Anti-human proacrosin antibody inhibits the zona pellucida (ZP)–induced acrosome reaction of ZP-bound spermatozoa

The anti-acrosin monoclonal antibody AcrC5F10 inhibited proacrosin activation, proacrosin-human zona pellucida glycoprotein A (ZPA) binding, and the zona pellucida (ZP)–induced acrosome reaction of the ZP-bound spermatozoa but had no significant effect on sperm-ZP binding. These results suggest that proacrosin-acrosin may play an important role in the ZP-induced acrosome reaction of spermatozoa after primary binding to the ZP. (Fertil Steril® 2010;93:2456–9. ©2010 by American Society for Reproductive Medicine.)

Key Words: Human, fertilization, spermatozoa, oocyte, proacrosin, acrosin, antibodies, acrosome reaction

Acrosin (EC 3.4.21.10) is a multifunctional protein localized to the acrosome of spermatozoa as the inactive zymogen proacrosin, converted to the active trypsin-like enzyme, and released during the acrosome reaction (AR) (1–3). Proacrosin activation involves endoproteolytic cleavage at both the N- and C-termini of the 53 KDa protein moiety, giving rise to the 35 KDa enzymatically active form, β-acrosin (4–6). Among the components that modulate proacrosin processing, boar zona pellucida (ZP) glycoproteins were reported to stimulate its conversion to β-acrosin (7–9). Evidence from studies done in animal experimental models and humans suggests the involvement of the proacrosin-acrosin system in several steps of fertilization, particularly in limited proteolysis and the release of other acrosomal contents during the AR, as well as in the interaction with ZP glycoproteins (10–12). Proacrosin-acrosin–deficient mouse spermatozoa generated by homologous recombination were found to have delayed ZP penetration and fertilization, they did not fertilize oocytes if challenged with wild-type spermatozoa, and they had a poor performance when exposed to oocytes with hardened ZP (13–15). In addition, $Acr^{-/-}$ spermatozoa showed an impairment in the release of their acrosomal proteins during the AR in spite of normal membrane vesiculation (16). Altogether, these results favor the notion of the involvement of proacrosin-acrosin in events that follow primary sperm binding to ZP.

Specifically regarding the human proacrosin-acrosin system, biochemical and molecular studies have been done to characterize structural and functional aspects of the active enzyme and the mechanism of the proenzyme activation with use of the purified proacrosin and whole sperm protein extracts (5, 6). Regarding its ability to interact with ZP glycoproteins, studies from our group performed with recombinant proacrosin (Rec-40) and truncated fragments revealed a key contribution of the sequence between positions 300–402 (DIII) to the interaction with ZP components (17), a region subjected to enzymatic processing during proenzyme activation (4, 6). Recently, our group also reported the detection of antibodies toward the human proacrosin-acrosin system in women consulting for infertility. Some of the antibodies specifically recognized the C-terminal protein region and inhibited the interaction between Rec-40 and rec-human zona pellucida glycoprotein A (hZPA), as well as the proenzyme activation (18); however, no further studies could be done to test their ability to block sperm-oocyte interaction because these antibodies are polyclonal in nature. The monoclonal antibody AcrC5F10 was developed toward human sperm proacrosin and was found to recognize the proenzyme only in acidic protein sperm extracts (6). In addition, AcrC5F10 was found to specifically recognize the recombinant proenzyme but not C-termini truncated products, and epitope mapping by the SPOTscan method identified the sequence spanning residues 305–309 (LPWYFQ) of the C-terminal region of human proacrosin (19).

On the basis of these findings, we proposed that interaction of human proacrosin with ZP glycoproteins, mainly hZPA, triggers

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or accelerates the proenzyme activation, and the active enzyme participates in ZP-induced sperm AR. The AcrC5F10 antibody may inhibit binding of human proacrosin to ZPA and proenzyme activation, resulting in an impaired sperm AR that would be evidenced by a decreased number of ZP-induced acrosome-reacted spermatozoa without changes in the number of sperm cells bound to the ZP. To test this hypothesis, the present study was carried out to assess the effects of AcrC5F10 anti-acrosin antibody on [1] h-proacrosin activation and enzymatic activity in sperm extracts, [2] interaction of recombinant h-proacrosin Rec-40 with rec-hZPA, [3] human sperm-ZP binding, and [4] ZP-induced AR of ZP-bound spermatozoa.

Unless specified, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), BioRad (Richmond, CA), Qiagen (Hilden, Germany), and Invitrogen Life Technologies (Carlsbad, CA). The monoclonal antibody AcrC5F10 was purchased from Biosonda (Santiago, Chile); it has been characterized as an IgG_1 isotype specific for human acrosin by indirect immunofluorescence, ELISA, and Western immunoblotting. Recombinant human ZPA (rec-hZPA) was expressed and purified from CHO cells, as previously described (20). Recombinant h-proacrosin (Rec-40) was expressed in *Escherichia coli* BL21 (DE3) with use of the pET22-b expression system and was purified by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (19). Human semen samples were provided by normozoospermic donors according to World Health Organization standards (21). All semen samples and oocytes were obtained with the donor's written consent, and protocols including human oocytes and spermatozoa were approved by the institutional review board (Royal Women's Hospital Research and Ethic Committee). In this study, only oocytes that failed to fertilize in IVF were used. Mouse IgG_1 antibodies (product M-7894, mouse IgG_1 κ (MOPC-21); Sigma), added at the same concentration as AcrC5F10, were used as control in all experiments.

BINDING OF REC-40 TO REC-HZPA

The effect of AcrC5F10 on binding of Rec-40 to rec-hZPA was evaluated as follows: Rec-40 (5 pmol per well) was immobilized in microtiter plates with 20 mmol/L of benzamidine, and, after blocking nonspecific binding sites, 5 μg/mL of AcrC5F10 (test) or MOPC-21 (control) antibodies and 1.0 μg per well of rec-hZPA were added. Binding of Rec-40 to rec-hZPA was revealed with use of a rabbit anti-hZPA antibody and an anti-rabbit IgG alkaline phosphatase conjugated secondary antibody; color reaction was developed by adding sodium *p*-nitrophenyl phosphate, and absorbance was measured at 450 nm (17). Experiments were repeated three times.

PROACROSIN ACTIVATION AND ACROSIN ACTIVITY

The effect of AcrC5F10 on activation of h-proacrosin or amidase activity of β-acrosin was assessed on human sperm acidic protein extracts, by adding 10 μg/mL of AcrC5F10 (test) or MOPC-21 (control) before or after proacrosin activation, respectively, followed by evaluation of DL-BAPNA hydrolysis with use of a colorimetric assay, as previously reported (6). Evaluations were done in duplicates in protein extracts from semen samples of three different donors.

SPERM–ZP BINDING AND ZP-INDUCED AR

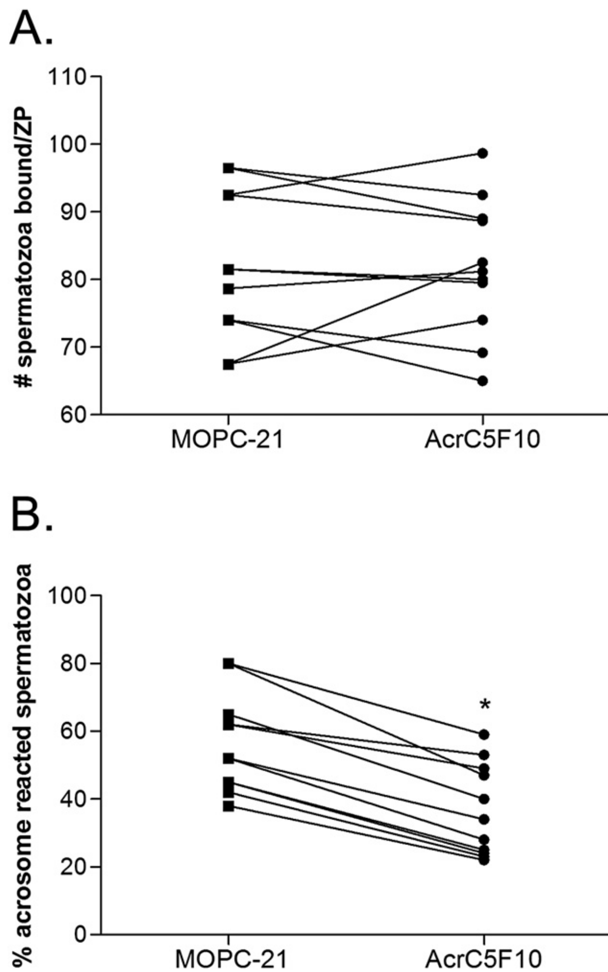
The effect of AcrC5F10 on human sperm-ZP binding and the ZP-induced AR of ZP-bound sperm was performed with use of unfertilized human oocytes from clinical IVF. Semen samples from normozoospermic men with normal sperm-ZP binding were used. Motile spermatozoa (2×10^6/mL) selected by the swim-up procedure were preincubated with 5 μg/mL of AcrC5F10 (test) or MOPC-21 (control) antibodies for 1 hour; then, four oocytes were added to both test and control sperm suspensions and incubated for an additional 2 hours. At the end of the incubation, each group of four oocytes was transferred to phosphate-buffered saline solution pH 7.4, containing 2 mg/mL bovine serum albumin, and washed by aspiration in and out of a glass pipette (inside diameter approximately 250 μm) to dislodge spermatozoa loosely adhering to the surface of the ZP. The number of spermatozoa tightly bound to the surface of the ZP of the oocytes was recorded. Then, all ZP-bound spermatozoa were removed from the ZP by vigorous aspiration in and out of a narrow-gauge micropipette with an inner diameter slightly smaller than that of the oocyte (approximately 120 μm) and smeared in a limited area (approximately 4 mm^2) of a glass slide. The acrosome status of spermatozoa was determined after cell staining with the lectin *Pisum sativum* agglutinin (PSA) labeled with fluorescein isothiocyanate (FITC-PSA), as follows: spermatozoa smeared onto the slide were fixed in 95% ethanol for 30 minutes after air drying and then stained in 25 μg/mL FITC-PSA in phosphate-buffered saline solution for 2 hours; the slide was washed and mounted. Two hundred spermatozoa per sample were counted with a fluorescence microscope and oil immersion at a magnification of $\times$400. Spermatozoa with more than half of the head showing a brightly and uniformly fluorescent signal were scored as acrosome intact, whereas sperm cells without fluorescence (a rare pattern) or a fluorescent band at the equatorial segment were considered to be acrosome reacted. Evaluations were done in a blind fashion (22). A total of 11 experiments were carried out.

Presence of AcrC5F10 antibody during proacrosin activation resulted in a decreased protease activity when compared with the control condition (AcrC5F10 = 76% of the activity in the presence of control MOPC-21; range: 72%–78%), suggesting the ability of the AcrC5F10 to impair, to some extent, proacrosin activation. In contrast to these findings, the addition of the AcrC5F10 after activation of sperm zymogens did not affect acrosin enzymatic activity (AcrC5F10 = 97% of control; range: 95%–99%). In addition to the negative effect of AcrC5F10 on proacrosin activation, a trend toward inhibition of Rec-40 binding to rec-hZPA was also observed when AcrC5F10 was added to the protein interaction assay (range: 37%–40%, compared with 0%–5% with control antibody). When added to the gamete interaction assay, the AcrC5F10 antibody was found to have no effect on sperm binding to the ZP (number of sperm bound/ZP = 81 [median value] with AcrC5F10 and 81 with MOPC-21, Fig. 1A). Contrasting with the lack of effect on sperm-ZP binding, presence of AcrC5F10 during the gamete interaction assay was associated with a significant ($P<.05$; Wilcoxon paired *t*-test) decrease in ZP-induced AR levels of ZP-bound spermatozoa (percentage of ZP-induced acrosome-reacted spermatozoa = 34% [median] with AcrC5F10 and 52% with MOPC-21, Fig. 1B).

The results describing the ability of the AcrC5F10 antibody to inhibit proacrosin activation are in agreement with previous findings

FIGURE 1

Effect of AcrC5F10 on spermatozoa-ZP binding and the ZP-induced AR of ZP-bound spermatozoa. Each line represents an individual assay. Wilcoxon paired *t*-test was used for statistical analysis. (**A**) Average number of spermatozoa bound/ZP calculated from four oocytes for each test (mean ± SEM; errors were omitted to diminish complexity of data representation). Differences between AcrC5F10 and control groups were not significant; $P > .05$. (**B**) Percentage of ZP-induced acrosome-reacted spermatozoa. $^*P < .05$.



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showing that AcrC5F10 recognizes a sequence localized in the proenzyme C-terminus, a protein region subjected to processing during proacrosin activation. Moreover, they agree with a previous report describing a blocking effect of AcrC5F10 on proacrosin activation induced by fucoidan in human sperm protein extracts (23). A relationship between abnormalities in proacrosin activation and altered sperm performance in in vitro fertilization was previously reported by our group (24). The decrease observed in the proacrosin-ZPA binding may result from steric hindrance caused by the antibody bound to the epitope located close to some of the motifs in the proenzyme C-terminal region involved in the interaction. These findings emphasize the relevance of C-terminal region of proacrosin in binding to ZPA and in proenzyme processing to the active form.

Sperm binding to ZP was not affected by AcrC5F10 anti-acrosin antibody; these results disagree with those from a previous study in which an inhibition of sperm binding to homologous ZP by AcrC5F10 antibody was reported (23). Differences between findings of these studies may be attributed to experimental protocols (longer gamete incubation time and 100 times higher concentration of the antibody) or to causes not identified by these authors. The results on the significant inhibitory effect of the anti-acrosin antibody on the acrosome reaction are in agreement with those describing the inhibition of acrosin activity and ZP-induced AR and sperm-ZP penetration by soybean trypsin inhibitor (25). Moreover, they agree with previous reports showing an impairment on the AR by antibodies from sera of patients receiving infertility treatment (26–28) or toward other specific proteins, such as actin (29), and with recent findings from our group showing an inhibitory effect of specific anti-acrosin antibodies generated by gene immunization on calcium ionophore–induced AR of murine spermatozoa (Veaute et al., unpublished observations). The AcrC5F10 antibody appears to be unable to access proacrosin molecules in noncapacitated intact sperm cells (6); membrane vesiculation may have occurred, as described in acrosin-deficient mice (16), allowing the antibody to access the proenzyme pool stored in the acrosome and inhibiting its activation. Alternatively, access of the antibody to the acrosomal compartment also may have occurred as the result of changes on the membranes during sperm capacitation, as reported by the release of some acrosomal matrix proteins (30, 31). The AR of ZP-bound human spermatozoa is critical for sperm-ZP penetration, and disordered ZP-induced AR causes failure of fertilization in conventional IVF and severe male infertility (32).

Results of the present study have shown the inhibitory effect of the anti-acrosin antibody AcrC5F10 on both the human proacrosin-acrosin activities and the ZP-induced AR of ZP-bound human spermatozoa but no effect on sperm-ZP binding, revealing the potential detrimental effect of specific antibodies toward the protease system on fertilization. Moreover, these findings suggest that the proacrosin-acrosin system plays an important role in the initial steps of the AR of ZP-bound human spermatozoa. The identification of sperm proteins involved in different events of gamete interaction, the mechanisms of their participation, and alterations in their functions is of great relevance toward understanding the molecular basis of human fertilization, as well as to unravel the causes of infertility.

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