Rat endometrial Vdup1 expression: changes related to sensitization for the decidual cell reaction and hormonal control

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Abstract

During implantation in rodents, attachment and invasion of embryonic trophoblast is accompanied by decidualization of the adjacent endometrial stroma. Decidualization can be initiated only when the endometrium is receptive, and this occurs for a short period in pregnancy. The molecular mechanisms underlying this phenomenon remain unclear. In the current study, using differential display and northern blot analysis, we found that steady-state levels of mRNA for vitamin D3 upregulated protein 1 (Vdup1) were significantly higher in ‘refractory’ and ‘delayed’ endometrium compared with ‘receptive’ endometrium or endometrium undergoing artificially induced decidualization. Conversely, thioredoxin (Txn), a ubiquitously expressed cellular redox regulator known to promote growth and proliferation, was found to have elevated transcript levels within the decidua- lizing endometrium. VDUP1 has previously been shown to bind TXN and inhibit its action. In an inverse, but cooperative, relationship, these molecules have been implicated in regulating cell growth and proliferation in a number of tissues and during transformation to cancer. The Vdup1 mRNA is localized to the uterine stroma in the nonreceptive endometrium, the site of increased Txn mRNA levels during decidualization. In addition, Vdup1 mRNA levels are inversely regulated by progesterone and estrogen; prolonged progesterone exposure stimulates an increase in Vdup1 mRNA levels whereas estrogen decreases Vdup1 transcript levels. Together, these results suggest a novel mechanism by which suppression of the decidual response in the nonreceptive endometrium may occur.


Introduction

Successful implantation depends upon the synchronized maturation of the embryo and the endometrium; the development of the embryo to the expanded blastocyst stage must coincide with hormone-dependent changes within the endometrium that render it receptive to implantation (Psychoyos 1973). Asynchrony between these events can delay or prevent implantation, as the uterus is receptive for only a short period in pregnancy or pseudopregnancy or when the uterus has been prepared with a specific regimen of hormones (Psychoyos 1973, Kennedy & Ross 1997). Interestingly, the nonreceptive uterus is one of the few tissues into which the embryo will not normally implant (Fawcett 1950, Kirby 1963, 1965). While the hormonal requirements for the onset of uterine receptivity in the rodent, as well as those underlying the phenomenon of delayed implantation, have been known in detail for over 30 years (Yochim & De Feo 1963, Psychoyos 1973), a complete picture of the molecular mechanisms orchestrating the transition from a nonreceptive to a receptive endometrium, and vice versa, remains elusive.

Through the use of mRNA differential display (ddRT-PCR), we exploited both the tight hormonal control for the onset of uterine receptivity and an established model for the induction of a sensitized endometrium in ovariectomized, hormone-treated rats (De Feo 1963, Psychoyos 1973, Kennedy & Ross 1997), and identified vitamin D3 upregulated protein 1 (Vdup1) as a gene upregulated in the nonsensitized rat endometrium. This was the case for both the refractory endometrium (the endometrial state immediately following receptivity) and during a model of delayed implantation, the prereceptive state that supports the survival of an embryo in diapause until sensitization is achieved. VDUP1 has been shown to interact with thioredoxin (TXN) by binding to its active site and inhibiting its biologic action...
(Nishiyama et al. 1999, Junn et al. 2000, Yamanaka et al. 2000). TXN has been implicated in numerous cellular processes, including the promotion of cell proliferation, the inhibition of apoptosis and the regulation of transcription factor binding and antioxidant activity (reviewed in Nishiyama et al. 2001). In addition, TXN is highly expressed in the uterus of mice (Osborne et al. 2001), rats (Sahlin et al. 1997) and humans (Perkins et al. 1995), as well as in the highly invasive cytrophoblast cells of the human placenta (Perkins et al. 1995). We show that Txn mRNA levels increase within the decidualizing endometrium, in contrast to Vdup1. The spatial and temporal expression for Vdup1 and Txn mRNA presented in the current study, and the opposing actions of these molecules reported in the literature, suggest that these molecules may interact within the endometrial stroma to modulate the potential for the decidual response in the rat.

Materials and Methods

Animals and tissue preparation

Female Sprague-Dawley rats (200–225 g body mass: Harlan Sprague-Dawley, Indianapolis, IN, USA) were housed in temperature- and light-controlled conditions (14 h light, 10 h dark with lights on from 0500 h to 1900 h) with free access to food and water. Animals were ovariectomized under ether anesthesia (BDH, Toronto, ON, Canada). To obtain rats with uteri differentially sensitized for the decidual cell reaction, estradiol (E2) and progesterone (P4) (Sigma) were administered subcutaneously (s.c.) in sesame oil as previously described (Kennedy & Ross 1997). Some rats received a bilateral injection of 100 μl sesame oil into the uterine lumen on the equivalent of day 5 of pseudopregnancy to induce decidualization (Finn & Keen 1963). Rats receiving intraluminal injections of sesame oil on day 5 and their controls continued to receive daily s.c. injections of E2 and P4 in sesame oil until they were killed (Kennedy & Ross 1997). Uteri were collected on the morning of each day of the equivalent of days 4–10 of pregnancy to induce decidualization (Finn & Keen 1963). Uteri were prehybridized in Church buffer (7% (w/v) SDS, 0.25 mol Na2HPO4/l (pH 7.2), 1 mmol EDTA/l and 1% (w/v) BSA) at 65°C for at least 30 min. The cDNA amplificons isolated from the ddRT-PCR gel was used as a template to synthesize 32P-labeled DNA probes for northern blot hybridization. An amount of 25 ng of cDNA was labeled by the random-priming technique in the presence of [α-32P]dCTP (Amersham) with an oligo-labeling kit (Random Primers Labeling System; Gibco-BRL). Hybridizations were carried out at 60°C for 20 h. The membranes were subsequently washed three times (15 min each) at 65°C in 20 mmol Na2HPO4/l (pH 7.2), with 4% (w/v) SDS and subjected to autoradiography at −70°C with a Biomax MS TranScreen-HE intensifying screen (Eastman Kodak) for the appropriate exposure time. Blots were then stripped in 1 mmol Tris/l, 1 mmol EDTA/l and 0.1 x Denhardt's reagent (1 x Denhardt's: 2% (w/v) BSA, 2% (w/v) Ficoll and 2% (w/v) polyvinylpyrrolidone, pH 8.0) for 2 h at 75°C, and sequentially reprobed with radiolabeled cDNAs for Txn, thioredoxin reductase 1 (Txnrd1) and 18S rRNA. The autoradiographs

northern blot analysis

An amount of 10 μg of total RNA was denatured and subjected to electrophoresis in a denaturing gel as previously described (Sambrook et al. 1989). RNA was then transferred to Hybond-N membrane (Amersham) by capillary transfer and cross-linked by exposure to 1.2 × 105 μJ/cm2 of UV energy (Hoefer Pharmacia Bio-tech, San Francisco, CA, USA). northern blot analysis was performed as described by Church and Gilbert (1984), with some modifications. Briefly, membranes were prehybridized in Church buffer (7% (w/v) SDS, 0.25 mol Na2HPO4/l (pH 7.2), 1 mmol EDTA/l and 1% (w/v) BSA) at 65°C for at least 30 min. The cDNA amplificon isolated from the ddRT-PCR gel was used as a template to synthesize 32P-labeled DNA probes for northern blot hybridization. An amount of 25 ng of cDNA was labeled by the random-priming technique in the presence of [α-32P]dCTP (Amersham) with an oligo-labeling kit (Random Primers Labeling System; Gibco-BRL). Hybridizations were carried out at 60°C for 20 h. The membranes were subsequently washed three times (15 min each) at 65°C in 20 mmol Na2HPO4/l (pH 7.2), with 4% (w/v) SDS and subjected to autoradiography at −70°C with a Biomax MS TranScreen-HE intensifying screen (Eastman Kodak) for the appropriate exposure time. Blots were then stripped in 1 mmol Tris/l, 1 mmol EDTA/l and 0.1 x Denhardt's reagent (1 x Denhardt's: 2% (w/v) BSA, 2% (w/v) Ficoll and 2% (w/v) polyvinylpyrrolidone, pH 8.0) for 2 h at 75°C, and sequentially reprobed with radiolabeled cDNAs for Txn, thioredoxin reductase 1 (Txnrd1) and 18S rRNA. The autoradiographs
were digitized using a Hewlett Packard Scanjet 4c/T scanner, and the densitometric intensity of each signal was quantified with Pharmacia ImageMaster VDS Video Documentation System Software, Version 2.0. The 18S rRNA signal was used to determine the relative amounts of RNA loaded into each well and transferred to the membrane and to normalize Vdup1, Txn and Txa1d1 signals (Smith & Hammond 1991).

**In situ hybridization**

At the time total RNA was isolated for the ddRT-PCR and northern blot analyses, some uterine horns were fixed by immersion in 4% (w/v) paraformaldehyde for 24 h. The horns were then rinsed in two changes of PBS and stored in 70% (v/v) ethanol until embedded in paraffin and sectioned at 6 μm.

The vector, containing an amplicon cloned from the differential display experiments, was linearized and cRNA sense and antisense probes were synthesized in the presence of digoxigenin (DIG)-labeled rUTP (Roche Molecular Biochemicals, Laval, QC, Canada) according to the manufacturer's instructions. The in situ hybridization technique used has been described in detail previously (Harvey et al. 1995). Briefly, uterine cross-sections were dewaxed in xylene, rehydrated through an alcohol series to 2×SSC, digested in proteinase K at 37°C for 5–7.5 min, and acetylated by incubation with freshly prepared 0.1 mol triethanolamine/l and 0.56% (v/v) acetic anhydride at room temperature. After prehybridization at 50°C for 4 h in a 50% (v/v) formamide, 5×SSPE, 1× Denhardt’s solution, the sections were hybridized with 100 ng of cRNA probe and 400 ng of RNA (in prehybridization solution) under a cover slip for 20 h at 50°C in a sealed humidified chamber. The sections were then washed extensively, treated with RNase and processed for immunologic detection of the DIG-labeled cRNA probe using anti-DIG antibodies at a dilution of 1:500 (Roche Molecular Biochemicals). Color development was allowed to continue until a signal (blue-purple precipitate) was detected. The color reaction for the sense cRNA-treated slides was terminated at the same time as for the antisense slides. Sections were then mounted using GVA-mount (Zymed Laboratories, San Francisco, CA, USA).

**Statistical analysis**

Experiments quantifying changes in mRNA levels by northern blot analysis were performed three times on separate groups of ovariectomized, hormone-treated rats (except when stated otherwise). The data were analyzed by within-blocks ANOVA, with experiments being considered blocks. Duncan’s multiple range test was performed to determine differences between groups. \( P < 0.05 \) was considered significant.

**Results**

**Identification of Vdup1 as a gene differentially expressed during the peri-implantation period**

Vdup1 was isolated from a differential display screen aimed at identifying changes in gene expression associated with the onset and subsequent disappearance of uterine sensitization for the decidual cell reaction. Specifically, total RNA (pooled from groups of 7 animals) at the equivalent of days 4, 5 and 6 of pseudopregnancy, as well as RNA from two groups of day 5 animals receiving inappropriate doses of E2 on the evening of day 4 (day 5 ‘high E2’ and day 5 ‘low E2’), were compared. Vdup1 was identified as a gene upregulated within the day 6, ‘refractory’ endometrium (Fig. 1). northern blot analysis confirmed the expression pattern seen by ddRT-PCR, as Vdup1 mRNA levels were significantly higher within the ‘refractory’ day 6 endometrium than within the ‘sensitized’ day 5 and ‘neutral’ day 4 endometrium (Fig. 2, \( P < 0.05 \)). Significantly higher levels of Vdup1 mRNA were also observed in the endometrium of animals in the day 5, low E2 group (low E2 group, Fig. 2, \( P < 0.05 \)). However, rats temporally correct for sensitization (equivalent of day 5 of pseudopregnancy) but with nonsensitized endometrium, as a consequence of a high dose of E2 on the evening of day 4, had no significant increase in Vdup1 mRNA levels (high E2 group, Fig. 2).

**Figure 1** mRNA differential display gel showing the cDNA profile for endometrial RNA for the primer combination T11MC (degenerate 3’ primer) and GATCTGACAC (5’ primer). The total RNA being compared (pooled from the uteri of seven animals for each group) includes endometrial RNA from the equivalent of days 4, 5 or 6 of pseudopregnancy and two endometrial RNA samples from day 5 rats that received inappropriate doses of E2 on the evening of day 4 (low and high E2). PCR reactions were run in triplicate, and the black arrow indicates the band representing Vdup1.
Northern blot analysis of Vdup1 and Txn mRNA expression during pseudopregnancy and oil-induced decidualization

Further characterization of Vdup1 mRNA expression from days 6–10 of pseudopregnancy, in the presence ('S') or absence ('N') of artificially induced decidualization, indicated a sustained increase in Vdup1 transcript levels within the refractory, nondecidualizing endometrium, whereas transcript levels remained low within the decidualizing endometrium (Fig. 3, P < 0.05). In contrast, mRNA levels for Txn increased during decidualization but remained low within the refractory endometrium (Fig. 3, P < 0.05).

Localization of Vdup1 and Txn mRNA expression within the refractory and decidualizing uterus by in situ hybridization

In order to elucidate a possible site of action for Vdup1, we localized the spatial expression pattern of Vdup1 mRNA within the day 7 pseudopregnant, 'nonstimulated' and 'stimulated' (decidualizing) endometrium by in situ hybridization. Vdup1 mRNA was highly expressed within the stromal compartment of nondecidualizing endometrium (Fig. 4A). However, by the equivalent of day 7 of pseudopregnancy, 48 h following the application of a decidualogenic stimulus, Vdup1 mRNA expression was detected in the stomal cells only on the periphery of the endometrium, while expression was absent within the decidualizing stroma immediately surrounding the uterine lumen (Fig. 4E). In contrast, Txn mRNA was expressed at low levels within the stroma and luminal epithelium of 'refractory' day 8 pseudopregnant endometrium (with slightly higher levels within the glandular epithelium – Fig. 4C) but was highly elevated within the decidualizing stroma on day 8 of pseudopregnancy (Fig. 4F). No signals were obtained with the sense probes (Fig. 4B and D).
**Hormonal control of Vdup1 and Txn mRNA expression in the pseudopregnant uterus**

A model of delayed implantation was used to explore further the regulation of Vdup1 mRNA expression by the ovarian steroids. Ovariectomized rats were given 96 h of P4 treatment (after 48 h of P4 priming, further P4 treatment in the absence of E2 results in a ‘delayed’ endometrium that will become sensitized only after E2 exposure) followed by either more P4 (‘delayed’) or P4 plus E2 (‘sensitized’). High levels of Vdup1 mRNA were evident within the uteri of delayed animals, whereas much lower levels of Vdup1 mRNA were seen in the ‘sensitized’ animals following the termination of delay (Fig. 5A and B).

The effects of the ovarian steroid hormones E2 and P4 on endometrial Vdup1, Txn and Txnrd1 mRNA levels were investigated in ovariectomized rats. Animals injected

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**Figure 4** *In situ* localization of Vdup1 and Txn mRNA within the pseudopregnant rat uterus. Paraffin-embedded uteri were cross-sectioned and analyzed by DIG *in situ* hybridization for Vdup1 and Txn mRNA signals. (A) × 10 magnification, antisense Vdup1 probe hybridized to ‘nonstimulated’ day 7 pseudopregnant uteri. (B) × 10 magnification, sense Vdup1 probe hybridized to ‘nonstimulated’ day 7 pseudopregnant uteri. (C) × 10 magnification, antisense Txn probe hybridized to ‘nonstimulated’ day 8 pseudopregnant uteri. (D) × 10 magnification, sense Txn probe hybridized to ‘nonstimulated’ day 8 pseudopregnant uteri. (E) × 10 magnification, antisense Vdup1 probe hybridized to ‘stimulated’ day 7 pseudopregnant uteri. (F) × 10 magnification, antisense Txn probe hybridized to ‘stimulated’ day 8 pseudopregnant uteri. LE = luminal epithelium, G = glandular epithelium, S = stroma and M = myometrium. Lower magnification panels are taken at × 4 magnification and black bar = 100 μm for all panels.

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**Figure 5** Hormonal control of Vdup1 and Txn mRNA levels within the rat uterus *in vivo* as determined by northern blot analysis. (A) Animals were ovariectomized and given 4 mg P4 daily for 4 days to induce a state of ‘delayed implantation’, and they then received 4 mg P4 plus 0.3 μg E2 to induce sensitization, or 4 mg P4 alone to maintain the ‘delay’. Animals were killed 18 h later, and total RNA from whole uterus was isolated. There were three animals in each group. In the northern blot analysis, 10 μg of RNA was used. 32P-labeled cDNA probes for Vdup1 and 18S rRNA were used as previously described. (B) Intensity of the signals was normalized with the signals for 18S rRNA (Vdup1/18S rRNA), and the averages of the two replicates are depicted in the bar graph. The values for the control groups were arbitrarily set at 1. (C) Animals were ovariectomized and given a single dose of either 1 μg E2, 4 mg P4, 1 μg E2 plus 4 mg P4, or vehicle (sesame oil). Uteri were collected 18 h later, and total RNA from whole uterus was isolated. There were three animals in each group. In the northern blot analysis, 10 μg of RNA was used. 32P-labeled cDNA probes for Vdup1, Txn and 18S rRNA were used for hybridization. (D) Intensity of the signals was normalized with the signals for 18S rRNA (Vdup1/18S rRNA), and the averages of the two replicates are depicted in the bar graph. The values for the control groups were arbitrarily set at 1.
with a single dose of E2 showed a significant decrease in Vdup1 mRNA levels while Txn mRNA levels were not significantly altered (Fig. 5C and D). P4 alone or P4 plus E2 in combination did not significantly alter expression of either Vdup1 or Txn mRNA levels over that of control, although it eliminated the E2-induced decrease in Vdup1 mRNA levels (Fig. 5C and D). Txnrd1 mRNA levels were significantly increased by treatment with E2 alone.

Discussion

Using differential display to uncover changes in gene expression associated with the onset and subsequent disappearance of uterine sensitization for the decidual cell reaction, the current study has identified Vdup1 as a gene upregulated within the ‘refractory’ endometrium (Fig. 1). Subsequent northern blot analysis confirmed the increase in endometrial Vdup1 mRNA levels following uterine sensitization (Fig. 2) and also indicated that this increase is sustained as long as the endometrium remains in a ‘refractory’ state (Fig. 3). In contrast, endometrial Vdup1 mRNA levels are not elevated following sensitization if decidualization has been initiated (Fig. 3). In situ hybridization experiments localized the site of Vdup1 mRNA expression to the stromal cells of the endometrium (Fig. 4). Together, these data suggest a possible role for VDUP1 in maintaining stromal cells of the endometrium in a refractory state.

While some studies have shown that an increase in Vdup1 expression is associated with the onset of various cancers, including breast (Butler et al. 2002, Goldberg et al. 2002), colon (Butler et al. 2003), and B-cell lymphoma (de Vos et al. 2003). In addition, some studies have shown that an increase in VDUP1 protects against cancer and inhibits tumor cell growth (Butler et al. 2002, Goldberg et al. 2003, Han et al. 2003).

VDUP1 has also been implicated as a binding protein for the redox regulator TXN, binding to its active site and inhibiting its biologic activity (Nishiyama et al. 2000). TXN has been shown to have a wide range of biologic activities, including growth promotion and proliferation, antioxidant activity, regulation of transcription factor binding and inhibition of apoptosis (reviewed in Nishiyama et al. 2001). It is possible that the TXN-mediated effects of VDUP1 are mediated through TXN inhibition. In fact, Schulze et al. (2002) recently described a system in which VDUP1 and TXN have opposing effects on human aortic smooth muscle cell proliferation. Specifically, overexpression of VDUP1 inhibited cell proliferation, in contrast to TXN-stimulated cell proliferation, as well as prevented nuclear translocation of TXN in response to platelet-derived growth factor, a potent stimulator of aortic smooth muscle cell proliferation and TXN activity (Schulze et al. 2002).

Because VDUP1 has been shown to regulate cell proliferation and growth negatively through inhibition of TXN activity, and because TXN has been shown to be highly expressed in the uterus of mice (Osborne et al. 2001) and humans (Perkins et al. 1995), as well as in the highly invasive cytotrophoblast cells of the human placenta (Perkins et al. 1995), we investigated whether Txn mRNA levels would have an opposite expression pattern to that of Vdup1 in the rat endometrium. Indeed, Txn mRNA levels increased within the decidualizing endometrium but remained low within the nondecidualizing, refractory endometrium (Fig. 3). While Vdup1 and Txn mRNA levels within the endometrium appear to have opposing expression patterns, they are both induced in the endometrial stroma (Fig. 4). This converse temporal expression but shared spatial expression suggests that they may have linked, but opposing roles in stromal cell proliferation within the uterus, with TXN stimulating the proliferation of decidual cells and VDUP1 inhibiting cell proliferation in the refractory or nonsensitized endometrial stromal compartment.

In addition to verifying the results obtained by ddRT-PCR, northern blot analysis also revealed a statistically significant increase in Vdup1 mRNA levels within the endometrium of animals in the day 5, low E2 group (low E2 group, Fig. 2). These animals, although temporally correct for sensitization (equivalent of day 5 of pseudopregnancy), were hormonally nonsensitized, as they did not receive E2 on the evening of day 4 but only an injection of P4. By contrast, rats with nonsensitized endometrium on day 5, as a consequence of a high dose of E2 on the evening of day 4, had low levels of Vdup1 mRNA (high E2 group, Fig. 2). These data would indicate that prolonged P4 exposure in the absence of E2, a situation that simulates lactational delay of sensitization/receptivity, results in an increase in Vdup1 mRNA levels. To investigate this observation further, we used a model of delayed implantation. Ovariectomized rats were given 96 h of P4 treatment (after 48 h of P4 priming, further P4 treatment in the absence of E2 results in a ‘delayed’ endometrium that will become sensitized only after E2 exposure) followed by either more P4 (‘delayed’) or P4 plus E2 (‘sensitized’). High levels of Vdup1 mRNA were evident within the uteri of delayed animals, whereas low levels of Vdup1 mRNA were seen in the ‘sensitized’ animals following the termination of delay (Fig. 5A and B). The fact that Vdup1 mRNA is upregulated within the endometrium during ‘delayed implantation’ further supports the hypothesis that VDUP1 may help to maintain a quiescent, nondecidualizing stromal cell population. In addition, E2 administration, which is itself a known promoter of cell growth, is sufficient significantly to downregulate Vdup1 mRNA levels within the...
Vdup1 expression in the rat endometrium


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