Progestins Prevent Apoptosis in a Rat Endometrial Cell Line and Increase the Ratio of \textit{bcl-X}\textsubscript{L} to \textit{bcl-X}\textsubscript{S}*

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Endometrial cell proliferation and cell death are regulated by ovarian hormones. The fall of ovarian progesterone in late secretory phase, or the artificial withdrawal of ovarian hormones during early pregnancy, are followed by programmed cell death of uterine epithelial cells. Aspects of this cell-specific response have been reproduced in a newly established rat endometrial cell line which expresses functional progesterone receptor. At low concentrations of serum and in the absence of glucocorticoids, these cells were dependent on progestins for survival. Removal of progesterone or addition of the antiprogestins RU38486 or ZK98299 led to a substantial increase of apoptotic cells indicated by the accumulation of internucleosomally degraded DNA. The hormonal control of cell proliferation and cell death correlated with the overall quantity and distribution of the different \textit{bcl-X} transcripts. Progesterone administration not only increased total \textit{bcl-X} mRNA level but also shifted the quantitative ratio between the different mRNA isoforms in favor for the apoptosis inhibiting form, \textit{bcl-X}\textsubscript{L}, compared with the apoptosis promoting form, \textit{bcl-X}\textsubscript{S}. These effects were rapid and could not be prevented by inhibitors of protein synthesis. As the low level of \textit{bcl-2} and \textit{bax} mRNA was not influenced by progesterone treatment, the observed changes in total amount of \textit{bcl-X} transcripts and spliced isoforms could represent the mechanism by which progesterone controls cell death in epithelial cells of the endometrium.

Sex steroid hormones control cell proliferation and cell differentiation in many organs, in particular in the genital tract and mammary gland. In the endometrium, growth of epithelial cells is dependent on estrogens and progesterone, and removal of ovarian hormones has been reported to cause cell death as exemplified in different species (1–3). Particularly, the degenerative response in the epithelium followed the characteristic morphological and biochemical features of programmed cell death, or apoptosis (4).

In the cycling human endometrium, apoptosis occurs in the glandular epithelium during the late secretory, the premenstrual, and the menstrual phases, and to a lesser extent during the proliferative phase (1). In the rabbit, estrogens induce proliferation of resting endometrial cells, whereas progesterone induces proliferation of already dividing cells and ultimately leads to differentiation and growth arrest. Ovariectomy of pseudopregnant rabbits, or administration of antiprogestins, cause intense apoptosis of endometrial epithelial cells (5, 6). Attempts to reproduce this behavior in simplified in vitro cultivation systems have only partially been successful using primary cells (7, 8).

The decision of cells to undergo apoptosis is controlled by external signals in combination with an autonomous genetic program. Cell death is regulated at several intracellular checkpoints by the action of members out of two gene families conferring opposite effects (9). The \textit{bcl-2} oncogene was first identified in human B-cell tumors with a chromosomal translocation placing it within the immunoglobulin locus. \textit{Bcl-2} was the founding member of a growing family of genes controlling apoptosis. These genes show homology clustered in two regions, BH1 and BH2 (10). \textit{Bcl-2} protects cells against some forms of apoptosis, but does not induce cell proliferation (9).

Another member of the family is \textit{bax} which promotes apoptosis and encodes a protein that can form homodimers or heterodimers with \textit{Bcl-2} (10). The ratio of \textit{Bcl-2} to \textit{Bax} determines the sensitivity of some cells to apoptotic stimuli and could be the target of various factors and signals that influence cell survival. Another homologue of \textit{Bcl-2}, \textit{Bcl-X}, exists in two isoforms generated by alternative splicing. The large form, \textit{Bcl-X}\textsubscript{L}, which contains BH1 and BH2, protects cells against cell death, whereas the short form, \textit{Bcl-X}\textsubscript{S}, promotes cell death by inhibiting \textit{Bcl-2} and \textit{Bcl-X}\textsubscript{L} function (11). Additional members of the \textit{bcl-2} family have been identified which influence the apoptotic pathway (12–15). Interestingly, another checkpoint on the apoptotic pathway is provided by the balance of two alternatively spliced isoforms encoding different species of interleukin-1β converting enzyme (9).

The finding that \textit{bcl-2} is expressed in the terminal differentiated syncytiotrophoblast and in endometrium suggested that Bcl-2 may be related to hormone-dependent apoptosis (16). In human epithelial endometrial cells Bcl-2 predominates at the end of the follicular phase and is low or absent in the secretory phase, when electron microscopic analysis shows apoptotic cells (17). These studies suggest that Bcl-2 may be involved in protection against apoptosis during the proliferative phase of the menstrual cycle, but does not seem to play a role in luteal phase (18). Presumably \textit{bcl-2} could be a direct target for steroid hormones, as there are potential hormone responsive elements in the \textit{bcl-2} gene promoter (17).

To investigate the regulation of endometrial cell proliferation and differentiation by ovarian hormones, in particular by progesterone, we have recently established cell lines from rat endometrial epithelium which exhibit some of the properties of the corresponding differentiated tissue (19). Whereas some lines established by immortalization with simian virus 40 large T antigen lost most of their epithelial markers and developed a
fibroblast-like phenotype, additional transformation by γ-Har
ras partly restored the epithelial phenotype giving rise to the
ancestor cell line RENTRO1 (20). Here we describe the stable
gene transfer of recombinant progesterone receptor to this en-
dometrial epithelial-like cell line to reconstitute progesterone
receptor response to the formerly non-responsive line. One of
the progeny cell line, RENTROP, was used to study the hor-
monal regulation of cell proliferation and cell death. In the
presence of low serum, a fraction of the cell population under-
goes apoptosis which leads to a progressive decrease in the
overall cell number. Survival of these serum-starved cells can
be restored by progesterone, as well as the synthetic glucocor-
ticoid, dexamethasone, which lead to a decrease in the propor-
tion of apoptotic cells. Consistently this effect can be antago-
nized by the antiprogestin RU486. Progestins and glucocorticoids also induce bcl-X transcripts. Induction of bcl-X
mRNA is accompanied by a shift toward a relative increase of
the bcl-Xi encoding isoform over the bcl-Xs isoform. As the cells
express low level of bcl-2 mRNA and as their level of bax mRNA is not influenced by hormonal treatment, our results suggest that the effect of progesterone deprivation on the apoptosis in the endometrial epithelium is mediated by a reversal of the
ratio between apoptosis-inducing and apoptosis-preventing iso-
forms of bcl-X.

**EXPERIMENTAL PROCEDURES**

*Generation of Stable Cell Lines—*An endometrial epithelial cell line that stably expresses the progesterone receptor was generated by ap-
plying two rounds of calcium phosphate precipitate-mediated gene
transfer to the rat endometrial RENTRO-1 cell line (20). First tetracy-
cline repressor-VP16 expression plasmid (21) was stably introduced into RENTRO-1 cells using the hygromycin-B phosphotransferase gene
as selection marker (22). One of the selected clones, which is referred to
as parental RENTRO-1, was stably transfected with a tetracycline-
inducible vector for the rabbit progesterone receptor (23) (expression
as selection marker (22). One of the selected clones, which is referred to
into RENTRO-1 cells using the hygromycin-B phosphotransferase gene
clone repressor-VP16 expression plasmid (21) was stably introduced
that stably expresses the progesterone receptor was generated by ap-
thesis in the presence of tetracycline at 10
m
[55x513]m
[55x31]PR, progesterone receptor.


### Fluorochrome DNA Nick End Labeling of Cells in Situ

Cells were fixed in situ in 4% phosphate-buffered saline-buffered formaldehyde.
After equilibration in terminal deoxynucleotide transferase buffer (30
mM Tris, 140 mM sodium cacodylate, pH 7.2, 1 mM CoCl2) for 30 min at
room temperature, cells were incubated in terminal deoxynucleotide
transferase buffer containing 40 μM fluorescein-15-DATP (Boehringer,
Mannheim, Germany) and 0.25 units/μl of terminal deoxynucleotide
transferase (Boehringer, Mannheim) in a humified atmosphere at
37 °C for 1 h. The reaction was terminated by incubating the cells in the solution containing 300 mM NaCl and 30 mM sodium citrate for 10
min at room temperature. Cells were counterstained with a solution of 4 μg/ml propidium iodide for 15 min. Cells were rinsed with water,
mounted in Mowiol (Hochst, Frankfurt) with 0.2 gliter para
phenylenediamine, and examined by fluorescence microscopy (Leitz,
Wetzlar).

### Genomic DNA Analysis—

For the detection of degraded DNA prod-
ucts, DNA from 3 × 106 cells was isolated according to Ref. 29. Briefly, the cells were lysed in 0.1 M lysis buffer (50 mM Tris, pH 7.5, 20
mM EDTA, and 1% Nonidet P-40) for 30 s. After centrifugation 2,000 × g
for 5 min at room temperature apoptotic DNA remained in the supernatant while intact nuclear DNA was recovered in the pellet. The supernatants
were treated at 56 °C for 2 h with RNase A (5 mg/ml) and SDS (1%) followed by digestion with proteinase K (2.5 mg/ml) at 37 °C for 18 h.
DNA was precipitated with ethanol, redissolved in water, and dissolved
in 0.5 μg/ml ethidium bromide and visualized under UV light. A
100-bp DNA ladder (Boehringer, Mannheim) was used as size marker.

### RNA Analysis—

Total cellular RNA was isolated by the guanidine
thiocyanate-phenol-chloroform extraction method (30). RNA se-
cretion analysis was performed as described below (31). For preparing the
bcl-X probe, plasmid pBCL2 was digested with PvuII and transcribed by T3 RNA
polymerase. The full-length transcript size of the bcl-X riboprobe was 294 bp, and the protected fragments for bcl-Xs and bcl-Xi
were 237 and 155 bp long, respectively. For preparing the bcl-2 probe, plasmid pbcl2 was digested with PvuII and transcribed by T3 RNA
polymerase. The full-length transcript size of the riboprobe was 416 bp, and the protected fragments for bcl-2a and bcl-2b were 371 and 234 bp long, respectively. The rat genome template PRGAPDH (Ambion, Austin,
TX) was digested with BglII and transcribed with T3 RNA polymerase.
The probe length was 204 bp and the size of the protected fragment was 145 bp. [-32P]CTP (Amersham, Braunschweig) radiola-
beled RNA probes were prepared using a kit according to the instruc-
tions of the manufacturer (Promega, Madison, WI). The probes were copurified with RNA and dissolved in hybridization buffer,
denatured at 95 °C for 10 min, and hybridized at 52 °C for 18 h. After
digestion with RNases A and T, followed by digestion with proteinase
K, the samples were precipitated, denatured, and subjected to electro-
phoresis on a 5% denaturing acrylamide gel. Quantitation was per-
formed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA)
using Image-Quant software. In all cases the quantitation was normal-
ized against the gapdh signal.

For Northern blot analysis 25 μg of total RNA were elec-
rophoresed in a 1% agarose-formaldehyde gel and transferred to nylon membrane, type NY 13N (Schleicher & Schuell). Membranes were hybridized with
[32P]-labeled 400-bp fragment of rat bax (32) for 16 h and washed in 2 ×
SSC, 0.1% SDS at 55 °C. Quantitation was done from densitometric
scans of autoradiographs; normalization was calculated referring to the
SSC signal.

### Incorporation of 16-35Methionine—

1.5 × 106 cells were incubated in 6
wells plates in medium containing 10% CS-FCS for 12 h. The medium
was removed and 1 ml of medium with 1% CS-FCS and 1 μCi of
[S]methionine (Hartmann Analytic, Braunschweig, Germany, 37
TBq/mmol), and incubation was continued for 5 h in the presence or
absence of cycloheximide (100 ng/ml). The incubation was stopped by

1 The abbreviations used are: FCS, fetal calf serum; bp, base pair(s); PR, progesterone receptor.
harvesting the cells in phosphate-buffered saline containing 0.2% SDS. Cells were lysed by treatment with 10% trichloroacetic acid for 10 min on ice and the lysates were filtrated through glass microfiber filters GF/C (Whatman). Filters were washed twice with 5% trichloroacetic acid and once with ethanol and radioactivity on the filters was quantitated by liquid scintillation counting. The results are expressed as incorporated disintegrations/min/well and correspond to the average of two independent experiments performed by triplicates.

Western Blots—Protein extracts were prepared by lysing cells at 4 °C for 1 h in 2 volumes of lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1% Nonidet P-40). The lysate was centrifuged at 13,000 rpm and 4 °C for 10 min, and the pellet discarded. Protein concentration in the supernatant was measured by Bradford assay (Bio-Rad). After adjusting to sample buffer and boiling for 5 min, 100 µg of protein were applied on a 15% SDS-polyacrylamide gel, and electrophoresis was performed at 25 mA for 2 h. The resolved proteins were transferred to an Hybond ECL membrane (Amersham) by electroblotting. Antibody incubation was performed in blocking buffer (1% skim milk, 0.5% Tween) in Tris-buffered saline at 4 °C. As primary antibodies were applied on a peroxidase-labeled anti-rabbit antibody was used (Amersham). As secondary antibody, a peroxidase-labeled anti-rabbit antibody was used (Amersham). Proteins bands were detected using the ECL kit (Amersham).

RESULTS

Generation of the RENTROP Cell Line—The previously characterized RENT-1 endometrial cell line, as well as the parental clone used in these experiments, express glucocorticoid receptor but do not contain significant levels of progesterone receptor (PR), as demonstrated in steroid binding assays (20) and transient transfection (Table I). To obtain a progestin-responsive endometrial epithelial-like cell line the parental RENTRO-1 cells were transfected with an expression vector for the rabbit PR (23). Several clones, including RENTROP, were shown to express a functional PR, as demonstrated in transient transfection experiments using the progesterone inducible reporter plasmid pGRE 5- tk- Luc (Table I). The level of induction with the synthetic progestin R5020 in RENTROP cells was comparable to values obtained in other PR expressing cell lines, as for example, mammary carcinoma-derived T47D (33) or diverse rabbit endometrial cell lines.2 Note that in RENTROP cells the response to dexamethasone was about 5-fold higher than the response to R5020, suggesting a high level of constitutive glucocorticoid receptor expression. Note that the differences in absolute value of glucocorticoid induction between RENTRO-1 and RENTROP cells do not reflect significant differences in glucocorticoid receptor activity between the cell lines, as independently confirmed by another series of reporter transfection experiments (data not shown).

In the absence of hormone, RENTROP cells cultivated in medium supplemented with charcoal-stripped FCS exhibited a morphology which did not differ significantly from that of the parental RENTRO-1 cells. The cells were well attached to the plastic surface, polygonal or elongated in shape, had an enlarged cytoplasm and a regular cell surface. While some cells showed a more elongated spindle-like appearance and exhibited dendritic protrusions, other cells rounded up and became only weakly attached to the plastic surface (Fig. 1, right panel). In addition, some enlarged cells appeared exhibiting a larger cell nucleus (see also ultrastructure, Fig. 3). Concomitant treatment with the antiprogestin RU486 antagonized the morphological changes induced by progestins. Interestingly a similar morphological change was observed in response to the synthetic glucocorticoid dexamethasone both in RENTROP cells as well as the parental RENTRO-1 cell line (data not shown).

Hormone Withdrawal Induces Apoptosis—The growth be-

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2 A. Scholz and M. Beato, unpublished data.
Behavior of RENTROP cells was influenced by the concentration of CS-FCS and by the presence of steroid hormones in the medium. At 10% CS-FCS the cell number was increased by addition of the synthetic progestin R5020 (Fig. 2, upper panel) or the synthetic glucocorticoid dexamethasone and this effect was reverted by the antagonist RU486 (data not shown). In the presence of 1% CS-FCS and under the influence of glucocorticoids or progestins, RENTROP cells maintained their number in culture. In the absence of hormone or in the presence of antiprogestional RU486 the cell number decreased due to a loss of dying cells (Fig. 2, middle panel). At 0.1% CS-FCS even addition of hormones was not sufficient to prevent cell death (Fig. 2, lower panel).

An electron microscopical analysis of cells cultivated at low serum in the absence of progestins or glucocorticoids (Fig. 3A), or in the presence of the antihormone RU486 (Fig. 3B), demonstrated apoptotic cells with condensed cell nucleus, shrunk cytoplasm, but with intact organelles. Furthermore, the cells exhibited membrane blebs at the cell surface, a characteristic feature of apoptotically dying cells. In the presence of either R5020 (Fig. 3C) or dexamethasone (data not shown), the cells showed an even surface, and some of them were enlarged and exhibited an extensively developed ergastoplasm, indicative of secretory cells (see higher magnification in Fig. 3D).

**FIG. 3.** Morphology of RENTROP cells in electron micrographs. A, control apoptotic cells grown in medium with 2.5% CS-FCS in the absence of added hormone for 24 h and further incubated in the absence or presence of the indicated hormones for 24 h. Note membrane blebs on the cell surface and intact membrane structures of organelles embedded in shrunk cytoplasm. B, apoptotic cells grown in the presence of RU486 for 24 h showing a morphology similar to the cells depicted in A. The cytoplasm is similarly shrunk and exhibits blebs on the surface. Left arrow indicates debris of a dead cell; right arrow points to a part of a nucleus of a dead cell with compact cytoplasm and condensed chromatin. C, representative cells cultivated in the presence of R5020 for 24 h. The magnification is the same as in A and B to emphasize the difference in size. Note the smooth cell surface with protrusions of microvilli-like structures. The lower cell is especially rich in metabolic organelles such as mitochondria and Golgi apparatus. D, details of the cytoplasm of the cell shown in C demonstrating the abundance of organelles characteristic of secretory cells. Bars at the bottom correspond to 5 μm; the left bar indicates the magnification used for A, B, and C, and the right bar applies to D.

**FIG. 4.** Identification of apoptotic RENTROP cells by fluorescence labeling of nicked DNA in situ. Upper left panel, cells grown without hormones; upper right panel, cells grown in the presence of R5020; lower left panel, R5020 and dexamethasone, 100 nM; lower right panel, R5020 antagonized with RU486.

**FIG. 5.** Analysis of DNA degradation. A, RENTROP cells were cultured in 10% CS-FCS for 9 h before the medium was changed to 1% CS-FCS and the cells were further incubated for 0 h (lanes 1 and 4), 12 h (lanes 2 and 5), or 24 h (lanes 3 and 6). Fragmented DNA was extracted and analyzed by electrophoresis in a 1.5% agarose gel (lanes 1–3). The residual nuclear DNA was also analyzed as a control of recovery (lanes 4–6). The amount of DNA loaded in each lane corresponds to 20% (solubilized fraction) and 10% (residual nuclear fraction) of total DNA. B, RENTROP cells were cultured in 10% CS-FCS for 9 h before changing to 1% CS-FCS in the absence of hormone (lane 2, EtOH) or in the presence of: dexamethasone (lane 3), RU486 (lane 4) or R5020 (lane 5). After 24 h total DNA was extracted and 5 μg were analyzed by electrophoresis in a 1.5% agarose gel. The gel was stained with ethidium bromide and photographed (inset). The negative was scanned with a densitometer and the density plotted against the migration distance in centimeters. The area corresponding to DNA fragments smaller than 2000 bp was quantified. A scan of the 100-bp ladder marker DNA (lane 1) is shown at the bottom.
Progesterone, Bcl-X Splicing, and Endometrial Apoptosis

The fraction of RENTROP cells that undergo apoptosis when cultivated under serum starvation (1% CS-FCS) was determined after incubation with various hormones (Fig. 5B). The numbers obtained from the densitometric scans confirmed the values found with the cytochemical assay: both dexamethasone and RU486 reduced the proportion of fragmented DNA as observed in the absence of hormone or in the presence of RU486.

We conclude that the effect of RU486 on the cell population at low serum as detected in the growth kinetics of RENTROP cells (Fig. 2), is due to a protection of cells otherwise undergoing programmed cell death.

Hormonal Effects on bcl-X Transcripts—To analyze the molecular mechanism by which hormones prevent apoptosis of RENTROP cells we first measured their effects on the expression of bcl-2 family members which are known to control apoptosis in many other cell types. Using RNase protection assays we could not detect expression of the two bcl-2 transcripts, which should correspond to the rat homologues of the human α and β isoforms of bcl-2 mRNA (35) (Fig. 6A). We conclude that Bcl-2 does not play an essential role in hormonal regulation of apoptosis in RENTROP cells.

Next we measured the expression of bax, as in many cells Bax promotes apoptosis by opposing the action of Bcl-2 (36). In total RNA from RENTROP cells bax was detectable by Northern blot analysis. After correction for RNA loading, based on quantitative estimation of 28 S ribosomal RNA from densitometric scans and confirmed by quantitation of the gapdh signal, no change in the expression level was detected after various hormonal treatments (Fig. 6B and Table II). Western blots performed with anti-Bax antibodies did not detect changes in the levels of the Bax protein following hormone treatment (Fig. 6C). These results suggest that the ratio of Bcl-2 to Bax is not likely involved in mediating apoptosis following hormone withdrawal.

An alternative possibility to control apoptosis would be to influence the ratio of the two Bcl-X isoforms. Whereas the large isoform, Bcl-XL, which contains all regions of homology to Bcl-2, protects against several forms of apoptosis, the short isoform, Bcl-XS, which lacks two of the homology regions, BH1 and BH2, promotes apoptosis by counteracting Bcl-2 and Bcl-XL function (36). Therefore, the ratio between the two isoforms could decide whether a given stimulus will cause apoptosis. In RNA from thymus and RENTROP cells both forms of bcl-X mRNA were detected by RNase mapping (Fig. 7). This result was also confirmed by semi-quantitative reverse transcriptase-polymerase chain reaction (data not shown).

Both in thymus and in RENTROP cells, transcripts of the large isoform were considerably more abundant than tran-
scripts of the short isoform (Fig. 7A, lanes 2 and 3). Following hormonal treatment, transcripts for both isoforms accumulated rapidly, the effect of dexamethasone being more pronounced than that of R5020, but the proportion of bcl-XL mRNA increased more markedly (Fig. 7A, lanes 8–11). As a consequence the ratio of short to large isoforms decreased by 2-fold, from 0.08 to 0.04, following treatment with either glucocorticoids or progestins (Fig. 7B, lower panel). The hormonal effects on the total level of bcl-X transcripts and on the ratio of both isoforms was almost maximal after 2 h of hormone treatment (Fig. 7B). Both effects were inhibited by the antihormone RU486 which probably not essential for this hormonal effect. 

The appearance of apoptosis in epithelial cells rapidly, the effect of dexamethasone being more pronounced than that of R5020, but the effects on bcl-X transcripts were similar. Cycloheximide, at concentrations which blocked protein synthesis by 85% (the incorporation of [35S]methionine without ethanol. 

To test whether the hormonal influence on the levels of the bcl-X transcripts are mediated by induction of an intermediary factor, we investigated the influence of inhibitors of protein synthesis. For these experiments, progestosterone was used instead of R5020, but the effects on bcl-X transcripts were similar. Cycloheximide, at concentrations which blocked protein synthesis by 85% (the incorporation of [35S]methionine without cycloheximide was 7.26 × 10^3 ± 0.6 × 10^3 dpm/well, and in the presence of 100 ng/ml cycloheximide, 1.5 × 10^3 ± 0.5 × 10^3 dpm/well) did not influence the levels of bcl-X transcripts nor the ratio of short to large isoforms (Fig. 8). As in the previous experiments, glucocorticoids and progestins increased the level of transcripts of the bcl-X gene by 2–3-fold and this effect was also prevented by treatment with cycloheximide (Fig. 8B, top panel). The hormonally induced decrease in the ratio of bcl-XS to bcl-XL was also not prevented by treatment with cycloheximide (Fig. 8B, bottom panel) demonstrating that ongoing protein synthesis is probably not essential for this hormonal effect.

**DISCUSSION**

**RENTROP Cells Reproduce the Apoptosis Response of Endometrial Tissue**—The appearance of apoptosis in epithelial cells of the endometrium following ovariectomy or treatment with the antiprogestin RU486 has been described in pseudopregnant rabbits (5, 6) and in primary cell culture (7, 8). Here we describe for the first time a hormone-dependent endometrial cell line. These rat endometrial cells, called RENTROP, as well as the parental RENTRO-1 cells, undergo spontaneous apoptosis by an intrinsic default mechanism when cultured under limiting concentrations of stripped serum, probably due to the lack of growth factors. The identification of apoptotic cells was based on light and electron microscopic morphology as well as on biochemical evidence for internucleosomal DNA degradation. In the presence of physiological concentrations of steroid hormones, namely glucocorticoids and progestins, apoptosis is prevented and the cell number is maintained, both effects being reversed by the antagonistic ligand RU486. Whereas the parental RENTRO-1 cell line lacks PR and only responds to dexamethasone, RENTROP cells express constitutively PR and respond to progestins as well. The behavior of RENTROP cells does not seem to represent a clonal exception as suggested by the observation of other independent cell clones derived from RENTRO-1 by stable transfection with PR which similarly respond to progestins. Moreover, a rabbit endometrial cell line, RBE7 (37), when transfected stably with an expression vector for the progesterone receptor, similarly exhibits apoptotic cell response to progestins and dexamethasone.

**Table III**

| Cell type | Treatment | bcl-X mRNA | bcl-XS/bcl-XL |
|-----------|-----------|------------|---------------|
| RENTROP   | Ethanol   | 1          | 1             |
|           | R5020     | 2.06 ± 0.30| 0.30 ± 0.06 (3)|
|           | Progesterone | 2.19 ± 0.20| 0.54 ± 0.15 (3)|
|           | Dexamethasone | 3.24 ± 0.37| 0.34 ± 0.12 (4)|
|           | RU486 | 1.07 ± 0.12| 1.64 ± 0.45 (3)|
|           | Progesterone + RU486 | 1.34 | 0.89 (1) |
|           | Dexamethasone + RU486 | 1.03 | 0.79 (1) |
| RENTRO-1  | Ethanol   | 1          | 1             |
|           | R5020     | 1.06 ± 0.06| 1.14 ± 0.41 (2)|
|           | Dexamethasone | 0.80 ± 0.14| 0.35 ± 0.11 (2)|

**DISCUSSION**

**RENTROP Cells Reproduce the Apoptosis Response of Endometrial Tissue**—The appearance of apoptosis in epithelial cells of the endometrium following ovariectomy or treatment with

**Effect of hormones on bcl-X transcripts in RENTROP and parental RENTRO-1 cells**

The total amount of bcl-X mRNA and the ratio of short to long isoform is given relative to the value obtained with solvent control, i.e. ethanol. Values of repeated experiments are indicated as the mean and standard deviation. The number of experiments is indicated in parentheses (n).
death in response to progesterone withdrawal.\(^3\)

The effects of progestins and glucocorticoids are additive in RENTROP cells, probably reflecting the fact that the cellular concentration of each hormone receptor is limiting\(^3\) (38). When comparing the two hormones, the effect of dexamethasone is more pronounced in agreement with a higher concentration of glucocorticoid receptor, as demonstrated in transient transfection assays with a hormone responsive reporter (Table 1). Even in the presence of saturating amounts of progestins, glucocorticoids, or both hormones, a small fraction of RENTROP cells still undergoes apoptosis when cultured under conditions of serum starvation. Therefore, either the receptor concentration is still limiting, or the steroid hormones cannot completely replace other survival factors present in serum. The availability of the cell culture system described here should facilitate the identification of these factors.

**Molecular Mechanism of Hormonal Regulation of Endometrial Apoptosis**—Bcl-2 is known to prevent apoptosis induced by a wide range of agents, suggesting that multiple pathways to cell death converge in a step that can be regulated by Bcl-2. Bcl-2 is mainly found in cell populations with a long life and/or proliferating ability, or in cells which undergo a transition from non-transcriptional effect on the activity of one or more components of the pathway leading to bcl-X transcription and processing.

The effects of progestins on bcl-X transcript levels are likely to be direct as they are rapid and cannot be affected by an inhibitor of protein synthesis such as cycloheximide. However, the concentration of cycloheximide tolerated by these cells as the concentration of cycloheximide affected by an inhibitor of protein synthesis such as cycloheximide. However, the concentration of cycloheximide tolerated by these cells is still limiting, or the steroid hormones cannot completely replace other survival factors present in serum. The availability of the cell culture system described here should facilitate the identification of these factors.

Another candidate for the regulation of apoptosis is Bax, a 21-kDa protein which can associate with Bcl-2 to which it exhibits extensive homology over the two conserved regions. Overexpression of Bax accelerates apoptotic death induced by cytokine deprivation, and counteracts Bcl-2 (10). However, we could not find indications for hormonal control of bax mRNA or Bcl-X in endometrial cells.

Bcl-X is also related to Bcl-2, and immunoreactivity against Bcl-X has been detected in a wide variety of cells (42). Among them were a variety of cells in the reproductive organs, including the mammary epithelium, the secretory epithelial and basal cells of the prostate, and the secretory cells of the uterine endometrium. In many cases, the patterns of bcl-X expression are strikingly different from those reported previously for bcl-2, suggesting that these two genes regulate cell survival at different stages of cell differentiation or in different cells (42).

Alternative splicing generates two isoforms of Bcl-X: a long form, Bcl-X\(_L\), similar in size to Bcl-2, which inhibits cell death (43–46), and a short form, Bcl-X\(_S\), that inhibits Bcl-2 function and promotes apoptosis (47). The latter form is highly expressed in proliferating cells, whereas the long form is found in long-living cells, such as brain neurons (11). However, Bcl-2 and Bcl-X may not be redundant, since in a B cell line Bcl-X\(_L\) blocked apoptosis induced by immunosuppressants whereas Bcl-2 was ineffective (48).

Our RNase mapping results indicate that in the presence of 1% serum, hormonal treatment with dexamethasone or R5020 for 2 h increases bcl-X expression 3- and 2-fold, respectively. The levels of expression of the short isoform are very low compared with those of the long isoform. The ratio bcl-X\(_S\)/bcl-X\(_L\) is 0.08 in cells cultivated with 10% CS-FCS and it is not affected when the cells are grown at low serum concentration. However, analysis of the ratio bcl-X\(_S\)/bcl-X\(_L\) after hormone treatment shows that it decreases significantly, from 0.08 in the control cells to 0.03 and 0.05 in cells treated with dexamethasone and R5020, respectively. The presence of the anti-hormone RU486 does not affect the total level of bcl-X message, but it increases the bcl-X\(_S\)/bcl-X\(_L\) ratio from 0.08 to 0.18. RU486 can antagonize completely both hormone effects: the quantity of total bcl-X message and the bcl-X\(_S\)/bcl-X\(_L\) ratio, demonstrating the specific action of the hormone.

The effects of progestins on bcl-X transcript levels are likely to be direct as they are rapid and cannot be affected by an inhibitor of protein synthesis such as cycloheximide. However, as the concentration of cycloheximide tolerated by these cells only blocks 85% of the total protein synthesis, we cannot exclude that the residual protein synthesis is sufficient to produce a protein involved in bcl-X transcription and/or splicing. It is also possible that the hormonal effect is mediated by a non-transcriptional effect on the activity of one or more components of the pathway leading to bcl-X transcription and processing.

There are indications for a direct effect of steroid hormones on the expression of the bcl-X gene in other cell types. For instance, in untreated myeloid cells bcl-X\(_S\) transcripts could not be detected, but bcl-X\(_L\) was up-regulated upon dexamethasone treatment (40). This situation is similar to that found in endometrial cells in which the bcl-X\(_S\) mRNA is hardly detect-
able, and the hormonal treatment leads to accumulation of \( bcl-X_L \) transcripts. A final elucidation of the molecular mechanisms underlying these hormonal effects awaits the cloning and functional analysis of the \( bcl-X \) promoter and the characterization of the regulated splicing of \( bcl-X \) transcripts. Unfortunately, \( bcl-X \) deficient mice will not be useful for elucidating the role of \( bcl-X \) in the endometrium, as the homozygous \( bcl-X^{-/-} \) mice die around embryonic day 13 due to extensive postmitotic neuronal death, apoptosis in the hematopoietic cells of the liver, and defective maturation of lymphocytes (49).

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