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SHORT NOTE

Cultured Microvascular Endothelial Cells Derived from the Bovine Corpus Luteum Possess NCAM-140

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Previously, five phenotypically different, stable types of microvascular endothelial cells (MVE) were isolated from the bovine corpus and cultured successfully. We found that three out of these five types of MVE express the neural cell adhesion molecule (NCAM). As shown by immunocytochemistry, weak NCAM immunoreactivity occurred mainly in the perinuclear area of cell type 1. Monolayers of types 2 and 5 revealed heavy NCAM immunoreactivity, which was localized predominantly at the lateral cell surface outlining the contact zones of adjacent cells. In contrast, cell types 3 and 4 were not NCAM immunoreactive. Western blot analyses substantiated these results: While cell type 1 showed a weak immunoreactive band, cell types 2 and 5 displayed strong NCAM-immunoreactive bands of a molecular weight of approximately 140 kDa (NCAM-140), which was absent in cell types 3 and 4. These results reveal for the first time that NCAM can be expressed by cultured MVE and may serve in mediating endothelial cell contacts. Since luteal cells also express NCAM-140, this adhesion molecule could in addition be involved in the interactions of luteal cells with MVE.

INTRODUCTION

The corpus luteum develops from a mature follicle after ovulation. Microvessels of the theca layer grow into the granulosa cell layer. Subsequently, a well-vascularized endocrine tissue is formed. The acquisition of its microvasculature corresponds to angiogenesis [1], which appears to be controlled by specific local factors, like the basic fibroblast growth factor [2] and/or a vascular endothelial growth factor [3]. It is conceivable that the life span and function of the endocrine-active, progesterone-producing luteal cells are heavily interwoven with the formation and maintenance of the microvasculature bed.

Recently, the expression of the neural cell adhesion molecule NCAM-140 has been reported in the corpus luteum of the rat [4] and mouse [5]. The NCAM glycoprotein family consists of three major members of molecular weights of 120, 140, and 180 kDa (cf. [6, 7]) and NCAMs were first found in nervous tissue and subsequently in endocrine cells. NCAMs allow cell adhesion by autoadhesion of neighboring NCAM-bearing cells. In the ovary, the cells expressing NCAM appeared to be the luteal cells [4, 5]. Yet, endothelial cells from microvessels may contribute to NCAM immunoreactivity, as well. Rat and mouse corpora lutea are not ideal for examining this question because they have abundant undifferentiated blood vessels due to a secretory lifespan of about 2 days. In contrast, corpora lutea of cows show well-developed arterioles, capillaries, and venules during an active lifespan of about 10 days. From this tissue, five different types of microvascular endothelial cells (MVE) can be isolated and cultured [8, 9]. In the present study we have addressed the question whether NCAM(s) can be expressed by these MVE and thus could mediate cell adhesion between endothelial cells and between endothelial cells and endocrine luteal cells, which also express NCAM.

MATERIALS AND METHODS

Cell culture. The method for isolation and culture of different phenotypes of MVE from the bovine corpus luteum has been described in detail [8, 9]. In brief, bovine corpora lutea of secretory stage were minced mechanically and sieved through meshes of 150- and 75-μm^2 pore sizes. Cell sediment was resuspended in 50% Percoll (Pharmacia, Freiburg, Germany) solution and centrifuged in 10-ml tubes at 16,000*g* for 20 min. While erythrocytes and luteal cells banded at the bottom and top of the gradient, MVE were found between these bands. The MVE fraction was washed and the cells were seeded onto collagen-coated (Vitrogen 100; Collagen Corp., Palo Alto, CA) 24-well culture plates. Medium was supplemented with 15 m*M* Hepes, 22 m*M* $NaHCO_3$, and 5% fetal calf serum. Growing colonies were classified

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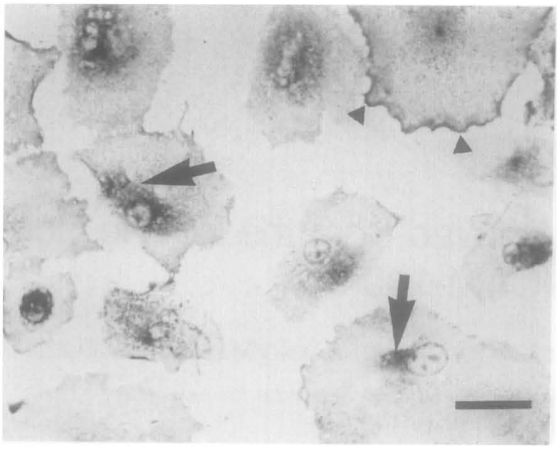

FIG. 1. Type 1 MVE: NCAM immunoreactivity is mainly associated with the perinuclear region (arrows) and only occasionally is surface staining seen (arrowheads). Note the wide intercellular gaps. Cells were stained with hematoxylin for 1 min to show the nuclei; phase-contrast micrograph; ×340; bar = 30 μm.

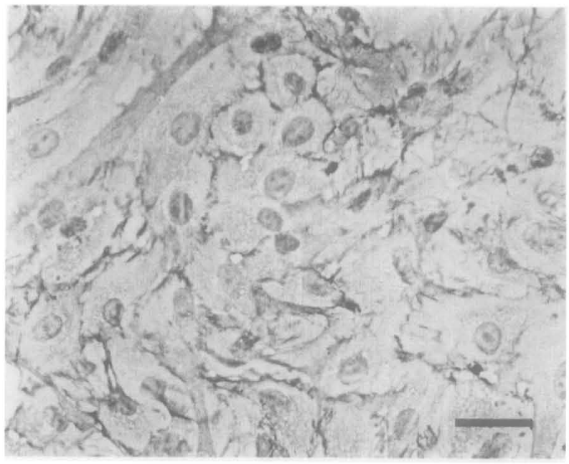

FIG. 2. Type 2 MVE: NCAM immunoreactivity is found at the lateral cell surface at contact sites of adjacent cells. Cells were stained with hematoxylin for 1 min to show the nuclei; phase-contrast micrograph; ×340; bar = 30 μm.

as types 1–5 according to own morphological criteria. Apparently pure colonies were subpassaged once or twice.

Immunocytochemistry. The immunocytochemical localization of NCAM was examined with a well-characterized NCAM antiserum directed against the N-terminal end of NCAM ([10]; a generous gift from Dr. G. Rougon, Marseille). The avidin–biotin (ABC) method was performed on paraformaldehyde-fixed cells (2% in 0.01 *M* PBS, pH 7.3). After preincubation with 0.01 *M* phosphate-buffered saline (PBS; pH 7.3) containing 0.5% bovine serum albumin (BSA), a biotinylated secondary antiserum (goat anti-rabbit IgG; 1:500 diluted in PBS with 1% BSA; Camon, Wiesbaden, Germany) and a commercial ABC kit (Vectastain; Camon) were used. Immunoreaction was made visible with 0.01% H_2O_2 and 0.05% DAB solution (in 0.05 *M* Tris–HCl, pH 7.6). Controls were performed by (i) omitting the first antisera and (ii) incubating with normal rabbit serum instead of the specific antisera (for details see [4]).

Western blotting. The Western blotting procedure has previously been described [4]. In brief, cells were scratched from the culture plates, transferred into 62.5 m*M* Tris–HCl buffer (pH 6.8) containing 10% sucrose and 2% sodium dodecyl sulfate (SDS), and sonicated before heating (100°C for 5 min). Samples (15 μg of protein per lane) were subjected to electrophoretical separation on 7.5% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE). The gels were blotted onto nitrocellulose membranes and probed with the same NCAM antiserum as used for immunocytochemistry [10] (1:1000 dilution, incubation overnight at 4°C). Immunoreaction was detected with the ABC method (nonradioactive detection) with biotinylated goat anti-rabbit IgG antiserum (1:500, 1 h, 20°C; Camon) as the second antibody according to the manufacturer's instructions (Vectastain ABC kit, Camon). The last step was incubation with 0.01% H_2O_2, 0.05% 3,3′-diaminobenzindine–tetrahydrochloride dihydrate solution (DAB; Aldrich, Milwaukee, WI; diluted in 0.05 *M* Tris–HCl, pH 7.6), and 0.03% $NiCl_2$.

RESULTS

Immunocytochemistry (Figs. 1–5)

Cultures of cell type 1 showed cytoplasmic NCAM immunoreactivity in the perinuclear area and only occasional NCAM immunoreactivity at the lateral cell surface (Fig. 1). In contrast, monolayers of type 2 and 5 cells (Figs. 2 and 5) revealed heavy NCAM immunoreactivity exclusively at the lateral cell surface. Thus, especially the contact zones of adjacent cells were markedly stained. Monolayers of types 3 and 4 (Figs. 3 and 4) displayed no NCAM immunoreactivity.

Western Blotting (Fig. 6)

Western blots confirmed these results: Types 1 (weak), 2, and 5 (strong) showed an NCAM-immunoreactive band of approximately 140 kDa, while types 3 and 4 did not.

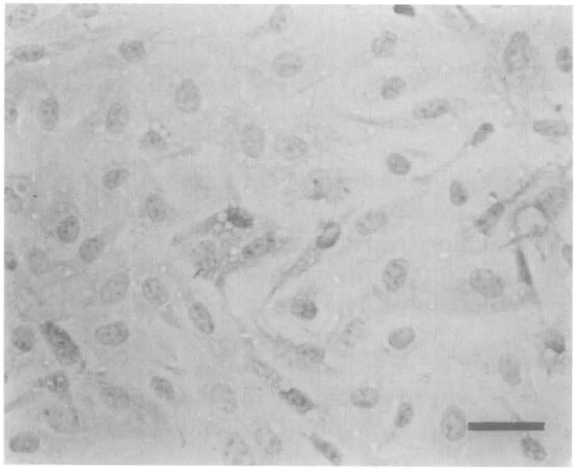

FIG. 3. Type 3 MVE: No NCAM immunoreactivity is found. Cells were stained with hematoxylin for 1 min to show the nuclei; phase-contrast micrograph; ×340; bar = 30 μm.

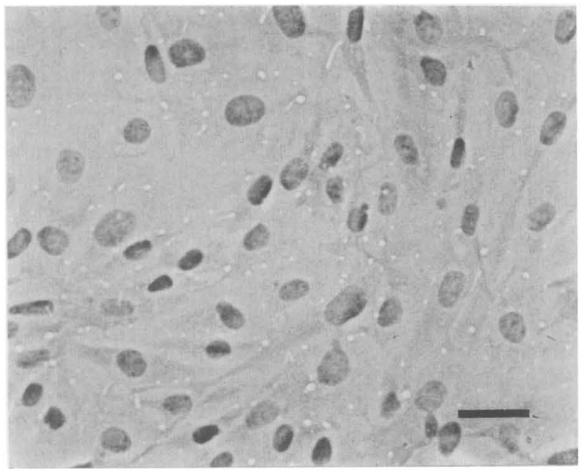

FIG. 4. Type 4 MVE: NCAM immunoreactivity is absent. Cells were stained with hematoxylin for 1 min to show the nuclei; phase-contrast micrograph; ×340; bar = 30 μm.

DISCUSSION

At present, numerous cell adhesion factors for blood cells are found on the apical side of vascular endothelial cells, including leukocyte adhesion molecules and lymphocyte function-associated antigens (cf. [11]). Several integrin receptors and receptors for heparin–sulfate glycosaminoglycan are localized at the basal side of vascular endothelial cells [12]. However, there is a lack of information concerning the presence of adhesion factors at the lateral cell side. Here we provide evidence that the neural cell adhesion molecule, NCAM-140, is expressed in culture by selected MVE derived from the bovine corpus luteum microvasculature.

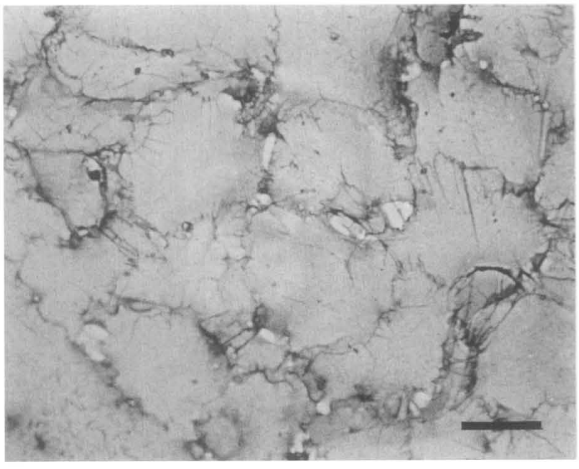

FIG. 5. Type 5 MVE: NCAM immunoreactivity is exclusively seen at the lateral cell surfaces, thus outlining the contact sites of neighboring cells. Although these cells, like the other types, were stained with hematoxylin for 1 min, the nuclei are inconspicuous; phase-contrast micrograph; ×340; bar = 30 μm.

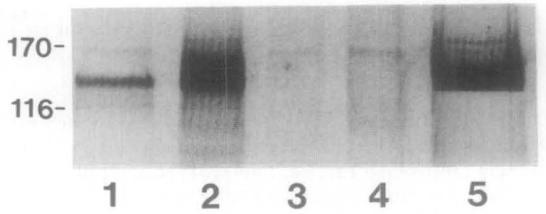

FIG. 6. Western blot analysis of NCAM in cultured MVE types 1–5. Immunoreactive bands of approximately 140 kDa were detected in cultured MVE types 1, 2, and 5. Size markers are indicated to the left.

Type 1 endothelial cells are only loosely attached to each other and become separated upon fixation. Cells of this type showed some surface and weak cytoplasmatic NCAM immunoreactivity. This predominant perinuclear NCAM immunoreactivity indicates that NCAM may not be transported efficiently to the cell surface of this type. In contrast, types 2 and 5 showing strong NCAM immunoreactivities at the lateral cell side, remained confluent after fixation. Strong surface-associated NCAM immunoreactivity, in the absence of intracellular NCAM immunoreactivity, argues for a functional importance of NCAM in the formation of cell-to-cell contacts. Interestingly, cell types 3 and 4 grew also as monolayers, but did not possess NCAM immunoreactivity. The cell adhesion factors involved in mediating their cell-to-cell contacts are presently unknown.

In previous studies, NCAM *in vivo* appeared to be associated mainly with luteal cells [4, 5]. We have obtained evidence by using homogenized bovine corpora lutea that NCAM-140 is present in the corpus luteum of cows (our unpublished data). This is in agreement with our results in rats [4] and the data from another group [5] studying mice. The results of the present study suggest that NCAM could provide a means of adhesion between endothelial cells and luteal cells, which can be viewed as functional units in an endocrine tissue. Clearly, additional studies are required to substantiate this assumption. Such studies are however presently hampered by the need to use frozen sections for NCAM immunocytochemistry (positive identification of MVE is difficult to carry out in frozen sections) and, moreover, molecular probes for bovine NCAM are not yet available (because the sequence of the bovine NCAM gene is presently unknown) to further analyze bovine tissue at the molecular level.

In summary, our results reveal for the first time that "endocrine" NCAM-140 can be expressed by MVE derived from the bovine corpus luteum. Endocrine luteal cells of mice, rats, and likely also cows possess NCAM-140. Therefore, the expression of this adhesion molecule by corpus luteum-derived MVE leads us to propose

that NCAM-140 may be involved in interactions between MVE and luteal cells.

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