GENERAL CONCLUSION

Several observations using morphological, physiological, and biochemical techniques have led investigators to propose that acrosin has multiple and integral functions in fertilization, including penetration of zona pellucida (ZP) by a sperm. However, using homologous recombination, Baba and coworkers have successfully produced male mice carrying a disruptive mutation in the acrosin gene (*Acr*). Surprisingly, they found that the $Acr^{-/-}$ mouse sperm completely lacking the acrosin protease activity still penetrate ZP and normally fertilize the egg. A unique phenotype observed in this $Acr^{-/-}$ mouse sperm showed a delay in sperm penetration of the ZP solely at the early stages after insemination as compared with homozygous and heterozygous mice sperm. These data provide evidence that acrosin is not essential for sperm penetration of the ZP.

To further elucidate the role of acrosin in fertilization, I have examined the involvement of acrosin in the acrosome reaction of sperm, using the $Acr^{-/-}$ mutant mice. When the ability of sperm to adhere (attach) and bind to the ZP of cumulus-free eggs was assessed *in vitro*, no significant difference was observed among $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ mouse sperm. Immunocytochemical analysis demonstrated that the release of several acrosomal proteins from the acrosome of $Acr^{-/-}$ mouse sperm was significantly delayed during the calcium ionophore- and solubilized ZP-induced acrosome reaction, in spite of normal membrane vesiculation. These data indicate that the delayed sperm penetration of the ZP in the $Acr^{-/-}$ mouse results from the altered rate of protein dispersal from the acrosome, and provide the first evidence that the major role of acrosin is to accelerate the dispersal of acrosomal components during acrosome reaction.

If proteolytic enzyme(s) sensitive to trypsin inhibitors are essential for the sperm penetration of ZP, as described everywhere, my findings lead to an additional question; does the sperm acrosome still contain trypsin-like protease(s), other than acrosin, that act on the limited proteolysis of ZP? Therefore, I have examined the effects of *p*-aminobenzamidine, a competitive inhibitor of trypsin and acrosin, on the function of $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm. It has been reported that a significant delay of protein

dispersal from the acrosomal matrix is observed in wild-type mouse sperm by adding *p*-aminobenzamidine, a trypsin/acrosin inhibitor, to the incubation medium. The pattern of this delayed release was similar to that of the $Acr^{-/-}$ mutant mouse sperm. However, no further delay of the protein dispersal was found when the $Acr^{-/-}$ sperm were treated with *p*-aminobenzamidine, indicating that among the *p*-aminobenzamidine-sensitive protease(s), only acrosin may function to accelerate this process. Although the $Acr^{-/-}$ sperm penetrated the ZP, the addition of *p*-aminobenzamidine in the fertilization medium caused a significant inhibition of fertilization *in vitro*. The result indicates that there is a *p*-aminobenzamidine-sensitive protease(s) other than acrosin participating in the zona penetration step. Indeed, I demonstrated that a protease with a size of 42 kDa was present in the supernatant of the acrosome-reacted sperm suspension. The enzyme was inhibited by *p*-aminobenzamidine, diisopropyl fluorophosphate and *N*-tosyl-L-lysine chloromethyl ketone, and was apparently activated by acrosin.

To elucidate the molecular mechanism of the sperm penetration through the egg ZP, it is thus important to characterize the acrosomal serine proteases further, including the 42-kDa protease. I have then examined gelatin-hydrolyzing enzymes present in two protein fractions: one is a fraction of proteins released from the acrosome of rodent sperm during calcium ionophore A23187-induced acrosome reaction (AR fraction), and another is a protein fraction obtained by extracting the acrosome-reacted sperm with Nonidet P-40 (NP fraction). A mixture of 42- and 41-kDa gelatin-hydrolyzing proteins was present in both AR and NP fractions of $Acr^{+/+}$ mouse sperm. In addition to the 42-kDa protease, the 41-kDa enzyme was also detected in $Acr^{-/-}$ mouse sperm by treatment of the sperm extracts with bovine pancreatic trypsin. Two-dimensional polyacrylamide gel electrophoresis revealed that the 42- and 41-kDa proteases were distinguished from acrosin by the isoelectric point. Surprisingly, the gelatin-hydrolyzing proteins corresponding to these two proteases were not detectable in rat and hamster sperm, and the amount of the gelatin-hydrolyzing activity in mouse was much smaller than those in rat and hamster. These findings may reflect the difference of the serine protease system for the sperm penetration through the egg ZP between mouse and other rodent animals.