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Variability of placental expression of cyclin E low molecular weight variants

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Variability of Placental Expression of Cyclin E Low Molecular Weight Variants¹

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ABSTRACT

Cyclin E, a G₁ cyclin serving to activate cyclin-dependent kinase 2, is the only cyclin gene for which alternative splicing leading to structurally different proteins has been described. Different cyclin E proteins are present in tumor tissues but absent from normal (steady) tissues. Cyclin E contributes to the regulation of cell proliferation and ongoing differentiation and aging. Because trophoblast has invasive properties and differentiates into syncytium and placental aging may develop at term, we examined cyclin E protein variants in human placenta. Placental samples were collected from 27 deliveries between 33 and 41 wk and were compared with ovarian cancer (positive control). Both placental and tumor tissues showed seven cyclin E low molecular weight (LMW) bands migrating between 50 and 36 kDa. Placental expression of cyclin E showed certain variability among cases. Lowest cyclin E expression was detected in normal placentas (strong expression of Thy-1 differentiation protein in villous core and low dilatation of villous blood sinusoids). Abnormal placentas (significant depletion of Thy-1 and more or less pronounced dilatation of sinusoids) showed significant increase either of all (early stages of placental aging) or only certain cyclin E proteins (advanced aging). Our studies indicate that a similar spectrum of cyclin E protein variants is expressed in the placental and tumor tissues. Low cyclin E expression in normal placentas suggests a steady state. Overexpression of all cyclin E proteins may indicate an activation of cellular proliferation and differentiation to compensate for developing placental insufficiency. However, an enhanced expression of some cyclin E LMW proteins only might reflect an association of cyclin E isoforms with placental aging or an inefficient placental adaptation.

developmental biology, placenta, pregnancy, trophoblast

INTRODUCTION

Placenta is required for mammalian reproduction. During early human pregnancy, there is a rapid proliferation of cytotrophoblast, resulting in the invasion of decidua, replacement of endothelial cells in uterine spiral arteries accompanying vascular dilatation, and terminal differentiation into the thin layer of syncytium at the villous surface [1]. Hence, the proliferating trophoblast exhibits invasive properties required for the implantation and for an adequate maternal blood supply to the intervillous space. The thin layer of terminally differentiated syncytium enables exchange of

nutrients and metabolites between the maternal and fetal blood.

Proliferation of eukaryotic cells is promoted by cyclin proteins, in association with cyclin-dependent kinases (cdk). Cyclin E, one of the G₁ cyclins, is expressed during mid- to late-G₁ phase. Kinase activities of cyclin E/cdk2 complexes are at maximum levels before S-phase entry. Functional knockout of cyclin E by injection of anticyclin E antibodies into fibroblast cells causes cell arrest in the G₁ phase. Conversely, the overexpression of cyclin E protein causes acceleration of progression through G₁. In addition to its important role in cell cycle progression, cyclin E also plays a key role in cellular differentiation and senescence (reviewed in [2]). Several studies with various differentiation systems have indicated postproliferative retention of significant levels of cyclins or cyclin E upregulation during cellular differentiation and aging [3–7].

The cyclin E gene produces a spectrum of alternatively spliced RNAs resulting in low molecular weight (LMW) cyclin E isoforms [8–10]. Cyclin E is the only cyclin gene for which alternative splicing leading to structurally different proteins has been described. When compared with the cyclin E protein originally described (cyclin E:E), a shorter splice variant (cyclin E:S) has been identified, which differs from cyclin E:E by a 49 amino acid deletion in the cyclin box. This shorter cyclin E:S isoform is defective in cdk2 binding, indicating that cdk2 binding is dependent on a functional cyclin box in cyclin E [8]. Other cyclin E variants are 15 amino acids longer (cyclin E:L) [10] or 45 amino acids shorter (cyclin E:T) isoforms [11].

The cyclin E:T has a molecular weight similar to cyclin E:S [11], and therefore they are seen in Western blots as a single (cyclin E:T/S) band. Although cyclin E:T contains an intact cyclin box, it is unable to function as a G₁ cyclin in yeast, pointing to a crucial functional role for sequences outside the cyclin box. However, during the cell cycle, cyclin E:T expression precedes up-regulation of the other isoforms and dramatically decreases in terminally differentiated cells. These observations suggest that cyclin E:T (and cyclin E:S) serve different functions when compared with the cyclin E isoforms stimulating progression of the cell cycle [11].

Additional splice variants of cyclin E have been identified to date. They include a 9-base pair (bp) in-frame deletion in the 5' domain of the message termed $\Delta 9$, a 148-bp deletion causing a C-terminal frameshift found in the 3' domain of the cyclin E mRNA termed $\Delta 148$, a 48-bp deletion from position 22 to 69 called $\Delta 48$, a 97-bp deletion from position 24 to 120 called $\Delta 97$, and an IN3 unspliced variant [12]. However, $\Delta 97$ and IN3 mRNA splice variants cannot be translated into proteins [12], leaving the identified cyclin E protein variants as cyclin E:L, cyclin E:E, cyclin E:T, cyclin E:S, $\Delta 9$, $\Delta 48$, and $\Delta 148$. The molecular weight of cyclin E proteins produced by $\Delta 9$, $\Delta 48$, and $\Delta 148$ mRNA splice variants remains, however, unknown.

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Most LMW cyclin E protein variants (except cyclin E: S) contain an intact cyclin box, bind to cdk2, and promote G₁-S transition (except cyclin E:T). The cyclin E:L variant is the predominantly expressed cyclin E protein isoform. This suggests that cyclin E:L is the major product of the cyclin E gene and cyclin E:E (originally considered to be a wild type) belongs to LMW variants with lower frequency of expression. The LMW cyclin E protein isoforms represent N-terminal truncations because the monoclonal antibody HE12, used to detect them on a Western blot, is directed toward a C-terminal epitope (residues 318–338) (data and review in [12]).

In human placental villi, cyclin E protein expression was found by immunohistochemistry in a higher percentage of cells than those that are actively dividing, including syncytiotrophoblast, and results of the Western blot analysis revealed that two forms of cyclin E protein were present [13]. In contrast, Bamberger et al. [14] recently reported that cyclin E expression is limited to dividing (Ki67+) villous cytotrophoblast and some cells in the mesenchymal villous core.

In an earlier study, we have found that expression of cyclin E in cryostat sections of a placental cotyledon was greatest overall within intravillous blood vessels and cytotrophoblast. However, cyclin E immunoreactivity was significantly reduced within large areas of syncytium [15]. We also reported that primary cytotrophoblast cultures from normal term pregnancies show high cyclin E expression at 24 h and that cyclin E protein forms complexes with cdk2. Subsequently, the expression of cyclin E alone decreased and cdk2-associated cyclin E was zero at 96 h of culture exhibiting syncytium formation. Accordingly, cyclin E gene expression was high at 24 h and diminished thereafter [15].

In this study, we used Western blot analysis to identify expression of cyclin E LMW protein variants in placental samples and compared it to the malignant tissue. We also quantified cyclin E expression and correlated the data with the development of placental senescence. Numerous studies have shown that after 34–36 wk of human gestation, even in otherwise uncomplicated pregnancies, the placenta may show advanced aging accompanied by villous degeneration while the fetus continues development [16–21]. In general, villous degeneration is characterized by fibrin deposits at the villous surface, marked interstitial fibrosis and fibrinoid degeneration of the stroma, and is associated with cellular apoptosis and congestion of villous sinusoids [17, 22–24]. Placental senescence affects some placentas during the third trimester, yet even the aging placenta may have a capacity to compensate for needs of the growing fetus [21]. Dilatation of villous sinusoids, with accompanying thinning of the villous membrane, is the principal adaptation to fetal hypoxia [25]. Immunohistochemically, aging and degeneration of terminal villi are associated with progressive diminution of Thy-1 differentiation protein expression in villous core [26].

MATERIALS AND METHODS

Tissues

Twenty-seven placentas from deliveries between 33 and 41 wk of pregnancy were investigated. The source of placental tissue was women with normal and abnormal pregnancies admitted to the University of Tennessee Medical Center. As the only University/teaching hospital in the area, we have a significant proportion of obstetric patients with abnormal pregnancies. Excluded were patients with blood transferable infections, e.g., hepatitis and HIV. Samples of ovarian cancer (positive control for cyclin E

expression) were obtained from NCI Cooperative Human Tissue Network, Columbus, OH. Samples of normal ovaries (control for cyclin E expression in normal tissue) were obtained from hysterectomy specimens in cooperation with our Department of Pathology. The study was approved by the Institutional Review Board.

Tissue Processing and Peroxidase Immunohistochemistry

The processing of the placenta started within 30–60 min after delivery. Several 10- × 10- × 5-mm blocks of tissue were collected, frozen, and 7- μ m cryostat sections incubated with monoclonal mouse anti-human Thy-1 antibody (1:100), clone F15-42-01 [27] (Dr. Rosemarie Dalchau of the Institute of Child Health, University of London, London, England), followed by Universal DAKO LSAB2 Peroxidase Kit (DAKO Corporation, Carpinteria, CA), as described previously [26].

For preparation of protein lysates from whole placenta, ovarian cancer, and normal ovaries, 300 adjacent tissue sections were collected into two microcentrifuge tubes and stored at -80°C . At least two distinct samples were processed from each case for immunohistochemistry and Western blotting.

Western Blotting

Tissue sections in microcentrifuge tubes were lysed by adding ice-cold lysis buffer (20 mM Tris pH 7.5, 200 mM NaCl, 0.25% Nonidet P-40; 400 μ l/100 mg) containing 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride. After 15 min on ice, the lysates were sonicated by Sonicator Cell Disruptor (Heat Systems-Ultrasonic, Inc., Plainview, NY) for 5 sec and centrifuged at $11\,000 \times g$ for 10 min at 4°C . Supernatants were stored at -80°C .

Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein (21 μ g) were loaded onto reducing 10% SDS-Tris-glycine polyacrylamide gels, transferred to nitrocellulose (Bio-Rad), and processed as described previously [15]. One of two identical membranes was first subjected to control staining (secondary antibody only), developed, washed, and stained for actin (0.25 μ g/ml in blocking reagent, clone C4; Boehringer Mannheim Corp., Indianapolis, IN). The second membrane was stained for cyclin E (0.1 μ g/ml in blocking reagent, clone HE12; Santa Cruz Biotechnology, Santa Cruz, CA).

Quantitative Analysis of Placental Villi

Placental villi were evaluated by 1) measurement of the density of Thy-1 expression in villous core, which diminishes with villous aging [26], and 2) measurement of dilatation of villous blood capillaries, which reflects an effort of the placenta to compensate the development of chronic fetal hypoxia [25, 28].

First, the video images of Thy-1 staining (original magnification $\times 200$ + camera factor 1.5) were captured from microscope via the RGB video camera into the Scion Image software (Scion Corporation, Frederick, MD). To determine the density of Thy-1 expression in villous core, we first measured raw optical density (OD) in the constant area (250 μm^2) in randomly selected terminal villi (120 measurements for each placental type—see *Results* for placental typing). To obtain net OD, each raw measurement was subtracted with the density of tissue background in the control staining. The net OD values were used for statistical calculations.

To evaluate the extent of dilatation of villous sinusoids, we used a freehand tool to delineate manually the margins of the blood vessels and then determined 1) the area in square micrometers and 2) the maximum axis, in micrometers, in 120 randomly selected sinusoids per placental type.

Quantitative Analysis of Cyclin E Expression

Blots were scanned into the Paint Shop Pro Version 5 software (JASC Software, Inc., Minneapolis, MN), saved in Windows Bitmap, and loaded into the Scion Image (Scion Corporation). Individual bands were manually selected using the freehand tool, and mean raw optical density and band area were measured. The raw optical density was subtracted with background value for the specific lane and net OD multiplied by the area to obtain the integrated density. Calculations were performed using Microsoft Excel 2002 (Microsoft Corporation). Sets of integrated densities for particular placental type were used for statistical calculations.

Statistical Analysis of Data

Statistical analysis of data was performed using the GraphPad InStat version 3.01 for Windows software (GraphPad Software, San Diego, CA).

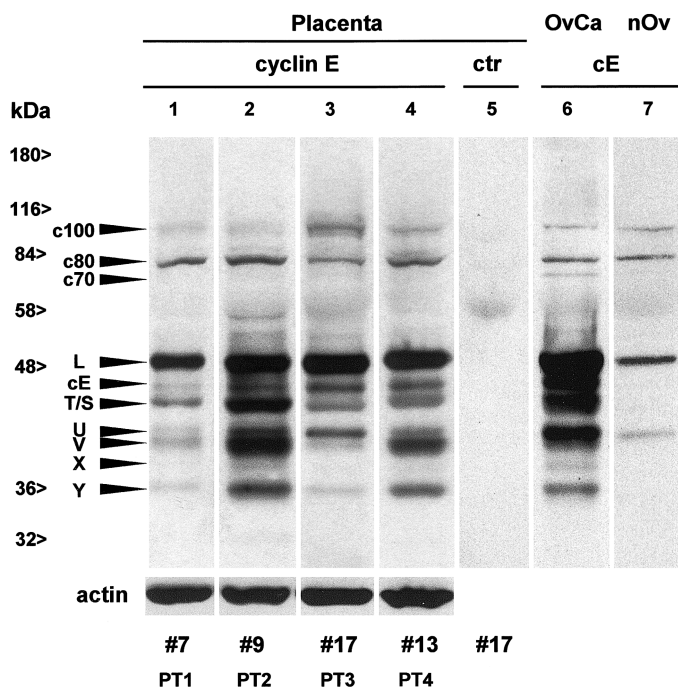


FIG. 1. Variability of cyclin E (cE) expression in placental samples and a comparison with ovarian cancer (OvCa; papillary cystadenocarcinoma, female 56 yr of age) and normal ovary (nOv; female 26 yr). Cryostat sections were lysed and extracts clarified by centrifugation. Equal amounts of protein (21 μ g) were separated by SDS-PAGE and immunoblotted with HE12 antibody to cyclin E (lanes 1–4, 6, and 7). Lane 5 (ctr) shows background staining with secondary antibody alone. Arrowheads (see text for the letters) indicate cyclin E protein variants. Rows at the bottom indicate case numbers (#) and placental types (PT; explained in the text to Fig. 2).

For evaluation of Thy-1 density, the net optical density values were subjected to one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test. The relationship of placental types to maternal age was evaluated in the same way. Maximum axis and area of sinusoids were evaluated by Kruskal-Wallis test (nonparametric ANOVA) followed by Dunn multiple comparisons test. For Western blots, the values of integrated density were transformed ($Y = \log[Y]$) and evaluated by one-way ANOVA, followed by Dunnett multiple comparisons test. The values of $P < 0.05$ were considered significant.

RESULTS

Comparison of Placental and Tumor Samples

We investigated cyclin E expression in placental samples and compared it with the ovarian carcinoma. Figure 1 shows that placental samples and ovarian carcinoma show seven distinct LMW protein variants, which migrate between 50 and 36 kDa. Cyclin E:L (L; 50 kDa) was strongly expressed in placental samples (lanes 1–4) and ovarian cancer (OvCa; lane 6). However, expression of cyclin E:E (cE; 45 kDa) was higher in ovarian cancer when compared with placental tissues. In placental samples, the cyclin E:T/S (T/S; 43 kDa) was highest in lane 2 and lowest in lane 1. The sample in lane 1 also showed the lowest and that in lane 2 the highest expression of the other four placental cyclin E LMW proteins, named cyclin E:U (U; \sim 39 kDa), cyclin E:V (V; \sim 38 kDa), cyclin E:X (X; \sim 37 kDa), and cyclin E:Y (Y; \sim 36 kDa). Expression of cyclin E:V in lanes 2 and 4 was even higher than that in ovarian cancer. Note low cyclin E expression in normal ovary (nOv, lane 7).

Placental samples, ovarian carcinoma, and normal ovary also showed several high molecular weight bands, which

were not detected in control staining (lane 5), possibly covalent complexes (c) of cyclin E with functionally related proteins. Two of them were shared among all samples, a weaker band at \sim 100 kDa (named cE:c100), and a stronger band at \sim 80 kDa (named cE:c80). Ovarian cancer also showed a distinct band at \sim 70 kDa (cE:c70).

Relationship of Cyclin E Expression to the Development of Placental Senescence

Variability of cyclin E expression among placentas raised a question of whether it is not somehow related to the development of placental senescence. We investigated two parameters: 1) expression of Thy-1 differentiation protein in villous core (high in normal mature terminal villi) and 2) extent of dilatation of villous blood sinusoids (low in normal mature terminal villi) [26]. Evaluation of these two parameters allowed the distinction of four placental types. Normal mature placentas (placental type 1; Fig. 2, a–c) showed strong Thy-1 in the core of terminal villi (net OD 68.5 ± 0.7 SEM) and small lumen of blood sinusoids (15.4 ± 0.4 μ m in diameter and area 121.7 ± 4.7 μ m²). Early development of placental senescence (placental type 2; Fig. 2, e and f) was characterized by significant diminution of Thy-1 staining (net OD 46.7 ± 0.9 ; $P < 0.001$) and moderate but significant ($P < 0.001$) dilatation of villous sinusoids (diameter 20.7 ± 0.6 μ m and area 225.9 ± 12.4 μ m²). Placentas showing further diminution of Thy-1 expression (net OD 31.6 ± 0.8 ; $P < 0.001$) and excessive dilatation of villous sinusoids (diameter 33.2 ± 1.0 μ m and area 556.3 ± 31.7 μ m²; $P < 0.001$) were classified as type 3 (Fig. 2, h and i). Finally, in placental type 4 (Fig. 2, k and l), the Thy-1 expression in terminal villi was very low (net OD 20.3 ± 0.8), but this was not accompanied by dilatation of villous sinusoids (diameter 15.4 ± 0.5 μ m and area 135.5 ± 8.5 μ m²). Quantitative evaluation is summarized in Figure 2, m–o. As anticipated, some placentas showed a mixture of villous types, suggesting transition between placental types 1 and 2 (Fig. 2d), types 2 and 3 (Fig. 2g), and types 3 and 4 (Fig. 2j). The right inset Figure 2g shows control staining.

Left inserts in Figure 2 show that low cyclin E expression accompanied normal placentas (a–c). Placental sample showing a transition to type 2 exhibited an increase of cyclin E expression (insert in Fig. 2d), which also accompanied genuine type 2 placentas (inserts in Fig. 2, e and f). The open arrow in Figure 2d indicates particularly high expression of cyclin E:T/S during type 1 to 2 transition, and bilateral arrows indicate the characteristic increase of other cyclin E LMW variants in placental type 2 (see also PT2, Fig. 3).

Transition to placental types 3 and 4 and genuine types 3 and 4 showed higher expression of some cyclin E LMW proteins when compared with type 1 (inserts in Fig. 2, g–l vs. a–c) but lower expression when compared with type 2 placentas (inserts in Fig. 2, g–l vs. e and f). From the total 27 placentas, 7 were classified as type 1, 4 in transition to type 2, 4 of genuine type 2, 2 in transition to type 3, 6 of genuine type 3, 2 in transition to type 4, and 2 of genuine type 4.

Type 1 placentas were found in younger women (mean age 19.8 ± 0.7 SEM) with either uncomplicated or complicated pregnancies (hypertension, drug abuse with limited prenatal care). Type 2 placentas were found in women with mean age 25.3 ± 1.5 with normal or abnormal pregnancies (gestational diabetes, anemia, drug abuse, heart diseases).

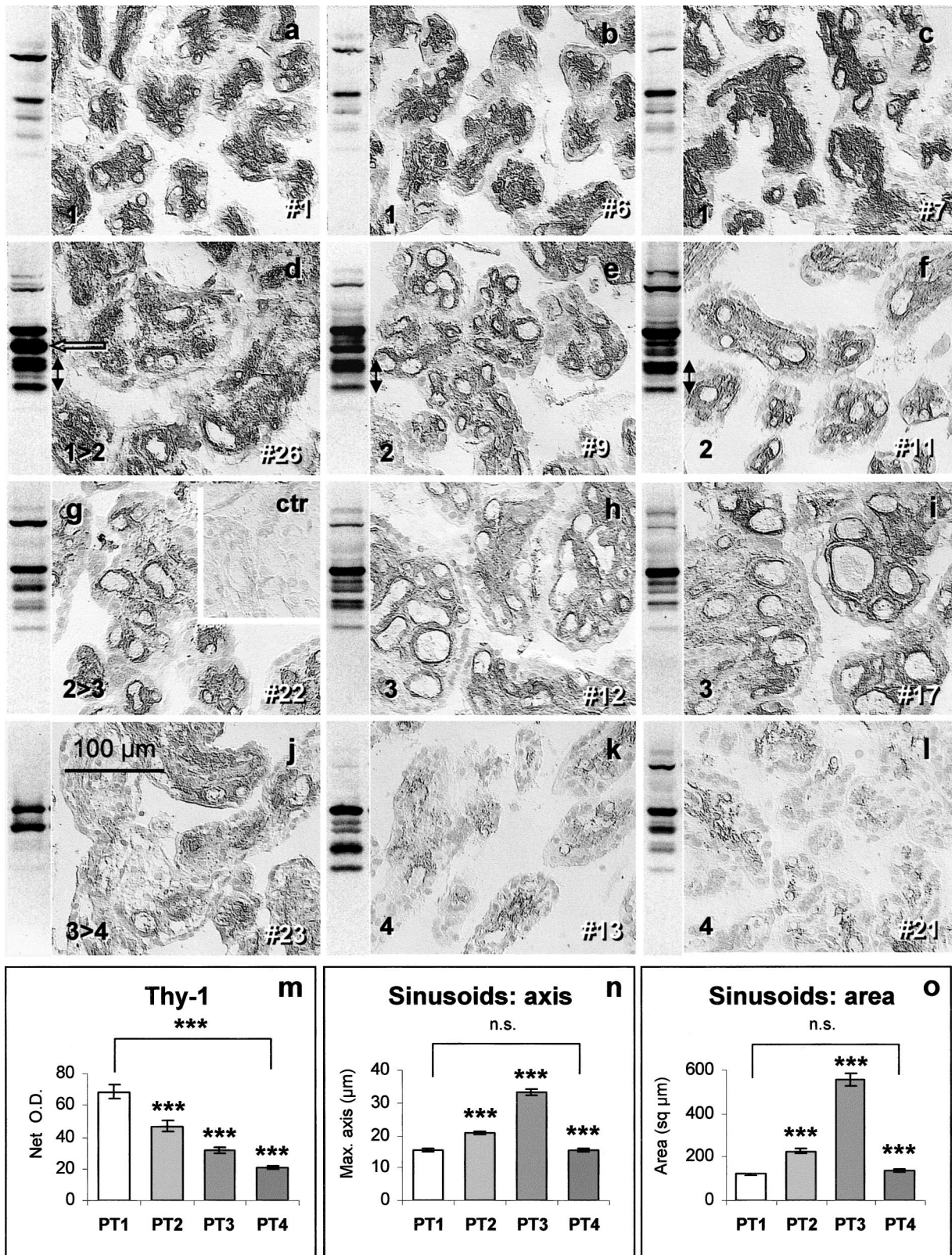


FIG. 2. A comparison of cyclin E expression with expression of Thy-1 differentiation protein (Thy-1) in villous core and dilatation of blood sinusoids. Slides with cryostat sections were fixed in acetone and stained for Thy-1 as described in *Materials and Methods*. Low cyclin E expression (inserts) is associated with type 1 placentas (1, a–c; note normal blood sinusoids and high Thy-1 expression in villous core). Transition to type 2 (1 > 2, d) and genuine type 2 placentas (2, e and f; note moderate dilatation of sinusoids and diminution of Thy-1 staining) show a marked increase of cyclin E:T/S in d (open arrow) and other cyclin E LMW variants (bilateral arrows). Transition to type 3 (2 > 3; g) and genuine type 3 placentas (3, h and i; note extreme dilatation of sinusoids and low Thy-1 in villous core) show higher cyclin E expression when compared with type 1 placentas but lower when compared with type 2. Transition to type 4 (j) and genuine type 4 placentas (4, k and l; note rare dilatation of sinusoids accompanied by weak or no Thy-1 expression in villous core) also show higher cyclin E expression when compared with type 1 but lower when compared with type 2. **m**) Quantitative evaluation of Thy1 staining (net optical density) in placental types (n = 120 measurements for each column). One-way ANOVA $P < 0.0001$; $***P < 0.001$, Tukey-Kramer multiple comparisons test between adjacent columns or as indicated. **n**) Demonstrates maximum axis (in μm), and **o**) area (in μm^2) of blood sinusoids (n = 120 measurements for each column). Nonparametric ANOVA, $P < 0.0001$; $***P < 0.001$; n.s., not significant, Dunn multiple comparisons test between adjacent columns, or as indicated; #, case number; right inset in g (ctr), control staining.

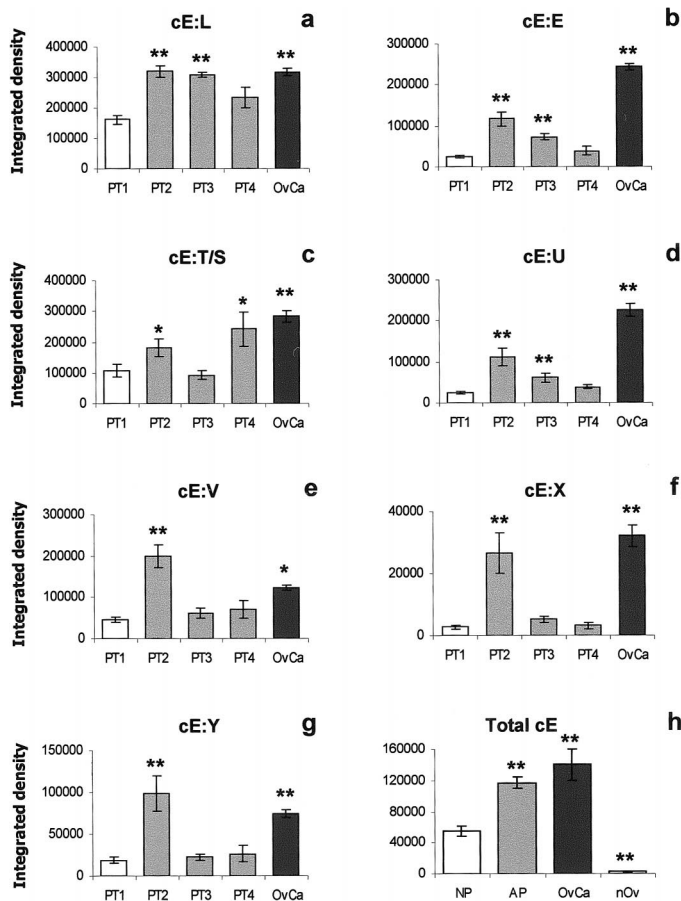


FIG. 3. Quantitative evaluation of cyclin E protein expression in placental and ovarian carcinoma samples. Blots were scanned and individual bands evaluated for integrated density (net optical density \times area) and statistically evaluated as indicated in *Materials and Methods*. Measurements for placental type 1 (PT1), $n = 20$; PT2, $n = 16$; PT3, $n = 16$; PT4, $n = 8$; ovarian carcinoma (OvCa), $n = 4$. Normal placenta (NP), $n = 140$, and abnormal placenta ([AN] PT2 + PT3 + PT4), $n = 252$ measurements; OvCa, $n = 28$, and normal ovary [nOv], $n = 42$ values in **h**. One-way ANOVA $P < 0.0001$ for transformed data ($Y = \log[Y]$) in all panels. * $P < 0.05$; ** $P < 0.01$ vs. first column, Dunnett multiple comparisons test. Details in text.

Type 3 placentas were associated with mean maternal age 26.3 ± 1.8 and abnormal pregnancies (diabetes mellitus on insulin, heart diseases, hypothyroidism, or preeclampsia). Placentas in transition to type 4 and genuine type 4 placentas were associated with more advanced maternal age (mean 36.0 ± 2.8). This group of women showed significantly higher maternal age (overall ANOVA: $P = 0.0014$; Tukey-Kramer multiple comparison test: $P < 0.01$ vs. women with PT1, PT2, and PT3).

One of two placentas classified as genuine type 4 (case 13, age 41 yr, gestational diabetes on insulin, cesarean section at $33 + 2$ wk of pregnancy) was a small placenta (380 g), associated with intrauterine growth retardation (δ 1250 g, Apgars 4 + 7) and reduced amniotic fluid volume. This placenta showed severe senescence, characterized by dominance of aged villi. The placenta from the other case (case 21, maternal age 35 yr, type II diabetes on insulin, repeated cesarean section at $37 + 2$ wk of pregnancy) was hypertrophic (820 g), associated with fetal macrosomia (δ 4900 g, Apgars 8 + 9). The placenta showed transformation of immature villi into the terminal villi persisting in an immature

state (low Thy-1 expression without fibrinoid degeneration).

Quantitative Evaluation of Cyclin E Expression

For statistical purposes, the placentas in transition from lower to the higher placental type were evaluated along with those showing higher placental type. Figure 3, a–g, shows quantitative evaluation of cyclin E LMW variants in placental types (PT1–PT4) and their comparison with the ovarian cancer (OvCa). When compared with normal placentas (PT1), the type 2 placental samples (and ovarian cancer) showed significantly higher expression of all cyclin E LMW variants. Type 3 placentas showed significantly higher expression of cyclin E:L, cyclin E:E, and cyclin E:U. Type 4 placentas showed significantly higher expression of cyclin E:T/S only.

To determine if placental abnormality is associated with higher cyclin E expression in general, all measurements of cyclin E LMW variants from abnormal placentas (types 2–4) were coupled and compared with measurements from placental type 1. Figure 3h shows that total cyclin E expression is significantly higher ($P < 0.01$) in abnormal placentas (APs) when compared with normal placentas (NPs). The total cyclin E expression in ovarian cancer was also significantly higher when compared with NPs ($P < 0.01$) but similar when compared with APs ($P > 0.05$). Total cyclin E expression in normal ovaries (six samples investigated) was significantly lower when compared with NPs ($P < 0.01$).

DISCUSSION

The determination of cyclin E LMW protein expression in placental samples may represent a novel aspect in discrimination between the placental physiology and pathology. Yet the meaning of differences in expression of cyclin E LMW variants among placental samples remains unknown. We speculate that the lower expression of cyclin E LMW protein isoforms represents a normal stable condition, where the placenta still has some reserve in its capacity to fulfill the demands of the progressively growing fetus. However, with pregnancy advancement and from the 34th to 36th wk in particular, the fetal demands still grow but the morpho-functional capacity of the placenta remains unchanged or even declines, and this is often associated with the development of placental senescence [17, 19–21, 29, 30].

Placental type 2 showed the highest expression of all cyclin E LMW variants. This may be interpreted as an effort of the placental structures to regenerate and increase the surface of the remaining mature terminal villi since high cyclin E expression is characteristic for early stages of differentiation in trophoblast cultures [15]. Hence, the type 2 placentas can be considered as capable of responding and fulfilling demands of the growing fetus by villous regeneration.

Relatively high expression of cE:X in type 2 placentas and ovarian cancer is of particular importance. The cE:X seems to be specific for activated mesenchymal cells, and its expression is enhanced during the early stages of trophoblast differentiation in vitro, as compared with the cE:X expression in corresponding placentas in vivo (unpublished observations). Early trophoblast cultures show marked activation of concomitant mesenchymal cells [31], and mesenchymal cells appear to participate in the regulation of differentiation of tissue-specific cells [32–39] and

in tumor progression [40–44]. Hence, an enhanced expression of cE:X in placental type 2 might reflect a contribution of mesenchymal cells to the stimulation of placental regeneration.

When compared with placental type 2, type 3 placentas showed an enhanced cyclin E expression of some LMW variants only. This correlated with the maximal dilatation of villous sinusoids and suggests that trophoblast regeneration is somehow inhibited. Hence, the excessive compensatory dilatation of sinusoids appears to represent an ultimate adaptive mechanism to fetal hypoxia, as suggested by others [25].

Type 4 placentas showed lower expression of cyclin E proteins when compared with types 2 and 3 but significantly higher cyclin E:T/S variant when compared with type 1 placentas. Because both cyclin E:S and cyclin E:T serve different functions when compared with the cyclin E isoforms stimulating progression of the cell cycle [11], our observations concur with those indicating that cyclin E upregulation (overexpression of cyclins E:S and E:T alone) also accompanies advanced cellular aging [2–7].

When compared with type 2 and 3 placentas, the type 4 placentas showed a lack of dilatation of villous sinusoids. This correlated with depletion of Thy-1 expression by vascular pericytes, i.e., cells communicating with endothelial cells and contributing to the dilatation of vascular lumen in response to hypoxia [45, 46]. This suggests that a lack of Thy-1⁺ (mature) pericytes might cause a failure of the placenta to compensate developing fetal hypoxia. As indicated in *Results*, a lack of differentiated pericytes may result either from accelerated villous aging in atrophic placentas or a lack of villous maturation (persisting immaturity) characteristic for the hypertrophic placentas of diabetic mothers [1]. In either case, however, the consequences for the fetus are similar—the development of fetal hypoxia, which is not compensated by placental adaptation (dilatation of villous sinusoids).

Enhanced cyclin E expression in vivo may reflect an existence of chronic fetal hypoxia and an attempt of the placenta to correct it. Even in the absence of normal villous regeneration, the aging placenta has a capacity to improve its function, e.g., by elongation and excessive dilatation of villous sinusoids [25, 47]. This may provide a larger surface for the maternal-fetal interface. This may help the fetus survive unaffected by an underlying disease process. However, although chronic adaptations may be successful for fetal survival, they may lead to adverse outcomes later in the life of an individual [21].

Our observations on Thy-1 depletion in villous core indicate that, in addition to pregnancy abnormalities, advanced maternal age plays an important role in cellular and molecular pathology of the placenta. Several studies have shown that advanced maternal age (>35 yr) is by itself a high risk factor [48–51]. Hence, even in otherwise uncomplicated pregnancies, advanced maternal age might affect the quality of the fetal outcome or quality of life of an individual.

Reactivity of HE12 antibody with high molecular weight bands (above the cyclin E:L) can also be detected, particularly in tumor cell extracts [12], yet the nature of these proteins is unknown. These bands are not detected by HE12 antibody in cdk2 immunoprecipitates [12]. This indicates that these high molecular weight bands, which appear to survive the preparation of protein lysates for Western blot technique, do not include cyclin E/cdk2 complexes. Hence, the high molecular weight cyclin E bands might represent

covalently bound cyclin E bi- or trimeric complexes with other functionally related proteins, such as proteins of the E2F transcription factor family, p18^{INK4c}, p21^{WAF1/Cip1}, and p27^{Kip1} (p27) [52–56]. Our recent observations indicate that the cE:c70 band in ovarian carcinomas in vivo and in MCF-7 breast carcinoma cells in vitro coincides with a similar band in p27 immunoblots (unpublished data). Hence, the cE:c70 might represent a complex of p27 with 43 kDa cE:T or cE:S.

In conclusion, because enhanced expression of cyclin E accompanies cell proliferation and ongoing cellular differentiation and aging, the low cyclin E expression in normal placentas suggests a steady state of the placenta, i.e., its ability to fulfill demands of the growing fetus. Overexpression of all cyclin E LMW proteins may indicate an activation of the adaptive mechanism (cellular proliferation and differentiation) that compensates for increasing fetal demands. However, an enhanced expression of fewer cyclin E LMW proteins might reflect an association of certain cyclin E isoforms with placental aging or an inefficient placental adaptation.

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