMRD, Pullman, WA) diluted with Tris-HCl at 1:100 at 4°C overnight. The sections were rinsed in Tris-HCl for 5 min before applying the secondary biotinylated horse anti-mouse antibody (Vector Laboratories) at a dilution of 1:500 for 30 min at room temperature. After rinsing, the sections were incubated with ABC-mouse reagent (Vector Laboratories) for 30 min at room temperature and rinsed again. Immunostaining was visualized using diaminobenzidine tetrahydrochloride (DAKO, Carpinteria, CA) as a substrate. The sections thus processed were counterstained with Mayer's haematoxylin. Tissues with known CD44 positivity (cumulus-oocyte complexes from full-grown pig follicles) served as positive controls, whereas in negative controls, the primary antibody was replaced by mouse IgG1 monoclonal antibodies (DAKO). All sections were examined by one operator scoring the relative intensity of the CD44 immunostaining (negative, feeble, intense, and very intense). Photomicrographs

were taken using a Nikon microphot-FXA microscope (Nikon, Tokyo).

HABP ligand blot and CD44 western blot analyses

Extracted proteins (10 μ g each lane) were separated by 7.5% SDS-PAGE under non-reducing conditions. After electrophoresis, one gel was stained with Coomassie brilliant blue R-250 to detect total proteins, and the other gels were transferred to Immobilon P^{SQ} membranes (Millipore, Bedford, MA) in 25 mmol Tris I^{-1} , containing 192 mmol glycine I^{-1} , 10% (v/v) methanol (pH 8.3) at 250 mA for 75 min at 4°C. The membranes were then washed with TBS-T containing 0.15 mol NaCl I^{-1} , 0.05% (v/v) Tween 20 in 20 mmol Tris-HCl I^{-1} (pH 7.5). For reaction with fluorescein isothiocyanate-conjugated hyaluronan (FITC-HA; Calbiochem, La Jolla, CA), the membranes were incubated with 5 μ g FITC-HA ml^{-1} in TBS-T at 37°C for 2 h and washed with TBS-T. After washing, the reaction sites were detected using an image analyser (LAS-1000 plus; Fuji, Tokyo). For CD44 immunoblotting, the membranes obtained from the same samples as the HABP analyses were blocked with 2% (w/v) skimmed milk in TBS-T overnight at 4°C and washed with TBS-T. The membranes were incubated with anti-porcine CD44 monoclonal antibody (PORC24A, VMRD) at a dilution of 1:1000 for 1 h at room temperature, and then reacted with FITC-conjugated goat anti-mouse immunoglobulins (Sigma) at a dilution of 1:1000 in TBS-T. After washing three times with TBS-T, the membranes were examined on an image analyser (LAS-1000 plus) for detection and allocation by molecular weight.

Semi-quantitative RT-PCR

RT-PCR was performed using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) that were optimized to allow the first strand of cDNA synthesis and PCR reactions to proceed sequentially as a single-step reaction using a PCR thermal cycler TP2000 (TaKaRa, Kyoto). Each primer was designed according to published mouse cDNAs, including regions that are highly conserved between mice and humans. For CD44, the sense primer (5'-GTACATCAGTCACAGACCTAC-3') and the antisense primer (5'-CACCATTTCCTAAGACTTGCT-3') generated a 598 bp cDNA fragment that corresponded to mouse CD44 nucleotides (accession number M27129). For β -actin (as an internal positive control), the sense primer (5'-GACCCAGATCATGTTTGAGACC-3') and the antisense primer (5'-ATCTCCTTCTGCATCCTGTGAG-3') generated a 593 bp cDNA fragment that corresponded to mouse β -actin nucleotides (accession number X03672). DNA sequencing was performed as described by Kimura *et al.* (2002a). The aliquot of total RNA (100 ng) extracted

from the oviductal epithelium was reverse-transcribed and then PCR-amplified in a total reaction volume of 50 μ l containing 10 pmol of each sense and antisense primer, 0.5 μ g of oligo (dT)_{12–18} primer, about 2.0 U of Taq DNA polymerase, 10 mmol Tris–HCl l^{–1} (pH 9.0), 60 mmol KCl l^{–1}, 1.5 mmol MgCl₂ l^{–1} and 200 μ m each of dNTP, moloney murine leukaemia virus reverse transcriptase (FPLCpure; Amersham Pharmacia Biotech), and RNAGuard ribonuclease inhibitor (pig) and stabilizer, including ribonuclease- and deoxyribonuclease-free BSA. This mixture, overlaid with mineral oil, was incubated at 42°C for 20 min for the RT reaction. PCR amplification proceeded after inactivation of the reverse transcriptase by heating for 5 min at 95°C. PCR cycling for β -actin and CD44 was carried out with 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 55°C and 1 min extension at 72°C. For semi-quantitative PCR, the number of cycles was optimized to ensure amplification of cDNA in the exponential phase of PCR. Genomic DNA contamination was detected by subjecting total RNA to RT–PCR without reverse transcriptase using β -actin primer pairs. Electrophoresis was carried out on the amplified product on 2% agarose gel and visualized by ethidium bromide staining. The intensity of the objective bands was quantified by densitometric scanning using NIH Image Version 1.62 free software (NIH, Bethesda, MD). The relative abundance of CD44 was normalized against that of β -actin by establishing a ratio of CD44: β -actin.

Statistical analyses

The densitometry ratio for CD44: β -actin used to determine the expression of CD44 mRNAs was examined using one-way factorial ANOVA and values are hereby presented as mean $\pm$ SEM. Differences between means were determined by a Student's *t* test. The percentage of positively CD44-stained sections and samples (scored for their relative intensities) were compared using a Student's *t* test. *P* values < 0.05 were considered significant.

Results

Immunohistochemical localization of CD44 in the pig oviduct

CD44 immunostaining was present on the surface of homologous cumulus cells (positive controls, Fig. 2a), whereas no staining was present on the negative controls (Fig. 2b).

In each oviductal sample, CD44-positive labelling was present on the surface of leucocyte-like cells located in the epithelium and the lamina propria of both control and inseminated individuals (see representative stained cells in Figs 3c,d and 4f,g, respectively).

In the lining epithelium, immunostaining varied in location and intensity among individuals. Intensity, as appreciated during examination under the light microscope, varied from weak (barely appreciable) to strong staining on the epithelial adluminal surface (Figs 3a,b,e,f and Figs 4a,b,e,f) as well as on the supranuclear domain of scattered epithelial cells, most often of the secretory-like cells (Figs 3a–h and 4a–h). Clearly stained samples (that is, strongly stained in the supranuclear domain) accounted only for 30–60% of the material examined from the UTJ and isthmus. A larger variation (albeit not fully quantified) was present in the AIJ and ampulla, and 20–50% of sections were strongly stained. The location and intensity of the CD44 immunostaining clearly varied among the oviductal segments examined. The adluminal surface appeared conspicuously stained in the sperm reservoir (UTJ–isthmus segments; see Figs 3a,b,e,f and 4a,b,e,f) but was barely visible in the AIJ or ampullar segments (Figs 3c,d,g,h and 4c,d,g,h). A similar trend of conspicuous surface immunostaining in the sperm reservoir was present in both control and inseminated sows, before or after ovulation. However, significantly (*P* < 0.05) more samples presented strong supranuclear staining in inseminated sows than in controls (54–63% versus 33–40% in the UTJ and I, respectively).

Morphology of the oviductal epithelial cells used for homogenates for blot and PCR analyses

Representative samples retrieved by scraping epithelium lining consisted largely of epithelial cell fragments that built, following fixation, conglomerates of spherical bodies clumped together (Fig. 1a). Contamination by the underlying connective tissue (mainly connective fibres), although difficult to rule out, was not evident. A view of a scraped isthmus segment (Fig. 1b) shows the scraped surface against a deeper area that was not touched by the scalpel blade.

HABP ligand blot and CD44 western blot analyses

The bands of proteins (electrophoresis) and of those determined as HABP (FITC–HA), extracted from oviductal epithelial cells of control and inseminated specimens, are shown (Figs 5a,b and 5c,d, respectively). Four bands of HABP (60, 90, 100 and 200 kDa) were detected in both treatment and control groups. It was clear that the intensity of the 200 kDa HABP band was stronger in the UTJ than in the other segments of the oviduct in both treatment and control groups and at all stages of oestrus. However, whereas the 90 kDa and 60 kDa HABP bands did not show obvious differences among the different segments of the oviducts, the 100 kDa band appeared to be more intense in the AIJ and ampulla among specimens. Although the specificity of the hyaluronan blot was

Fig. 2. Control sections for CD44 immunostaining. Homologous cumulus-oocyte complexes (COCs) aspirated from fully grown preovulatory pig follicles were used as positive controls. (a) Arrows show the surface CD44 staining. (b) Omission of the primary antibody and replacement by a mouse IgG1 monoclonal antibody was used as negative control (pig ampulla). Scale bar represents 10 μ m.

assessed in preliminary studies (M. Yokoo, unpublished), the lack of variation of the 60 and 90 kDa HABP bands might suggest some degree of non-specific interactions with the antibody. However, the presence of major bands stained by Coomassie blue without any counterpart in the

ligand blotting strongly indicates that the binding was specific. The western blotting for CD44 (Figs 5e and f, for control and inseminated sows, respectively), showed that the 200 kDa band, detected in the epithelial cells from both control and treated groups, particularly before

Fig. 3. Immunohistochemical localization of CD44 in the epithelium of the (a,e) uterotubal junction, (b,f) isthmus, (c,g) ampullar-isthmus junction and (d,h) ampulla of the oviduct of control (non-inseminated) sows (a–d) before ovulation and (e–h) after ovulation. Arrowheads indicate immunolabelling on the epithelial surface; arrows indicate positive staining on the supranuclear domain. Open arrowheads in a, c, d and f indicate immunoreaction on leucocyte-like cells in the lamina propria (LP) and the epithelium (EP). LU, lumen. Scale bar represents 10 μ m.

Fig. 4. Immunohistochemistry of CD44 in the epithelium of the (a,e) uterotubal junction, (b,f) isthmus, (c,g) ampullar-isthmic junction and (d,h) ampulla of the oviduct of inseminated sows (a–d) before ovulation and (e–h) after ovulation. Arrowheads indicate immunolabelling on the epithelial surface; arrows indicate supranuclear staining and open arrowheads indicate the leucocyte-like cell staining. EP, epithelium; LU, lumen; SPZ, spermatozoa. Scale bar represents 10 μ m.

Fig. 5. Electrophoretic, ligand blotting and western blotting analysis of epithelial homogenates of representative oviducts from non-inseminated (control) and inseminated sows, before and after ovulation. Proteins were subjected to 7.5% SDS-PAGE and gels were (a,b) stained with Coomassie blue R-250, or (c,d) transferred to membranes and incubated with fluorescein isothiocyanate-conjugated hyaluronan for detecting hyaluronan-binding proteins (HABP) bands (the four HABP bands are marked with arrowheads), or (e,f) with anti-CD44 antibody (the CD44 band is indicated with an arrowhead), respectively. UTJ, uterotubal junction; IST, isthmus; AIJ, ampullary-isthmic junction; AMP, ampulla.

ovulation, corresponded to the hyaluronan receptor. The intensity of this CD44 band was again higher in the UTJ than in the other tubal segments, and was independent of treatment group (control versus inseminated).

CD44 expression by RT-PCR in the oviductal epithelium

The expression bands of CD44 mRNA appeared more conspicuous during preovulatory oestrus than after

ovulation (RT-PCR shown in Fig. 6a,b; densitometry data shown in Fig. 6c) in the control and inseminated groups. In particular, the preovulatory UTJ and AIJ from the control group expressed significantly higher amounts of CD44 mRNA ($P < 0.05$ and $P < 0.01$, respectively) compared with the same segments of the oviduct from the inseminated group (Fig. 4c). In addition, when considering all samples, CD44 expression was higher before than after ovulation (27.26 ± 4.14 and 15.37 ± 3.13 , respectively; $P < 0.05$).

Fig. 6. CD44 mRNA expression (a,b: RT-PCR bands, c: scanning densitometry quantitation of band intensities) in oviductal epithelium before ovulation (left panels) and after ovulation (right panels) from (a) control (non-inseminated) and (b) inseminated sows. The β-actin specific band (internal positive control) shows that the intensity was equal among RNA samples. (c) The densitometry analysis shows the ratio (%) of CD44:β-actin for the various tubal segments in control (black bars) and inseminated (shadow bars) sows (mean ± SEM, asterisks denote significantly different ratios: * $P < 0.05$, ** $P < 0.01$). UTJ, uterotubal junction; IST, isthmus; AIJ, ampullary-isthmic junction; AMP, ampulla.

Discussion

The results of the present study indicate that CD44 immunostaining is present in the tubal epithelium, particularly in the UTJ and the caudal part of the isthmus (the so-called sperm reservoir) of pigs. The intensity of immunostaining, located at the adluminal epithelial surface and the supranuclear cell domain, was relatively higher during the preovulation stage of standing oestrus. In addition, using ligand blot analysis and RT-PCR techniques, this study confirmed that specific HABPs (especially the CD44 receptor) and CD44 mRNA are also expressed in the oviduct epithelial cells of the sperm reservoir. Occurrence of spontaneous ovulation and the presence of inseminated spermatozoa affected the expression.

CD44 immunostaining was consistently present in leucocyte-like cells throughout samples, pertaining to the nature of the antibody. In the epithelial cells, CD44 immunostaining was localized to the apical cell surface and the supranuclear region of, mostly, non-ciliated cells. The apical cell surface localization would correspond to the cell membrane hyaluronan receptor, and the supranuclear localization might represent either the Golgi region processing of CD44 not as yet inserted into the plasma membrane, or CD44 bound to hyaluronan that has been internalized into endosomes (lysosomes) to undergo degradation (Culty *et al.*, 1992; Knudson *et al.*, 2002). A screening of the samples for acid phosphatase presence (a commonly used indicator for lysosomes) (Barka, 1960) confirmed the co-localization of the CD44 immunostaining and lysosomes in the

supranuclear domain (P. Tienthai, unpublished), thus indicating an intracytoplasmic CD44 localization in the pig oviduct either at the synthesis packaging stage or during degradation. Considering mRNA CD44 was higher in samples taken before ovulation, the supranuclear staining might represent enhanced synthesis and packaging of the protein. Tienthai *et al.* (2000) localized hyaluronan immunohistochemically in the supranuclear epithelium domain, indicating that an endosome-mediated inclusion mechanism for the receptor ligand might also be present in the pig oviduct. However, a substantial variation in the relative intensity of the CD44 immunostaining was present among samples and sows in the present study, with barely half of the preparations showing strong supranuclear staining. There is not a clear explanation for this variation. Considering the antibody consistently stained leucocyte-like cells in all samples examined and major specific bands were detected by Coomassie blue, a problem of specificity should be ruled out. Some samples remained longer than overnight (days) in the fixative. This could have diminished the immunostaining and account for the variation in the intensity of immunolabelling. Finally, although not investigated in the present study, the hormonal status of the tissue samples may differ, and these differences may convey changes in the type and amount of the CD44 molecule present at a given time, as has been determined for human normal and pathological endometrium (Saegusa *et al.*, 1998; Durst *et al.*, 2001).

After rapid transport of spermatozoa through the uterus, spermatozoa colonize the UTJ-caudal isthmus before aliquots of the sperm population are released towards the site of fertilization (Hunter, 1981, 1984). One of the main functions of the sperm reservoir is to maintain normal ultrastructure and fertilizing ability of the spermatozoa before the time of fertilization (Rodriguez-Martinez *et al.*, 1990; Mburu *et al.*, 1997). Many studies have reported that the microenvironment in the intraluminal fluid of the sperm reservoir influences spermatozoa, for instance by inhibiting sperm metabolism and motility and stabilizing their plasma-lemma, by specific signalling (Suarez *et al.*, 1991; Rodriguez-Martinez *et al.*, 1998). *In vivo*, most spermatozoa residing in the preovulatory sperm reservoir in pigs do not show changes in membrane destabilization, such as lipid scrambling that defines the first stages of capacitation (Harrison *et al.*, 1993), thus indicating that the function of the sperm reservoir before ovulation is to delay capacitation, extending the viability and fertilizing capacity of the stored spermatozoa (Rodriguez-Martinez *et al.*, 2001).

Glycosaminoglycans have been able to maintain sperm motility and to induce capacitation *in vitro* in cows (Parrish *et al.*, 1994), dogs (Kawakami *et al.*, 2000), pigs (Suzuki *et al.*, 2002) and humans (Huszar *et al.*, 1990). In particular, human sperm motility is almost unchanged

by the addition of 0.25 mg hyaluronan ml⁻¹ during a 30 h observation period (Sbracia *et al.*, 1997). In pigs, exposure of frozen-thawed spermatozoa to hyaluronan (500 µg ml⁻¹) *in vitro*, under IVF conditions, diminishes polyspermy (Suzuki *et al.*, 2000, 2002). Taken together, the available information supports the contention that hyaluronan has a protective effect on sperm viability, although the mechanism of action is still unknown. Hyaluronan and other sulphated glycosaminoglycans are conspicuously present in the pig tubal fluid and there is a tendency to reach maximum amounts during oestrus (Tienthai *et al.*, 2000). Hyaluronan in pigs is localized in the oviductal preovulatory sperm reservoir (Tienthai *et al.*, 2000) and it is specifically produced by the epithelium as indicated by the expression of hyaluronan synthase 3 (has-3, Kimura *et al.*, 2002b). Lastly, as shown by the present results, the specific hyaluronan receptor CD44 is present in, and perhaps synthesized by, the same epithelium.

Pig oviduct epithelia were analysed by ligand blotting and western blotting for HABPs and CD44 protein respectively, to increase the understanding of a presumed interaction between hyaluronan and CD44. Four HABP bands of 60, 90, 100 and 200 kDa were present in each tubal sample. Those of 100 and 200 kDa varied among oviductal segments. Although there is a risk of non-specific interactions between the HA-FITC and hyaluronan, preliminary assessments of specificity using unlabelled hyaluronan (M. Yokoo, unpublished) ruled out non-specific binding. This finding implies that the oviduct epithelium possesses the binding proteins required to modulate eventual biological activities by hyaluronan, with one of the larger HABP group belonging to the CD44 family (Toole, 1990). In the present study, the anti-CD44 antibody used for both immunohistochemistry and western blotting was a mouse anti-pig CD44 monoclonal antibody termed wCD44, which is a very valuable reagent in studies of the immune system in pigs (Zuckermann *et al.*, 1994). The most common form of CD44, the so-called standard CD44, with a molecular mass of about 85–90 kDa, has been found in many tissues (Culty *et al.*, 1990), including the human cumulus granulosa cells (Ohta *et al.*, 1999). CD44 isoforms vary in size from 80 to 250 kDa, a variability that might arise from the alternative splicing of variant exons which, in turn, possibly modulates some of the functions of the receptor (Ponta *et al.*, 1998). In the present study, the CD44 antibody reacted specifically with a single band of protein, with a predicted molecular mass of 200 kDa, when isolated from oviduct epithelial homogenates, especially from the sperm reservoir. The findings should not be surprising as Terpe *et al.* (1994) reported that CD44 variant isoforms (CD44v) are mostly restricted to basal and luminal epithelia of human endometrium. For instance, the CD44E (epithelial isoform), with an approximate molecular mass of 130 kDa, was found in this location in the human endometrium (Behzad *et al.*,

1994). CD44 in the oviductal epithelium of the sperm reservoir may be similar to CD44v, building a specific pathway between hyaluronan and CD44. However, the presence of such a 200 kDa CD44 isoform in genital epithelia has not been reported. Western blotting and RT-PCR with the different CD44 antibodies, primers and probes is necessary to elucidate definitively the type of epithelial CD44 isoforms.

In the present study, the expression of CD44 mRNA was more intense in the sperm reservoir during pre-ovulatory oestrus than after ovulation ($P < 0.05$). CD44 was also more clearly localized in the UTJ-caudal isthmus (the sperm reservoir) than in the other segments. As described for non-inseminated sows (Tienthai *et al.*, 2000), the amounts of hyaluronan in the intraluminal fluid follow a similar pattern to that found here for CD44. Therefore, CD44 might bind or anchor hyaluronan to the epithelium and thereby affect the function of the oviduct. Whether sperm storage is related to this pathway remains to be determined, as only circumstantial evidence is so far provided (Rodriguez-Martinez *et al.*, 2001). Although the significantly higher expression of CD44 mRNA before ovulation might be a consequence of a particular hormone stimulation (for example, oestrogen), there is no explanation for the significant decrease in CD44 mRNA in relation to the presence of spermatozoa in the lumen. Although spermatozoa interact by binding to the epithelium (Suarez *et al.*, 1991), further studies are needed to ascertain whether such binding implies biological signalling of such a nature that could explain a downregulation of the receptor. In the present study, more samples showed strong CD44 immunolabelling in the inseminated sows compared with controls, indicating that these specimens have a higher amount of the receptor ligand compound in endosomes.

The use of RT-PCR in this study demonstrates that the oviduct epithelium of the sperm reservoir contains CD44 mRNA, with amounts corresponding to the presence of a distinct CD44 immunolabelling during the pre-ovulation stage. In general, the mammalian oviductal epithelium consists of two types of cell, ciliated and secretory cells which, in primates and cows, undergo cyclic variations in their relative numbers (Abe, 1996). In pigs, secretory cells are abundant in the isthmus, a ratio that changes markedly towards the ad-ovarian tubal segments (Rodriguez-Martinez *et al.*, 1988). Ovarian steroids modify, via specific receptors (Rodriguez-Martinez *et al.*, 1988), the synthetic and secretory capacity of the oviductal epithelium during the cycle (Abe and Oikawa, 1993) which, in turn, has led to suggestions that ovarian factors directly or indirectly affect sperm function in the sperm reservoir (Hunter *et al.*, 1998). In the human endometrium, CD44 mRNA expression in the normal menstrual cycle is closely related to the secretory differentiation of the glandular epithelium (Saegusa *et al.*, 1998). As stated,

the present study has shown that CD44 is most often found in secretory cells, more intensely in the sperm reservoir at the preovulatory stage, with corresponding findings in CD44 immunoblotting and amounts of CD44 mRNA. These findings confirm that the oviductal epithelium cells produce CD44, and indicate that variations in ovarian hormones may regulate CD44 mRNA during oestrus, as shown by the variability of the CD44 immunostaining in the epithelium at different stages of oestrus. For instance, Kimura *et al.* (2002a) reported that equine chorionic gonadotrophin and pig follicular fluid stimulate CD44 expression of cumulus cells during *in vitro* maturation. However, no attempts were made in the present study to determine hormonal patterns in the tissues of the experimental animals.

A conspicuous CD44-positive labelling was also present on the surface of intraepithelial leucocyte-like cells of the tissue specimens examined. There is a possibility, albeit not explored in the present study, that these leucocytes are intraepithelial lymphocytes and macrophages (Rodriguez-Martinez *et al.*, 1990; Abughrien *et al.*, 2000). CD44 is not only involved in the inflammatory response (Pure and Cuff, 2001), but is also highly expressed on macrophages during lung inflammation (Taylor *et al.*, 2000) and expressed on lymphocytes (Westermann *et al.*, 1996). Whether intraepithelial leucocytes would act as part of the mucosal defence in an immunologically privileged oviduct has not been determined in pigs. To the best of the authors' knowledge, no studies have ascertained whether sperm removal in pigs is effective in the oviduct, except for some morphological indications (Rodriguez-Martinez *et al.*, 1990).

In conclusion, the results of the present study indicate for the first time that CD44 in pigs is expressed by the oviductal epithelium during well-defined stages of spontaneous oestrus, varying in relation to the occurrence of spontaneous ovulation and the presence of inseminated spermatozoa. The presence of the hyaluronan receptors and the hyaluronan ligand in the organ calls for a signal pathway of hyaluronan CD44 that could play an important role during sperm storage in the tubal reservoir, fertilization and early embryo development. Additional studies are, however, required to define in detail the roles of hyaluronan during sperm transport and fertilization.

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References

- Abe H (1996) The mammalian oviductal epithelium: regional variations in cytological and functional aspects of the oviductal secretory cells *Histology and Histopathology* **11** 743–768
- Abe H and Oikawa T (1993) Effects of oestradiol and progesterone on the cytodifferentiation of epithelial cells in the oviduct of the newborn golden hamster *Anatomical Record* **235** 390–398
- Abughrien BM, Dore MA, McGeady TA and Fitzpatrick E (2000) Intraepithelial leucocytes in the bovine uterine tube *Cells, Tissues, Organs* **166** 20–30
- Barka T (1960) A simple azo dye method for histochemical demonstration of acid phosphatase *Nature* **187** 248–250
- Behzad F, Seif MW, Campbell S and Aplin JD (1994) Expression of two isoforms of CD44 in human endometrium *Biology of Reproduction* **51** 739–747
- Culty M, Miyake K, Kincade PW, Sikorski E, Butcher EC, Underhill C and Silorski E (1990) The hyaluronate receptor is a member of CD44 (H-CAM) family of cell surface glycoproteins *Journal of Cell Biology* **111** 2765–2774
- Culty M, Nguyen HA and Underhill C (1992) The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan *Journal of Cell Biology* **116** 1055–1062
- Durst B, Sorg RV, Röder G, Betz B, Beckmann MW, Niederacher D, Bender HG and Dall P (2001) The influence of hormones on CD44 expression in endometrial and breast carcinomas *Oncology Reports* **8** 987–993
- Harrison RAP, Mairet B and Miller NG (1993) Flow cytometric studies of bicarbonate-mediated Ca^{2+} influx in boar sperm populations *Molecular Reproduction and Development* **35** 197–208
- Hunter RHF (1981) Sperm transport and reservoirs in the pig oviduct in relation to time of ovulation *Journal of Reproduction and Fertility* **63** 109–117
- Hunter RHF (1984) Pre-ovulatory arrest and peri-ovulatory redistribution of competent spermatozoa in the isthmus of the pig oviduct *Journal of Reproduction and Fertility* **72** 203–211
- Hunter RHF, Huang WT and Holtz W (1998) Regional influences of the fallopian tubes on the rate of boar capacitation in surgically inseminated gilts *Journal of Reproduction and Fertility* **114** 17–23
- Huszar G, Willetts M and Corrales M (1990) Hyaluronic acid (Sperm Select) improves retention of sperm motility and velocity in normospermic and oligospermic specimens *Fertility and Sterility* **54** 1127–1134
- Kano K, Miyano T and Kato S (1998) Effects of glycosaminoglycans on the development of *in vitro*-matured and -fertilized porcine oocytes to the blastocyst stage *in vitro*. *Biology of Reproduction* **58** 1226–1232
- Kawakami E, Arai T, Oishi I, Hori T and Tsutsui T (2000) Induction of dog capacitation by glycosaminoglycans and glycosaminoglycan amounts of oviductal and uterine fluids in bitches *Journal of Veterinary Medical Science* **62** 65–68
- Kimura N, Konno Y, Miyoshi K, Matsumoto H and Sato E (2002a) Expressions of hyaluronan synthases and CD44 mRNAs in porcine cumulus–oocyte complexes during *in vitro* maturation *Biology of Reproduction* **66** 707–717
- Kimura N, Yokoo M, Tienthai P, Heldin P, Kjellén L, Sato E and Rodriguez-Martinez H (2002b) Localization of hyaluronan synthases, hyaluronidase and hyaluronan binding proteins in the epithelium of the pig oviduct *Theriogenology* **57** 500 (Abstract)
- Knudson W, Chow G and Knudson CB (2002) CD44-mediated uptake and degradation of hyaluronan *Matrix Biology* **21** 15–23
- Mburu JN, Einarsson S, Dalin A-M and Rodriguez-Martinez H (1995) Ovulation as determined by transrectal ultrasonography in multiparous sows: relationships with oestrous symptoms and hormonal profiles *Journal of Veterinary Medicine* **42** 285–292
- Mburu JN, Rodriguez-Martinez H and Einarsson S (1997) Changes in sperm ultrastructure and localization in the porcine oviduct around ovulation *Animal Reproduction Science* **41** 137–148
- Ohta N, Saito H, Kuzumaki T, Takahashi T, Ito MM, Saito T, Nakahara K and Hiroi M (1999) Expression of CD44 in human cumulus and mural granulosa cells of individual patients in *in vitro* fertilization programmes *Molecular Human Reproduction* **5** 22–28
- Parrish JJ, Susko-Parrish JL, Uguz C and First NL (1994) Differences in the role of cyclic adenosine 3',5'-monophosphate during capacitation of bovine sperm by heparin or oviduct fluid *Biology of Reproduction* **51** 1099–1108
- Pollard JW, Plante C, King WA, Hansen PJ, Betteridge KJ and Suarez SS (1991) Fertilizing capacity of bovine sperm may be maintained by binding to oviductal epithelial cells *Biology of Reproduction* **44** 102–107
- Ponta H, Wainwright D and Herrlich P (1998) The CD44 protein family *International Journal of Biochemistry and Cell Biology* **30** 299–305
- Pure E and Cuff CA (2001) A crucial role for CD44 in inflammation *Trends in Molecular Medicine* **7** 213–221
- Ranganathan S, Ganguly AK and Datta K (1994) Evidence for presence of hyaluronan binding protein on spermatozoa and its possible involvement in sperm function *Molecular Reproduction and Development* **38** 69–76
- Rodriguez-Martinez H (2001) Oviduct function in cows and pigs: with special reference to sperm capacitation *Asian–Australasian Journal of Animal Science* **14** 28–37
- Rodriguez-Martinez H, Stanchev P and Eriksson H (1988) Ultrastructure of the endosalpinx in relation to oestradiol and progesterone receptors during the porcine oestrous cycle *Proceedings of the 11th International Congress of Animal Reproduction and AI (ICARAI)*, Dublin **2** 64–66
- Rodriguez-Martinez H, Nicander L, Viring S and Larsson K (1990) Ultrastructure of the uterotubal junction in preovulatory pigs *Anatomia, Histologia, Embryologia* **19** 16–36
- Rodriguez-Martinez H, Han Y, Song X, Funahashi H and Niwa K (1997) Additive effects of sodium hyaluronate as inducer of capacitation of boar spermatozoa *in vitro*. *Proceedings of the 5th International Conference on Pig Reproduction*, Keekrade p 142 (Abstract)
- Rodriguez-Martinez H, Larsson B, Pertoft H and Kjellén L (1998) GAGs and spermatozoon competence *in vivo* and *in vitro*. In *Gametes: Development and Function* pp 239–274 Eds A Lauria, F Gondal, G Enne and L Gianaroli. Spono Symposia, Spono
- Rodriguez-Martinez H, Tienthai P, Suzuki K, Funahashi H, Ekwall H and Johannisson A (2001) Oviduct involvement in sperm capacitation and oocyte development *Reproduction Supplement* **58** 129–145
- Saegusa M, Hashimura M and Okayasu L (1998) CD44 expression in normal, hyperplastic, and malignant endometrium *Journal of Pathology* **184** 297–306
- Sbracia M, Grasso J, Sayme N, Stronk J and Huszar G (1997) Hyaluronic acid substantially increases the retention of motility in cryopreserved/thawed human spermatozoa *Human Reproduction* **12** 1949–1954
- Simonsson A (1994) Näringsrekommendationer och fodermedel till svin. Swedish University of Agricultural Sciences, Research Information Centre, Uppsala *Husdjur* **75** 1–61
- Smith TT and Yanagimachi R (1990) The viability of hamster spermatozoa stored in the isthmus of the oviduct: the importance of sperm–epithelial contact for sperm survival *Biology of Reproduction* **42** 450–457
- Suarez SS, Redferm K, Raynor P, Martin F and Philips DM (1991) Attachment of boar sperm to mucosal explants of oviduct *in vitro*: role in formation of a sperm reservoir *Biology of Reproduction* **44** 998–1004
- Suzuki K, Eriksson B, Shimizu H, Nagai T and Rodriguez-Martinez H (2000) Effect of hyaluronan on monospermic penetration of porcine oocytes fertilized *in vitro*. *International Journal of Andrology* **23** 13–21
- Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T and Rodriguez-Martinez H (2002) Capacitation status and *in vitro* fertility of boar spermatozoa: effects of seminal plasma, cumulus–oocyte complexes-conditioned medium and hyaluronan *International Journal of Andrology* **25** 84–93
- Taylor ML, Noble PW, White B, Wise R, Liu MC and Bochner BS (2000) Extensive surface phenotyping of alveolar macrophages in interstitial lung disease *Clinical Immunology* **94** 33–41
- Terpe HJ, Stark H, Prehm P and Gunthert U (1994) CD44 variant isoforms are preferentially expressed in basal epithelial of non-malignant human fetal and adult tissues *Histochemistry* **101** 79–89

- Tienthai P, Kjellén L, Pertoft H, Suzuki K and Rodriguez-Martinez H** (2000) Localization and quantitation of hyaluronan and sulfated glycosaminoglycans in the tissues and intraluminal fluid of the pig oviduct *Reproduction, Fertility and Development* **12** 173–182
- Toole BP** (1990) Hyaluronan and its binding proteins, the hyaladherins *Current Opinion in Cell Biology* **2** 839–844
- Turley EA, Austen L, Vandelight K and Clary C** (1991) Hyaluronan and a cell-associated hyaluronan binding protein regulate the locomotion of ras-transformed cells *Journal of Cell Biology* **112** 1041–1047
- Underhill C** (1992) CD44: the hyaluronan receptor *Journal of Cell Science* **103** 293–298
- Viring S, Einarsson S, Nicander L and Larsson K** (1980) Localization of the sperm 'reservoir' at the uterotubal junction of the pig *Proceedings of the 9th International Congress of Animal Reproduction and Artificial Insemination, Madrid* **3** 331 (Abstract)
- Westermann J, Walter H, Nagahori Y, Heerwagen C and Miyasaka M** (1996) Blood leucocyte subset of the rat: expression of adhesion molecules and localization within high endothelial venules *Scandinavian Journal of Immunology* **43** 297–303
- Woerner SM, Givehchian M, Durst M, Schneider A, Costa S, Melsheimer P, Lacroix J, Zoller M and Doeberitz MK** (1995) Expression of CD44 splice variants in normal, dysplastic and neoplastic cervical epithelium *Clinical Cancer Research* **1** 1125–1132
- Yokoo M, Tienthai P, Kimura N, Niwa K, Sato E and Rodriguez-Martinez H** (2002) Localisation of the hyaluronan receptor CD44 in porcine cumulus cells during *in vivo* and *in vitro* maturation *Zygote* **10** 317–326
- Zuckermann FA, Binns RM, Husmann R, Yang H, Carr MM, Kim YB, Davis WC, Misfeldt M and Lunney JK** (1994) Analyses of monoclonal antibodies reactive with porcine CD44 and CD45 *Veterinary Immunology and Immunopathology* **43** 293–305

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