Presence of the complement-regulatory protein membrane cofactor protein (MCP, CD46) as a membrane-associated product in seminal plasma

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The seminal plasma complement regulator membrane cofactor protein (MCP) was examined by sequential centrifugation and phase separation in the detergent Triton X-114. The presence of MCP components in seminal plasma depleted of the 40 kDa sperm MCP product by low speed centrifugation was confirmed. Subsequent centrifugation at 2200 g recovered a pellet containing a prominent 60 kDa and a weak 50–55 kDa MCP component. A 60 kDa MCP product remained detectable in the supernatant fraction after this centrifugation step but this was depleted by ultracentrifugation. Recovery of the seminal plasma MCP components in the pellet fraction obtained by ultracentrifugation suggested that seminal plasma MCP is membrane-associated. Seminal plasma fractions were also subjected to phase separation in Triton X-114. MCP components in both pellet and supernatant fractions partitioned to the detergent phase, confirming that seminal plasma MCP is membrane associated. The origin of these proteins was investigated by analysing MCP products in seminal plasma from vasectomized men. The 40 kDa sperm MCP protein was absent but a 60 kDa MCP component, which partitioned to the detergent phase in Triton X-114, was evident. Seminal plasma therefore contains typical membrane-associated MCP products that appear to be derived distal to the ductus deferens.

Introduction

Spermatozoa are at risk from complement-mediated damage during their transit through the female reproductive tract which contains functionally active complement (Price and Boettcher, 1979; Perricone et al., 1992). It has been demonstrated that spermatozoa express three membrane bound complement regulatory proteins, decay-accelerating factor (DAF), membrane cofactor protein (MCP) and CD59 that, in other systems, function to prevent autologous complement-mediated damage (Anderson et al., 1989; Cervoni et al., 1992; Rooney et al., 1992; Cervoni et al., 1993; Simpson and Holmes, 1994). These regulators act at two levels within the complement pathway. DAF and MCP both act at the level of the C3 convertases (Lublin and Atkinson, 1989; Seya and Atkinson, 1989), while CD59 interacts with the terminal complement pathway components C8 and C9 directly regulating the formation of the cytolytic membrane attack complex (MAC) (Rollins et al., 1991). Each of the complement regulators displays a distinct distribution on mature spermatozoa and they have all been shown to be functionally active. However, while DAF and MCP are expressed on spermatozoa as novel low molecular mass forms of 55 and 40 kDa, respectively, CD59 is expressed as an 18–20 kDa protein typical of other systems (Anderson et al., 1989; Cervoni et al., 1992; Rooney et al., 1992; Cervoni et al., 1993; Simpson and Holmes, 1994). The distribution of the proteins suggests that C3 convertase regulators play an important role during fertilization when the inner acrosomal membrane is exposed following capacitation and acrosome reaction, while protection of spermatozoa from complement in the lower regions of the female reproductive tract is focused primarily at the MAC (Simpson and Holmes, 1994).

It is evident that seminal plasma also contains a number of complement regulatory factors. Rooney et al. (1993) demonstrated that extracellular vesicular organelles termed prostasomes, present in seminal plasma, express CD59 and they proposed that spermatozoa may acquire additional surface CD59 by interacting with prostasomes. In addition, Hara et al. (1992) reported that MCP is present in seminal plasma and suggested that the protein represented a soluble form of MCP because it was present in seminal plasma from which spermatozoa had been removed by low speed centrifugation. Seya et al. (1993) demonstrated that seminal plasma MCP is a 60 kDa protein with the functional characteristics of MCP isolated from cells. Other MCP species that have been characterized are generally expressed as two transmembrane components which function intrinsically, protecting only the cell on which they are expressed (Seya and Atkinson, 1989). This raises the question of how a soluble MCP component in seminal plasma could confer protection from complement-mediated damage to spermatozoa. The origin of seminal

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plasma MCP is also unclear. Seya et al. (1993) reported that the protein comigrates with a testis MCP product in SDS-PAGE, but it is also known that an MCP protein of similar molecular mass is expressed in the prostate where MCP is coexpressed with CD59 on the prostatic epithelium (Seya et al., 1993; Simpson and Holmes, 1994). The aim of this study was to examine the nature of the 60 kDa MCP protein present in seminal plasma by sequential centrifugation and phase separation in the detergent Triton X-114. In view of the known contribution of the prostate to seminal plasma, we also investigated the origin of this MCP product by comparing seminal plasma from normal fertile donors and from vasectomized men.

Materials and Methods

Tissues, antibodies and membrane and seminal plasma preparations

Adult human testes were obtained fresh at surgery from patients undergoing orchidectomy. Prostate glands were obtained as surgical specimens from men undergoing transurethral resection of the prostate; membranes were prepared as previously described (Simpson and Holmes, 1994). Supernatants recovered after ultracentrifugation were retained and designated the soluble fraction.

Human semen was obtained from normal fertile donors and from patients returning for semen analysis following vasectomy. Specimens were liquefied at 37°C for 30 min and seminal plasma prepared by centrifugation at 1600 g for 5 min to remove spermatozoa, followed by a further centrifugation step at 2200 g for 10 min as described by Seya et al. (1993). The resulting seminal plasma was supplemented with 1 mmol phenylmethyl sulfonyl fluoride l−1 and then subjected to one or two further ultracentrifugation steps at 100 000 g for 1 h. At each centrifugation step, a sample of supernatant and pellet material was retained. With the exception of the pellets obtained from the initial centrifugation step of semen from vasectomized men, the pellets from each centrifugation step were resuspended in similar volumes of either nonreducing sample buffer or tris-buffered saline (TBS). Pelleted material obtained by centrifugation of vasectomized semen at 1600 g was resuspended directly into a small volume, typically 100 µl, of nonreducing sample buffer, to ensure that any contamination with spermatozoa could be excluded in subsequent blotting experiments.

The following monoclonal antibodies (mAbs) were used: J4.48 (purified ascites, Serotec, Oxford) anti-MCP mAb (Pesando et al., 1987) diluted to a final concentration of 0.2 µg ml−1 in TBS containing 2% (w/v) BSA and BRIC 229 anti-CD59 mAb (Fletcher et al., 1992) as undiluted tissue culture supernatant. All secondary and tertiary antibodies were supplied by Dako Ltd (Glossop).

Gel electrophoresis, immunoblotting and phase separation in Triton X-114

Western blotting was carried out using a modification of the method described by Towbin et al. (1979). Briefly, membrane preparations and pellet and supernatant fractions derived from semen were subjected to SDS-PAGE under nonreducing conditions on gels containing 10% (w/v) acrylamide according to the method of Laemmli (1970) and electro-transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, UK Ltd., Watford). The membranes were blocked with 5% (w/v) dried milk powder in PBS containing 0.2% (v/v) Tween 20, incubated for 2 h at room temperature with primary mAb, and mAb binding detected using an enhanced immunoperoxidase technique (Wainwright and Holmes, 1993).

Membrane preparations of testis and prostate, and supernatant and pellet fractions of semen were separated into detergent and aqueous phases by condensation in Triton X-114 as described by Bordier (1981).

Results

Examination of MCP products in semen from normal fertile donors by immunoblotting

Semen from normal fertile donors was subjected to sequential centrifugation and MCP components were examined in the pellet and supernatant fractions by immunoblotting (Fig. 1a). The initial 1600 g centrifugation step recovers spermatozoa from semen and, consistent with previous reports, a novel 40 kDa sperm MCP component was detected in this fraction (Fig. 1a, lane 1) (Anderson et al., 1989; Cervoni et al., 1992; Seya et al., 1993; Simpson and Holmes, 1994). Subsequent centrifugation at 2200 g generated a pellet that contained a prominent 60 kDa MCP component and, in some specimens, additional weak components of 50–55 kDa and 40 kDa (Fig. 1a, lane 2). The 40 kDa component is most probably derived from the residual spermatozoa. The presence of both a 50–55 kDa and a 60 kDa product in this pellet fraction suggests that seminal plasma contains membrane-associated MCP proteins typical of other isolated MCP species. MCP typically migrates as two components in SDS-PAGE and, as a result of an expression polymorphism, there is an inherited variability in the relative quantity of each component (Ballard et al., 1987). The upper component predominant phenotype is most frequent and in these individuals the quantity of the lower component varies up to a maximum of 35% total MCP. This variability may account for the apparent absence of the lower component in some individuals as the concentration of this protein may be below the limit of detection. In addition, Seya et al. (1993) have shown that the concentration of MCP in seminal plasma of individuals varies between 250 and 700 ng ml−1 and this may influence the profile of detectable MCP components.

Analysis of the supernatant fraction generated following centrifugation at 2200 g revealed a component of 60 kDa (Fig. 1a, lane 3). This is consistent with the report of Seya et al. (1993) who, under the same conditions, identified a product of similar molecular mass in seminal plasma, which they designated soluble MCP. Supernatants obtained from the 2200 g centrifugation step were subjected to ultracentrifugation to determine whether extracellular vesicles in seminal plasma are likely to contribute to MCP in this fluid. The profile of MCP components observed in the pellet after ultracentrifugation of seminal plasma at 100 000 g was similar to that present in the pellet obtained after centrifugation at 2200 g (compare Fig. 1a,
lanes 2 and 4). Thus although the 2200 g supernatant appeared to contain only a 60 kDa MCP component, additional 50–55 and 40 kDa MCP proteins were clearly evident in the pellet recovered when this supernatant was subjected to ultracentrifugation. The most likely explanation is that ultracentrifugation enriches for MCP components as a proportion of total protein and, consistent with this, the 60 kDa MCP in the pellet fraction is increased by comparison with the supernatant from which it was derived (Fig. 1a, lanes 4 and 3, respectively). Some MCP components are not detectable in unconcentrated seminal plasma and this may explain why Seya et al. (1993) observed only a single 60 kDa MCP component in seminal plasma. Because the 40 kDa MCP product unique to spermatozoa is a transmembrane protein, its detection in the pellet fraction most probably reflects the presence of membrane fragments derived from damaged spermatozoa. Hence, the 60 and 50–55 kDa MCP components in the same pellet fraction are also likely to be membrane-associated. Moreover, seminal plasma CD59, which is known to be present on prostaticsomes, was also found predominantly in the pellet rather than the supernatant fraction after ultracentrifugation (Fig. 1b, lanes 1 and 2, respectively). A weak 60 kDa MCP component detectable in the supernatant fraction recovered after the first ultracentrifugation step (Fig. 1a, lane 5) was depleted after a second ultracentrifugation step (Fig. 1a, lane 7). MCP proteins in seminal plasma therefore appear to be membrane-associated and, to confirm this, we conducted phase separation studies using Triton X-114.

**Phase separation of seminal plasma MCP in Triton X-114**

Upon condensation in Triton X-114, amphiphilic or integral membrane proteins partition to the detergent phase while hydrophilic or soluble proteins remain exclusively in the aqueous phase. In pelleted material obtained after centrifugation of seminal plasma at 2200 g, both 60 kDa and 40 kDa MCP components detectable in the specimen illustrated were present only in the detergent phase (Fig. 2, lane 2); the aqueous phase was devoid of detectable MCP (Fig. 2, lane 3). Similarly, the 60 kDa MCP product detected in the supernatant fraction after centrifugation at both 2200 and 100 000 g (Fig. 2, lanes 4 and 7, respectively) also partitioned exclusively to the detergent phase (Fig. 2, lanes 5 and 8, respectively); again, no MCP products were detected in the aqueous phase (Fig. 2, lanes 6 and 9).

**Examination of MCP products in seminal plasma from vasectomized men**

The origin of seminal plasma MCP components is unclear. Seya et al. (1993) suggested that the 60 kDa component which they designated soluble MCP is derived from the testis. However, our own studies (Simspon and Holmes, 1994) have shown that both testis and prostate membrane preparations contain MCP proteins similar in molecular mass to seminal plasma MCP. In the present study, MCP components detected in testis (Fig. 3, lane 1) and prostate (Fig. 3, lane 5) membrane preparations were found to partition exclusively to the detergent phase (Fig. 3, lanes 2 and 6, respectively); no MCP components were detected in the corresponding aqueous phase (Fig. 3, lanes 3 and 7, respectively). Either of these two tissues could therefore contribute membrane-associated MCP components to seminal plasma, although the profile of MCP components present in the pellet recovered from seminal plasma by ultracentrifugation closely resembles that of MCP proteins detected in prostate membranes (Fig. 1, lane 4 and Fig. 3, lane 6).
Fig. 2. Separation of the seminal plasma membrane cofactor protein (MCP) in Triton X-114. MCP components in pelleted material and supernatant fraction from the 2200 g centrifugation step (lanes 1 and 4, respectively) and supernatant from the 100 000 g step (lane 7) were phase separated in Triton X-114. After partitioning, anti-MCP mAb detected MCP components in the detergent phases (lanes 2, 5 and 8). MCP products were undetectable in the corresponding aqueous phases (lanes 3, 6 and 9).

Fig. 3. Immunoblot of membrane cofactor protein (MCP) components in membrane and soluble fractions of human testis and prostate. Anti-MCP mAb detects three MCP products in membrane preparations of normal human testes (lane 1) and two components in prostate membrane preparations (lane 5). On separation of testis and prostate membranes in Triton X-114, all components partitioned to the detergent phase (lanes 2 and 6, respectively); the corresponding aqueous phases were devoid of MCP components (lanes 3 and 7, respectively). Soluble fraction obtained from testis contained no MCP products (lane 4). A very weak 60 kDa MCP component was evident in the soluble fraction of some prostate specimens (lane 8), although this partitioned to the detergent phase in Triton X-114 (data not shown).

This suggests that seminal plasma is unlikely to contain soluble MCP. However, to determine whether soluble MCP proteins are generated by testis and prostate, we examined the soluble fraction obtained from these tissues. No MCP components were detected in the soluble fraction of testis (Fig. 3, lane 4), but a very weak 60 kDa component was evident in the soluble fraction of some prostate specimens tested (Fig. 3, lane 8) although, in common with residual MCP observed in seminal plasma following centrifugation at 100 000 g, this partitioned to the detergent phase in Triton X-114 (data not shown).

The origin of seminal plasma MCP was investigated further by subjecting semen samples from vasectomized men to sequential centrifugation. The 40 kDa sperm MCP component observed in semen from normal fertile donors was absent from the pellet obtained after centrifugation of semen from 5, respectively).
vasectomized men at 1600 g (Fig. 4, lane 2). However, this pellet contained a 60 kDa MCP component that was only weakly evident in the pellet obtained from normal donors (compare Fig. 4, lanes 1 and 2). This is due to technical differences in the preparation of pellets from normal and vasectomized donors; the 60 kDa MCP component is over-represented in samples from vasectomized as opposed to normal donors because these samples were concentrated to ensure that spermatozoa were completely absent. In the remaining centrifugation steps, the MCP profile of seminal plasma from vasectomized donors was similar to that of normal fertile donors. Thus, a 60 kDa component was detected in the supernatant after centrifugation at 2200 g (Fig. 4, lane 4) and this was depleted by ultracentrifugation; the MCP component was detected in the pellet obtained from centrifugation at 2200 g (Fig. 4, lane 5) rather than in the supernatant fraction (Fig. 4, lane 6). Moreover, the 60 kDa MCP component in the pellet obtained by centrifugation at 2200 g (Fig. 5, lane 1) and in supernatants from both 2200 g and 100 000 g centrifugation steps (Fig. 5, lanes 4 and 7, respectively) partitioned to the detergent phase when separated in Triton X-114 (Fig. 5, lanes 2, 5, and 8). A similar 60 kDa membrane-associated MCP protein is therefore present in seminal plasma from both fertile and vasectomized donors.

Discussion

In this study we set out to investigate MCP proteins in human seminal plasma further. We confirmed that seminal plasma contains an MCP protein of approximately 60 kDa and demonstrated that seminal plasma may also contain a 50–55 kDa MCP component. MCP proteins were depleted from seminal plasma by ultracentrifugation and partitioned to the detergent phase when extracted with Triton X-114, suggesting that these products are membrane-associated. We show that seminal plasma from vasectomized donors contains MCP products with similar separation characteristics in Triton X-114 and that seminal plasma MCP is therefore derived distal to the ductus deferens.

MCP is a transmembrane protein and as an intrinsic complement regulator it binds only to C3b/C4b deposited on the cell surface on which it is expressed, acting then as a cofactor for the serine protease factor I (Seya and Atkinson, 1989). The reported presence of apparently soluble forms of MCP in a number of body fluids including tears, blood plasma and seminal fluid (Hara et al., 1992) appears paradoxical in view of the known functional and biochemical properties of this protein in other systems. Hence, soluble MCP in seminal plasma would be unlikely to confer protection against the deposition of activated complement on the surface membranes of spermatozoa. The present study shows that seminal plasma contains 60 and 50–55 kDa MCP components that are depleted by ultracentrifugation and partition to the detergent phase in Triton X-114. Seminal plasma MCP is therefore typical of other characterized MCP species in that it is membrane-associated.

Seminal plasma contains components derived from multiple male accessory glands as well as from the testis. Seya et al. (1993) suggested that the origin of the 60 kDa MCP component detected in seminal plasma, and designated soluble MCP, was most probably the testis. This was based on the observation that an MCP protein of this molecular mass was present in a lysate of testis. However, it was subsequently demonstrated that membrane preparations of human testis contain both an MCP protein of approximately 60 kDa and also components of 50–55 kDa and 45 kDa (Simpson and Holmes, 1994). The hydrophobic nature of the testis membrane MCP components was confirmed in the present study and this, together with the apparent absence of MCP products from the soluble fraction of testis, further suggests that soluble MCP is unlikely to be present in seminal plasma but rather that the testis could contribute membrane-associated MCP products.

In the present study, however, the presence of MCP in seminal plasma from vasectomized men suggests that this protein is derived from a location distal to the ductus deferens rather than...
from the testes. None the less, Rooney et al. (1993) noted that the concentration of CD59 in seminal plasma from vasectomized donors appeared lower than that from normal fertile donors, indicating that the testis may contribute in part to seminal plasma CD59. We have not directly compared concentrations of MCP in seminal plasma from vasectomized and fertile donors and therefore a contribution by the testis to seminal plasma MCP cannot be entirely excluded. A more complete assessment of the status of the male genital tract for complement regulatory proteins is now necessary to establish the contribution of the various male accessory glands to MCP in seminal plasma.

Although membrane-associated MCP in seminal plasma could be present on cell debris, prostasomes are more likely candidates because these organelles are already known to contain CD59 (Rooney et al., 1993). In addition, both CD59 and MCP are co-expressed on prostatic epithelium (Seya et al., 1993; Simpson and Holmes, 1994) and, in the present study, the majority of MCP detected in seminal plasma was pelleted in the same centrifugation step as CD59, which is known to be associated with prostasomes. Therefore MCP, like CD59, may be present on prostasomes. It has been shown that, by virtue of its GPI-anchor, spermatozoa may acquire additional surface CD59 through interaction with prostasomes (Rooney et al., 1993). However, since MCP is a transmembrane protein it is unlikely that seminal plasma could act as a reservoir for MCP and, in any case, spermatozonal MCP is confined solely to the inner acrosomal membrane (Anderson et al., 1989; Cervoni et al., 1992; Simpson and Holmes, 1994). Rather than protecting spermatozoa it is more likely that MCP, consistent with its known intrinsic activity, confers protection from complement-mediated damage directly on prostasomes or other organelles on which it is expressed. Although their function in seminal plasma is currently unclear, prostasomes have been shown to possess immunosuppressive activity and may also contain enzymes (Ronquist and Brody, 1985; Skibinski et al., 1992) and, overall, they are considered beneficial to fertility. Through interaction with spermatozoa, prostasomes could be capable of ascending the female reproductive tract where, at multiple locations, they may be at risk from complement-mediated damage. We suggest that expression of membrane-associated MCP, in addition to CD59, may contribute to the maintenance of the integrity of prostasomes and therefore potentially to reproductive success.

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