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A specific immunoassay for proAMH, the uncleaved proprotein precursor of anti-Müllerian hormone

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ABSTRACT

The utility of serum anti-Müllerian hormone (AMH) assays in assessment of female fertility have been investigated extensively but little is known about the biological activity of the hormone being studied. ProAMH is the proprotein precursor and is incapable of binding to the AMH-specific type II receptor. Proteolytic cleavage generates receptor-competent $AMH_{N,C}$ which is a non-covalent complex of the N- and C-terminal cleavage fragments. Commercially available AMH assays do not differentiate the two forms of AMH. Techniques were developed to dissociate the $AMH_{N,C}$ complex and abolish its two-site immunoassay immunoreactivity. This allowed specific quantification of proAMH. The surfactant sodium deoxycholate (DOC) dissociated $AMH_{N,C}$ without disrupting binding of proAMH to the capture-antibody with an optimal concentration of 0.1–0.2%w/v. The incorporation of a DOC incubation step into the AMH Gen II ELISA detected proAMH, with $AMH_{N,C}$ cross-detection conservatively estimated at 6.0% ± 2.5% (mean ± S.D.). The intra-assay and inter-assay variability were estimated at 8.0%CV and 13.0% CV respectively. The levels of proAMH and total AMH were assessed in 5 boys and 5 men and the proportion of proAMH was found to be significantly higher in boys ($p = 0.005$). This study will facilitate further investigation of the role of proteolytic cleavage in AMH signalling.

1. Introduction

Anti-Müllerian hormone (AMH) is a TGFβ superfamily cytokine produced by granulosa cells in the ovary and Sertoli cells within the testis. The clinical utility of serum AMH assays is established in paediatric males and adult females (Seifer and Maclaughlin, 2007; Visser et al., 2012) while uses in adult males are emerging (Dennis et al., 2013). AMH levels in women are highly correlated with antral follicle counts and can therefore be used to predict ovarian reserve non-invasively (de Vet et al., 2002; Fanchin et al., 2003; van Rooij et al., 2002). Very high levels of AMH are associated with ovarian pathologies such as polycystic ovary syndrome (Iliodromiti et al., 2013; Pigny et al., 2003) or increased risk of ovarian hyperstimulation syndrome during IVF protocols (Broer et al., 2011; Yates et al., 2011). Serum AMH assays are utilised in paediatrics to determine the presence of testes in incidences of ambiguous genitalia and intersex conditions (Lee et al., 2003). There is also a sharp decline in AMH levels between tanner stages 2 and 3 during the pubertal transition in males, but the clinical significance of this phenomenon remains undetermined (Aksglaede et al., 2010).

AMH is synthesised as a glycoprotein precursor (proAMH) that is incapable of binding to the AMH-specific receptor, AMHR2 (di Clemente et al., 2010; MacLaughlin et al., 1992). The receptor-binding form, $AMH_{N,C}$, is formed by proteolytic cleavage of proAMH by subtilisin/kexin proprotein convertases (Nachtigal and Ingraham, 1996). $AMH_{N,C}$ is a non-covalently associated complex of the N-terminal and C-terminal cleavage fragments (AMH_N and AMH_C, respectively) (Pepinsky et al., 1988). $AMH_{N,C}$ and AMH_C are both capable of activating AMH receptor complexes (MacLaughlin et al., 1992) but free AMH_C is yet to be detected in serum and may not be a physiological form. The forms of AMH in human blood were first described recently, with both proAMH and $AMH_{N,C}$ being present (Pankhurst and McLennan, 2013). The physiological

Abbreviations: AMH, anti-Müllerian hormone; ELISA, enzyme-linked immunosorbent assay; DOC, sodium deoxycholate; CV, coefficient of variation; proAMH, proprotein precursor AMH; AMH_N, N-terminal AMH cleavage fragment; AMH_C, C-terminal AMH cleavage fragments; $AMH_{N,C}$, noncovalent complex of the N-teminal and C-terminal cleavage fragments; rh-AMH, recombinant human AMH; TMB, 3,3′,5,5′-tetramethylbenzidine; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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significance of proAMH in circulation is currently unknown (McLennan and Pankhurst, 2015; McLennan et al., in press) but some proproteins are synthesised at one site and activated at another (Fyhrquist and Saijonmaa, 2008) which enables the activity of the protein to be modulated by multiple cell types. There are other examples (e.g. proNGF) where the proprotein has functions that are independent of the cleaved form (Hempstead, 2014; Nykjaer et al., 2004). Neither of these possibilities have been examined for proAMH, as the current AMH ELISAs do not differentiate the two forms and instead measure an aggregate of proAMH and $AMH_{N,C}$ (total AMH) (Gassner and Jung, 2014; Pankhurst et al., 2014).

The aim of the present study was to produce a specific immunoassay for proAMH. ProAMH and $AMH_{N,C}$ have antigenic similarity as their amino acid sequences differ only in the presence or absence of the amide bond at position 451/452. Therefore, development of a method to abolish the detection of $AMH_{N,C}$ was favoured over the generation of cleavage-site-specific antibodies. The strategy involved the use of the surfactant sodium deoxycholate (DOC) which dissociates the non-covalent bonds between the $AMH_{N,C}$ fragments (Pepinsky et al., 1988). A two-site immunoassay with a capture-antibody binding to AMH C-terminal epitopes and a detection antibody binding to AMH N-terminal epitopes was used. The DOC incubation step produced a proAMH-specific assay by dissociating $AMH_{N,C}$ which led to removal of the N-terminal fragment in subsequent wash steps (Fig. 1). The detection of proAMH was retained as the antibody binding-sites in the N- and C-terminal domains are connected via covalent bonds.

2. Materials and methods

2.1. Participants and serum collection

All study participants were healthy volunteers recruited from the community in Dunedin, New Zealand. The age and sex of each participant is listed in Table 1 or Supplementary Table 1. This project was approved by the University of Otago Human Ethics Committee (Health). Written informed consent was obtained directly from all participants and from the legal guardian of the 5 boys that were under the age of consent. Venous blood was drawn and clotted for 1 h at room temperature. Serum was obtained by centrifugation at 2000 ×g for 5 min and samples were snap-frozen and stored at −80 °C.

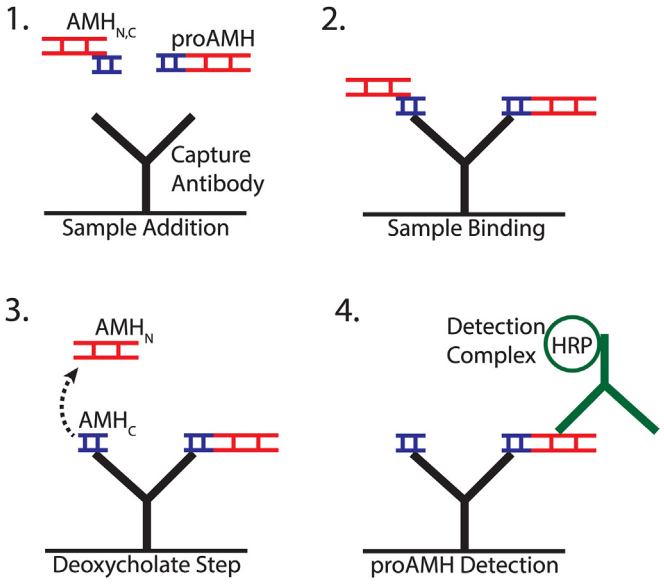

Fig. 1. Schematic diagram of the proAMH-specific ELISA technique. During sample addition (step 1), proAMH and $AMH_{N,C}$ are both present and available to bind to the capture antibody. In sample binding (step 2), both $AMH_{N,C}$ and proAMH bind to the capture antibody on the ELISA plate via epitopes in the C-terminal domain. Deoxycholate (step 3) is added to dissociate the $AMH_{N,C}$ complex, liberating AMH_N to the solution to be removed in subsequent wash steps. The detection complex (step 4) consisting of the biotinylated detection antibody and streptavidin-conjugated horseradish peroxidase (HRP) binds to epitopes in the AMH N-terminal domain. ProAMH remains the only AMH species available for the capture *and* detection antibodies.

2.2. Recombinant proteins and proAMH standards

Recombinant human AMH (rh-AMH) was isolated from HEK293 cells transfected with a proAMH vector (Px'Therapeutics, Grenoble, France, under contract to the University of Otago). This proAMH preparation contains trace amounts of $AMH_{N,C}$ (Pankhurst and McLennan, 2013). The concentration of the rh-proAMH standard was calibrated to the AMH Gen II standards (Beckman Coulter, Cat# A79766) using the AMH Gen II ELISA kit (Beckman Coulter, Cat# A79765). $AMH_{N,C}$ standards were produced according to a previously described method (Pankhurst et al., 2014). Briefly, 2.75 nM rh-proAMH was treated with 200 IU/mL furin (New England Biolabs, Cat# P8077) for 24 h at 37 °C. A similarly treated rh-proAMH sample was prepared without the addition of furin for comparison (Fig. 2). The proAMH standard (Fig. 2) was used in the proAMH-specific assays and the AMH Gen II standards (Beckman Coulter, Cat# A79766) were used in the AMH Gen II (total AMH) assays unless otherwise stated.

2.3. $AMH_{N,C}$ dissociation via DOC pre-incubation

AMH was diluted to 140 pM in 0.01 M phosphate buffer containing 1% bovine serum albumin and were mixed 1:1 with sodium deoxycholate (DOC, Sigma, Cat# D6750) dissolved in 0.01 M phosphate buffer, pH 7.4. Tubes were incubated at room temperature for 1 h and were then applied to an AMH Gen II ELISA plate for 1 h. The wells were washed 10 times with 400 μL of AMH Gen II kit wash buffer and the bound proteins were recovered from the plate by adding 40 μL of SDS-PAGE loading buffer (0.06 M Tris–HCl, pH 6.8, 2% SDS, 40% Glycerol, 10% 2-Mercaptoethanol) followed by heating to 95 °C for 5 min. The samples were then run on SDS-PAGE for western blotting.

2.4. $AMH_{N,C}$ dissociation via DOC post-incubation

The AMH from human serum was bound to an AMH Gen II ELISA plate by adding 20 μL of serum from an adult male (participant B, see Supplementary Table 1) and 100 μL of AMH Gen II assay buffer. For application of rh-proAMH or rh-$AMH_{N,C}$, samples were diluted to required concentrations and 20 μL was applied to the ELISA plate with 100 μL of AMH Gen II assay buffer. Serum or recombinant protein samples were incubated on the ELISA plate for 1 h followed by 5 washes with 400 μL of AMH Gen II assay wash buffer. DOC was dissolved in 0.02 M phosphate buffer at pH 6.8 that had been pre-warmed to 37 °C and was stored at this temperature until the time of use. DOC solutions were applied for 30 min consisting of one application of 150 μL (Fig. 3B) or two sequential applications of 150 μL, incubated for 15 min each (Fig. 4A and B). The ELISA plates were washed 5 times with 400 μL of AMH Gen II assay buffer and proteins were recovered for analysis with SDS-PAGE loading buffer, as described above.

2.5. ProAMH ELISA protocol

The following protocol utilises all reagents supplied in the AMH