Histopathology 2004, **45**, 511–517

Cellular retinol-binding protein-1 expression in endometrial stromal cells: physiopathological and diagnostic implications

A Orlandi, A Ferlosio, A Ciucci, F Sesti,[1] B Lifschitz-Mercer,[2] G Gabbiani,[3] L G Spagnoli & B Czernobilsky[4]

Institute of Anatomical Pathology and [1]Institute of Gynaecology, Tor Vergata University, Rome, Italy, [2]Institute of Pathology, Tel Aviv Medical Centre, Ichilov Hospital, Tel Aviv, Israel, [3]Institute of Pathology, University of Geneva, Geneva, Switzerland, and [4]PATHO-LAB Laboratories, NESS-Ziona, Israel

Date of submission 8 April 2004
Accepted for publication 22 April 2004

Orlandi A, Ferlosio A, Ciucci A, Sesti F, Lifschitz-Mercer B, Gabbiani G, Spagnoli L G & Czernobilsky B (2004) *Histopathology* **45**, 511–517

Cellular retinol-binding protein-1 expression in endometrial stromal cells: physiopathological and diagnostic implications

Aims: Cellular retinol-binding protein-1 (CRBP-1) contributes to the maintenance of the differentiated state of the endometrium through retinol bioavailability regulation. The aim was to analyse CRBP-1 expression in endometrial stromal cells at eutopic and ectopic sites in different physiopathological conditions.

Methods and results: Antibodies to CRBP-1, CD10 and α-smooth muscle actin were applied to proliferative ($n = 10$), secretory ($n = 9$) and atrophic ($n = 7$) endometrium, decidua ($n = 4$), adenomyosis ($n = 5$), endometriosis ($n = 10$), endometrial polyps ($n = 9$), simple endometrial hyperplasia ($n = 6$), well-differentiated endometrioid carcinoma ($n = 6$) and submucosal leiomyomas ($n = 5$). In some cases, Western blotting and reverse transcription-polymerase chain reaction were also applied. CRBP-1 was expressed by eutopic and ectopic endometrial stromal cells more markedly during the late secretory phase and in decidua of pregnancy. CRBP-1 expression was low in the stroma of atrophic endometrium and absent in myometrium, leiomyomas and cervical stroma. CD10 immunoreactivity was weak in atrophic endometrium and in decidua.

Conclusions: CRBP-1 expression characterizes endometrial stromal cells at eutopic and ectopic sites and appears to be more specific than CD10. The level of CRBP-1 varies in intensity according to hormonal variations, reaching its maximum in predecidua and decidua. Thus, immunodetection of CRBP-1 may help to elucidate the physiopathological changes which occur in endometrial stroma and can also be applied as an adjuvant stromal marker.

Keywords: actin, adenomyosis, CD10, endometriosis, physiopathology of endometrium

Abbreviations: CRBP, cellular retinol-binding protein; FABP, fatty acid-binding protein

Introduction

Retinol and its derivates participate in a wide range of biological processes and play a significant role in the control of endometrial growth and differentiation.[1–3] Retinol bioavailability is regulated by the presence of specific cytoplasmic receptors; among these, cellular retinol-binding protein 1 and 2 (CRBP-1 and -2) which are members of the fatty acid-binding protein (FABP)/CRPB family, have distinct physiological roles.[1,4] CRBP-1 regulates uptake, subsequent esterification and bioavailability of retinol.[5] Studies based on transcript analysis suggest that endometrium expresses significant levels of CRBP-1 throughout the menstrual cycle, whereas levels of CRBP-2 vary and mediate

Address for correspondence: Professor Augusto Orlandi, Institute of Anatomical Pathology, Department of Biopathology and Image Diagnostics, Tor Vergata University of Rome, Via Montpellier 1, 00133 Rome, Italy. e-mail: orlandi@uniroma2.it

the effects of ovarian steroids.[6,7] The recognition of endometrial stromal cells at eutopic and ectopic sites is important for gynaecological pathologists. In fact, endometrial stromal cells show a variable appearance according to hormonal stimuli[8] and may be morphologically confused with myometrial or leiomyomatous cells in uterine curettage specimens. Indeed, morphological overlapping with other mesenchymal-derived cells may make the recognition of ectopic endometrial stromal cells at ectopic sites difficult, but this constitutes an important morphological feature for the diagnosis of endometriosis. Finally, identification of endometrial stromal cells surrounding glands helps to differentiate adenomyosis from minimally invasive, well-differentiated endometrial adenocarcinoma.[8,9] CD10 has been recently introduced as an immunohistochemical marker of endometrial stroma,[10] although with some limitations.[11,12] In the present study we investigated the expression of CRBP-1 endometrial stromal cells in normal eutopic sites and in various physiopathological states, and compared it with that of CD10.

Materials and methods

SPECIMENS

Tissue samples were obtained from diagnostic biopsy specimens and surgical pathology material over the previous 3 years. Clinical data are reported in Table 1. Freshly excised samples were formalin-fixed and prepared for histology and immunohistochemistry and in some cases (see Table 1) were frozen in isopenthane, previously cooled in liquid nitrogen and stored in pools at −80°C for protein and mRNA extraction (see below).

MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

Paraffin serial sections (4 μm thick) were stained with haematoxylin and eosin or employed 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 monoclonal anti-α-smooth muscle actin (α-actin 1 : 100; Dako Cytomation, Glostrup, Denmark), anti-AE1/AE3 cytokeratins (1 : 40; Ylem, Avezzano, Italy), anti-CD10 (1 : 10; Ylem) and a polyclonal rabbit anti-CRBP-1 antibody[13] (1 : 100, supplied by G. Gabbiani, Geneva, Switzerland). Before incubation with antibodies, heat-mediated antigen retrieval with a 10-mM sodium citrate buffer solution (pH 6.0) in a microwave oven (three cycles of 5 min) was

Table 1. Clinical data and experimental procedures used in the study

Histology	Age (years)	Site
Eutopic endometrium	34	Uterus (a)*
	41	Uterus (a)*
	39	Uterus (a)
	43	Uterus (b)*
	39	Uterus (b)
	39	Uterus (a)*
	40	Uterus (b)*
	25	Uterus (a)
	31	Uterus (b)
	37	Uterus (a)
	44	Uterus (a)
	29	Uterus (b)*
	55	Uterus (c)
	62	Uterus (c)
	65	Uterus (c)
	33	Uterus (a)
	33	Uterus (a)
	38	Uterus (a)
	46	Uterus (a)
	31	Uterus (b)
	40	Uterus (b)
	43	Uterus (b)
	44	Uterus (b)
	52	Uterus (c)
	66	Uterus (c)
	67	Uterus (c)
	46	Uterus (c)
Decidua	31	Uterus
	32	Uterus
	38	Uterus
	43	Uterus

Table 1. (*Continued*)

Histology	Age (years)	Site
Adenomyosis	44	Uterus
	51	Uterus
	39	Uterus
	29	Uterus
	41	Uterus
	52	Uterus
Endometriosis	51	Abdominal wall
	44	Bowel
	37	Peritoneum
	39	Bowel
	33	Ovary
	41	Endocervix
	41	Skin
	49	Endocervix
	30	Skin
	33	Appendix
Endometrial polyp	44	Uterus
	55	Uterus
	39	Uterus
	68	Uterus
	54	Uterus
	70	Uterus
	76	Uterus
	56	Uterus
	73	Uterus
	70	Uterus
Simple endometrial hyperplasia	29	Uterus
	40	Uterus
	46	Uterus
	47	Uterus
	51	Uterus
	54	Uterus
Well-differentiated endometrioid carcinoma	45	Uterus
	55	Uterus
	55	Uterus
	67	Uterus
	71	Uterus
	71	Uterus

Methods used: histology and immunohistochemistry for all cases, *also Western blotting and RT-PCR.
(a), Proliferative; (b), secretory; (c), atrophic endometrium.

performed. Diaminobenzidine was used as the final chromogen. All immunohistochemical procedures were performed at room temperature. Semiquantitative CRBP-1 immunoreactivity was estimated at $\times 200$ magnification in at least 10 fields by two of the authors who used the following grading system in arbitrary units: negative (0), weak and focal (0.5) and positive (from 1+ to 3+). The interobserver reproducibility was >95%. For each case, the ratio of the score with the number of fields analysed was calculated. Results were analysed by means of Student's *t*-test. The differences were considered statistically significant at a value of $P < 0.05$.

WESTERN BLOTTING

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed as follows. Tissue samples were finely fragmented in the presence of liquid nitrogen and mixed in buffer according to Laemmli,[14] immediately sonicated, boiled for 3 min and centrifuged. Protein content was determined according to the Bradford method[15] and verified by an electrophoretic gel stained with Coomassie Brilliant Blue (R 250; Fluka, Buchs, Switzerland) in methanol, acetic acid and water (45 : 10 : 45). For Western blotting,[16] 5–50 μg of proteins were electrophoresed on a 5–20% gradient gel and transferred to nitrocellulose filters (0.45 mm; Schleicher & Schuell, Dassel, Germany). The latter were then incubated with anti-α-actin (1 : 500) or anti-CRBP-1 (1 : 200) followed by a goat anti-mouse and anti-rabbit IgG ($1 : 10^5$), respectively. Enhanced chemiluminescence was used for detection (Amersham Biosciences, Little Chalfont, UK). Quantification of X-Omat R films was performed as previously reported.[17]

RNA EXTRACTION AND REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Tissue samples were finely homogenized for 60 s using a T25 Ultra-Turrax (Janke & Kunkel, Staufen, Germany) and total RNAs isolated by Tri-Reagent (Molecular Research Centre, Cincinnati, OH, USA), according to the manufacturer's instructions. Each RNA sample was quantified spectrophotometrically at 260 nm. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using specific primers based on a published sequence, as previously reported.[11] PCR products were analysed by electrophoresis on 2.5% agarose gel using 1 μg of pGEM DNA (Promega, Madison, WI, USA) as the marker. The amount of amplified PCR products was analysed by a densitometric reading in triplicate and the final value was expressed as the ratio of each gene amplified to that of β_2-microglobulin.[11,18]

Results

The semiquantitative evaluation of CRBP-1 and CD10 immunostaining of stromal cells is shown in Figure 1. In general, eutopic endometrial stromal cells were positive for CRBP-1 and negative for α-actin (Figure 2). In contrast, underlying myometrial tissue was CRBP-1 negative and α-actin positive, as has been previously reported.[13] CRBP-1 positivity changed during the menstrual cycle (Figure 2). CRBP-1 immunoreactivity was present in all stromal cells during the proliferative phase (Figure 2d). In this phase, CRBP-1 immunoreactivity was less intense than that of CD10 ($P < 0.02$, Figure 1). CRBP-1 expression progressively increased during the secretory phase (Figure 2b,e). The most marked CRBP-1 immunoreactivity was observed in stromal cells with predecidual changes and in decidua of pregnancy (Figure 2g). When incubated with CD10, predecidual cells and decidua showed only weak positivity with scattered cells that were more strongly positive (Figure 2h). This positivity was markedly less than that of CRBP-1 ($P < 0.002$; Figure 1). Stromal cells were still CRBP-1 positive in atrophic endometrium, although the expression was somewhat reduced (Figure 2j). In atrophic endometrium, CD10 expression was low, focal and mostly periglandular (Figure 2k). CRBP-1 positivity of stromal cells in adenomyosis (Figure 2a) was similar to that of endometriosis and comparable to that of eutopic proliferative endometrium. CD10 positivity in endometriosis and adenomyosis was similar to that of proliferative endometrium and somewhat more than that of CRBP-1, although this difference was not significant (Figure 1). In endometrial polyps, endometrial stromal cells were CRBP-1 positive whereas thick-walled vessels and fibrous stroma were CRBP-1 negative (Figure 2h). Also in endometrial polyps, CRBP-1 positivity decreased as atrophic changes increased (Figure 2i). CD10 staining of endometrial polyps was somewhat less than with CRBP-1, although this difference was not significant (Figure 1). In simple hyperplasia, CRBP-1 and CD10 immunostaining of endometrial stromal cells was similar to that of polyps. In well-differentiated endometrioid carcinoma, stromal CRBP-1 positivity was scarce and less than that of CD10 ($P < 0.004$). Concerning CRBP-1 immunoreactivity of glands, proliferative and atrophic endometrium showed weak cytoplasmic staining. The latter was also observed in simple endometrial hyperplasia and in well-differentiated endometrioid carcinoma (data not shown).

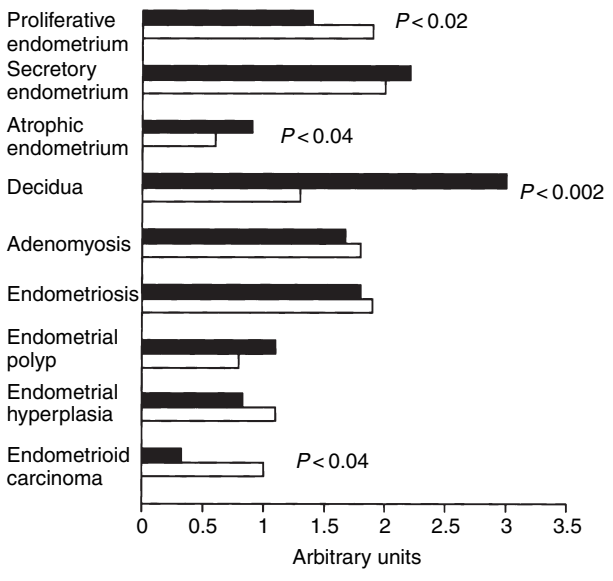

Figure 1. Bar graph showing a comparative evaluation of cellular retinol-binding protein-1 (CRBP-1) (■) and CD10 (□) immunoreactivity of endometrial stromal cells in different physiopathological conditions. Semiquantitative immunoreactivity was estimated using the following grading system in arbitrary units: negative (0), focal (0.5) and positive (from 1+ to 3+) and results analysed by means of Student's *t*-test.

Densitometric analysis of Western blotting and RT-PCR confirmed the greater CRBP-1 protein and transcript levels in secretory compared with proliferative endometrium and the almost absent expression in myometrial and leiomyomatous tissues ($P < 0.01$, Figure 3). Leiomyomatous tissue and myometrium showed a high α-actin protein and transcript content whereas they were scarce in endometrium, as previously reported.[13]

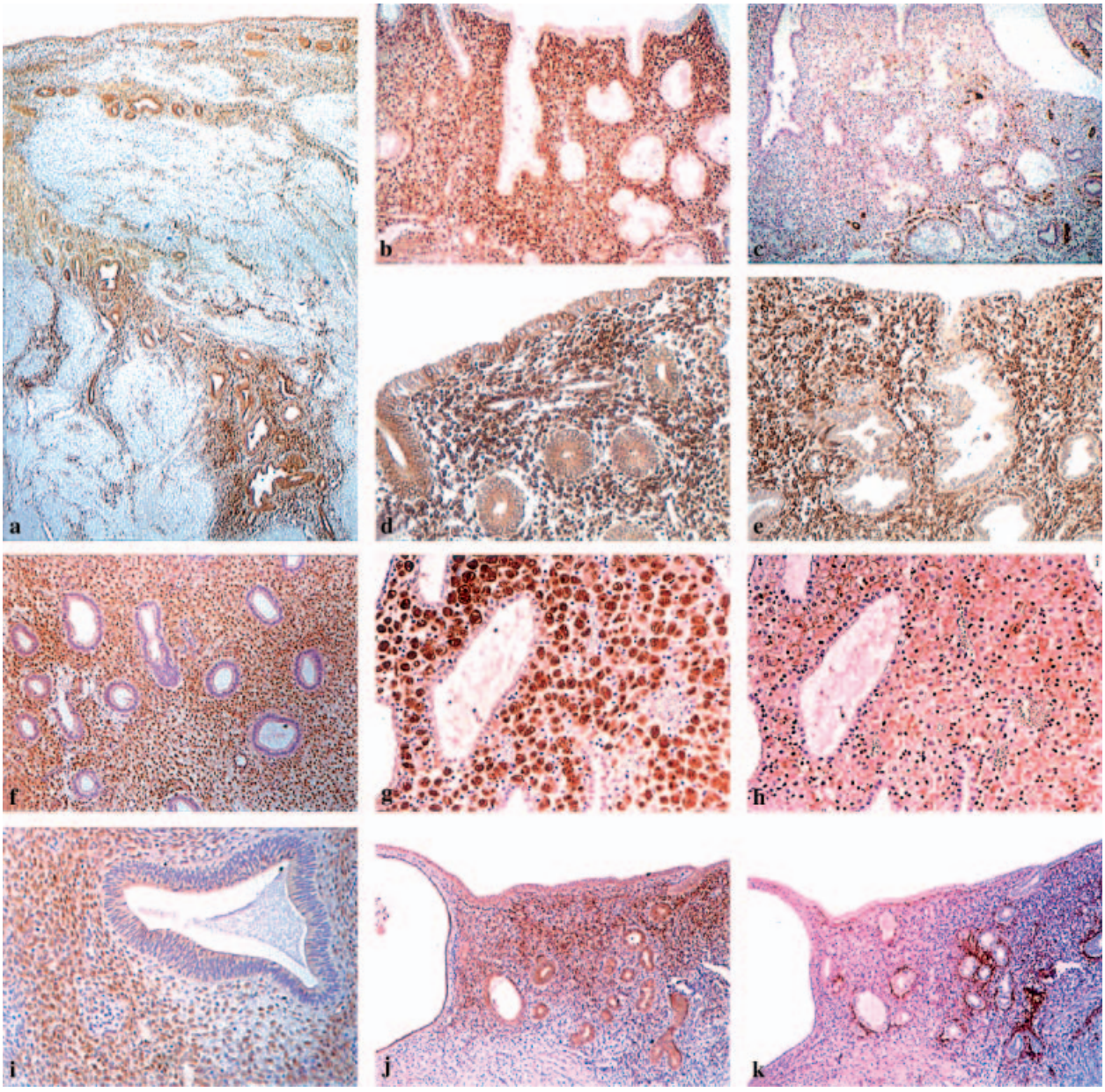

Figure 2. a, Cellular retinol-binding protein-1 (CRBP-1)-positive immunostaining clearly recognizes stromal cells of adenomyosis and of eutopic secretory endometrium which are (**b**) CRBP-1 positive and (**c**) α-actin negative. At higher magnification, CRBP-1 stromal cells are positive with variably intensity in (**d**) proliferative, in (**e**) secretory endometrium and in (**f**) endometrial polyp. Cells of decidua are markedly CRBP-1 positive (**g**) and only focally CD10+ (**h**). In the presence of atrophic changes, stromal cells are still positive in endometrial polyp (**i**) and eutopic endometrium (**j**), whereas in the latter they are only weakly and focally positive for CD10 (**k**).

Discussion

Our results document that eutopic and ectopic endometrial stromal cells express CRBP-1 in all physiopathological conditions examined. Immunohistochemical and biomolecular data showed that CRBP-1 proteins and transcript levels were higher during the secretory phase compared with those of proliferative endometrium. The highest CRBP-1 immunoreactivity was observed in decidua. CRBP-1 expression was retained in the stroma surrounding adenomyosis and in endometrial stroma of endometriosis and it was similar to that of CD10. In curettage or biopsy specimens or in the absence of endometrial glands, the morphological overlapping between stromal and other mesenchymal cells such as smooth muscle cells

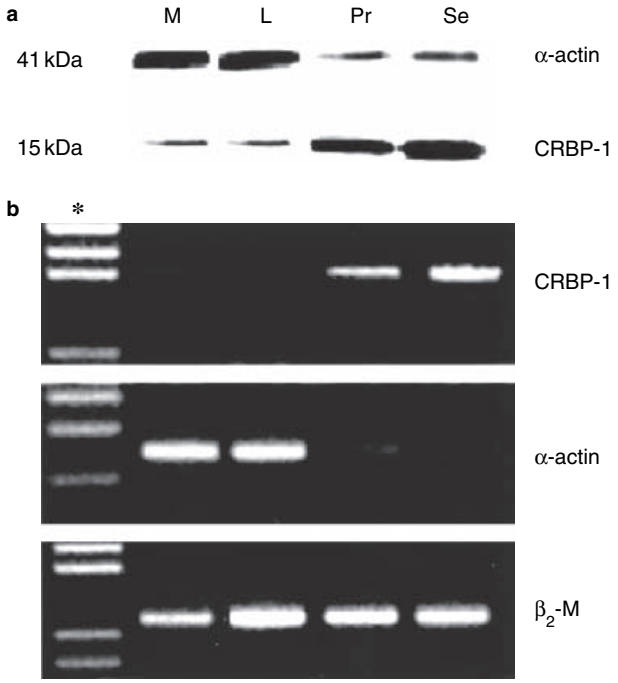

Figure 3. Cellular retinol-binding protein-1 (CRBP-1) and α-actin protein expression and transcript in proliferative (Pr) and secretory endometrium (Se), myometrium (M) submucosal leiomyomatous tissue (L), examined by (**a**) reverse transcription-polymerase chain reaction and (**b**) Western blotting. Densitometric analysis reveals in both cases that CRBP-1 protein and transcript levels are higher in secretory compared with proliferative endometrium and almost absent in leiomyoma and myometrium; lane *, pGEM DNA marker; α-actin, α-smooth muscle actin; β_2-M, β_2-microglobulin.

(SMCs) and fibroblasts may make their distinction difficult.[8,9] In particular, SMCs may resemble endometrial stromal cells because of cytoplasmic eosinophilia and the acquisition of round nuclei. This is further complicated by the morphological changes of stromal cells during the menstrual cycle.[8] Conventional and new cytoskeletal markers (α-actin, desmin, and h-caldesmon) have proven to be highly capable of recognizing smooth muscle differentiation in uterine cells.[19,20] Unfortunately, unlike SMCs, endometrial stromal cells lack a well-characterized immunophenotype. Consequently, the definitive confirmation of eutopic endometrial stromal cells and even more so in the context of adenomyosis and endometriosis is occasionally hindered by the lack of reliable markers for stromal cells. Our results suggest that CRBP-1 immunostaining can help to recognize endometrial stromal cells in the latter conditions.

In recent years, CD10 has been introduced as a marker of endometrial stromal cells.[10,21] However, CD10 can be also detected in morphologically typical fibroblasts and SMCs[22] and desmin may be found in endometrial stromal cells.[20] Moreover, CD10 positivity has been reported in normal cervical stroma.[11,12] Thus, CRBP-1 positivity could be a useful marker of cervical endometriosis because, in contrast to CD10, it does not stain cervical stroma. Our results also indicate that CD10 immunostaining is decreased in the stroma of atrophic endometrium and in decidua. In these conditions, CRBP-1 is expressed in the stromal cells.

Presently, the biological role of CRBP-1 in endometrial cells as well as the significance of its variations during the menstrual cycle have not been established. Retinoic acid, the most active retinol derivative, induces a transient increase of CRBP-1 expression in myofibroblasts *in vitro*,[23] suggesting a link between CRBP-1 and the intracellular conversion of retinol in retinoic acid. In vascular SMCs, high levels of CRBP-1 are associated with increased expression of RAR-α, the receptor that mediates the effects of retinoids at the nuclear level.[24] CRBP-1 expression has been associated with SMC phenotypic differentiation and susceptibility to apoptosis.[25] Expression of CRBP-1 is also an important signal during implantation of the embryo.[26] Moreover, decidual cells, isolated from the uterus and provided with retinol, synthesize and release retinoic acid in the medium.[26] One can thus envisage that CRBP-1 expression could represent a target for pharmacological strategies aimed at influencing endometrial stromal cell growth through the control of retinoic acid bioavailability. Further studies are needed to clarify these points and to evaluate the role of CRBP-1 expression in endometrial glandular cells.

In conclusion, CRBP-1 immunodetection can help in understanding the physiopathological changes which occur in endometrial stroma. In addition, CRBP-1 expression may represent a highly sensitive and diagnostically useful additional marker of endometrial stromal cells in eutopic and ectopic sites.

Acknowledgements

The authors thank S. Cappelli, A. Colantoni, C. Marcelli and L. Santangelo for their technical assistance. This work was partially supported by a 60% grant of Tor Vergata University (2001) and ICRETT fellowship no. 481/2001 from UICC.

References

1. Blomhoff R. Transport and metabolism of vitamin A. *Nutr. Rev.* 1994; **52;** S13–23.
2. Underwood BA. Vitamin A and cancer prevention conference—an introduction. *J. Natl. Cancer. Inst.* 1984; **73;** 1371–1372.

3. Boettger-Tong HL, Stancel GM. Retinoic acid inhibits oestrogen-induced uterine stromal and myometrial cell proliferation. *Endocrinology* 1995; **136;** 2975–2983.
4. Napoli JI, Posh KP, Fiorella PD, Boerman MH. Physiological occurrence, biosynthesis and metabolism of retinoic acid: evidence for roles of cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) in the pathway of retinoic acid homeostasis. *Biomed. Pharmacother.* 1991; **45;** 131–143.
5. Napoli JL. Biosynthesis and metabolism of retinoic acid: roles of CRBP and CRABP in retinoic acid homeostasis. *J. Nutr.* 1993; **123;** 362–366.
6. Loughney AD, Kumarendran MK, Thomas EJ, Redfern CP. Variation in the expression of cellular retinol binding proteins in human endometrium throughout the menstrual cycle. *Hum. Reprod.* 1995; **10;** 1297–1304.
7. Siddiqui NA, Loughney A, Thomas EJ, Dunlop W, Redfern CP. Cellular retinoid binding proteins and nuclear retinoic acid receptors in endometrial epithelial cells. *Hum. Reprod.* 1994; **8;** 1410–1416.
8. Dallenbach-Hellweg G. *Histopathology of the Endometrium.* Berlin, New York, Heidelberg: Springer-Verlag, 1981; 159–216.
9. Silverberg SG, Kurman RJ. Tumors of the uterine corpus and gestational trophoblastic disease. In: *Atlas of Tumor Pathology.* Washington, DC: AFIP, 47–89.
10. McCluggage WG, Sumathi VP, Maxwell P. CD10 is a sensitive and diagnostically useful immunohistochemical marker of normal endometrial stroma and of endometrial stromal neoplasms. *Histopathology* 2001; **39;** 273–278.
11. Toki T, Shimizu M, Takagi Y, Ashida T, Konishi I. CD10 is a marker for normal and neoplastic endometrial stromal cells. *Int. J. Gynecol. Pathol.* 2002; **21;** 41–47.
12. McCluggage WG, Oliva E, Herrington CS, McBride H, Young RH. CD10 and calretinin staining of endocervical glandular lesions, endocervical stroma and endometrioid adenocarcinomas of the uterine corpus: CD10 positivity is characteristic of, but not specific for, mesonephric lesions and is not specific for endometrial stroma. *Histopathology* 2003; **43;** 144–150.
13. Orlandi A, Francesconi A, Clement S, Ropraz P, Spagnoli LG, Gabbiani G. High levels of cellular retinol binding protein-1 expression in leiomyosarcoma: possible implications for diagnostic evaluation. *Virchows Arch.* 2002; **441;** 31–40.
14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227;** 680–685.
15. Bradford MM. A rapid and sensitive method for the quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 1976; **72;** 248–254.
16. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 1979; **76;** 4350–4354.
17. Orlandi A, Ehrlich HP, Ropraz P, Spagnoli LG, Gabbiani G. Rat aortic smooth muscle cells isolated from different layers and at different times after endothelial denudation show distinct biological features *in vitro*. *Arterioscler. Thromb.* 1994; **14;** 982–989.
18. Wu MH, Chuang PC, Chen HM, Lin CC, Tsai SJ. Increased leptin expression in endometriosis cells is associated with endometrial stromal cell proliferation and leptin gene up-regulation. *Mol. Hum. Reprod.* 2002; **8;** 456–464.
19. Schurch W, Skalli O, Seemayer TA, Gabbiani G. Intermediate filament proteins and actin isoforms as markers for soft tissue tumor differentiation and origin. I. Smooth muscle tumors. *Am. J. Pathol.* 1987; **128;** 91–103.
20. Rush DS, Tan J, Baergen RN, Soslow RA. h-Caldesmon, a novel smooth muscle-specific antibody, distinguishes between cellular leiomyoma and endometrial stromal sarcoma. *Am. J. Surg. Pathol.* 2001; **25;** 253–258.
21. Sumathi VP, McCluggage WG. CD10 is useful in demonstrating endometrial stroma at ectopic sites and in confirming a diagnosis of endometriosis. *J. Clin. Pathol.* 2002; **55;** 391–392.
22. Oliva E, Young RH, Amin MB, Clement PB. An immunohistochemical analysis of endometrial stromal and smooth muscle tumors of the uterus: a study of 54 cases emphasizing the importance of using a panel because of overlap in immunoreactivity for individual antibodies. *Am. J. Surg. Pathol.* 2002; **26;** 403–412.
23. Xu G, Redard M, Gabbiani G, Neuville P. Cellular retinol-binding protein-1 is transiently expressed in granulation tissue fibroblasts and differentially expressed in fibroblasts cultured from different organs. *Am. J. Pathol.* 1997; **151;** 1741–1749.
24. Neuville P, Yan Z, Gidlof A *et al.* Retinoic acid regulates arterial smooth muscle cell proliferation and phenotypic features *in vivo* and *in vitro* through an RARalpha-dependent signaling pathway. *Arterioscler. Thromb. Vasc. Biol.* 1999; **19;** 1430–1436.
25. Orlandi A, Francesconi A, Cocchia D, Corsini A, Spagnoli LG. Phenotypic heterogeneity influences apoptotic susceptibility to retinoic acid and cis-platinum of rat arterial smooth muscle cells *in vitro*: implications for the evolution of experimental intimal thickening. *Arterioscler. Thromb. Vasc. Biol.* 2001; **21;** 1118–1123.
26. Zheng WL, Sierra-Rivera E, Luan J, Osteen KG, Ong DE. Retinoic acid synthesis and expression of cellular retinol-binding protein and cellular retinoic acid-binding protein type II are concurrent with decidualization of rat uterine stromal cells. *Endocrinology* 2000; **141;** 802–808.