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Cellular retinol binding protein-1 expression in endometrial hyperplasia and carcinoma: diagnostic and possible therapeutic implications

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Cellular retinol binding protein-1 (CRBP-1) contributes to the maintenance of the differentiative state of endometrial glandular cells through the regulation of bioavailability of retinol and derivatives, but its role in endometrial oncogenetic process remains unclear. Antibodies to CRBP-1, estrogen and progesterone receptors (ER and PR) were applied to paraffin sections of proliferative ($n=10$) and secretory endometrium ($n=9$), and to endometrial polyps ($n=6$), simple ($n=7$), complex ($n=3$) and atypical endometrial hyperplasias ($n=9$) as well as to 47 endometrioid carcinomas of different histological grade (G) (G1, $n=18$; G2, $n=19$; G3, $n=10$). Four serous and two clear cell carcinomas were also examined. In glandular cells, CRBP-1 positivity was mainly cytoplasmic and rarely in the nuclei. CRBP-1 immunodetection was weakly positive in proliferative and low and focal in secretory endometrium and higher in atypical as compared to simple and complex hyperplasias. CRBP-1 expression in G1 endometrioid carcinomas was similar to that in atypical hyperplasias. In the latter, the highest CRBP-1 expression was observed in areas of squamous differentiation. Semiquantitative evaluation revealed a significant decrease of cytoplasmic CRBP-1 immunoreactivity with the increase of tumor grade. Among G3 endometrioid carcinomas, 60% were CRBP-1 negative, whereas the remaining cases showed a very low and focal positivity. Serous carcinomas were also CRBP-1 negative. When areas of different grading were present within the same tumor, less differentiated areas retained a lower CRBP-1 immunoreaction. The progressive decrease of CRBP-1 paralleled that of ER and PR immunodetection. RT-PCR in eight endometrioid carcinomas suggested a decrease of CRBP-1 with the increase of tumor grade also at transcriptional level. Our results indicate that CRBP-1 immunodetection may constitute an additional tool for histological grading of endometrial carcinoma. The CRBP-1 loss during the progression of endometrial cancer suggests a new potential target for pharmacological strategies aimed to counteract its progression by increased intracellular retinol bioavailability.
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Retinol (the prototypic vitamin A) and its metabolites called retinoids play important physiologic roles in a wide range of biological processes and participate in the control of cell growth and differentiation.[1–5] Intracellular retinoid bioavailability is regulated by the presence of specific receptors; among these, cellular-retinol-binding proteins (CRBPs), which are members of the fatty acid-binding proteins (FABP)/CRPB family and are prominent among mammals.[6] CRBPs have an important role in the retinol metabolism presenting the ligand to specific enzymes.[1,7] In humans, three CRBPs types have been described: CRBP-1 is widely distributed through the body, whereas CRBP-2 is mainly restricted to the small intestine and CRBP-3 to the cardiac and skeletal muscle.[8] CRBP-1 is a 15 000 Da cytosolic protein that regulates the uptake and subsequent esterification of retinol and its bioavailability.[9] CRBP-1 acts as a 'chaperone' that

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prevents the interaction between retinol and intracellular enzymatic milieu.[10]

In normal human breast epithelium, CRBP-1 is uniformly expressed but appears downregulated in about 24% of breast cancers.[11] A loss of CRBP-1 gene expression is also reported in human ovarian cancers and ovarian cancer cell lines.[12] All together, these data suggest that a reduction of CRBP-1 expression significantly contributes to the oncogenetic process.[13]

In the endometrium, transcript analysis documents significant levels of CRBP-1 throughout the menstrual cycle, whereas those of CRBP-2 vary and possibly mediate the effects of ovarian steroids.[14,15] The availability of a specific antibody[16] allowed to establish that a CRBP-1 positive immunodetection is characteristic of endometrial decidual type stromal cells.[17]

Endometrial cancer represents the eighth commonest cause of death from cancer in the female population.[18] The overall-5-year survival for endometrial cancer is around 80%, but substantial prognostic differences exist between the different histological types of endometrial carcinomas.[19] Endometrioid type represents about 80% of all endometrial carcinomas.[19,20] Most endometriod carcinomas are well to moderately differentiated,[20] with various degrees of endometrial type glandular differentiation,[21] arise in a background of endometrial hyperplasia and are also known as type 1 endometrial carcinomas.[19,22] About 10% of endometrial cancers are type 2 (high grade) lesions, are not estrogen driven and histologically include serous and clear cell carcinomas.[19,20] Endometrioid carcinomas, from a molecular view point, as well as morphologically, usually resemble proliferative rather than secretory endometrium.[23] In a previous study, we reported some preliminary observations documenting cyclically regulated CRBP-1 expression in normal endometrial epithelium, which was somehow more prominent in proliferative than in secretory glands.[24] The present study deals with CRBP-1 expression in a series of endometrial hyperplasias and carcinomas. In the latter, we correlated CRBP-1 levels to tumor grade. According to our results, it appears that a loss of CRBP-1 expression is associated with the development of less differentiated endometrial carcinomas.

Materials and methods

Specimens

Tissue samples were obtained from diagnostic biopsies and operative procedures over the last 3 years (Institute of Pathology of Tor Vergata University and Patho-Lab Laboratories); 13 cases of simple hyperplasia without atypia, six cases of endometrial polyps, three cases of complex hyperplasia without atypia, nine cases of complex hyperplasia with atypia, 53 of endometrial carcinomas, including 47 endometrioid carcinomas (Grade 1, $n=18$; Grade 2, $n=19$; Grade 3, $n=10$), two clear cell and four serous adenocarcinomas, were investigated. We also studied 10 biopsies of proliferative and nine of secretory endometrium. Grading was according to F.I.G.O. and WHO criteria (5). The mean age of patients with endometrial carcinoma was 61 years, with a minimum of 45 and a maximum of 79 years. In eight cases of large tumors (four G1, two G2 and two G3 endometrioid carcinomas), after intraoperatory diagnosis, small freshly excised samples were frozen in isopentane, cooled in liquid nitrogen and stored at -80°C. Experimental procedures were approved by local Ethical Committees.

Morphological and Immunohistochemical Study

Paraffin serial sections (4-μm-thick) were stained with Hematoxylin–Eosin and used for immunohistochemistry. For the latter, after deparaffinization, blocking of endogenous peroxidase activity with 0.2% H_2O_2 (20 min) and incubation with normal goat serum (30 min), sections were exposed for 1 h to a polyclonal rabbit anti-CRBP-1 (15) (1:100, furnished by G Gabbiani, Geneva, Switzerland), monoclonal antiestrogen (ER; clone 1D5, Ylem, Italy) and antiprogesteron receptors (PR; clone 1A6, Ylem). Before incubation with primary antibodies, heat-mediated antigen retrieval with a solution of 10 mM sodium citrate buffer (pH 6.0) in a microwave oven (three cycles of 5 min) was performed. Diaminobenzidine was used as final chromogen. All immunohistochemical procedures were performed at room temperature. Semiquantitative CRBP-1, ER and PR immunoreaction was estimated at $\times 200$ magnification in at least 10 fields by two the authors, who used a grading system in arbitrary units as follows: absent (0), low and focal (0.5), positive (weakly positive, 1+; moderately positive, 2+; strongly positive, 3+).[24] The interobserver reproducibility was $>95\%$. For each case, the ratio of the score with the number of fields analyzed was calculated. Results were analyzed by means of Student's *t*-test. The differences were considered statistically significant for value of $P<0.05$.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions and the amount of RNA was determined by measuring OD_{260}. RNA (4 μm) were digested with 1 U of Amplification Grade DNAse I (Invitrogen) for 15 min at room temperature and the reaction was stopped by incubation with EDTA 25 mM for 10 min at 65°C. Reverse transcription was performed using random hexamers pdN_6 and SuperScript II (Invitrogen); samples were incubated at 37°C for 90 min and the reaction was heat-inactivated for the following 5 min. Negative controls were also prepared by

omitting reverse transcriptase in the reaction mixture. The primers used for cDNA amplification were: 5′CGC TTG TGG CCA AAC TGG CTC C (sense) and 5′ACA CAT CCT GCT GAT TGG TTG G (antisense) for CRBP-1 and 5′CTT GTC TT TCA GCA AGG ACT GG (sense) and 5′CCT CCA TGA TGC TGC TTA CAT GTC (antisense) for β2-microglobulin. Reaction conditions were: 5 min at 95°C, 35 cycles each cycle consisting of 45 s at 95°C, 1 min at 60°C and 1 min at 72°C, followed by further 5 min at 72°C for final elongation; amplicons were 486 and 157 base pairs, respectively.

Results

In normal proliferative endometrium, glandular cells displayed a weak CRBP-1 immunopositivity (Figure 1a), whereas in secretory endometrium their CRBP-1 staining was low and focal (Figure 1b). In simple (Figure 1c) and complex hyperplasias, CRBP-1 expression was almost similar to that of proliferative endometrium. This degree of CRBP-1 expression was retained in endometrial polyps (Figure 1d). In the latter and in simple hyperplasia, stromal fibrous tissue cells were only focally CRBP-1 positive and thick vessel walls negative, as previously reported.[17]

In atypical hyperplasia, CRBP-1 expression was more evident (Figure 1e) and similar to that detected in G1 endometrioid carcinomas (Figure 1f). The highest expression of CRBP-1 was observed in areas of squamous differentiation (Figure 1g). A decrease of CRBP-1 immunostaining was detected with the progressive increase of histological grade. In G2 carcinomas, CRBP-1 expression was less diffuse (Figure 1h and i) and, in some glands, CRBP-1 positive neoplastic cells alternated with others displaying an absent CRBP-1 immunoreaction, in a sort of tumor 'cell mosaicism' (Figure 1j), suggesting a progressive loss of CRBP-1 expression. In G3 carcinomas, the positivity was low and focal or absent (Figure 1k and l); 60% of G3 endometrioid carcinomas as well as all serous carcinomas were CRBP-1 negative. Less differentiated areas in otherwise G1 or G2 endometrioid carcinomas retained a lower CRBP-1 expression (Figure 2). CRBP-1 immonodetection was low and focal in clear cell carcinomas.

Results of semiquantitative evaluation of CRBP-1 immunostaining in endometrial carcinomas are shown in Figure 3. CRBP-1 expression was higher in complex atypical compared to simple hyperplasia ($P<0.0009$). CRBP-1 immunodetection decreased in less differentiated carcinomas with significant differences (G1 *vs* G2: $P<0.017$; G1 *vs* G3: $P<0.0004$ and G2 *vs* G3: $P<0.0009$, respectively). No statistical difference was observed comparing CRBP-1 expression in atypical hyperplasias and G1 carcinomas. In a more restricted number of cases, RT-PCR showed a decrease of CRBP-1 transcript content in G3 endometrial as compared to G2 and G1 carcinomas (Figure 4).

In some G2 carcinomas, a nuclear CRBP-1 immunoreactivity was also observed but its frequency did not reflect that of cytoplasmic staining (data not shown). Nuclear CRBP-1 positivity was extremely rare in G1 and absent in G3 carcinomas.

As shown in Figure 5, CRBP-1 decreased expression in endometrial carcinomas associated with reduced staining for ER (G1 *vs* G2: $P<0.002$; G1 *vs* G3: $P<0.04$; G2 *vs* G3: $P<0.04$) and PR expression (G1 *vs* G2: $P<0.02$; G1 *vs* G3: $P<0.0002$; G2 *vs* G3: $P<0.03$). Examination of serial sections demonstrated a good correlation between overall CRBP-1 and ER and PR tissue expression in endometrial carcinomas (not shown).

Discussion

In the present study, we analyzed the expression of CRBP-1 in endometrial carcinomas in comparison with that of normal and hyperplastic endometrium. Our results showed that epithelial glandular CRBP-1 imunodetection in simple hyperplasia was weakly positive and similar to that of proliferative endometrial cells. CRBP-1 expression increased in atypical hyperplasia and this feature was retained in G1 carcinomas. Importantly, we observed a progressive decrease of CRBP-1 immunoreactivity in less differentiated endometrial carcinomas, with a low or absent CRBP-1 expression in G3 endometrioid carcinomas. CRBP-1 was completely absent in serous carcinomas as well as in clear cell carcinomas. Our findings suggest that CRPB-1 may represent an additional phenotypic marker for the grading of endometrial carcinomas in which other morphological parameters, including nuclear pleomorphism and/or architectural abnormalities, do not allow a univocal grading. In fact, in G2 carcinomas with focal less differentiated areas, the latter retained a lower or negative CRBP-1 expression. A similar mosaicism has been recently demonstrated in G2 endometrial carcinomas for MLH1 expression.[25] The striking overall difference in the expression of CRBP-1 in type I and type II endometrial carcinomas reflects the differences in their risk factors and molecular pathogenesis.[26–28] Type I tumors display various alterations that may coexist, including microsatellite instability and PTEN, k-ras and β-catenin gene mutations.[22,29–32] Type II tumors are usually not associated with estrogen stimulation or hyperplasia[32] and are characterized by mutation of p53 tumor suppressor gene.[33] The similar CRBP-1 expression in atypical hyperplasia and well-differentiated endometrioid carcinoma can be considered an additional proof of the close relationship of these two entities which has been well documented in the literature. Furthermore, atypical hyperplasia progresses to carcinoma in a high percentage of cases.[30,34,35] The low or

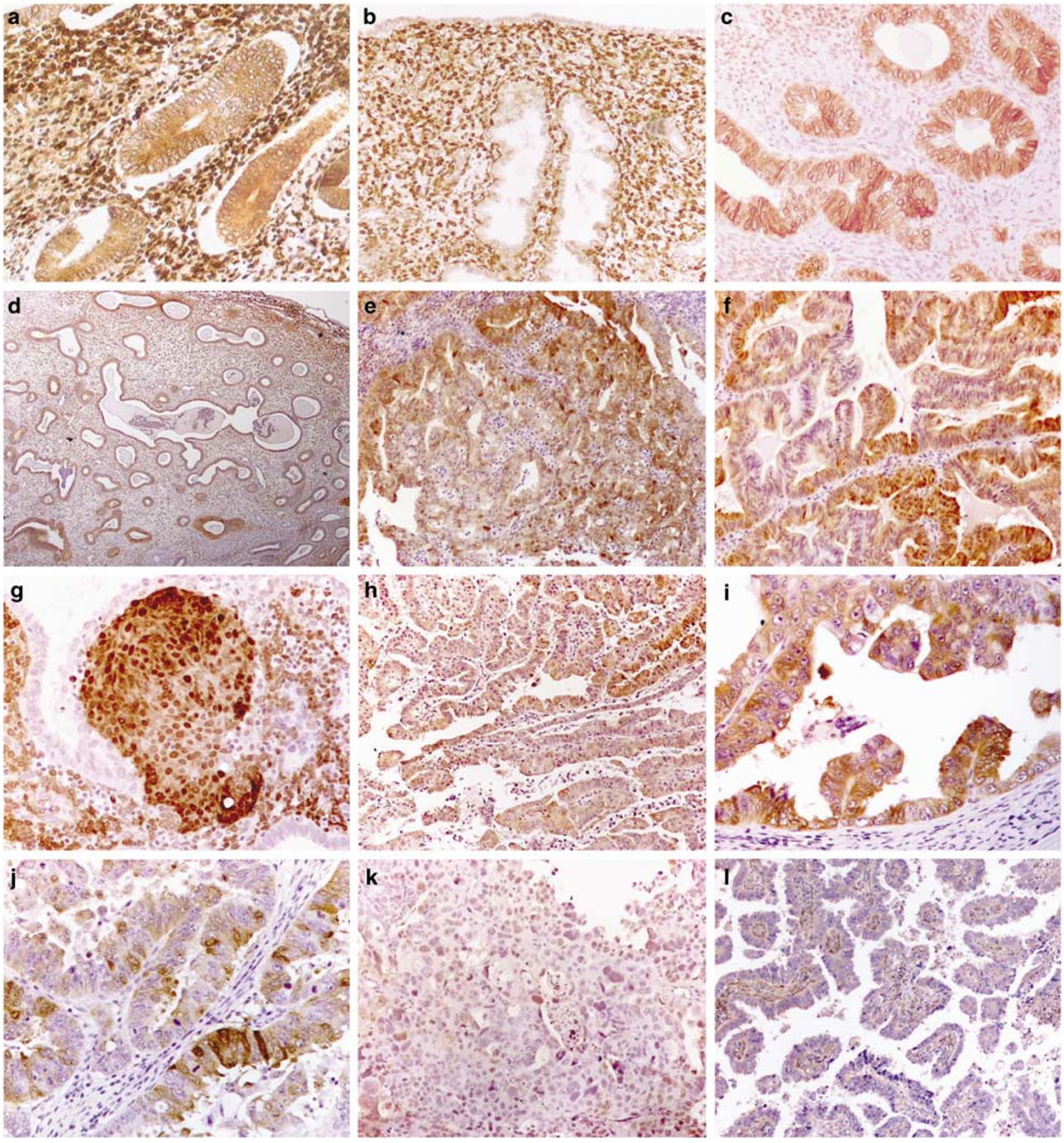

Figure 1 Immunohistochemical detection of CRBP-1 expression in normal and neoplastic endometrial cells. (**a**) A weakly cytoplasmic positivity is observed in proliferative endometrial glandular cells whereas (**b**) in secretory epithelium CRBP-1 is low, in contrast to endometrial stromal cells which are markedly positive. Cytoplasmatic CRBP-1 expression is present in (**c**) typical hyperplasia as well as in (**d**) endometrial polyps; in the latter, stromal cells are only focally CRBP-1 positive and thick vessel walls negative. CRBP-1 expression is more marked in (**e**) complex atypical hyperplasia as well as in (**f**) well-differentiated endometrioid carcinoma, particularly in (**g**) areas of squamous differentiation. (**h** and **i**) CRBP-1 expression is still evident in moderately differentiated carcinoma. (**j**) A detail of G2 carcinoma showing CRBP-1 positive glandular cells alternate with negative ones. (**k**) G3 endometrioid carcinoma shows a poor CRBP-1 expression, which is completely absent in (**l**) serous carcinoma. Original magnification, $\times 100$ for **d**; $\times 200$ for **c**, **e**, **f**, **h** and **l**; $\times 250$ for **a**, **g**, **i**, **j** and **k**.

absent CRBP-1 expression in serous and clear cell carcinomas further supports the distinct molecular pathogenetic pathways in type I as compared to type II endometrial carcinomas.[22]

The role of CRBP-1 in retinoid signaling during cancer progression has become relevant during the last years[13] but it is far from being completely understood. In the uterus, CRBP-1 expression

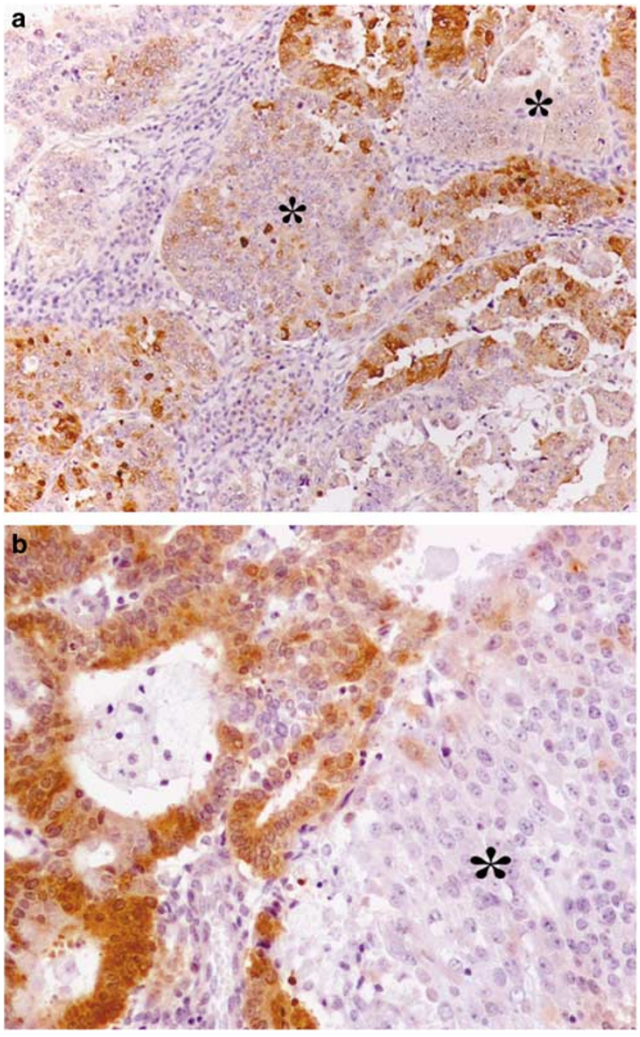

Figure 2 (**a** and **b**) In poorly differentiated areas (*) of an otherwise G2 endometrioid carcinoma, CRBP-1 expression is markedly reduced, whereas in adjacent more differentiated areas with a glandular pattern CRBP-1 expression is retained. Original magnification, ×200 for **a**; ×250 for **b**.

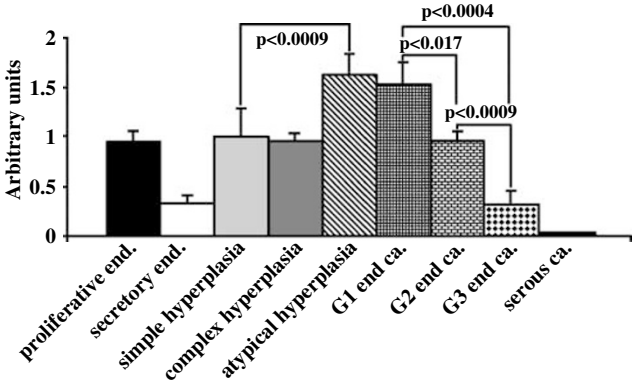

Figure 3 Bar graphs showing semiquantitative evaluation of CRBP-1 expression in proliferative and secretory endometrium (end.), simple, complex and atypical hyperplasia, well differentiated (G1), moderately differentiated (G2), poorly differentiated (G3) and serous carcinomas.

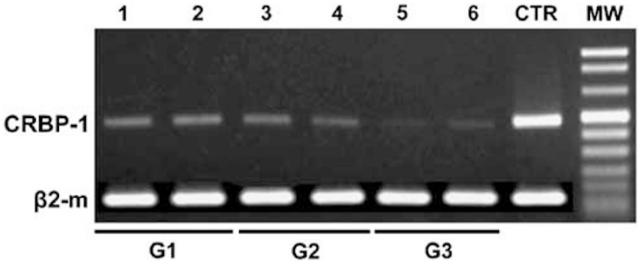

Figure 4 Demonstrative gel of CRBP-1 transcript level examinated by Reverse-transcriptase polymerase chain reaction. Transcript levels are progressively reduced in less differentiated endometrioid carcinomas; β2 microglobulin (β2-m) is used as control gene.

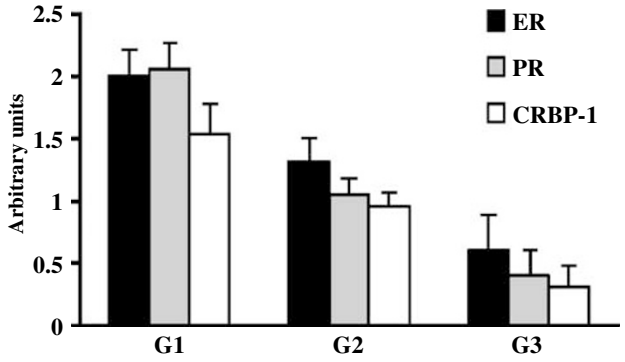

Figure 5 Bar graphs showing semiquantitative evaluation of CRBP-1, estrogen and progesterone receptor (ER and PR) expression in well differentiated (G1), moderately differentiated (G2) and poorly differentiated (G3) endometrioid carcinomas.

represents an important signal during normal implantation of the embryo.[36] Decidual cells, isolated from the uterus and provided with retinol, synthesize and release retinoic acid in the medium.[36] Endometrial epithelial cells differ in their retinoid physiology from stromal cells; since the latter appear to be more responsive to retinol than to retinoic acid.[14] Differences in CRBP-1 expression may reveal the necessity to maintain higher concentrations of retinoic acid through the intracellular retinol conversion in endometrial epithelial cells to facilitate their differentiation.[6,7,14]

Our present data did not allow to explain why CRBP-1 expression is increased in atypical hyperplasia compared to proliferative endometrial epithelium. Nevertheless, some considerations can be made. In squamous epithelium of the cervix, CRBP-1 expression is mainly limited to basal layers and varies according to hormonal stimulation[37] and increases with the presence of dysplasia.[38] In atypical hyperplastic endometrium, increased CRBP-1 expression may represent the presence of an exaggerate epithelial response to estrogen stimulation, which requires an increase in the biosynthesis and metabolism of retinol.[7] Our results in endometrial carcinoma are in agreement with

previous reports documenting a loss of CRBP-1 expression in carcinoma of the breast[39] and ovarian cancers.[12] Nevertheless, present data alone do not suffice to clarify how the loss of CRBP-1 expression relates to endometrial cancer progression. CRBP-1 downregulation occurring with cancer progression is likely to contribute to the loss of cell differentiation and control of proliferation.[11] Reduced levels of CRBP-1 may interfere with retinoic acid metabolism by reducing retinol bioavailability and blocking retinol esters.[6] One of the possible mechanisms of CRBP-1 silencing is hypermethylation-associated inactivation, as reported in breast[40] and prostate cancer[41] but also in several cancer cell lines *in vitro*.[42] Moreover, the parallel and progressive decrease in ER and PR status and CRBP-1 in less differentiated endometrioid carcinomas further supports a link between these receptor patterns in type I endometrial carcinomas.[43] The role of CRBP-1 in the regulation of rationed bioavailability[1,2,6,11] suggests that during the progression of type 1 endometrial carcinogenesis the prodifferentiative function of retinoids is progressively lost. The absence of CRBP-1 in less differentiated carcinomas is likely to result in a sort of intracellular hypovitaminosis, since normal or even excess of retinol levels cannot promote epithelial differentiation.[13] Conversely, dietary retinal intake can influence the extent of CRBP-1 silencing in human cancer.[42] The maintenance of CRBP-1 expression in G1 and partially in G2 endometrioid carcinomas suggests possible adjuvant pharmacological interventions using retinoid derivatives. In this sense, screening for CRBP-1 expression may represent an important prerequisite for pharmacological strategies aimed at influencing endometrial cancer cell growth through an increase of retinoic acid bioavailability.[13] Moreover, modulation of CRBP-1 expression may represent a potential target of therapeutic strategies aimed to increase intracellular retinol bioavailability and thus arrest the progression of endometrial cancer.

In conclusion, reduced CRBP-1 expression is associated with the progression of endometrial carcinoma to higher grades. Furthermore, CRBP-1 immunodetection can be considered an additional useful tool for grading of endometrial carcinoma and may help to detect areas of differentiation, which cannot be easily identified by routinary histological examination. Further studies are necessary to define the biological role of different patterns of CRBP-1 expression and possible implications in pharmacological strategy aimed to counteract the progression of endometrial cancer.

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