

Human oocyte-derived sperm chemoattractant is a hydrophobic molecule associated with a carrier protein

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Objective: To characterize the nature of the human oocyte-derived chemoattractant.

Design: Laboratory in vitro study.

Setting: Academic research institute.

Patient(s): Ten healthy sperm donors. Oocyte-conditioned media from women undergoing IVF treatment because of male factor infertility.

Intervention(s): Sperm samples were processed by the migration-sedimentation technique. Oocyte-conditioned media were collected 2–3 hours after oocyte stripping.

Main Outcome Measure(s): Sperm chemotaxis was assayed in a μ -slide chamber according to the direction of swimming relative to that of the chemical gradient.

Result(s): Oocyte-conditioned media treated with proteases did not lose their chemotactic activity; on the contrary, they became more active, with the activity shifted to lower concentrations. When oocyte-conditioned media were subjected to hexane extraction, chemotactic activity was found in both the hydrophobic and aqueous phases. Known mammalian sperm chemoattractants were ruled out as oocyte-derived chemoattractants.

Conclusion(s): Our results suggest that the oocyte-derived chemoattractant is a hydrophobic nonpeptide molecule that, in an oocyte-conditioned medium, is associated with a carrier protein that enables its presence in a hydrophilic environment. (*Fertil Steril*® 2014;102: 885–90. ©2014 by American Society for Reproductive Medicine.)

Key Words: Sperm chemotaxis, chemotaxis, oocyte-derived chemoattractant

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In recent years it became clear that mammalian spermatozoa must be guided for reaching the oocyte (1). So far, three potential sperm guidance mechanisms have been recognized in humans: chemotaxis (2), thermotaxis (3), and rheotaxis (4). All of these mechanisms are effective only in capacitated

spermatozoa, i.e., ripe spermatozoa that have acquired the ability to fertilize the egg (3–5). Thermotaxis and rheotaxis are assumed to be long-range mechanisms (1, 4) guiding spermatozoa to the fertilization site, and chemotaxis is thought to be a short-range and more accurate mechanism that acts in the im-

mediate surroundings of the oocyte-cumulus complex, guiding spermatozoa to the oocyte (1). This short-range mechanism appears to be a two-step process: first chemotaxis to the cumulus cells that surround the oocyte, and then chemotaxis within the cumulus mass toward the oocyte itself. This is based on the finding that, in humans, there are two origins of sperm chemoattractants: the mature oocyte and the surrounding cumulus cells (6). The cumulus cells secrete a single chemoattractant, progesterone (P) (7, 8). This chemoattractant is highly active, with chemoattraction being observed at a concentration range as low as 10^{-11} – 10^{-10} mol/L (7, 8). The identity of the

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chemoattractant(s) secreted from the oocyte is not known, despite intensive efforts to identify it. It is known, however, that it is more potent than the cumulus-derived chemoattractant progesterone (6), meaning that it is probably active at concentrations lower than 10^{-11} mol/L. Realizing that the identification of a substance present at such a low concentration is extremely difficult, our aim in this study was to characterize the nature of the oocyte-derived chemoattractant.

MATERIALS AND METHODS

Chemicals

Modified human tubal fluid (HTF) medium and human serum albumin (HSA) solution were obtained from Irvine Scientific; polyvinylpyrrolidone (PVP; 25K) was from Fluka; P, pronase E, and undecanal were purchased from Sigma; proteinase K and n-hexane were from Merck; and prostaglandin E1 was purchased from Avanti Polar Lipids.

Spermatozoa

Human semen samples were obtained from ten healthy donors after 3 days of sexual abstinence. The study was approved by the Bioethics and Embryonic Stem Cell Research Oversight Committee of the Weizmann Institute of Science. Informed consent was obtained in writing from each sperm donor. Semen samples were allowed to liquefy for 30–60 minutes at room temperature and verified for normal sperm density, motility, and morphology (according to World Health Organization guidelines [9]). Human spermatozoa were separated from the seminal plasma by the migration-sedimentation technique (10) with the use of the commercially available HTF medium supplemented with 0.3% (w/v) HSA. Following this procedure, the sperm concentration was adjusted to $4\text{--}5 \times 10^5$ cells/mL in HTF medium containing 0.3% HSA and 3.5% (w/v) PVP. The sperm suspensions were incubated under an atmosphere of 5% (v/v) CO₂ at 37°C for an additional 1 hour (2 hours in total with the separation procedure) to obtain capacitated spermatozoa (5).

Oocyte-conditioned Media

Oocyte-conditioned media and the respective control medium (G-1; Vitrolife) were obtained from the In Vitro Fertilization Unit at Assaf Harofeh Medical Center (with the approvals of the hospital's Helsinki committee and the Weizmann Institute Bioethics and Embryonic Stem Cell Research Oversight Committee). Cumulus-oocyte complexes were retrieved from women undergoing transvaginal oocyte aspiration for IVF. The women were treated with GnRH analogue and gonadotropin for ovarian stimulation. Oocyte retrieval was performed 35–38 hours after hCG injection. The cumulus cells were stripped from the cumulus-oocyte complexes with the use of both mechanical pipetting and 10–30 seconds treatment with 40 units/mg hyaluronidase (type IV-S; Sigma) followed by washing, thus avoiding leftovers of cumulus cells. Groups of stripped oocytes (6–8 per group) were cultured in 25- μ L drops of G-1 medium supplemented with 10% (v/v) HSA for 2–3 hours before undergoing intracytoplasmic sperm

injection. The conditioned media were collected and frozen just before the injection.

Progesterone Determination

Oocyte-conditioned media were analyzed for the presence of P with the use of a P ELISA kit (Enzo Life Sciences) according to the manufacturer's instructions. The concentration of P was calculated according to an experimental standard curve. The lowest concentration of the standard was 15.62 pg/mL, which set up the lowest limit of detection. Media from six different women pooled in pairs (total of three measured samples) were subjected to analysis. Samples were diluted 1:14 and run in duplicates. The negative control was G-1 medium supplemented with 10% (v/v) HSA.

Hexane Extraction

An equal volume of n-hexane was added to the samples of oocyte-conditioned medium and control medium. The tubes were vortexed for 2 minutes and then spun (3,000g) until a clear differentiation between two phases was observed. The upper phase, containing the hexane fraction, was transferred to another tube. Both fractions were then subjected to chemotaxis assays.

Chemotaxis Assays

Chemotaxis assays were performed as previously described (11) with the use of a disposable μ -slide chemotaxis chamber (Ibidi). Each assay included a negative control experiment in the absence of the test substance. In experiments with oocyte-conditioned media, this negative control consisted of a gradient of the oocyte-incubating medium (G-1 medium supplemented with 10% [v/v] HSA) treated the same way as the oocyte-conditioned medium (e.g., protease treatment). In experiments with prostaglandin E1, the negative control contained a gradient of the diluent.

Chemotaxis Assessment and Statistics

Chemotaxis was assessed, as previously described (12), according to the distribution of the instantaneous directionality angles (γ_{inst} , the angle between the vector of the cell frame-to-frame displacement and the gradient direction; the video frequency was 25 frames/s). For each treatment the total number of angles in the gradient direction (N_+) and in the opposite direction (N_-) were summed from a number of experiments. A combined odds parameter was calculated as the ratio between these sums (combined Odds = $\Sigma N_+ / \Sigma N_-$). The odds parameter yields values close to 1 when the swimming is random; it is >1 when the swimming is biased in the gradient direction. The intensity of the chemotactic response was reflected in the combined odds ratio (OR) parameter (combined OR = combined Odds_{treatment}/combined Odds_{control}).

To assess the statistical significance of elevated OR, we used a block-bootstrapping algorithm as described earlier (13). Briefly, we resampled a large control data set to build a distribution of control OR for different sample sizes.

The distribution provided the probability that a treatment OR is above a control OR randomly drawn from this distribution. The assay variability was assessed by the width of the control confidence intervals, assuming similar variability for the treatment and control samples. The dependence of the confidence intervals of the control OR values on the sample size is presented in Table 1.

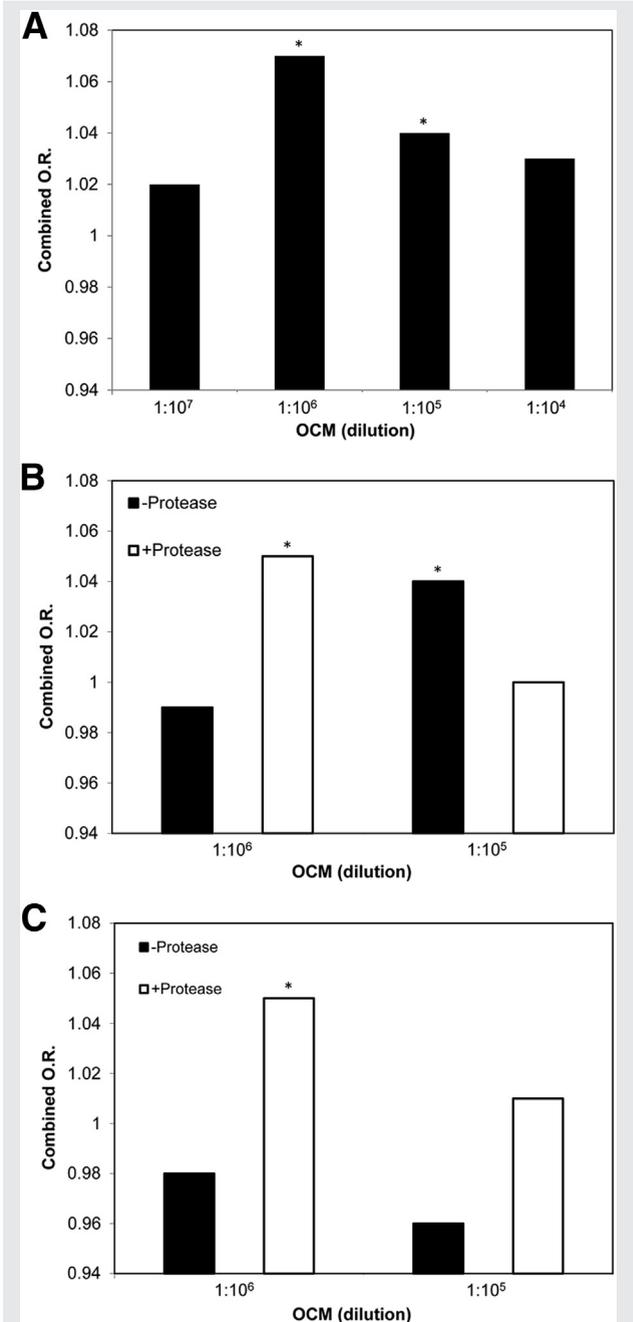
RESULTS

To use the most physiologically relevant source of oocyte-derived chemoattractant for characterization, we used human oocyte-conditioned media, i.e., media in which mature oocytes stripped from the surrounding cumulus cells were incubated for 2–3 hours. We assayed chemotaxis by determining the swimming bias in the direction of the chemoattractant gradient, and calculating the ratio between the biases in the presence and absence of a gradient (combined odds ratio). A value of 1 means no chemotaxis; a value of >1 means chemotaxis toward the chemoattractant (12, 13). Figure 1A shows a representative chemotactic response of oocyte-conditioned media at various dilutions, demonstrating that an oocyte-conditioned medium is usually chemotactically active at 10⁵–10⁶ dilution.

To verify that these conditioned media had no remains of P from the cumulus cells, we performed a highly sensitive P-specific ELISA assay. We assayed three different samples of oocyte-conditioned media, each being a mixture of oocyte-conditioned media obtained from two women (6–8 oocytes each). In two of the samples, no P was detected by the assay. In the third sample, P at a concentration of 2 × 10⁻⁹ mol/L was detected. Because oocyte-conditioned media are chemotactically active at 10⁵–10⁶ dilutions (Fig. 1A), the effective concentration of P in the diluted sample was in the range of 2 × 10⁻¹⁵–10⁻¹⁴ mol/L, which is four orders of magnitude below the threshold P concentration that can be sensed by human spermatozoa and stimulate a chemotactic response [10⁻¹¹–10⁻¹⁰ mol/L (7, 8)].

To characterize the nature of the oocyte-derived chemoattractant, we first determined whether or not it is a peptide. We incubated oocyte-conditioned media with a broad-spectrum protease, proteinase K. In each experiment we verified the activity of the protease by running the oocyte-conditioned media on sodium dodecyl sulfate–polyacrylamide gel electrophoresis before and after the proteolysis. The chemoattractant activity of the

FIGURE 1



Chemotactic response of human spermatozoa to oocyte-conditioned media (OCM) in the absence and presence of proteases. (A) Dependence of the chemotactic response on the OCM dilution. (B) Chemotactic activity of OCM after treatment with proteinase K (20 µg/mL, 3 h, 37°C). (C) Chemotactic activity of OCM after treatment with a mixture of proteinase K and pronase E (50 µg/mL each, 3 h, 37°C). The data are presented as combined odds ratios (ORs) of 6,900–9,500 tracks (91–121 × 10⁴ angles [A]) and 5,000–5,800 tracks (70–80 × 10⁴ angles [B, C]). The negative control is defined as OR = 1. Note that error bars are missing, because the combined OR value is calculated from a pool of the whole data and is not an average. Also note that, owing to the large variations between sperm samples (even of the very same sperm donor—a well-known phenomenon in sperm biology (23–25), a quantitative comparison is allowed within each panel but not between panels. *P < .05 vs. the appropriate control. Armon. Oocyte-derived sperm chemoattractant. Fertil Steril 2014.

TABLE 1

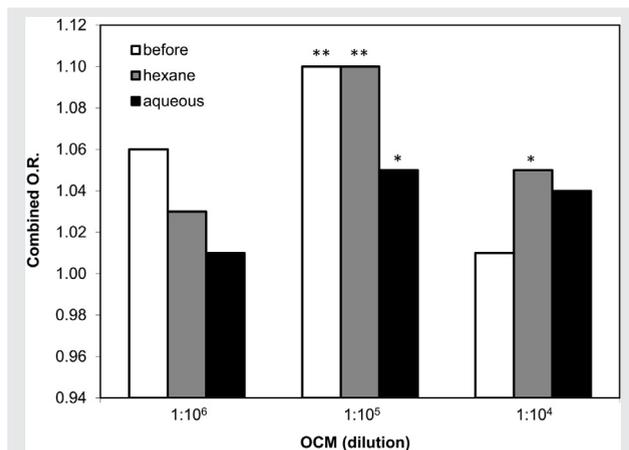
Confidence intervals (95% CIs) of odds ratio values according to sample size.

Sample size (no. of angles)	CI ^a
5 × 10 ⁴	0.86–1.16
10 × 10 ⁴	0.90–1.11
100 × 10 ⁴	0.97–1.03

Note: The CIs were calculated with the use of block-bootstrapping.

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FIGURE 2



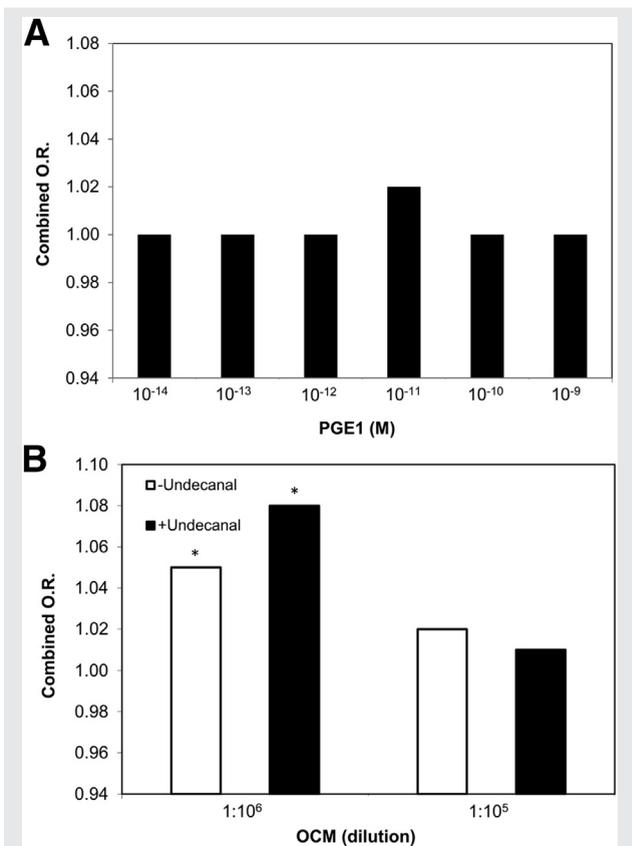
Chemotactic response of human spermatozoa to oocyte-conditioned medium (OCM) before and after extraction with hexane. The data are presented as combined odds ratios (ORs) of 1,400–2,900 tracks ($18\text{--}35 \times 10^4$ angles). Note that error bars are missing, because the combined OR value is calculated from a pool of the whole data and is not an average. * $P \leq .06$; ** $P < .01$ vs. the appropriate control.

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oocyte-conditioned media was not lost by the proteolysis (Fig. 1B; similar results were obtained with trypsin [data not shown]). On the contrary, the activity shifted to a lower concentration (i.e., higher dilution). Because proteinase K cleaves peptide bonds adjacent to the carboxyl group of both aliphatic and aromatic amino acids, these results suggest that the chemoattractant in oocyte-conditioned media is not a peptide (unless a very short peptide).

To verify the unexpected observation that the proteolysis increased the chemotactic activity of the medium from $1:10^5$ to $1:10^6$ dilution, we carried out another set of experiments, this time using a mixture of two proteases, proteinase K and pronase E in higher concentrations than those used for proteinase K alone, thus eliminating any possibility of peptide leftovers (excluding very small peptides). In this set of experiments, the sample of oocyte-conditioned media (before treatment) happened to be chemotactically inactive. (This happened occasionally owing to the fact that we used a pool of media from different women.) Nevertheless, these inactive media became active after proteolysis (Fig. 1C). Clearly, proteolysis elevated the chemoattractant concentration. This could be the outcome of two scenarios. One is that the chemoattractant is an amino acid or a very short peptide (such as a di- or tripeptide) and proteolysis produces more of it. The other is that the chemoattractant is a hydrophobic molecule which, in an oocyte-conditioned medium, is associated with a carrier protein that enables its presence in a hydrophilic environment. According to this scenario, proteolysis destroys the carrier protein and frees the hydrophobic chemoattractant to the medium (the high concentration of albumin in the medium likely enables presence of the chemoattractant in the aqueous solution).

FIGURE 3



Examination of sperm receptors suspected to be involved in human sperm chemotaxis. (A) Chemotactic response to prostaglandin E1 (PGE1). The data are presented as combined odds ratios (ORs) of 4,200–5,300 tracks ($58\text{--}73 \times 10^4$ angles). (B) Chemotactic response to oocyte-conditioned medium (OCM) in the presence of undecanal. The data are presented as combined ORs of 2,500–3,600 tracks ($9\text{--}15 \times 10^4$ angles). Note that error bars are missing, because the combined OR value is calculated from a pool of the whole data and is not an average. * $P \leq .02$ vs. the appropriate control.

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If the oocyte-derived chemoattractant is indeed hydrophobic and it is chemotactically active both when it is in complex with its carrier protein and when unbound, it should exhibit chemotactic activity in both hydrophilic and hydrophobic environments. To determine whether or not this is the case, we extracted with the use of hexane the hydrophobic substances from the oocyte-conditioned media and examined the chemotactic activity of both the hexane fraction and the remaining aqueous fraction. Consistently with the carrier-protein scenario, both fractions were chemotactically active (Fig. 2).

Two sperm receptors apparently can act as chemotaxis receptors. One of them is the CatSper channel (or a protein closely associated with it), shown to be the sperm receptor for the chemoattractant P (14, 15). The other is OR1D2, which is suggested to mediate the chemotactic response to the odorant bourgeonal (16) (although a later study raised the possibility that the response to bourgeonal is mediated

by CatSper [17]). Because the ligands of these receptors are quite hydrophobic, we examined whether any of them is the chemoattractant in oocyte-conditioned medium. CatSper is also activated by prostaglandin E1 (but via a binding site different from that of P) (14, 15) and is known to be secreted from the cumulus-oocyte complex (18). Prostaglandin E1 exhibited no chemotactic activity at any tested concentration (Fig. 3A), even though it bound to its receptor (reflected in stimulating intracellular Ca^{2+} rise [data not shown]). Likewise, preincubation of oocyte-conditioned media with undecanal—an antagonist of the OR1D2 receptor (16), did not affect the chemotactic activity of the medium (Fig. 3B). These results indicate that neither prostaglandin E1 nor an OR1D2 ligand is the oocyte-derived chemoattractant.

DISCUSSION

The results of this study show that the human oocyte-derived sperm chemoattractant does not lose its activity under the action of proteases (Fig. 1), can be present in both hydrophobic and hydrophilic environments (Fig. 2), and is not one of the known ligands of the recognized chemotaxis receptors (Fig. 3). The first two observations could be explained by a number of scenarios: a hydrophobic chemoattractant in complex with a carrier protein; two (or more) chemoattractants, one hydrophobic and one hydrophilic; a chemoattractant of an amphipathic nature; or a chemoattractant that is an amino acid or a very short peptide. The finding that the activity of the oocyte-conditioned media increased after proteolysis, together with the conservation of activity in both hydrophobic and hydrophilic environments, is consistent with the possibility of a hydrophobic chemoattractant associated with a carrier protein in an aqueous environment. According to this possibility, both the hydrophobic molecule and its complex with the carrier protein are chemotactically active. The free hydrophobic molecule is expected to be more active because otherwise proteolysis would not increase the activity. The other mentioned scenarios do not seem to be probable. The possibility of an amphipathic chemoattractant or two chemoattractants, one hydrophobic and one hydrophilic, is inconsistent with the proteolysis-dependent increase in chemotactic activity. Likewise, the possibility of an amino acid chemoattractant or a very short chemoattractant peptide is inconsistent with the proteolysis results with trypsin, which generates relatively long peptides. The expected result in such a case would be no change in activity. The results further indicate that the oocyte-derived chemoattractant is not one of the peptides reported to act *in vitro* as chemoattractants for human spermatozoa (e.g., CCL5 [19], allurin [20], CCL20 [21], or A-type natriuretic peptide [22]), because all of these are expected to undergo proteolysis by the proteases used. Therefore, it seems to be probable that the chemoattractant is a hydrophobic molecule associated with a carrier protein.

It is reasonable to speculate that the receptor for the oocyte-derived chemoattractant is different from the known receptors for sperm chemoattractants. Otherwise, the receptor for the oocyte-derived chemoattractant might be saturated

with cumulus-derived P when passing through the mass of cumulus cells, thus interfering with the binding of the oocyte-derived chemoattractant to this receptor.

In conclusion, the chemoattractant is likely a hydrophobic nonpeptide molecule that, when secreted from the oocyte, is in complex with a carrier protein. Apparently, the extreme potency of the chemoattractant compensates for its low concentration in the medium, and the carrier protein enables the presence of the chemoattractant in aqueous solutions and prevents its adsorption to nonspecific locations.

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