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Regression of the Decidualized Mesometrium and Decidual Cell Apoptosis Are Associated with a Shift in Expression of Bcl2 Family Members[1]

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ABSTRACT

The purpose of this study was to determine whether regression of the decidua basalis (DB), which begins on Day 14 of pregnancy in the rat, results from an intrinsic program of apoptosis regulated by Bax and Bcl2. Expression of Bax and Bcl2 and the incidence of apoptosis were evaluated throughout gestation by Western blot analysis and detection of DNA fragments. Antiprogestin (RU486) was also administered during proliferation of DB to study progesterone regulation of Bax/Bcl2 balance. Bax, the pro-apoptotic protein, was expressed at a low level throughout pregnancy, whereas Bcl2, the pro-survival partner, was most abundantly expressed on Days 8 and 10, which are a time of proliferation and decidualization, and declined to barely detectable levels thereafter. These changes resulted in a 12-fold increase in the Bax:Bcl2 ratio on Day 17 as compared with Day 8 of pregnancy ($P < 0.05$). DNA laddering and in situ staining of DNA fragments first became visible on Day 14 and involved 2% of cells by Days 17 and 21 ($P < 0.05$). Treatment with RU486 on Day 9 enhanced Bax and suppressed Bcl2 within 6 h, increasing the Bax:Bcl2 ratio sixfold ($P < 0.05$). Apoptosis was minimal at 6 h and increased to 9% of cells by 24 h ($P < 0.05$). Thus, progesterone appears to regulate the apoptotic threshold of stromal cells by modulating Bax and Bcl2 expression.

apoptosis, cytokines, decidua, pregnancy, progesterone, uterus

INTRODUCTION

Decidualized stromal cells of the mesometrium (decidua basalis [DB]) proliferate during early pregnancy and later undergo a cycle of regression, which begins during the last third of pregnancy and continues until parturition [1, 2]. The period of proliferation is characterized by appropriate expression of cell cycle regulators and receptors for progesterone, estrogen, and epidermal growth factor [2–6]. Although the DB regresses during later pregnancy (i.e., beginning at approximately Day 14) [7], the mechanisms controlling DB regression and the ultimate fate of the decidualized stromal cells in normal pregnancy are not known. Several studies have established that regression of the decidualized endometrium of pseudopregnancy occurs by apoptosis, and that this process is mediated by products of the *bcl2* gene family [8–10]. The present study was undertaken to determine whether these proto-oncogene products play a similar role during pregnancy and if their relative abundance is regulated by progesterone.

The Bcl2 family of proteins is represented by at least 16 homologues, some of which suppress apoptosis whereas others promote apoptosis when activated by phosphorylation [11–13]. An excess of Bax promotes cell death, but expression of Bcl2 can neutralize this effect [12, 14]. The competing actions of these homologues led Oltvai and Korsmeyer [15] to view this system as a biological ''rheostat,'' functioning to set the apoptotic threshold of the cell. The survival function of Bcl2 is explained, at least in part, by its ability to form heterodimers with its pro-apoptotic partner and, thus, to prevent cell death [11, 12, 14, 16]. The aim of this study was to determine whether DB regression during normal pregnancy resulted from an intrinsic program of cell death or apoptosis regulated by Bax and Bcl2.

We expected that the Bax/Bcl2 rheostat would provide a high threshold for apoptosis (i.e., a low Bax:Bcl2 ratio) during early pregnancy, but that a lower threshold would be observed (i.e., a higher Bax:Bcl2 ratio) during later pregnancy. To test this notion, we used Western blot analysis to determine changes in the expression of Bax and Bcl2 proteins and immunohistochemistry to determine the incidence of apoptosis. Antiprogestin (RU486) was also administered to normal pregnant rats during the proliferative stage of DB development to investigate the role of progesterone in regulation of the Bax/Bcl2 balance.

MATERIALS AND METHODS

Animals

Adult female Holtzman rats weighing 225–250 g were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and bred in the laboratory animal care facility of our institution. All animal care and use were conducted in accordance with NIH guidelines for the care and use of laboratory animals and with a protocol approved by the Medical College of Georgia Committee on Animal Use in Research and Education. Pregnancy (Day 1) was identified by the presence of vaginal sperm after overnight exposure to a fertile male. Rats were killed at 0800–0900 h between Days 8 and 21 of pregnancy (term is on Day 22) or as otherwise mentioned for specific experiments.

Antiprogestin Treatment

Normal pregnant rats received a 25-mg pellet of crystalline RU486 (Mifepristone: 11β-[4-dimethylaminophenyl]-17β-hydroxy-17α[prop-1-ynyl]-estra-9,9-dien-3-one; Sigma Chemical Co., St. Louis, MO) intraperitoneally at 0830–0900 h on Day 9. Rats also received 0.5 mg of RU486 in saline/40% ethanol intraperitoneally immediately after implantation of the pellet to ensure rapid onset of RU486 action [17]. Animals were killed 3, 6, 12, and 24 h

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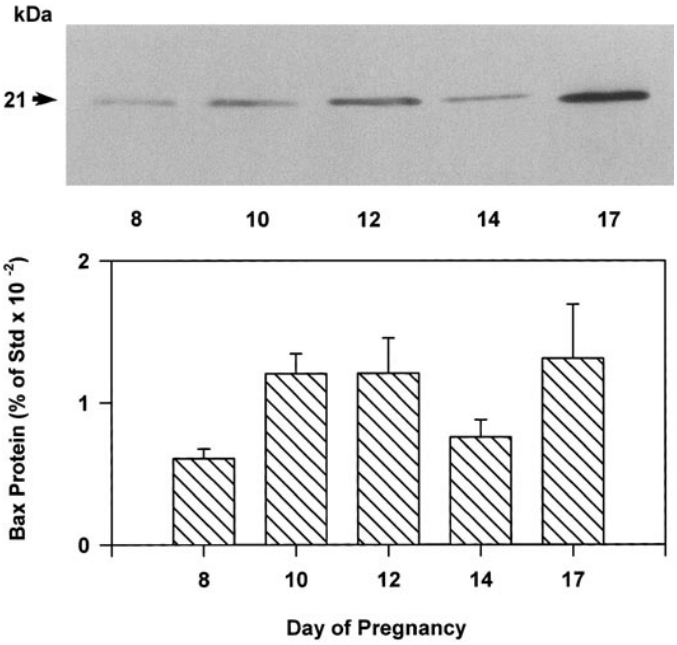

FIG. 1. The upper panel is a representative immunoblot of Bax expression in DB at various stages during pregnancy. The lower panel summarizes the densitometric analysis of replicate experiments (mean ± SEM of values expressed as a percentage of the Day 10 standard preparation, n = 4–6). Refer to *Materials and Methods* for detailed explanations. Statistical analysis was performed using ANOVA followed by the Student-Newman-Keuls multirange test. No significant changes in Bax expression were detected ($P > 0.05$).

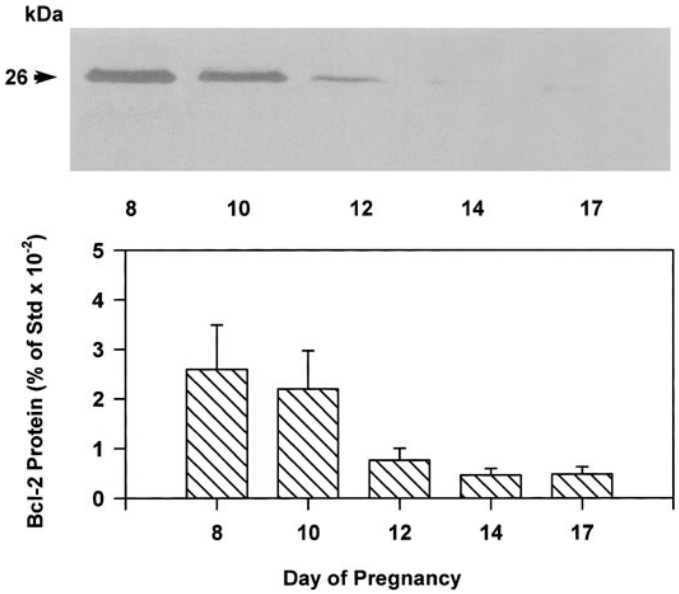

FIG. 2. The upper panel is a representative immunoblot of Bcl2 expression in DB at various stages during pregnancy. The lower panel summarizes the densitometric analysis of replicate experiments (mean ± SEM of values expressed as a percentage of the Day 10 standard preparation, n = 4–6). Refer to *Materials and Methods* for detailed explanations. Statistical analysis was performed as in Figure 1. One-way ANOVA indicated that Bcl2 expression changed with the progression of pregnancy ($P < 0.05$), but pairwise comparisons of individual means were not different ($P > 0.05$).

later. This regimen of RU486 treatment does not alter the serum progesterone [17]. Although RU486 is most potent as an antiprogestin, it has significant antiglucocorticoid activity [18]. However, blockade of progesterone action most likely accounts for the effects of RU486 on the DB, because these effects were similar to those induced by ovariectomy and were reversed by progesterone [2, 4, 5, 16]. Furthermore, glucocorticoids do not appear to influence decidualization [19].

Tissue Preparation for Western Blot Analysis

The DB were isolated by gently separating the placenta and myometrial regions with 23-gauge needles. Tissues from Days 8 and 10 of pregnancy contain some chorioallantoic cells, but antimesometrial decidua, choriovitelline, fetal, and myometrial tissues were removed. In addition, the DB begins to regress on Day 14 and becomes too thin to reliably dissect after Day 17. Other procedures and validations used have been detailed elsewhere [5]. Tissues from a single litter were pooled to yield 0.1–1.0 mg DNA/ml homogenate. Occasionally, it was necessary to combine DB from more than one litter (e.g., as many as 30 DB were required per pool during early pregnancy) and for some treatment groups (e.g., RU486 for 24 h). Tissue pools were snap-frozen in liquid nitrogen and stored at approximately −80°C until homogenized. Four to 6 tissue pools were analyzed per treatment group.

Each pool of DB tissues was homogenized in 2 ml of TEDG buffer (10 mM Tris; 1 mM EDTA; 1 mM dithiothreitol; 30% glycerol (v/v); pH, 7.4), with aprotinin (2 μg/ml) and leupeptin (0.5 μg/ml) added just before use. An aliquot of the whole homogenate was taken for DNA determination [20]. The cytosol fraction was isolated by differential centrifugation, and a volume equivalent to 40 μg of DNA (to normalize samples on a ''per cell'' basis) was prepared for electrophoresis on 12% polyacrylamide gels under denaturing conditions according to procedures described elsewhere [5].

After electrophoresis, gels were electroblotted to nitrocellulose membranes (Hybond-ECL; Amersham Life Science, Inc., Buckinghamshire, UK). The blots were incubated for 18 h at 4°C in the presence of rabbit anti-mouse Bax polyclonal immunoglobulin (Ig) G (sc-526), diluted to 1:400, or rabbit anti-human Bcl2 polyclonal IgG (sc-492), diluted to 1:400, both of which were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Specificity of the reaction was verified by incubating membranes in the same solution but with normal rabbit serum instead of antisera. Blots were washed, incubated with second antibody,

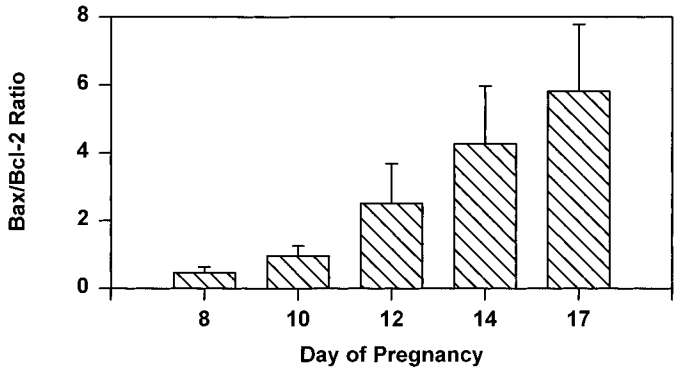

FIG. 3. Summary analysis of changes in the Bax/Bcl2 ratio in DB during pregnancy (mean ± SEM of values determined from data in Figures 1 and 2 and calculated for each tissue preparation, n = 4–6). Statistical analysis was performed as in Figure 1. One-way ANOVA revealed significant differences between mean values and day of pregnancy ($P < 0.05$), but pairwise comparisons of individual means were not different ($P > 0.05$).

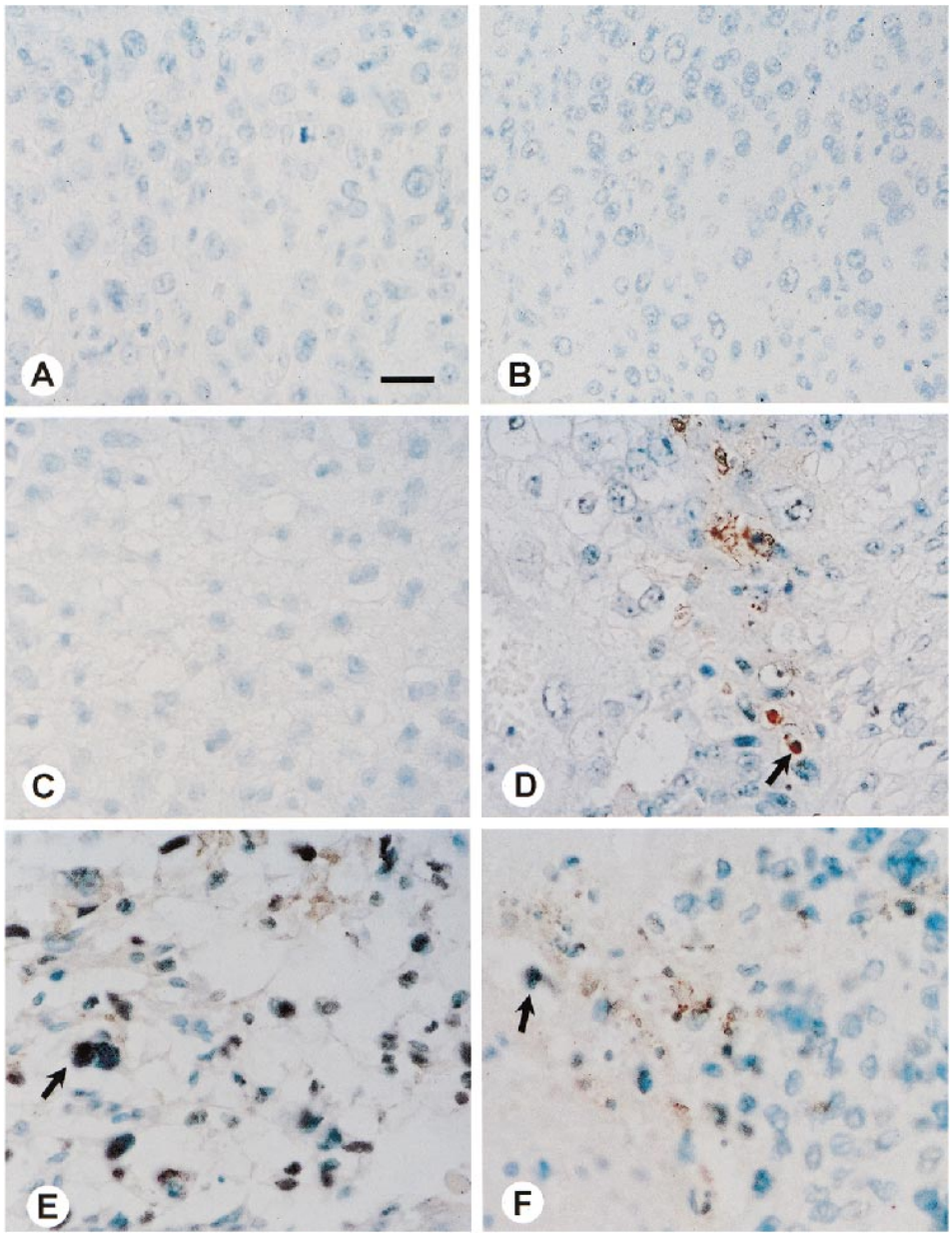

FIG. 4. Representative photomicrographs depicting in situ staining of DNA fragments in stromal cells of the DB with progression of pregnancy. Dark-brown staining indicates a positive reaction. **A**) Day 8. **B**) Day 10. **C**) Day 12. **D**) Day 14. **E**) Day 17. **F**) Day 21. The scale bar in **A** equals 23 μm and applies to all photographs. Tissues were counterstained with 1% methyl green. Arrows show examples of dense accumulation of reaction product indicative of the apoptotic process. Negative control tissue sections (not shown) showed no reaction product and appeared exactly as seen in **A**–**C**, which confirm that no nonspecific staining occurred because of endogenous peroxidase activity or nonspecific binding of the anti-digoxigenin peroxidase complex.

and exposed to chemiluminescent detection substrates as described elsewhere [3, 4]. The membranes were exposed to X-Omat AR imaging film (Eastman Kodak Co., Rochester, NY) for 3 to 60 min. The autoradiographs were then scanned and quantified by densitometry using Intelligent Quantifier software (Bio Image Systems Corp., Ann Arbor, MI).

The molecular size of immunoreactive bands was determined by comigration of a ladder of biotinylated SDS-PAGE molecular weight standards (Bio-Rad Laboratories, Hercules, CA) applied to a lane in each gel. Prestained standards were also applied to gels to assess the transfer efficiency of samples. The relative abundance of Bax and Bcl2 proteins was determined by comparing immunostained bands from experimental samples with the corresponding immunostained bands from a pool of DB (Day 10) that was similarly prepared and applied to each gel. We have found that this pooled reference preparation is the most reliable way to compare relative changes in protein expression [3, 21].

In Situ Detection of Apoptosis

The DB tissues were fixed in neutral buffered 10% formaldehyde solution for 10–12 h, embedded in paraffin wax, and sectioned at 6 μm. Tissue sections were mounted on ProbePlus slides (Fisher Scientific Co., Pittsburgh, PA), and the tissue slides were prepared for digoxigenin labeling of genomic DNA and subsequent immunoperoxidase reaction using procedures described elsewhere [2, 5]. The proce-

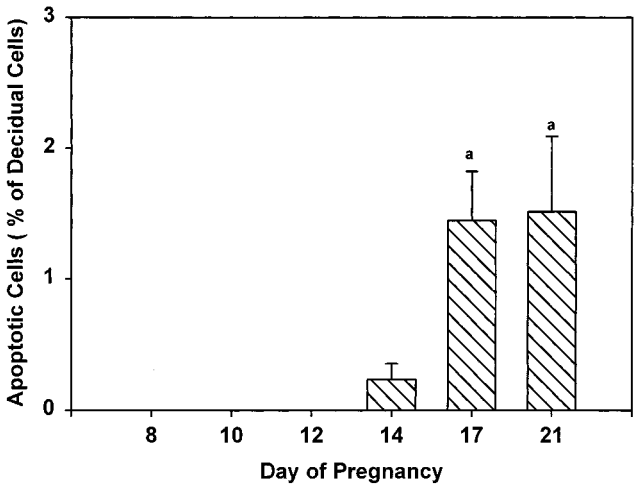

FIG. 5. Summary analysis of the frequency of apoptosis in DB during pregnancy (mean ± SEM of values expressed as a percentage of total decidual cells, n = 3–4). Two thousand decidual cells were classified in randomly selected microscopic fields. The number of apoptotic cells is expressed as a percentage of all decidual cells counted. Statistical analysis was performed as in Figure 1. [a]Means differ from all others except each other ($P < 0.05$).

dures for the immunostaining reaction itself, which targets 3′-hydroxyl DNA terminal ends generated by DNA fragmentation, followed instructions provided by the manufacturer (ApopTag Plus In Situ Apoptosis Detection Kit; Oncor, Inc., Gaithersburg, MD). Diamino-benzidene served as substrate to visualize the anti-digoxigenin-peroxidase complex. Tissues were counterstained with 1% methyl green and then destained briefly in 100% butanol before dehydration and mounting under glass coverslips with Permount. Negative control slides were prepared from tissues taken at each time point using exactly the same procedures, except that water was added instead of the terminal deoxynucleotidyl transferase. Tissues provided by the manufacturer served as positive controls.

The incidence of apoptosis was evaluated by examining stained tissue sections under a microscope (magnification, ×400). Two thousand decidual cells were counted in randomly selected microscopic fields. The number of apoptotic stromal cells was expressed as a percentage of all decidual cells counted in each section. Tissues from at least three animals in each treatment group were analyzed, and mean values were calculated. Cells exhibiting dark-brown staining of nuclei and apoptotic bodies were considered to be apoptotic. However, nuclei showing only a diffuse, light-brown staining reaction were considered to be in the early stage of apoptosis and were not counted as being apoptotic (see Fig. 10 for examples).

Detection of Apoptosis by DNA Laddering

Cells undergoing apoptosis cleave their DNA between nucleosomes, yielding fragments in multimers of 180–200 bp [22, 23]. The DNA was prepared from dissected DB by phenol-choloroform-isoamyl alcohol extraction (24:24:1) following the method described by Moulton [24]. The DNA samples (15 μg) were electrophoretically separated on 1.8% agarose gels in 0.5× TPE (45 mM Tris-phosphate and 1 mM EDTA; pH, 8.0) at 70 V for 2 h using a 100-base pair (bp) DNA ladder (2 μg) as a molecular weight standard. The gel was stained with ethidium bromide (1 μg/ml), and photographs were taken. The intensity of band fluorescence

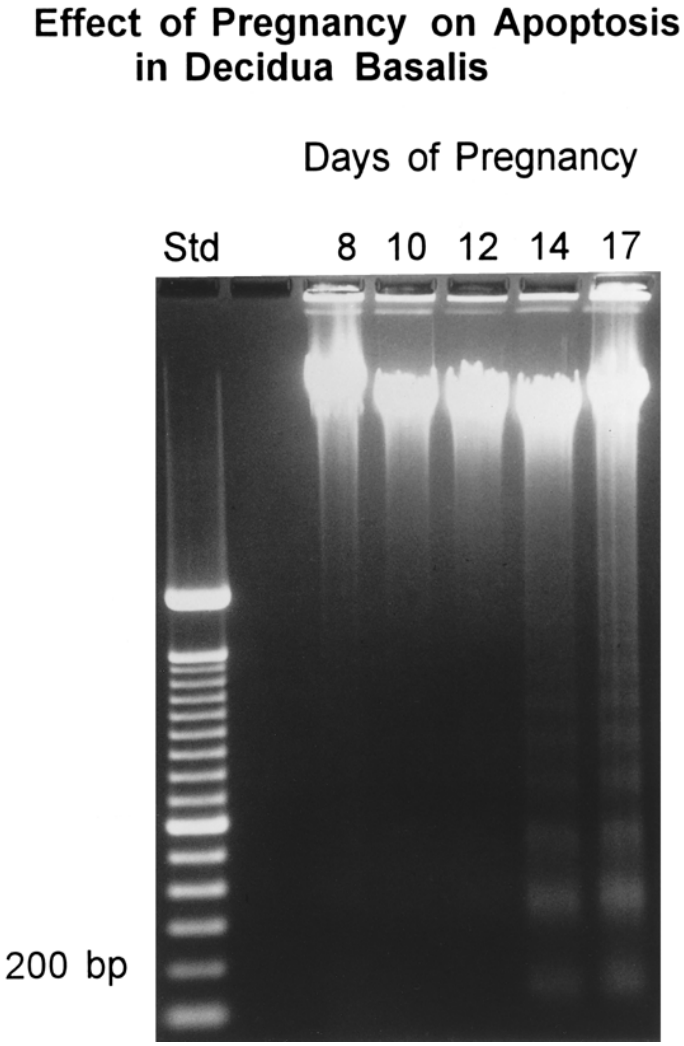

FIG. 6. Representative gel of three experiments showing DNA fragmentation in decidualized stromal cells of the DB during pregnancy. Fifteen micrograms of DNA were applied to each lane of a 1.8% agarose gel and electrophoresed as described in *Materials and Methods.* A 100-bp DNA ladder was used to reference the molecular size (Std).

was measured using an Image-Pro Plus image analyzer (Media Cybernetics, Silver Spring, MD). Nucleosomal DNA fragmentation values are expressed as the ratio of the intensity of the 200-bp band to the intensity of the DNA band excluded from entering the gel (>15 kbp) to correct for gel loading.

Statistical Analysis

Values are reported as the mean ± SEM. Statistical analysis was made by one-way ANOVA, and when significant treatment effects were indicated, the Student-Newman-Keuls multirange test was employed to make pairwise comparisons of individual means.

RESULTS

Figure 1 shows that Bax, the cell death signaling partner, was rather uniformly expressed throughout pregnancy. On the other hand, Bcl2 was most abundant on Days 8 and 10, a time of proliferation and differentiation of the mesometrium, and declined markedly thereafter (Fig. 2). The functional significance of these expression patterns derives from

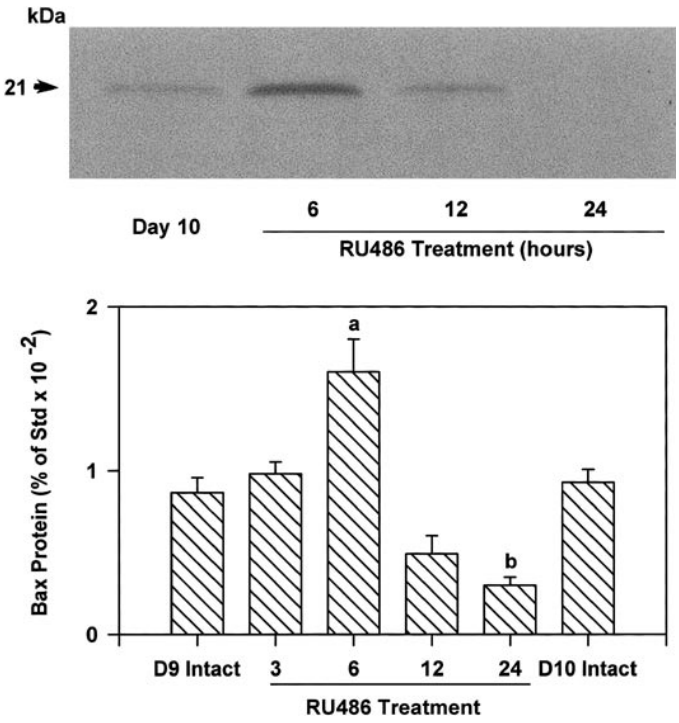

FIG. 7. The upper panel is a representative immunoblot showing the effects of RU486 treatment on Bax expression in DB. The lower panel summarizes the densitometric analysis of replicate experiments (mean ± SEM of values expressed as a percentage of the Day 10 standard preparation, n = 4–6). Rats were sacrificed at the indicated intervals after administration of RU486 at 0830–0900 h on Day 9 of pregnancy. The D9 Intact control group shows the level of Bax expression at the time of RU486 pellet implantation, and the D10 Intact control group shows the level of expression 24 h later in the absence of treatment. Refer to *Materials and Methods* for a more detailed explanation of the experimental design. Statistical analysis was performed as in Figure 1. [a]Mean differs from all others ($P < 0.05$). [b]Mean differs from all others except RU486 treatment for 12 h ($P < 0.05$).

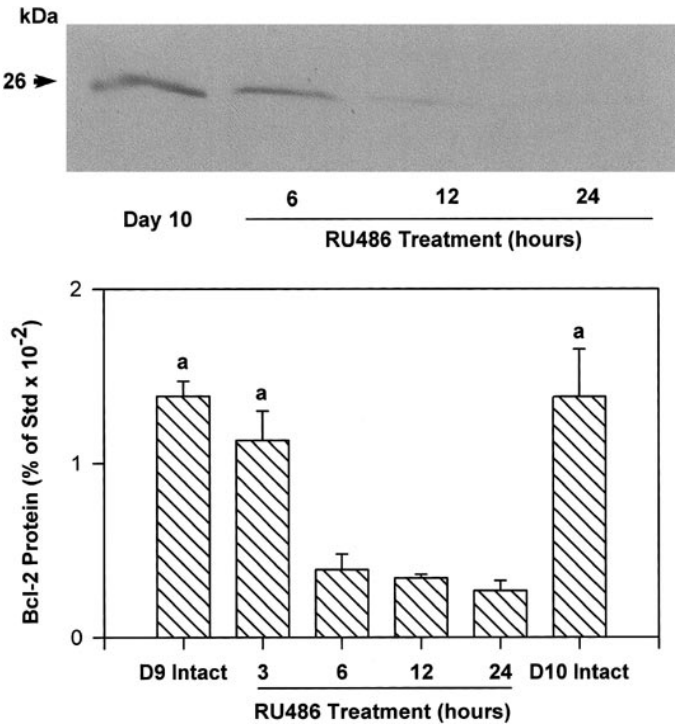

FIG. 8. The upper panel is a representative immunoblot showing the effects of the antiprogestin RU486 on Bcl2 expression in DB. The lower panel summarizes the densitometric analysis of replicate experiments (mean ± SEM of values expressed as a percentage of the Day 10 standard preparation, $n = 4–6$). The experimental design was the same as in Figure 7, and the statistical analysis performed as in Figure 1. [a]Mean values differ from 6, 12, and 24 h after treatment ($P < 0.05$).

the potential for heterodimer formation between Bax and Bcl2. A high Bax:Bcl2 ratio enhances the probability for Bax homodimer formation and cell death signaling, whereas a relative abundance of Bcl2 favors formation of Bax/Bcl2 heterodimers and Bcl2 homodimers, which promote cell survival [11, 12]. Thus, the ratio of Bax expression to Bcl2 expression was calculated for each DB sample, and an average value was calculated for each day of pregnancy. Figure 3 shows that Bax became increasingly more dominant during late pregnancy, suggesting an ever-increasing preponderance of death signals. Thus, we predicted an increase in decidual cell apoptosis in conjunction with Bax dominance.

To evaluate this notion, tissue sections of DB were prepared and stained to detect DNA fragmentation by the terminal deoxynucleotidyl transferase-mediated nick end-labeling method. Figure 4 shows representative tissue sections of DB and the frequency of DNA fragmentation. Positive staining was never noted in stromal cells on Days 8, 10, and 12 of pregnancy; however, by Day 14, a few stromal cells exhibited dense deposits of reaction product (≈0.2%). The frequency of stromal cells undergoing DNA fragmentation increased sixfold by Day 17 and remained elevated to within 18 h of parturition (Day 21). These findings are summarized in Figure 5. We confirmed the late-pregnancy onset of apoptosis more directly by observing enhanced intranucleosomal cleavage of DNA into multimers of 168–200-bp fragments (Fig. 6). Summary analysis of DNA from samples of DB prepared from three to four rats (i.e., n = 3–4) at each day of pregnancy showed that DNA fragmentation on Days 14 and 17 was significantly elevated over that during the earlier stages ($P < 0.05$; data not shown).

Because progesterone plays an absolutely crucial role in the decidualization and proliferation of stromal cells [25–28], we hypothesized that withdrawal of progesterone action by administration of antiprogestin (RU486) during differentiation of the DB (Day 10) would lead to suppression of Bcl2 expression and up-regulation of Bax. To test this idea, a 25-mg pellet of RU486 was implanted i.p. on the morning on Day 9 of pregnancy. Rats were sacrificed 3, 6,

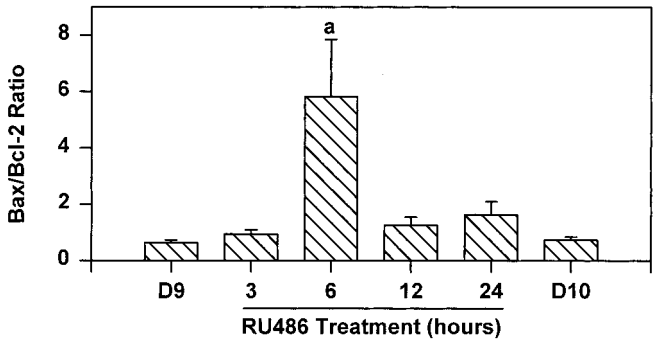

FIG. 9. The effects of RU486 treatment on the Bax:Bcl2 ratio (mean ± SEM of values as determined from data in Figures 6 and 7 and calculated for each tissue preparation, n = 4–6). Statistical analysis was performed as in Figure 1. [a]Mean differs from all others ($P < 0.05$).

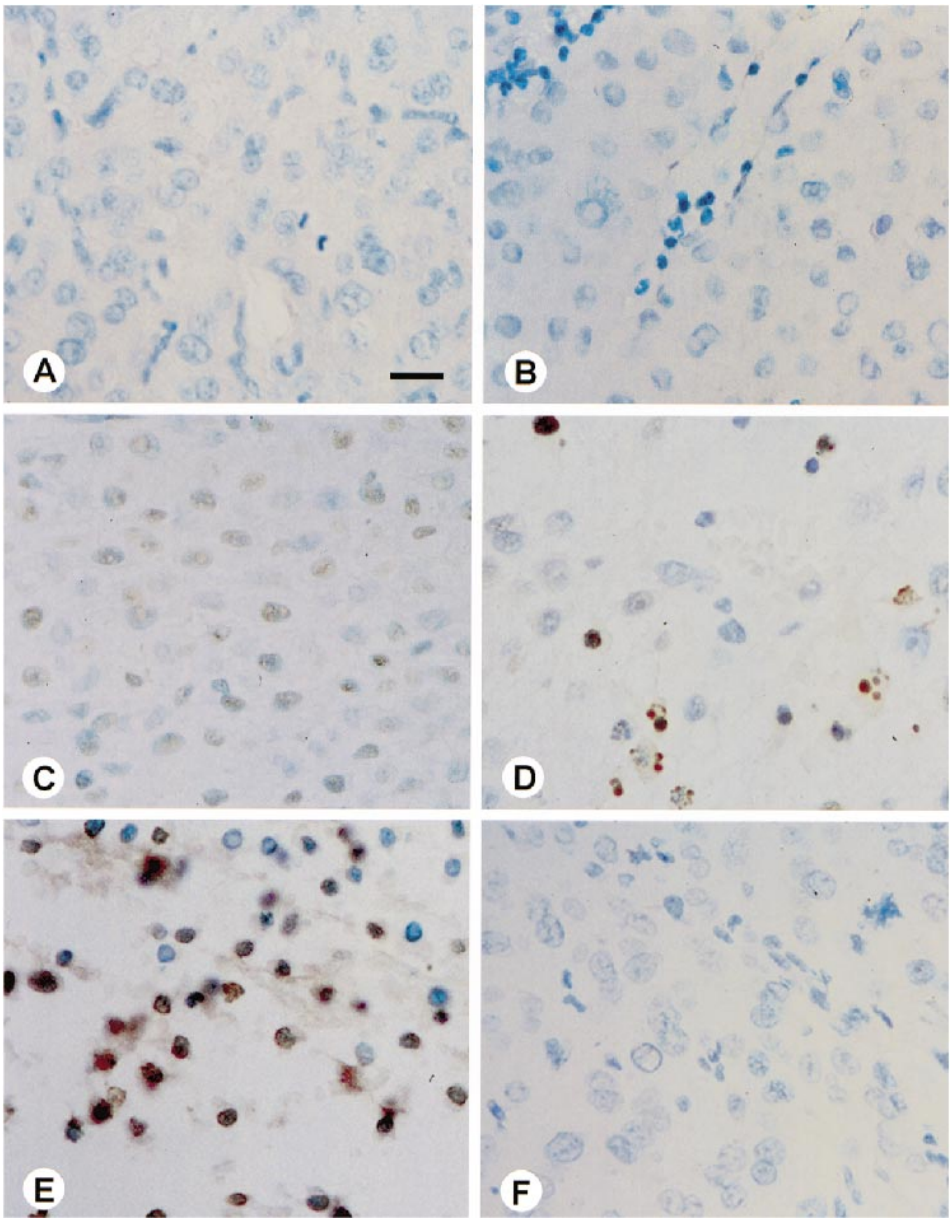

FIG. 10. Representative photomicrographs demonstrating in situ staining of DNA fragments in stromal cells of the DB after treatment with the antiprogestin RU486. Brown staining indicates a positive reaction. **A**) Day 9 intact shows the condition of the DB at the time of RU486 treatment. **B**) Three hours of exposure to RU486. No apoptotic cells were noted. The nuclei of the intravascular polymorphonuclear leukocytes show an intense reaction to the counterstain. **C**) Six hours of exposure to RU486. The diffuse, light-brown nuclear staining seen in many stromal cells was considered to be early stage apoptosis. Such cells were not counted as being apoptotic. **D**) Twelve hours of exposure to RU486. Dense accumulation of reaction product in nuclei and apoptotic bodies (dark-brown staining) indicate apoptotic cells. Note the marked change in stromal cell morphology and loss of histological integrity of the deciduum. **E**) Twenty-four hours of exposure to RU486. Histological integrity of the deciduum is severely compromised, and many stromal cells show an advanced stage of apoptosis. **F**) Day 10 intact group showing the condition of the DB 24 h later in the absence of RU486. The scale bar in **A** equals 23 μm and applies to all photographs. The experimental design was the same as in Figure 7, and tissue staining procedures were the same as in Figure 4. No visible reaction product was noted in negative control tissue sections (not shown), which appeared exactly as **A** and **B**.

12, and 24 h thereafter. Figure 7 shows that the antiprogestin enhanced Bax by 150% within 6 h, whereas Bcl2 expression declined by 60% (Fig. 8). Thus, the antiprogestin brought about a readjustment in the Bax/Bcl2 rheostat to favor Bax and the initiation of cell death signals (Fig. 9). Both the frequency and intensity of DNA fragmentation in stromal cells were examined in sections of DB to ascertain whether the changing rheostat was ultimately associated with stromal cell death (Fig. 10). No reaction product was noted in the untreated controls on Days 9 and 10 or in rats treated with antiprogestin for 3 h. However, by 6 h, diffuse, light staining was noted in many cells, which was interpreted as early stage apoptosis; mature apoptotic bodies were rarely observed (~0.5% of cells). Densely stained nuclei and mature apoptotic bodies were noted with increasing frequency thereafter, eventually involving more than 9% of cells by 24 h (Fig. 11).

DISCUSSION

It has been long recognized that histological regression of the DB begins at approximately Day 14 in the pregnant rat [7], but the signaling pathways and mode of regression remain unresolved. Our study shows that Bax and Bcl2 are

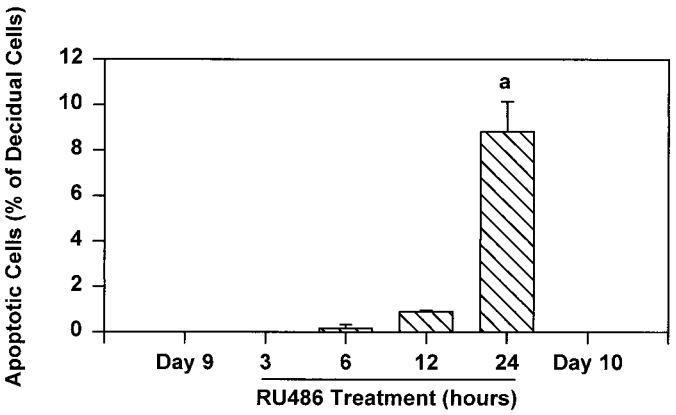

FIG. 11. Summary analysis of frequency of apoptosis in DB after treatment with RU486 (mean ± SEM of values expressed as a percentage of total decidual cells, n = 3–4). Methods of analysis were as described in Figure 5. [a]Mean differs from all others ($P < 0.05$).

highly expressed in decidualized mesometrial stromal cells, and that they appear to form a functional cell survival/death rheostat. During early pregnancy, the rheostat favored decidual cell survival, whereas after Day 10, Bax increasingly became the more dominant partner and was associated with stromal cell apoptosis and DB regression.

Our findings also suggest that Bax dominance may be the default condition, because it was expressed at a relatively constant level throughout pregnancy. Thus, variable expression of Bcl2 may control modulation of the rheostat ''setting,'' wherein elevated Bcl2 expression during early pregnancy effectively rescues stromal cells from regression and, in turn, allows them to proliferate and differentiate to the decidualized phenotype in preparation for nidation and placentation. In this way, the repressor of cell death may provide one type of signal for maternal recognition of pregnancy. During late pregnancy, the relative decline in Bcl2 expression permits Bax to gain dominance, the downstream effects of which most likely involve both direct and indirect activation of the caspase family of cell death proteases that form the final steps in the apoptotic pathway [12, 16, 29].

The events triggering the late-pregnancy switch in the rheostat setting are obscure considering that it occurs despite continued high levels of serum progesterone and of progesterone-binding sites [3, 30, 31]. Serum levels of progesterone do not begin to fall until after Day 21, in preparation for parturition [30]. Thus, progesterone must be less able to maintain a favorable Bax:Bcl2 ratio for stromal cell survival. We have proposed that DB regression is initiated during late pregnancy by a physiologic withdrawal of progesterone action resulting from differential expression of PR isoforms. The transactivationally active receptors (i.e., PR-A and -B) are abundantly expressed and maintained by progesterone in ovariectomized pregnant rats during early pregnancy; expression of these isoforms is specifically lost during midpregnancy and after RU486 treatment [3, 4]. On the other hand, PR-C, an inactive form that binds progesterone, is abundantly expressed throughout pregnancy and is progesterone-independent [4, 32]. Thus, we propose that PR-C may function as a progesterone-binding sink and cause a relative physiologic withdrawal of progesterone action.

Progesterone is an important modulator of the rheostat setting in an endometrial epithelial cell line [33]. The present study shows that progesterone plays a similar role in the mesometrium of normal pregnancy, wherein antiprogestin treatment not only suppressed Bcl2 but also enhanced Bax, resulting in a sixfold increase in the Bax:Bcl2 ratio. These events preceded the onset of visible apoptosis. Expression of both proteins was lost at later time points, perhaps as a consequence of Bax activation of the caspase cascade, because Bcl2 family members are also caspase substrates [12, 29]. Progressive degradation of DB histology was also evident after 6 h and was associated with an ever-increasing number of apoptotic cells. After 24 h of RU486 treatment, DB integrity was entirely lost, and fully 9% of the stromal cells were apoptotic. This is a very huge fraction given that the visible stages of apoptosis have a short duration (≈3 h). It has been calculated that apoptosis in only 2%–3% of cells at a given time can represent a cell loss of 25% per day [34].

Thus, blockade of progesterone action in decidual cells not only results in the deprivation of cell survival signals (e.g., growth factors, cell cycle regulators, Bcl2) but also appears to augment cell death signals (e.g., p27, protein kinase C, and Bax), which conspire to produce a strong, programmed cell death response [1, 2, 5, 6].

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