β 1C Integrin Expression in Human Endometrial Proliferative Diseases

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Integrins are ubiquitous cell adhesion molecules that are involved in maintaining normal tissue morphology and have been implicated in the aggressive behavior of several malignancies. β 1C integrin is an alternatively spliced variant of the β 1A integrin subunit that, at variance with β 1A, inhibits epithelial cell proliferation. β 1C integrin is expressed in non-proliferative, benign prostatic epithelium and is down-regulated in prostatic adenocarcinoma. In the current study, we examined β 1C expression at mRNA and protein levels in 18 endometrial adenocarcinoma and in 20 endometrial hyperplastic tissues, using Northern and Western blotting analysis and immunohistochemistry. The pattern of integrin expression was compared to that of the endometrium of 14 normal cycling women. The results of this study document inhibited β 1C integrin expression in endometrial adenocarcinoma, both at the mRNA and protein levels, at variance with significantly up-regulated β 1C mRNA expression in endometrial hyperplasia, in comparison with normal proliferative endometria. Our data suggest a key role of the regulation of β 1C integrin expression in the pathogenesis of endometrial proliferative diseases: β 1C integrin may act as growth modulator in cancer cells, playing a role in downstream intracellular signaling. (*Am J Pathol* 2003, 163:2543–2553)

The endometrium is a complex system, which involves interactions among different cell types, steroid hormones, cytokines, adhesion molecules, and growth factors and which probably relies on an integrated network of intercellular and intracellular signaling pathways in which all of the above molecules act in a paracrine, autocrine, or endocrine fashion.¹ Moreover, the normal process of cell growth requires a delicate balance between hormones,

growth factors, and adhesion molecules, including integrins, and apoptosis-related genes; an alteration of the synthesis and/or expression of these factors can contribute to the genesis of benign or malignant proliferative diseases. Among the above factors, integrins, which belong to a large superfamily of cell adhesion receptors,² coordinate the cell-extracellular matrix adhesive recognition and undergo dynamic alterations during the menstrual cycle in the human endometrium.

Integrins are heterodimeric transmembrane complexes of α and β subunits, in which the cytoplasmic tails modulate the receptor ligand binding affinity, distribution, surface expression, cell adhesion, and spreading.³ Moreover, they play a fundamental role in the regulation of cell proliferation and differentiation by their effects on different pathways of signal transduction in cooperation with other molecules, such as hormones, cytokines and growth factors. In fact, integrin expression is differentially regulated in tumors and altered expression in cancer cells, such as in prostatic carcinoma,^{4,5} has been correlated with changes in invasiveness, tumor progression, and metastatic potential.⁶

Integrins display dynamic temporal and spatial patterns of expression in the endometrium during the menstrual cycle and pregnancy.⁷ In addition, previous studies have documented an inverse correlation between integrin expression and tumor differentiation in adenocarcinoma cells.¹

Among the ubiquitous β 1 integrin family, an alternatively spliced variant of the human β 1 integrin subunit, designated β 1C, containing a unique 48 amino acid cytoplasmic domain, has been identified.⁸ Recent studies have demonstrated a unique function of β 1C in the regulation of cell growth^{2,9}; expression of recombinant β 1C in mammalian cells completely inhibited DNA synthesis and cell proliferation, while the wild-type β 1 had no effect.¹⁰ Furthermore, *in vivo* expression of β 1C is thought to correlate with a “non-proliferating cell” phenotype, since β 1C is expressed in normal prostatic cells, while it is down-regulated in prostatic adenocarcinoma.^{11,12}

While in a recent study we have shown that β 1 and β 1C integrins¹³ are present in the human endometrium throughout the menstrual cycle, in the postmenopausal

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Table 1. Clinicopathological Features of 18 Patients with Endometrial Carcinoma and 20 Patients with Endometrial Hyperplasia

Patients	Age (years)	Postmenopausal age (years)	Histology	Grade	Stage (F.I.G.O.)
K1	50	—	Adenosquamous carcinoma	1	IIb
K2	59	2	Endometrioid adenocarcinoma	3	IIIc
K3	65	10	Serous papillary carcinoma	1	Ic
K4	77	29	Clear cell carcinoma	3	IVb
K5	65	25	Endometrioid adenocarcinoma	3	IVb
K6	69	17	Serous papillary carcinoma	2	Ic
K7	74	22	Endometrioid adenocarcinoma	2	Ic
K8	68	12	Endometrioid adenocarcinoma	1	Ib
K9	64	18	Endometrioid adenocarcinoma	1	Ic
K10	76	22	Serous papillary carcinoma	3	Ic
K11	85	34	Endometrioid adenocarcinoma	1	Ib
K12	50	—	Serous papillary carcinoma	1	Ib
K13	58	4	Endometrioid adenocarcinoma	1	IIb
K14	66	15	Endometrioid adenocarcinoma	1	Ib
K15	54	2	Adenosquamous carcinoma	3	IIIa
K16	66	14	Endometrioid adenocarcinoma	2	IIb
K17	68	18	Endometrioid adenocarcinoma	2	Ib
K18	67	13	Endometrioid adenocarcinoma	1	Ia
H1	45	—	Simple hyperplasia		
H2	72	22	Simple hyperplasia		
H3	35	—	Simple hyperplasia		
H4	56	1	Simple hyperplasia		
AH5	53	—	Atypical hyperplasia		
H6	50	—	Simple hyperplasia		
H7	54	9	Simple hyperplasia		
H8	51	—	Simple hyperplasia		
H9	43	—	Simple hyperplasia		
H10	52	2	Simple hyperplasia		
H11	62	12	Simple hyperplasia		
AH12	49	—	Atypical hyperplasia		
AH13	62	12	Atypical hyperplasia		
AH14	46	6	Atypical hyperplasia		
AH15	83	33	Atypical hyperplasia		
AH16	76	26	Atypical hyperplasia		
AH17	55	2	Atypical hyperplasia		
H18	75	25	Simple hyperplasia		
H19	41	—	Simple hyperplasia		
H20	45	—	Simple hyperplasia		

K, endometrial carcinoma; H, endometrial hyperplasia; AH, atypical hyperplasia; —, data unknown.

age, and pregnancy, no data are available on the expression of $\beta 1C$ integrin in proliferative diseases of the human endometrium such as adenocarcinoma and hyperproliferative lesions.

In consideration of the supposed pivotal role of the adhesion molecules in the regulation of endometrial growth and differentiation, the study of their expression in hyperplastic and neoplastic conditions may allow a better understanding of endometrial biology and tumorigenesis.

Based on these premises, the aim of this study was to explore the expression of $\beta 1C$ integrin, both at mRNA and protein levels by means of Northern and Western blotting and immunohistochemistry to determine its altered expression in hyperplastic and neoplastic endometrial tissues, in comparison with normal proliferative endometria.

Materials and Methods

Study Population

The study population included 18 patients with endometrial adenocarcinoma, 20 patients with endometrial (sim-

ple or complex) hyperplasia (Table 1), and 14 patients with proliferative endometria (3 in the early-, 4 in the mid-, and 5 in the late-proliferative phase undergoing hysterectomy for subserosal leiomyoma; for two patients it was not possible to establish the date of the last menstrual cycle). For the latter group of patients, the dating was based both on patients history and on histological examination.

RNA Extraction and Northern Blot Analysis

Fresh endometrial samples were collected under sterile conditions from the above cohort of patients and snap-frozen in liquid nitrogen. The frozen tissue samples were pulverized to a fine powder and cellular RNA was extracted using the guanidinium isothiocyanate-cesium chloride procedure.¹⁴ Total RNA (25 μ g) isolated from the tissues was electrophoresed through 1% denaturing agarose gel containing 660 mmol/L formaldehyde, and transferred¹⁵ to a nylon membrane (Hybond N⁺; Amersham, Milan, Italy). The filters were subsequently pre-hybridized overnight at 42°C with a buffer consisting of 50% formamide, 5X Denhardt's solution (1% Ficoll 400,

1% polyvinylpyrrolidone, 1% bovine serum albumin), 5X SSPE [3 mol/L NaCl, 200 mmol/L $\text{Na}_2\text{H}_2\text{PO}_4$ (pH 7.0), 19 mmol/L ethylenediaminetetraacetic acid (EDTA)], 0.5% sodium dodecyl sulfate (SDS) and 100 $\mu\text{g}/\text{ml}$ sonicated salmon sperm DNA. The filters were then hybridized for 20 hours at 42°C by adding 3×10^6 cpm of ^{32}P -labeled probe per milliliter to the pre-hybridization solution. Hybridization was carried out as already described by Perlino et al.¹² The filters were washed once with 2X SSPE, 0.1% SDS, for 10 minutes at room temperature, then with 1X SSPE, 0.1% SDS at 42°C, followed by several washes in 0.1X SSPE, 0.1% SDS at 65°C and finally exposed at -80°C overnight or longer to Kodak X-OMAT AR 5 film (Kodak, Rochester, NY). Radiolabeled probes were generated using the Megaprime DNA labeling kit (Amersham), 5 μl of α -[^{32}P]-dCTP (3000 Ci/mmol, Amersham)¹⁶ and 25 ng of double-stranded either 116-bp fragment specific for the β 1C integrin or a full-length human β 1 cDNA.⁸ The specific 116-bp β 1C fragment (nucleotides 2435-2550)⁸ was generated by PCR using pBluescript β 1C plasmid as template and the resulting fragment was subcloned in the pBluescript vector as previously described.¹²

mRNA levels were normalized using a cDNA probe¹⁷ corresponding to the ribosomal 28S rRNA, a constitutively expressed gene, expressed at invariant levels. For this purpose, the blots were stripped in 0.1% boiling SDS and re-probed with the radiolabeled- ^{32}P 28S cDNA probe. Quantitative analysis was performed by densitometric scanning of the autoradiographs using a Bio-Rad GS-700 densitometer (Bio-Rad, Richmond, CA); multiple exposures of the same Northern blots in a linear range were performed. The ratio between the 4.3-kb long β 1C mRNA levels and the 28S rRNA levels was calculated for each sample to take into account for differences in RNA loading. The average of either β 1C or β 1 mRNA expression levels in control normal endometrium, derived from 12 of the 14 patients, was set at 100 (arbitrary units). The variability of β 1C or β 1 mRNA levels in the normal tissue specimens, measured by comparing the percentage of mRNA level from different normal endometrial tissues present on the same filter, was always less than 10% in the different measurements.

β 1C or β 1 mRNA levels in neoplastic and hyperplastic endometria were calculated as percentage of normal endometria mRNA levels hybridized on the same filter. For each specimen, the mean value ($\pm$ SEM) of results obtained in at least three experiments was calculated.

Immunoblotting

Either normal (7 cases), tumoral (8 cases), and hyperplastic (9 cases) endometrial frozen tissue samples obtained from hysterectomy were homogenized in lysis buffer containing 0.1% SDS, 1% Nonidet P-40, 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 200 mmol/L LiCl, 5 mmol/L EDTA, 10% glycerol, 10 $\mu\text{g}/\text{ml}$ aprotinin, 120 $\mu\text{g}/\text{ml}$ leupeptin, and 170 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride. The homogenate was sonicated for 20 seconds then centrifuged for 30 minutes at $14,000 \times g$ at 4°C.

2-mercapto-ethanol (1%) was added to each lysate for 30 minutes at 4°C to further solubilize potentially cross-linked molecules; it was then centrifuged, and supernatants were collected. One hundred and fifty micrograms of tissue extracts were electrophoresed on 7.5% SDS-polyacrylamide gel under reducing conditions and immunoblotting was carried out as previously described^{13,18} using either 5 $\mu\text{g}/\text{ml}$ rabbit polyclonal affinity-purified antibody to β 1C integrin (provided by Dr. Languino) or 1 $\mu\text{g}/\text{ml}$ mouse monoclonal antibody to β 1 integrin (Transduction Laboratories, Lexington, KY) or 10 $\mu\text{g}/\text{ml}$ antibody to β -tubulin (α -Aldrich Italia, Milan, Italy). The blots were incubated with antibody for 16 hours at 4°C in TBS-T [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.2% Tween-20] containing 5% nonfat dry milk. The membrane was then washed three times in TBS-T and incubated with horseradish peroxidase-conjugate goat affinity-purified antibody to rabbit or mouse IgG (Amersham Pharmacia Biotech, Milano, Italy), for β 1C integrin or β 1 integrin, respectively, diluted 1:20,000 in TBS-T for 1 hour at room temperature. After three washes in TBS-T, the proteins were visualized using the Amersham enhanced chemiluminescence system according to the manufacturer's instructions. Protein levels were normalized using the constitutively expressed β -tubulin protein. For this purpose, the blots were stripped at 55°C for 20 minutes with stripping buffer [2% SDS, 10 mmol/L β -mercaptoethanol, 6 mmol/L Tris-HCl, (pH 6.8)] and hybridized, as described above, with 10 $\mu\text{g}/\text{ml}$ antibody to β -tubulin. Densitometric values for immunoreactive bands were quantified using a GS-700 Imaging Densitometer (Bio-Rad). β 1C and β 1 protein levels were calculated as a percentage of the control (proliferative endometrium) taken as 100 in arbitrary units, on normalization using β -tubulin as control for protein loading. The variability of β 1C or β 1 protein levels in the normal tissue specimens, measured by comparing the percentage of protein level from different normal endometrial tissues present on the same filter, was always less than 5% in the different measurements.

For each specimen, the mean value ($\pm$ SEM) of the results obtained in at least three experiments was calculated.

Histopathology and Immunohistochemistry

The study material included all surgical samples from which tissue fragments for the molecular investigations had been taken, with the exception of one case of endometrial carcinoma for which tissue blocks were not available for the immunohistochemical procedures.

The surgical samples were fixed in 10% neutral buffered formalin for 12 to 24 hours, embedded in paraffin, cut, and stained with hematoxylin and eosin (H&E). The histological preparations were reviewed by two pathologists (E. M. and L. R.) to compare the histological dating with the clinical dating in women with normal cycles, to specify the histological subtype of endometrial carcinomas and define tumor grade.

A single paraffin block per case was selected for immunostaining based on good morphological preserva-

tion. Five-micrometer-thick sections were cut, collected on positively charged slides, dewaxed, and rehydrated. Following quenching of endogenous peroxidase with 3% H₂O₂ for 15 minutes at room temperature, the sections were immunostained for β 1 integrin and its spliced variant β 1C integrin using an avidin-biotin peroxidase technique (ABC) with an automated immunostainer (TecMate 500; Dako, Glostrup, Denmark). Before the staining procedure, the sections to be incubated with anti- β 1 integrin antibodies were immersed in 1% SDS in TBS for 10 minutes at room temperature. A mouse monoclonal antibody against β 1 integrin (clone: 18, dilution 1:20; Transduction Laboratories) and a rabbit antiserum against β 1C integrin (provided by Dr. L. Languino and used at a 1.7 μ g/ml dilution, as previously reported)¹² were used as primary antibodies with overnight incubations at 4°C.

Control sections for specificity included staining of positive controls (normal and carcinomatous breast) and of negative control sections, which were incubated with the immunoglobulin fraction of normal mouse or rabbit sera in place of the specific immunoreagent (Fig 11).

Evaluation of β 1 Integrin and β 1C Integrin Immunoreactivity

In all cases β 1 integrin and β 1C integrin immunoreactivity was independently evaluated by two pathologists (E. M. and L. R.) by separately counting the relative number of immunoreactive stromal and epithelial cells in 10 different microscopic fields, observed at $\times$ 400 magnification; the extent of the immunoreactivity within each cell component (stromal *versus* epithelial), meant as the percentage of immunoreactive cells, was semiquantitatively scored as follows: 0, absence of immunoreactive stromal/epithelial cells; 1, 1% to 10% immunoreactive stromal/epithelial cells; 2, 11% to 25% immunoreactive stromal/epithelial cells; 3, 26% to 50% immunoreactive stromal/epithelial cells; 4, >50% immunoreactive stromal/epithelial cells.

HL60 Cells

Human leukemia HL60 cells were grown in RPMI 1640 (Life Technologies, Milan, Italy), with 50 μ g/ml gentamicin, 2 mmol/L glutamine, and 15% inactivated fetal calf serum, at 37°C in presence of 5% CO₂. Total RNA from differentiated cells was prepared 24 hours after incubation with 160 nmol/L TPA (phorbol-12-myristate-13-acetate; Sigma), as previously described.¹⁹ A sample of normal human liver, obtained during cholecystectomy, was also used to generate mRNA.

Statistical Analysis

The levels of β 1, β 1C, and 28 S mRNA among the different endometrial samples are expressed as means ($\pm$ SEM) of normalized densities of cDNA bands. Regulation of β 1, β 1C, and 28 S mRNA expression with respect to controls in the separate measurements recorded for each patient was assessed by paired Student's *t*-test. Differ-

ences between samples (endometrial carcinoma tissues and hyperplastic tissues) were evaluated by unpaired Student's *t*-test comparing the mean differences respect controls. Semiquantitative evaluation of immunoreactivity in the different groups was performed by χ^2 test; mean values of β 1 and β 1C immunoreactivity in epithelial and stromal cells, among the different groups, were compared by ANOVA followed by Duncan post hoc test.

Results

Patients

The salient clinicopathological features of patients with hyperproliferative endometrial disease are illustrated in Table 1. Overall, there were 11 endometrioid adenocarcinomas (6 well-differentiated, G1; 3 moderately, G2; and 2 poorly differentiated, G3), 4 serous adenocarcinomas (G1: 2 cases, G2 and G3: one case each), 2 adenosquamous (one G1 and one G3) and 1 clear cell carcinoma (G3) and, with the exception of 2 G1 carcinomas from premenopausal patients, all endometrial adenocarcinomas (K) occurred in postmenopausal women. The mean age of patients with histologically proven endometrial adenocarcinoma was 66 ($\pm$ 9) (range, 50 to 85 years of age).

Among the endometrial hyperplasias, 13 cases of simple and 7 cases of complex hyperplasia with cytologic atypia were histologically identified.²⁰ The mean age of the patients with simple or atypical hyperplasia was 55 ($\pm$ 13) (range, 35 to 83 years of age). None of the patients had received any adjuvant treatment (radiotherapy, chemotherapy, or hormone therapy) before surgery.

RNA Expression Analysis in Endometrial Carcinoma Tissues

Steady-state levels of β 1C mRNA were quantified by Northern blotting of RNA from 12 endometrial neoplastic and 12 non-neoplastic tissues. Because of the very low amount of RNA obtained from the tissue samples it was necessary to analyze total RNA rather than poly(A⁺)RNA.

Figure 1 shows the results of a typical Northern analysis of β 1C mRNA. A 4.3-kb-long transcript corresponding to the β 1C mRNA length, was found in all samples. Total RNA extracted from HL60 cells (lane 1) and human liver RNA (lane 2) were used as positive and negative controls of β 1C mRNA expression, respectively, since β 1C is produced by HL60 cells but not from normal human liver. As expected, β 1C mRNA levels in endometrial tissues was lower (37%) than the mRNA levels in HL60 cells (data not shown); moreover, a threefold increase of β 1C mRNA was documented in normal proliferative endometrial samples, in comparison with normal human liver (12% of HL60 mRNA level; data not shown).

The autoradiographic analysis of Northern hybridization (Figure 1) showed variable expression of β 1C mRNA in the different neoplastic tissues (lanes 6 to 10), in comparison with the RNA from samples of proliferative endometrium (lanes 3 to 5).

Figure 1. $\beta 1C$ mRNA expression is down-regulated in human endometrial carcinoma and up-regulated in endometrial hyperplastic tissues. Total RNA was isolated from 12 normal proliferative endometria, 12 endometrial carcinomas, and 17 endometrial hyperplasias. $\beta 1C$ mRNA expression was evaluated by Northern blotting using the 116-b $\beta 1C$ -specific probe. Twenty-five micrograms of total RNA were used for each sample. **Lane 1:** RNA from HL60 cells used as positive control. **Lane 2:** RNA from human liver used as negative control. **Lanes 3 to 5:** RNA from normal proliferative tissues. **Lanes 6 to 10:** RNA from neoplastic endometrial tissues. **Lanes 11 to 12:** RNA from hyperplastic tissues. As described in Material and Methods, after hybridization with the $\beta 1C$ cDNA probe, the filter was stripped and hybridized again with the full-length $\beta 1$ probe. To normalize for the amounts of total RNA loaded for each sample, the blot was stripped and rehybridized once again using a 28S rRNA probe.

To normalize the differences due to mRNA loading and transfer, the same blots were dehybridized and rehybridized again with a human 28S rRNA cDNA probe. The values of $\beta 1C$ mRNA levels in all tissues were related to the 28S RNA levels, accounting for the RNA loading in each sample and calculated as a percentage of control (normal proliferative tissue). Finally, the mean values of at least three separate measurements for each patient were recorded and the average values $\pm$ SEM were reported in Figure 2. Down-regulation of $\beta 1C$ mRNA levels in comparison with the controls resulted in statistical signifi-

Figure 2. $\beta 1C$ and $\beta 1$ mRNA expression in neoplastic endometria. $\beta 1C$ and $\beta 1$ mRNA expression was analyzed as described in Figure 1. The average of either $\beta 1C$ or $\beta 1$ mRNA expression levels in 12 normal proliferative endometrial tissue samples (NP) was set at 100. $\beta 1C$ and $\beta 1$ mRNA levels in neoplastic endometria (K1-K12) were calculated as percentage of NP. Mean values $\pm$ SEM from at least three different experiments are illustrated. Down-regulation of $\beta 1C$ mRNA levels, in comparison with controls, was statistically significant ($T = -3.03$, $P = 0.01$). Down-regulation of $\beta 1$ mRNA levels in comparison with the controls was not statistically significant ($T = -0.33$, $P = 0.74$).

cance ($T = -3.03$, $P = 0.01$). As illustrated in Figure 2, the percentile expression of $\beta 1C$ integrin in this subset of samples ranged from 38% to 137%, with an average value of $70\% \pm 9$. A decrease greater than 10% of $\beta 1C$ steady-state level mRNA expression ($61\% \pm 9$) was detected in 10 of 12 endometrial carcinomas (83%), as compared to proliferative endometrium. In particular, in eight of the carcinoma samples the difference, with respect to the control, was statistically significant ($0.05 < P < 0.001$); the remaining two carcinoma samples, though still showing down-regulated $\beta 1C$ mRNA expression, were not considered statistically evaluable, because only a few measurements per case were available. Of the two carcinoma samples (K2 and K3) that did not show decreased $\beta 1C$ mRNA levels, one (K2) demonstrated a statistically significant ($P < 0.05$) increased $\beta 1C$ mRNA level ($137\% \pm 13$) in comparison with normal proliferative tissue, whereas the $\beta 1C$ mRNA level of the remaining case (K3) was comparable ($110\% \pm 12$) to normal endometrial tissue ($P > 0.05$).

Since $\beta 1C$ is a spliced variant of the $\beta 1$ integrin subfamily, alterations of its mRNA level could simply reflect an altered splicing mechanism occurring in endometrial cancer. To test such possibility, total $\beta 1$ mRNA level in all endometrial specimens was also measured using a full-length $\beta 1$ cDNA probe that hybridizes with all $\beta 1$ integrin variants.⁸ The 4.2-kb-long transcript corresponding to the $\beta 1$ mRNA length is illustrated in Figure 1. The $\beta 1$ mRNA steady-state levels ranged between 34% and 150%, with an average value of $97\% \pm 10$; the difference with the control group was not statistically significant ($T = -0.33$, $P = 0.74$).

In consideration of the relatively low number of endometrial carcinoma samples included in this study, a statistical evaluation of the relationships between $\beta 1C$ mRNA expression and tumor histotype, grade, and stage was not feasible. Nevertheless, a trend toward a direct correlation between $\beta 1C$ mRNA expression and tumor grade was apparent, as illustrated in Figure 3, and may deserve further investigation in larger series of cases.

RNA Expression Analysis in Endometrial Hyperplastic Tissues

$\beta 1C$ mRNA levels were evaluated in a cohort of 17 endometrial hyperplastic samples, seven of which showed simple hyperplasia and the remaining being atypical hyperplasia based on histopathological examination. A statistically significant increase of $\beta 1C$ steady-state mRNA levels with respect to the controls was detected ($T = 3.72$, $P < 0.001$). As illustrated in Figure 4, the percentile increase of $\beta 1C$ mRNA ranged from $102\% \pm 2$ to $322\% \pm 38$, with an average value of $157\% \pm 15$. In 12 of the 17 endometrial hyperplastic samples examined, the increased $\beta 1C$ mRNA levels detected in individual measurements of each case, in comparison with controls, were statistically significant ($0.05 < P < 0.001$). One of the samples was not suitable for statistical evaluation due to the limited number of measurements available, while the remaining four cases (H4, AH5, H8, and H9) showed

Figure 3. $\beta 1C$ mRNA levels and tumor grade and stage in endometrial carcinoma. $\beta 1C$ mRNA expression was calculated as described in Figure 1; the average of $\beta 1C$ mRNA expression levels in 12 normal proliferative endometrial tissues specimens (NP) was set at 100. $\beta 1C$ mRNA levels in neoplastic tissues were calculated as percentage of NP. Mean values $\pm$ SEM from at least three different experiments are shown. **A:** $\beta 1C$ mRNA levels in 6 well-differentiated (G1), 2 moderately differentiated (G2) carcinomas, and 4 poorly differentiated (G3) endometrial carcinomas. **B:** $\beta 1C$ mRNA levels in 8 stage I, 1 stage II endometrial carcinoma, 1 stage III, and 2 stage IV endometrial carcinomas (according to International Federation of Gynaecology and Obstetrics).

decreased ($77\% \pm 2$) $\beta 1C$ mRNA levels in comparison with controls, but the difference was not statistically significant.

Figure 4. $\beta 1C$ and $\beta 1$ mRNA expression in hyperplastic endometrial tissues. $\beta 1C$ and $\beta 1$ mRNA expression was analyzed as described in Figure 1. The average of either $\beta 1C$ or $\beta 1$ mRNA expression levels in 12 normal proliferative endometrial tissues (NP) was set at 100. $\beta 1C$ and $\beta 1$ mRNA levels in 17 hyperplastic endometrial tissues were calculated as percentage of NP. Mean values $\pm$ SEM from at least three different experiments are shown. Increase of $\beta 1C$ steady-state mRNA level with respect to controls resulted in statistical significance ($T = 3.72$, $P < 0.001$). Increase of $\beta 1$ mRNA steady-state level with respect to the controls resulted in statistical significance ($T = 4.43$, $P < 0.001$).

Figure 5. $\beta 1C$ mRNA level in endometrial hyperplasia (H: simple hyperplastic tissues, 10 cases; AH: atypical hyperplastic tissues, 7 cases) ($T = 2.05$, $P = 0.058$). $\beta 1C$ mRNA expression was calculated as described in Figure 1: the average of $\beta 1C$ mRNA expression levels in 12 normal proliferative endometrial tissues specimens (NP) was set at 100. $\beta 1C$ mRNA levels in hyperplastic tissues were calculated as percentage of NP. Mean values $\pm$ SEM from at least three different experiments are shown.

Furthermore, as illustrated in Figure 5, $\beta 1C$ mRNA levels were higher in the 7 atypical hyperplastic (AH) samples ($191\% \pm 29$ of the controls) than in the 10 samples of simple hyperplasia (H) ($134\% \pm 13$ of the controls), ($T = 2.05$, $P = 0.058$).

As for the carcinoma samples, the full-length $\beta 1$ integrin mRNA expression was also measured in the same 17 hyperplastic samples. The $\beta 1$ mRNA steady-state levels, with respect to controls, ranged between 71% and 362% with an average value of $179\% \pm 18$; the difference was highly significant ($T = 4.43$, $P < 0.001$). As for $\beta 1C$, the mean value of $\beta 1$ mRNA expression was higher in the atypical hyperplasia subgroup ($219\% \pm 34$) than in the simple hyperplasia subgroup ($151\% \pm 15$) ($T = 2.07$, $P = 0.055$).

Quite obviously, the difference between $\beta 1C$ and $\beta 1$ integrins mRNA expression in carcinomatous and hyperplastic tissues was highly statistically significant ($\beta 1C$: $T = 4.77$, $P < 0.0001$; $\beta 1$: $T = 4.03$, $P < 0.001$).

$\beta 1$ and $\beta 1C$ Protein Expression in Normal and Diseased Endometrial Tissues

Seventeen endometrial samples harboring endometrial proliferative diseases were selected for immunoblotting analysis of $\beta 1C$ and $\beta 1$ integrins, the results of which are shown in Figure 6. Both $\beta 1C$ and $\beta 1$ proteins were expressed in normal endometrial samples (Figure 6A, lanes 1 and 2), in 8 carcinoma samples (lanes 3 to 10), and in 9 hyperplastic tissues (lanes 11 to 19). The values of $\beta 1C$ and $\beta 1$ protein expression were calculated as a percentage of proliferative endometrium, after normalization for β -tubulin protein expression. The mean value of at least three separate measurements for each patient were recorded and the average $\pm$ SEM are reported in Figure 6B.

Drastically down-regulated $\beta 1C$ protein levels, with respect to the normal endometria, were detected in all pathological samples of this set (tumoral tissues: $T = -27.06$, $P < 0.0001$; hyperplastic tissues: $T = -10.6$, $P < 0.0001$). In particular, the percentile expression, with respect to the control, ranged from 4% (± 1) to 27% (± 5) (mean value: $13\% \pm 3$) in the 8 carcinomatous tissues and from 20% to 73% (mean value: $38\% \pm 6$) in the 9 hyperplastic tissues examined.

Figure 6. β 1C and β 1 protein expression in endometrial carcinoma and hyperplasia. **A:** Normal (lanes 1 and 2), tumor (lanes 3 to 10), and hyperplastic (lanes 11 to 19) tissue detergent extracts were electrophoresed on 7.5% SDS-polyacrylamide gel under reducing conditions, and immunostained using antibody either to β 1C integrin or to β 1 integrin or to β -tubulin. **B:** The mean values $\pm$ SEM of either β 1C or β 1 levels in neoplastic and hyperplastic tissues, normalized using β -tubulin, were calculated as a percentage of the levels detected in 7 normal proliferative tissues (NP). At least three separate measurements for each specimen were performed. Down-regulated β 1C protein levels, with respect to the normal endometria, were detected in all pathological samples of this set (tumoral tissues: $T = -27.06$, $P < 0.0001$; hyperplastic tissues: $T = -10.6$, $P < 0.0001$). Total β 1 protein content expressed in neoplastic endometrial tissues and in hyperplastic tissues with respect to controls were not statistically different (carcinoma tissues: $T = -2.04$, $P = 0.09$; hyperplastic tissues: $T = -0.93$, $P = 0.38$).

In contrast, the total β 1 protein content expressed in neoplastic endometrial tissues and in hyperplastic tissues, with respect to controls, was not statistically different (carcinoma tissues: $T = -2.04$, $P = 0.09$; hyperplastic tissues: $T = -0.93$, $P = 0.38$).

β 1C Immunohistochemistry

The results of the semiquantitative evaluation of β 1 and β 1C immunoreactivity are schematically illustrated in Figure 7, and show a strong association between percentage of immunoreactive epithelial cells and the type of lesion (β 1: $\chi^2 = 22.61$, $P = 0.0009$; β 1C: $\chi^2 = 56.52$, $P < 0.001$); mean values and ANOVA results are reported in Table 2.

Overall, β 1C immunoreactivity in epithelial cells was localized in the cytoplasm and showed staining reinforcement at the poles of the cells. Epithelial cells of normal proliferative endometria (Figure 8) demonstrated the highest percentages of β 1C immunoreactive cells in the whole series of cases and in these cases the percentage of β 1C-positive epithelial cells (mean, 74.57%, range = 64% to 90%) was comparable to that of β 1 immunoreactive cells (mean, 73.93%, range = 28% to 91%). Also, β 1C immunoreactivity was comparatively more intense and the number of immunoreactive cells higher in the epithelial compartment, in comparison with the stromal compartment of both normal and diseased endometria (Table 2).

Figure 7. β 1C and β 1 immunoreactivity in endometrial tissues. The results of semiquantitative evaluation of β 1C (**A**: $\chi^2 = 56.52$, $P < 0.001$) and β 1 (**B**: $\chi^2 = 22.61$, $P = 0.0009$) immunoreactive epithelial cells are reported for the endometrial tissues from 14 patients in the proliferative phase (NP), 17 patients affected by endometrial carcinoma (K), and 20 patients affected by endometrial hyperplasia (H).

Reduced β 1C immunoreactivity, in terms of number of immunoreactive epithelial cells, was detected both in endometrial hyperplasia (mean value = 37.5%, range = 21% to 65%) (Figure 9) and in carcinomas (mean value = 16.88%, range 8% to 28%) (Figure 10) and this difference was statistically significant ($F = 136.3$, $P < 0.001$); the post hoc test, in fact, detected a significant difference both between hyperplastic and carcinomatous samples and between each of these groups in comparison with normal proliferative endometria.

Interestingly, the highest percentage of β 1C immunoreactive cells was detected in two well-differentiated carcinomas while the lowest percentages were demonstrated in two poorly differentiated cancer cells. Nevertheless, a correlation between β 1C immunoreactivity and tumor differentiation, tumor stage, or histotype, could not be statistically assessed due to the relatively low number of cases.

Stromal cells exhibited an increase of both β 1C and β 1 immunoreactive cells in endometrial carcinoma and hyperplasia while β 1C and β 1 immunoreactive stromal cells were almost undetectable in normal proliferative endometria, but the difference was not statistically significant ($F = 0.62$, $P = 0.54$ and $F = 2.35$, $P = 0.11$ for β 1C and β 1, respectively). Figure 11 shows positive and negative controls as reported in the Materials and Methods section.

Table 2. β 1C and β 1 Immunoreactivity in Endometrial Tissues

Tissue	β 1C		β 1	
	Epithelium	Stroma	Epithelium	Stroma
NP (<i>n</i> = 14)	74.57 (1.9)	1.36 (0.6)	73.93 (4.8)	3.21 (1.3)
K (<i>n</i> = 17)	16.88 (1.5)	2 (0.4)	36.76 (5.3)	6.06 (0.9)
H (<i>n</i> = 20)	37.5 (2.9)	1.7 (0.2)	58.1 (5.3)	4.75 (0.6)
ANOVA results	*	n.s.	*	n.s.

**P* < 0.001.

The mean values of β 1C and β 1 immunoreactive cells are reported in the epithelial and stromal compartments of endometrial tissues. S.E.M. is reported in brackets. n.s., not significant.

Discussion

The results of the current study show for the first time that a down-regulation of β 1C integrin-spliced variant expression, both at mRNA and protein level, takes place in endometrial adenocarcinoma in comparison with normal endometrial samples. It should be noted that all endometrial adenocarcinomas analyzed occurred in postmenopausal women; thus, the down-regulation of β 1C integrin expression in these tissues is very significant considering that normally, β 1C integrin expression is maximal in atrophic endometrium compared with proliferative and secretive endometria.¹³ These data parallel those obtained by our group and by others documenting a down-regulation of β 1C expression in prostate, breast, and lung cancer.^{12,18,21,22}

Interestingly, we documented a significantly up-regulated β 1C mRNA expression in hyperplastic endometrial tissues. These same tissue samples also demonstrated decreased β 1C protein levels, although this decrease was less intense ($38\% \pm 6$ of protein expression in comparison with the normal proliferative endometrial tissue) when compared to the carcinomatous tissues ($13\% \pm 3$ of protein expression). These results are further supported by those obtained by immunohistochemistry, documenting reduced percentages of immunoreactive epithelial cells in endometrial hyperplasia and even more reduced percentages in endometrial carcinomas, in comparison with normal proliferative tissues.

Since β 1C is one of the four known β 1 variants, representing a spliced variant of the full-length β 1 integrin molecule, one might argue that variations of β 1C protein expression might recapitulate modified expression of

Figure 8. β 1C integrin immunoreactivity in proliferative endometrium. Most epithelial glandular cells exhibit moderate to strong immunostaining that is mainly localized at the basal and apical pole of the cells. Occasional stromal cells also display weak immunoreactivity. (ABC method anti- β 1C, $\times 200$).

Figure 9. β 1C integrin immunoreactivity in atypical endometrial hyperplasia. Several epithelial cells demonstrate weak to moderate immunostaining, without apparent intracellular compartmentalization. (ABC method anti- β 1C, $\times 100$).

Figure 10. β 1C integrin immunoreactivity in endometrioid carcinoma. Groups of neoplastic epithelial cells show moderate to strong intracytoplasmic immunoreactivity. (ABC method anti- β 1C, $\times 100$).

the full-length protein. Therefore, we also measured full-length β 1 integrin expression in the same series of tissues and found no significant alterations in neoplastic and hyperplastic samples, in comparison with controls.

Interestingly, we observed decreased β 1C integrin mRNA expression in the carcinoma tissues, indicating that the control of β 1C integrin expression might be regulated at a transcriptional and/or posttranscriptional level in such a fashion as to maintain selectively reduced mRNA and/or protein levels of β 1C. In contrast, β 1 protein levels, which are likely to reflect β 1A levels, although decreased at the mRNA level, may remain constant in normal and neoplastic tissues by compensatory mechanisms. In fact, considering that β 1B and β 1D variants are unlikely to be expressed in endometrial tissues, since β 1B expression is restricted to skin and liver tissues, whereas β 1D is detectable in striated muscle cells only,^{23,24} the contribution of β 1A variant is essential for the evaluation of the overall β 1 integrin expression.

The up-regulated β 1C mRNA expression, despite decreased β 1C immunoreactivity observed in endometrial hyperplasia, however, suggests that the synthesis of β 1C by epithelial cells might be regulated at a posttranscriptional level.

On the other hand, β 1C immunoreactivity may be more closely related to protein stability than to transcriptional activity; in fact, processing of heterologous nuclear RNA, modulation of the nucleo-cytoplasmic transport of mature mRNA and changes in mRNA stability may regulate the posttranscriptional control of mRNA,²⁶ and raised

Figure 11. Controls of immunostaining specificity. **A:** Negative control for β 1C integrin immunoreactivity in endometrial carcinoma obtained by substituting the primary antibody with non-immune serum. Both the epithelial and stromal cells do not show any immunoreactivity. (ABC method anti- β 1C, $\times 160$). **B:** Positive control for β 1C integrin immunoreactivity in normal breast. The ductules show consistent immunoreactivity that is mainly compartmentalized at the apical pole of the epithelial cells. (ABC method anti- β 1C, $\times 200$).

steady-state protein levels can occur without parallel increase of transcriptional activity. Furthermore, prolonged protein storage may justify increased β 1C immunoreactivity notwithstanding decreased mRNA.

Steady-state expression of mRNA is controlled by both transcriptional and posttranscriptional processes; moreover, protein levels may not accurately reflect the expression of their respective mRNA. Altered mRNA translation can regulate the expression of heat-shock proteins, membrane immunoglobulins, and other proteins, as well as cellular proteins in vesicular stomatitis virus-infected cells. In such instances, either the existence of specific inhibitors of mRNA translation or the lack of components of the translational machinery has been implicated.²⁵

On these premises and based on the results of the current study, we can postulate that β 1C integrin expression may be down-regulated at the level of protein synthesis, by reducing the translation efficiency, as supported by the detection of almost equal mRNA amounts in carcinoma and in normal endometria tissues.

The study of adhesion molecule expression in human tissues and cancer cell lines may be relevant for better understanding the role of these factors in cell cycle regulation and in tumor genesis and progression and, in-

deed, the adhesion pathway is very important in moderating neoplastic progression and aggressiveness.²⁷ Alterations of adhesion molecules seem to commonly occur in tumors as they progress to malignancy, as demonstrated by reduced tumorigenicity and motility in several different cells overexpressing $\alpha 5\beta 1$.²⁸ In addition, stepwise loss of integrins, such as $\alpha 1$, $\alpha 4$, and $\alpha v\beta 3$, with increasing tumor grade has been shown.²⁹ In this regard, the results of the current study document progressively decreased $\beta 1C$ expression, at protein level, in endometrial hyperplasia, atypical hyperplasia, and carcinomas, with the exception of increased $\beta 1C$ mRNA levels in hyperplastic tissues. These differences, in comparison with normal proliferative endometria, suggest that the inhibitory effects of this integrin on cell proliferation are increasingly reduced or lost with the progression from low proliferative toward highly proliferative hyperplastic lesions and finally, to malignant lesions. Consequently, a pivotal role for $\beta 1C$ can be postulated in the progression from hyperproliferative conditions, such as simple endometrial hyperplasia, to pre-cancerous lesions, such as atypical hyperplasia, and to invasive carcinoma. Possibly, the active role of $\beta 1C$ might be exerted on tumor differentiation as well since we documented a trend for a direct correlation between $\beta 1C$ expression and the grade of endometrial carcinoma. The relative rarity of atypical hyperplastic lesions and the necessity of examining both cryopreserved and formalin-fixed tissues did not allow the inclusion of a large number of these cases in the present study, thus making it unsuitable to perform adequate statistical evaluation. Nevertheless, these preliminary results may be an incentive to further investigate this issue in larger series of cases.

In consideration of the counteracting roles of $\beta 1$ and $\beta 1C$ in cell cycle regulation, the results of the current study add further insight for the understanding of the molecular control of cell growth and the mechanisms of intracellular signal transduction. Although the regulatory mechanisms in endometrial tissues is a very complex scenario with multiple controllers, the present demonstration of a key role of the $\beta 1$ and $\beta 1C$ integrin molecules may be critical to devise novel effective diagnostic and/or prognostic approaches.

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