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Expression and alternative splicing of the neural cell adhesion molecule NCAM in human granulosa cells during luteinization

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Abstract

Freshly aspirated human granulosa cells from pre-ovulatory follicles and granulosa cells luteinized in culture possess the neural cell adhesion molecule (NCAM) of approximate molecular mass of 140,000 and NCAM mRNA as confirmed by S1-nuclease protection assays and RT-PCR. Moreover, in the process of luteinization the NCAM isoform pattern is modified. Isoforms containing an insert of 10 amino acids (termed VASE) in the extracellular domain of NCAM were supplemented by alternatively spliced isoforms without this insert. NCAM immunoreactivity, at light and electron microscope levels, was associated with the cell membrane of most granulosa cells which formed clusters. During time in culture an increasing subpopulation of granulosa cells, devoid of NCAM immunoreactivity, spread out and formed monolayers. This differential expression and the alternative splicing of NCAM during luteinization of granulosa cells raise the possibility that NCAM could be involved in folliculogenesis and the formation of the corpus luteum in the human.

Key words: Neural cell adhesion molecule; NCAM; Adhesion; Folliculogenesis; Granulosa cell; Human; Luteinization; Alternative splicing

1. Introduction

The neural cell adhesion molecule (NCAM) glycoprotein family consists of three major members with molecular masses of 120, 140 and 180 kDa, which are generated from a single gene by alternative splicing (cf. [1,2]). At least 20 major exons code for these different isoforms [2,3]. In addition, further small exons can give rise to additional NCAM isoforms. Between exons 12 and 13, four additional independent alternatively spliced exons of 3–48 bp [3–6] and at the exon 7/8 splice junction a 30 bp exon (termed VASE) [5,7] were discovered. The alternatively spliced exon VASE is close to the NCAM domain that mediates cell–cell adhesion [8]. The biological relevance of VASE expression still remains obscure, however, in the nervous system, VASE has been shown to down-regulate the neurite growth-promoting effect of NCAM [9]. NCAM mRNAs containing VASE were also present in rodent endocrine tissues and cells [10].

NCAMs were first found in the nervous system, but subsequently were also found in polypeptide hormone- and steroid-producing endocrine cells [10–15]. NCAMs allow the formation of cell clusters by auto-adhesion of neighboring NCAM-bearing cells. We have previously described the presence of NCAM-140 and NCAM mRNA in the rat ovary [14]. We found that NCAM was most abundant in granulosa cells and luteal cells. These results were confirmed by another study performed in the mouse [16]. Within the granulosa, forming large follicles of the rat, we noticed [14] a marked difference in the degree of NCAM immunoreactivity: the cells lining the follicular antrum showed stronger immunoreactivity than the mural granulosa cells. Moreover, follicular fluid stained with a NCAM antiserum. We have therefore proposed a role for NCAM in the dynamic processes of folliculogenesis, namely in the segregation of granulosa cells. We also suspected involvement of NCAM in the formation of the corpus luteum, which depends on the concomitant development of its vasculature. The last assumption is based on a recent study in the bovine species, in which we showed that subtypes of corpus luteum-derived microvasculature endothelial cells express NCAM-140 [17], extending our idea of an involvement of NCAM in the formation of the corpus luteum.

Thus, accumulating evidence for a functional significance of NCAM in the ovary led us to examine whether ovarian NCAM expression also occurs in the human ovary. Because of the possible clinical relevance of granulosa cells' expression of NCAM during folliculogenesis and luteinization, in the present study we have examined NCAM expression by aspirated human granulosa cells and by cultured human granulosa-lutein cells.

2. Materials and methods

2.1. Culture of human granulosa-lutein cells and lung tumor cell lines

Granulosa cells were cultured in DMEM:Ham's F12 medium (1:1; v/v; Sigma, Munich, Germany) [18–20]. We found that coating of culture dishes with laminin (Sigma, Munich, Germany) supported the

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adherence of the cells during immunocytochemistry, while other differences were not seen. Therefore cells grown on both laminin-coated and non-coated dishes were examined in the present study. The human small cell carcinoma cell line, SCLC H69 (Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany) was grown under serum-free conditions (thus termed H69$^-$) in RPMI 1640 medium (Biochrom Beteiligungs GmbH, Berlin, Germany) supplemented with 5 μg/ml bovine insulin, 10 μg/ml human transferrin, 30 nM sodium selenite (Sigma) and 2 mM L-glutamine. The human adenocarcinoma cell line, A549 (DKFZ), was grown in RPMI 1640 medium containing 10% FCS (Biochrom Beteiligungs GmbH).

2.2. Cloning of human NCAM cDNA

Total RNA was isolated from human SCLC H69$^-$, human adenocarcinoma cells A549, as well as from freshly aspirated human granulosa and cultured granulosa-lutein cells, as described [21]. For cDNA synthesis and its amplification by the polymerase chain reaction (PCR) the primers E7s (5′ ACTCTTACCTGTGAAGCCTC 3′, 'sense') and E13a (5′ CATCTGCCCTTCAAGCTTAG 3′, 'antisense', with the exception of the point mutation underlined, which introduces a *Hin*dIII restriction site) were used.

Synthesis of cDNA and PCR amplification modified after [22] was carried out as described by [10] with 1 μg total RNA extracted from the SCLC H69$^-$ cell line using 50 pmol of each primer in a DNA Thermal Cycler 480 (Perkin-Elmer; Applied Biosystems Weiterstadt, Germany). PCR amplified cDNA was separated on a 2% agarose gel. The DNA fragment (exon 7–13) was eluted and cloned into the *Sma*I site of Bluescribe [M13$^-$] vector (Stratagene, La Jolla, CA) after phosphorylation with T4 polynucleotide kinase and repair synthesis ('fill-in') with Klenow enzyme (both enzymes: AGS, Angewandte Gentechnologie Systeme) using standard methods [23]. This clone containing the 884 bp human NCAM cDNA fragment is referred to as 6hNCAM. DNA was sequenced by the desoxynucleotide chain termination method [24] using the sequencing kit from Pharmacia (Pharmacia, Freiburg, Germany).

2.3. Synthesis of cRNA-probe and S1 nuclease protection assay

The cDNA clone 6hNCAM was used as a template for cRNA synthesis. Synthesis of ^{32}P-labelled cRNA (specific activity: 3.9×10^7 cpm/μg) for S1 nuclease protection assay (S1-NPA) was carried out according to the T3-polymerase protocol of Promega Biotec (Madison, Wisconsin, USA) using 20 μCi of [α-^{32}P]UTP (specific activity: 15 TBq/mmol) and 2 μg of 6hNCAM cut with *Eco*RI (using the internal *Eco*RI site, as well as the *Eco*RI site in the polylinker sequence of the vector). The resulting cRNA probe contained 354 nt of human NCAM (exons 10–13) corresponding to the published sequence (positions 1576–1933 plus an additional triplet AAG between exon 12 and 13) of human NCAM [25] and 45 nt of vector sequences.

S1-NPA with labelled cRNA probe was carried out as described previously [10,26].

2.4. Analysis of the alternative exon VASE by RT-PCR

cDNA synthesis from RNA isolated from granulosa cells harvested after different time periods in culture was carried out with 20 μg extracted total RNA. A random-primed reverse transcription (RT) was carried out for 30 min at 37°C in a 50 μl reaction mix according to the manufacturers protocol (GIBCO/BRL, Eggenstein, Germany) using 200 U M-MLV-reverse transcriptase, 2 μl of 10 × hexanucleotide mixture (Boehringer-Mannheim, Mannheim, Germany), and 29 U RNA guard (Pharmacia, Freiburg, Germany).

PCR amplification was performed with a 2 μl aliquot immediately after inactivation of reverse transcriptase in a 50 μl reaction mix containing 0.5 U of *Taq* DNA polymerase according to the protocol of AGS (Angewandte Gentechnologie Systeme, Heidelberg, Germany), using 2 mM dATP, dGTP, dCTP, dTTP ('cold PCR', i.e. unlabelled), or 1 mM dCTP and 5 μCi [α-P^{32}]dCTP ('hot PCR', i.e. radioloabelled), and 20 pmol of each primer E7s (see above) and E9a (5′ ACCTCACAGGTGATGTTCAC 3′) 'antisense' (see also [10]).

2.5. Immunocytochemistry and Western blotting

The immunocytochemical localization of NCAM [27] on cells at day 1 and 5 after isolation was examined with a well-characterized NCAM antiserum directed against the N-terminal end of NCAM (1:500–1:1,000; generous gift from Dr. G. Rougon, Marseille) and a commercial NCAM antiserum (1:100–1:500; Serva, Heidelberg, Germany). The details of the immunocytochemical techniques have been described [14,17]. The avidin-biotin (ABC -method was performed (reagents included a 1:500 dilution of a goat anti-rabbit IgG (Camon, Wiesbaden, Germany) and an ABC kit (Camon)) after paraformaldehyde fixation of the cells (4% in 0.01 M phosphate-buffered saline, PBS, pH 7.3). Controls, performed by (i) omitting the first antisera, and (ii) incubation with normal rabbit serum instead of the specific antisera, were negative.

To obtain further information on NCAM localization, immunocytochemistry at the electron microscope level was performed. After immunostaining (see above), cells were immediately post-fixed with 5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 1 h. Cells were treated with 1% OsO_4 for 30 min and then embedded into Epon. Thin sections were cut, most were viewed unstained but some were stained with uranyl acetate for 15 s, lead citrate for 1 min and were then examined with a Zeiss EM 10 transmission electron microscope.

As described previously [14,17] medium and cells (which were scratched from the culture plates) were centrifuged and the pellets were transferred into 62.5 mM Tris-HCl (pH 6.8) containing 10% sucrose and 2% sodium dodecyl sulfate. After sonication and heating (100°C for 5 min), samples (15 μg of protein per lane) were subjected to electrophoretic separation on 7.5% SDS-polyacrylamide gels. The gels were blotted onto nitrocellulose membranes and probed with the same NCAM antisera as used for immunocytochemistry (1:500 dilution of antiserum donated by Dr. Rougon; 1:100 dilution of Serva antiserum).

3. Results

During the first 2 days of culture NCAM immunoreactivity was found to be associated with the cell membrane of cells sticking closely together and forming clusters (Fig. 1a,b). Cells spreading out and forming monolayers, a process increasing with time in culture, exhibited low or no NCAM immunoreactivity. However, with increasing length of culture period, strong NCAM immunoreactivity was present on cells segregating from the (NCAM-immunonegative) monolayer (Fig. 1c). The faint cytoplasmic staining of attached cells in Fig. 1a and c was not observed in controls and thus may represent an intracellular pool of NCAM. While NCAM was not readily detectable in immunoblots of freshly aspirated granulosa cells, NCAM of approximately 140,000 molecular mass was found in cultured granulosa-lutein cells on culture day 5 (Fig. 2), if adherent cells and floating cells (see below) were sampled together.

Further evidence for the presence of NCAM in human granulosa cells was obtained by S1 nuclease assays. The large differences in sequence between rodent NCAM and human NCAM excluded the use of probes for rodent NCAM for S1 nuclease analyses for the positive identification of NCAM mRNA in human granulosa cells. Therefore, we isolated mRNA from human small cell carcinoma cells, synthesized cDNA, amplified it by PCR, and cloned it into a suitable vector (see Fig. 3 and section 2). Sequence analysis revealed that the clone (6hNCAM) obtained differs in sequence from the published human NCAM derived from a human neuroblastoma cDNA library [25] at two positions (Fig. 3). At the exon10/exon 11 splice junction at position 1589, a G is replaced by a C. Whether this transversion at position 1589 in clone

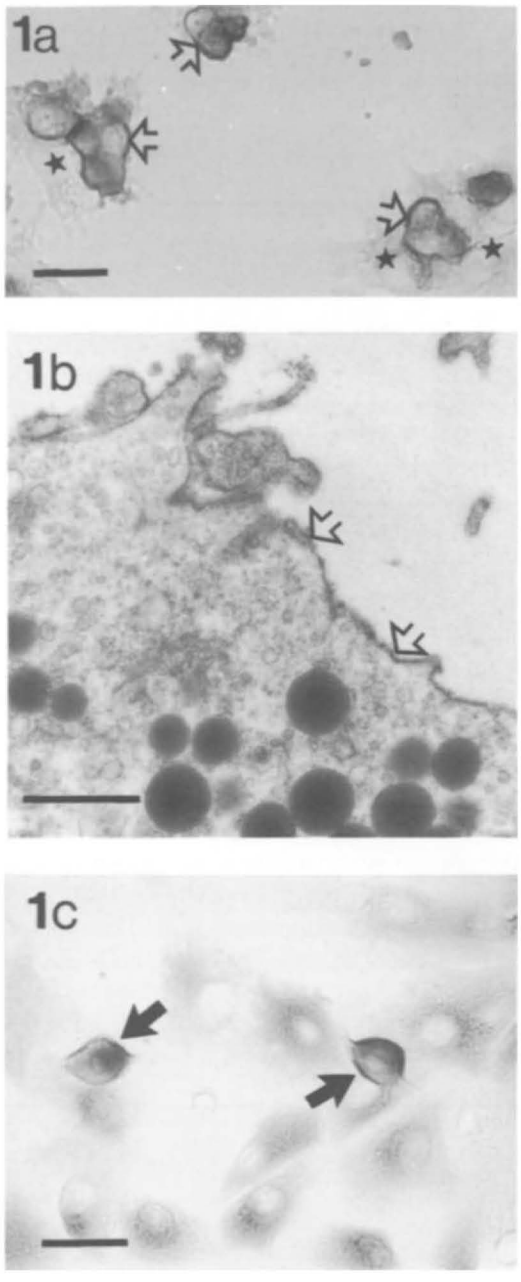

Fig. 1. NCAM-immunoreactivity on cultured granulosa-lutein cells. (a) NCAM immunoreactivity (arrows) was detected in adhering cells which form clusters, but cells spreading out on the plastic surface (marked with an asterisk) were unstained (culture day 1; antiserum from Serva, Heidelberg was diluted 1:100; bar = 40 μm). (b) At the electron microscope level NCAM immunoreactivity was associated with the cell membrane (arrows; culture day 1; antiserum from Dr. G. Rougon was diluted 1:500; bar = 1 μm). (c) After 5 days in culture, granulosa-lutein cells formed monolayers. The adhering granulosa-lutein cells had no detectabe NCAM immunoreactivity. However, NCAM-immunoreactive cells (arrows) rounding up and segregating from the monolayer were found (antiserum from Dr. G. Rougon, 1:500; bar = 40 μm).

6hNCAM is due to a PCR- or cloning artifact remains unclear. At position 1720, G is replaced by T, a transition having no effect on the amino acid composition. Since T at position 1720 was also found in the human muscle sequence [28] it can be concluded that it may be characteristic for human muscle and small cell lung cancer, whereas the G nucleotide at position 1720 [25] may be characteristic for human neural tissue. Furthermore the AAG trinucleotide at the exon 12/exon 13 splice junction observed in the sequence of human brain NCAM [25] is not present in clone 6hNCAM. These small differences in sequence do not exclude the use of the probes for protection analyses, since the enzym S1 nuclease does not recognize up to three consecutive mismatches [6].

The structure of the cDNA, the cRNA probe for S1 nuclease assays and the protected cRNA fragment indicating co-linearity with the NCAM cRNA probe are shown in Fig. 3. Results of S1 nuclease assays showed that freshly aspirated human granulosa cells from preovulatory follicles possess mRNA coding for NCAM (Fig. 4). NCAM mRNA was also present in these cells during culture, when they underwent luteinization. NCAM mRNA was identified among RNA isolated from human small lung cell carcinoma cells ($H69^-$), which were used as a positive control, but was absent in the human adenocarcinoma cell line A549 (negative control) [29].

The microsplicing event of the alternatively spliced extra exon, VASE, was analysed by PCR analysis using oligonucleotide primers downstream and upstream of the exon 7/8 splice junction after cDNA synthesis and PCR amplification (Fig. 5). As indicated by the band of 337 bp, freshly isolated human granulosa cells and cells after 1 day in culture express mainly NCAM mRNAs with VASE. Non-VASE mRNAs (307 bp band), augmented during the culturing period (2 days in culture) until similar amounts of NCAM with and without VASE were detected after 4 days in culture.

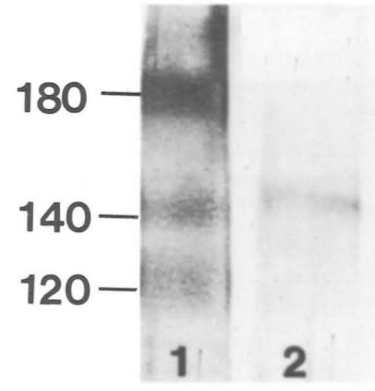

Fig. 2. Western blot analyses of NCAM in granulosa cells. NCAM of approximately 140,000 molecular mass was found in cultured granulosa-lutein cells on culture day 5 (lane 2). Human olfactory bulb (lane 1) was used as a positive control containing NCAM isoforms of 120,000, 140,000 and 180,000 Da. (Antiserum, diluted 1:500, was obtained from Dr. Rougon.)

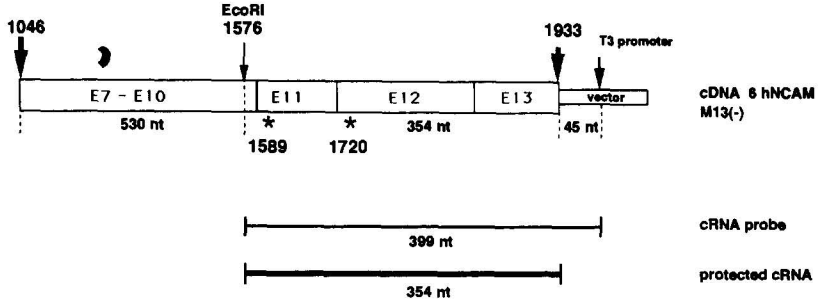

Fig. 3. Scheme of the sequenced human NCAM cDNA, the NCAM cRNA probe used for S1 nuclease protection assay and its protected fragment. The 887 bp hNCAM cDNA fragment amplified by PCR between exons 7 and 13 cloned into Bluescribe M13(−) vector is shown on the top. The T3 promoter, the *Eco*RI restriction site and the vector sequences are indicated. During sequencing we observed a transversion (G→C; see asterisk at position 1589) at the exon 10/exon 11 splice junction, which would change the amino acid sequence from an alanine to a proline. We found, in addition, a transition (G→T; see asterisk at position 1720), which is also present in human muscle (see section 4). Note that the sequenced clone contains no AAG triplet at the exon 12/13 junction, which was present in the published sequence of human NCAM derived from a neuroblastoma cDNA library [25]. The cRNA probe (399 nt) synthesized from cDNA 6hNCAM M13(−) spans the NCAM sequence between the internal *Eco*RI restriction site in exon 10 and exon 13. The 354 nt RNA fragment (nucleotide position 1576–1933), protected from S1 nuclease hydrolysis, is shown at the bottom.

4. Discussion

The principal finding of the present study is that NCAM and its mRNA are present in human granulosa cells and in granulosa-lutein cells in culture. In addition, we observed that during differentiation in culture, cells lose NCAM during the process of adhesion and spreading. However, cells express NCAM again when they segregate from the monolayers. Differential alternative splicing occured during culture. NCAM mRNAs without the additional exon, VASE, were generated in addition to NCAM mRNAs including VASE, which were present thoughout the culturing period.

We have previously reported the expression of the neural cell adhesion molecule, NCAM-140, in follicles and in the corpus luteum of the rat ovary [14]. These results were confirmed by a study in the mouse ovary [16]. In our previous study we speculated that NCAM could be a factor involved in the formation of the follicular antrum and of the corpus luteum [14]. The present study largely extends our previous work and focuses on human granulosa and cultured granulosa-lutein cells. We present evidence that the expression of NCAM changes: while early after seeding granulosa-lutein cells adhering to each other showed NCAM immunoreactivity, NCAM immunoreactivity vanished with time in culture in cells spreading out and was not detectable on the cells forming monolayers. However, strong NCAM immunoreactivity was present on cells segregating from the monolayers, floating in the medium, or only loosely adherent cells. Many of the last mentioned cells presumably were washed off during the immunocytochemical procedure and therefore might have escaped observation. For most Western blots both adherent and floating cells were used, allowing the identification of NCAM with a molecular weight of 140 kDa in granulosa-lutein cells in culture. The behavior of granulosa-lutein cells in losing NCAM after spreading is reminiscent of the NCAM down-regulation observed in a NCAM-expressing tumor line: a subpopulation of a human small cell lung cancer line down-regulated its NCAM and became adherent to the culture dish [30].

In the present study we have used a human small cell lung cancer line as a positive control for human NCAM mRNA. It is well documented that these tumor cells express NCAM [29,31–33]. The use of a cDNA probe for human NCAM obtained from this cell line became essential for the positive identification of human NCAM mRNA in human granulosa cells, because the sequences

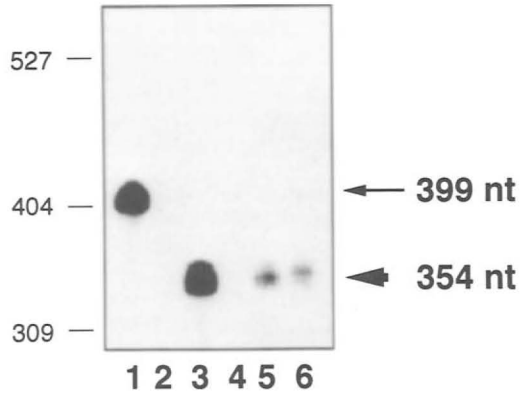

Fig. 4. S1 nuclease protection analysis of extracted RNA of human granulosa cells and human lung tumor cell lines. Human NCAM mRNA is present in freshly aspirated granulosa cells (lane 5), in granulosa cells cultured for 5 days (lane 6), in a human small cell carcinoma cell line ($H69^-$) (lane 3), but not in a human adenocarcinoma cell line (A549) (lane 4). As a size marker, *Hpa*II-digested pBR322 was used and the fragment sizes are indicated at the left side. In lane 1 the probe containing flanking vector sequences is shown. The thin arrow depicts the undigested probe of 399 nt, whereas the bold arrow points to the protected cRNA fragment of 354 nt. Lane 2 = tRNA control.

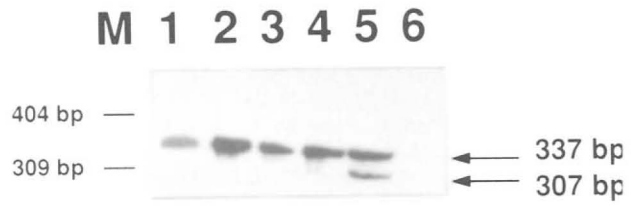

Fig. 5. PCR analysis of the expression of the alternative exon VASE in extracted RNA of human granulosa cells. The use of oligonucleotide primers downstream and upstream the exon 7/8 splice junction in PCR amplification revealed that mainly human NCAM mRNA containing alternative exon, VASE, is present in freshly isolated granulosa cells (lane 2) and granulosa cells cultured for 1 day (lane 3). This is indicated by the band of 337 bp. Non-VASE mRNAs (307 bp band) augmented during the culturing period (lane 4; 2 days in culture) until similar amounts of NCAM with and without VASE can be detected after 4 days in culture, when cells had undergone luteinization (lane 5). As size marker (indicated at the left side), *Hpa*II-digested pBR322 radioactively labelled at its 5' end under standard conditions was used. Lane 1, control cDNA clone N1 [5], which contains alternative exon VASE (indicated by the presence of a 337 bp band); lane 6, PCR control (H_2O).

of rodent NCAM and human NCAM differ too much and thus can not be used in S1 nuclease analysis of NCAM in human cells.

The similarities of the molecular form of NCAM (endocrine NCAM-140; [2]) in the present study, the studies in the rat [14] and the bovine species [17] are evident. Based on the data of all these studies, NCAM-140 could be involved in cell-to-cell adhesion (granulosa cells in the growing follicle), as well as cell separation (antrum formation). In most previous studies NCAM has been suggested to play a role in the autoadhesion of NCAM-bearing cells of the same kind via a homophilic binding, such as in the endocrine cells of the pancreatic islet, in the adenohypophysis, in adrenal cortex, adrenal medulla and in the testis [10,15]. In these cases the 'recognition site' of NCAMs, which was identified as lying within the third Ig domain [8], may be modulated by alternative splicing of the neighbouring fourth Ig domain. It is conceivable that altered NCAM forms could account for various degrees of cellular 'adhesiveness', and that the change in the amounts of NCAM isoforms with or without exon VASE, when granulosa cells undergo luteinization, could affect NCAM function and cell behavior. Such a regulatory influence has been observed in neurons, where VASE down-regulated the neurite outgrowth-promoting effect of NCAM [9,34].

Besides isoform switching (e.g. exclusion of the VASE exon), changes in the function of NCAM may also be accomplished by post-translational changes (e.g. loss of polysialic acid modifications) [35,36]. Thus the alternatively spliced exon VASE in the fourth Ig domain, which is physically close enough to the regions of the molecule thought to mediate cell–cell interaction, may modulate interaction of granulosa cells. In addition, soluble NCAM forms observed in different body fluids [37], possibly including the follicle fluid [14], could participate in the regulation of cell separation by saturating cell-bound NCAM.

In conclusion, the present results showing that specific isoforms of the adhesion molecule NCAM are expressed by human granulosa cells in vivo and by granulosa-lutein cells in culture hint for the first time at the involvement of NCAM in the processes of folliculogenesis and formation of the corpus luteum in the human ovary. Additional studies are required to pinpoint the precise roles and the regulation of NCAM expression by ovarian cells, especially because now the question of the function(s) of NCAM in the developing follicle and in the corpus luteum have become of potential clinical relevance.

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