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# Creatine Kinase Activity as an Indicator of Unopposed Estrogen Action in the Mouse Uterus Associated with Anti-progesterone Treatment

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**The brain isozyme of creatine kinase (CKB) is a major component of the estrogen-induced proteins in the rat uterus. Hormonal specificity of this response was studied in cotransfection assays using the rat CKB promoter linked to the bacterial chloramphenicol acetyltransferase gene. Response was specific for estrogen as 17β-estradiol in the presence of estrogen receptor dramatically stimulated the CKB promoter. This induction was completely blocked by the estrogen antagonist ICI 164,384. Nuclear receptors for progesterone, androgen, glucocorticoid and vitamin D did not significantly activate the CKB promoter in the presence of their respective ligands. Creatine kinase (CK) activity was analyzed in decidualized mouse uterus to assess estrogenic activity *in vivo*. Upon oil stimulation, uterine horns of day 4 pseudopregnant mice underwent a dramatic outgrowth in response to endogenous progesterone. This response was accompanied by a significant decrease in CK activity from a control value of 1.44 ± 0.25 to 0.38 ± 0.08 IU/mg protein ($P < 0.001$), indicating that the action of estrogen was suppressed. Treatment of females one day prior to oil-stimulation with progesterone receptor antagonists, RU486 (Mifepristone) or ZK299 (Onapristone), or with a monoclonal antibody to progesterone (DB3), abolished decidualization, and also restored the CK activity to the control value. These results suggest that CK can be used as a specific cellular marker to detect unopposed estrogen action in the mouse uterus associated with progesterone withdrawal or receptor blockade.**

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## INTRODUCTION

Creatine kinase (CK) is a ubiquitous enzyme that catalyzes the reversible transfer of a phosphate moiety between adenosine triphosphate and creatine [1]. The brain isoform of CK (CKB) is one of the major induced proteins (IP) found in the rat uterus following estrogen treatment [2]. It follows that CKB may be useful as a sensitive marker for hormonal responsiveness because its abundance in the uterus, at both mRNA and protein levels, is rapidly detected following estrogen administration into immature rats [3–7]. Contrary to data generated in rats, the specific activity of human CKB in normal, hyperplastic and neoplastic endometrium has been found to be regulated by progesterone rather than estrogen [8, 9].

It is known that progesterone specific antibodies and progesterone receptor antagonists block progesterone-mediated functions such as implantation and decidualization in a variety of mammalian species [10–13]. While inhibition of estrogen-induced endometrial proliferation was observed in RU486-treated monkeys [14, 15], unopposed estrogen action, manifested by enhanced epithelial mitotic activity at day 3 of pregnancy, has been reported in the mouse uterus after passive immunization against progesterone [16]. Furthermore, RU486 has been shown to reverse progesterone-mediated inhibition of estrogen-inducible glycoprotein (USP-1) synthesis and secretion in rat uterine epithelial cells [17]. This paradox may relate specifically to the species examined or to differences in

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the way each of these blockades affect progesterone action [18].

The above cited studies, however, did not address two fundamental questions. The first concerns the hormonal specificity of CKB gene activation. To assess whether hormones other than estrogen have any direct effects on the rat CKB gene promoter, cotransfection assays were performed with various nuclear receptors, and their activity on the CKB promoter in response to cognate ligands was measured. To further clarify whether there exists a direct interaction between estrogen and progesterone receptors upon activation, both receptors were coexpressed in HeLa cells and the influence of liganded progesterone receptor on CKB promoter was examined. The second question relates to the relationship between activity of estrogen-induced uterine proteins (e.g. CKB) and inhibition of progesterone actions which results from ligand withdrawal or receptor blockade. Because decidual cell reaction of the rodent uterus is a classic response to progesterone after priming by estrous estrogen [19], progesterone antagonists should, in theory, inhibit or interrupt this process when given *in vivo*. In the studies presented here, we employed a mouse decidualization model to examine the effects of a monoclonal antibody to progesterone (DB3) and two anti-progestins (RU486 and ZK299) on endometrial sensitivity. CK activities were measured in uterine tissues of these animals with the aim of utilizing CK as a cellular marker to assess the status of estrogen action during progesterone withdrawal or receptor blockade.

## EXPERIMENTAL

### *Construction of plasmids*

The estrogen receptor expression plasmid pKCR2-ER has been described previously by Green *et al.* [20], and the expression vector pRS and the plasmid pRShGR containing the cDNA for the human glucocorticoid receptor by Giguère *et al.* [21]. The pRShAR and pRShVDR plasmids were constructed by cloning the cDNAs for the human androgen receptor [22] and the human vitamin D receptor [23] into the BamH1 site of the PRS vector, respectively. The human progesterone receptor (B form) cDNA [24] was cloned into the pRST7 vector [25]. The reporter plasmid pLPwtCAT was made by subcloning a 2.9 kb fragment of the rat CKB gene promoter from LPwt [26] into pUCPLCAT [27].

### *Gene transfer*

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) without phenol red, supplemented with 10% fetal bovine serum. Cells were plated at a density of $5 \times 10^5$ cells per 100 mm plate and transfected 24 h later using the method of calcium phosphate precipitation [28]. To correct for differences in transfection and harvesting efficiency, 2.0 $\mu$g of the $\beta$-galactosidase expression plasmid pCH110 (Pharmacia Biotech Inc., Piscataway, NJ) was cotransfected with 1.5 $\mu$g of the reporter plasmid (pLPwtCAT). Various amounts of expression plasmids for the receptors were used, i.e. 1.0 $\mu$g of pKCR2-ER, 0.5 $\mu$g of pRST7hPRB, 0.1 $\mu$g of pRShAR, 5.0 $\mu$g of pRShGR, or 5.0 $\mu$g of pRShVDR. Total amounts of DNA were standardized to 20 $\mu$g using Bluescript PSK+ (Stratagene Cloning Systems, La Jolla, CA). Fourteen to 16 h post-transfection, the medium was changed and various ligands at concentrations of $10^{-7}$ M, or vehicle alone (ethanol) were added. Cells were harvested 24 h later and extracts prepared as described previously [29]. CAT assays [30, 31] were performed and $\beta$-galactosidase activity was measured [32]. To increase sensitivity, chlorophenol red $\beta$-galactoside (CPRG; Boehringer Mannheim Biochemicals, Indianapolis, IN) was used as substrate for the $\beta$-galactosidase assay [33]. CAT activity was normalized by expressing percent conversion of chloramphenicol into acetylated chloramphenicol divided by the $\beta$-galactosidase activity. Fold stimulation is the ratio of CAT activity in the presence of hormone to that without hormone.

### *Reagents*

Progesterone, 17$\beta$-estradiol, dihydrotestosterone, dexamethasone (Sigma Chemical Co., St Louis, MO), 1,25$(OH)_2D_3$ (Biomol Research Labs Inc., Plymouth Meeting, PA), and ICI 164,384 (gift from A. E. Wakeling, ICI Pharmaceuticals, Cheshire, England) were dissolved in ethanol and diluted in tissue culture medium to appropriate concentrations before use. RU486 (Mifepristone; gift from Roussel-Uclaf, Romainville, France) and ZK299 (Onapristone; gift from D. Henderson, Schering AG Pharmaceutical Research, Berlin, Germany) were dissolved in sesame oil and administered subcutaneously (s.c.) at a dose of 0.1 mg/0.1 ml. Monoclonal antibody ($IgG_1$) directed against progesterone (DB3) was prepared in BALB/c mice and purified as reported previously [34]. DB3 (9.0 nmol or 1.35 mg) was injected intraperitoneally (i.p.) in 1.0 ml phosphate-buffered saline (PBS).

### *Animals*

Mature virgin BALB/c mice (10–12 weeks old; Harlan Sprague Dawley Inc., Indianapolis, IN) were housed in a light (14 h light:10 h darkness; lights off at 20.00 h) and temperature (22°C) controlled room, and fed and watered *ad libitum* (Diet LM-485, Teklad, Madison, WI). Females were caged with vasectomized males of the same strain between 17.00 and 10.00 h and pseudopregnancy was dated from the morning when a vaginal plug was detected (day 1). Mating was presumed to have taken place at 02.00 (time 0) [35].

*Decidualization*

Pseudopregnant mice were treated with 0.1 mg RU486 (s.c.), 0.1 mg ZK299 (s.c.), or 9.0 nmol (1.35 mg) DB3 (i.p.) at 10.00 h on day 3 of pseudopregnancy. Control animals received (s.c.) an equivalent volume of oil. On day 4 (16.00 h), 10 $\mu$l of sesame oil was injected intraluminally into the right uterine horn (stimulated) and the left horn was left undisturbed to serve as an internal control (non-stimulated). At necropsy 72 h after application of the decidual stimulus, the uterine horns were removed, trimmed, blotted and weighed. Decidual response to trauma at the injection site was easily distinguished from the response to oil which stimulated deciduomata along the length of the uterus. Uterine wet weight gain was calculated by subtracting the weight of the non-stimulated horn from that of the stimulated horn. The horns were then frozen at $-70$°C in freezing buffer containing glycerol (20 g/l), aprotinin (0.85 IU/l; Sigma) and NaCl (2.92 g/l).

*CK assay*

Uterine samples were thawed on ice and homogenized in 1.0 ml of the assay buffer (1:10 freezing buffer in bis–tris electrophoresis solution). Homogenate was centrifuged at 45,000 rpm for 75 min at 4°C. The supernatant was collected and diluted 1:10 with the assay buffer for CK analysis using the quantitative kinetic kit from Sigma (47-UV) on a Uvikon spectrophotometer at 340 nm. CK values measured in international units (IU) per ml were then divided by protein concentrations [36] of the same preparation to provide CK activity as IU per mg protein. Intra- and interassay coefficients of variation were 2.3 and 7.5%, respectively.

*Numerical methods*

Pairwise statistical analyses were performed using Student's $t$-test. Unless stated otherwise, all results are reported as mean $\pm$ standard error of the mean and $P < 0.05$ is used as the level of significance.

## RESULTS

*Hormonal specificity of the CKB gene activation*

In order to determine the hormonal specificity of the CKB promoter response, HeLa cells were cotransfected with different intracellular receptor expression plasmids and a CKB reporter construct. These cells do not contain endogenous receptors for estrogen, progesterone, androgen or vitamin D, but do contain low levels of the glucocorticoid receptor. Various titration experiments were carried out to decide optimal conditions for each receptor construct. Therefore, the amount of expression plasmids (ranging from 0.1 to 5.0 $\mu$g) used in the transfections represents the maximum level of ligand-dependant activity demonstrated by each receptor on their respective responsive elements. As shown in Fig. 1, only the estrogen receptor was able to activate the CKB promoter in a hormone-dependent manner: the level of stimulation was approx. 100-fold higher in the presence of 17$\beta$-estradiol than in its absence. Progesterone and androgen (dihydrotestosterone) in conjunction with their receptors had little effect on the CKB promoter, whereas liganded glucocorticoid and vitamin D receptors elicited very weak activation (1.6- and 1.7-fold increase, respectively). The CKB activation by estrogen and its receptor was completely blocked by the estrogen antagonist ICI 164,384, which itself had no agonist effect (Fig. 2).

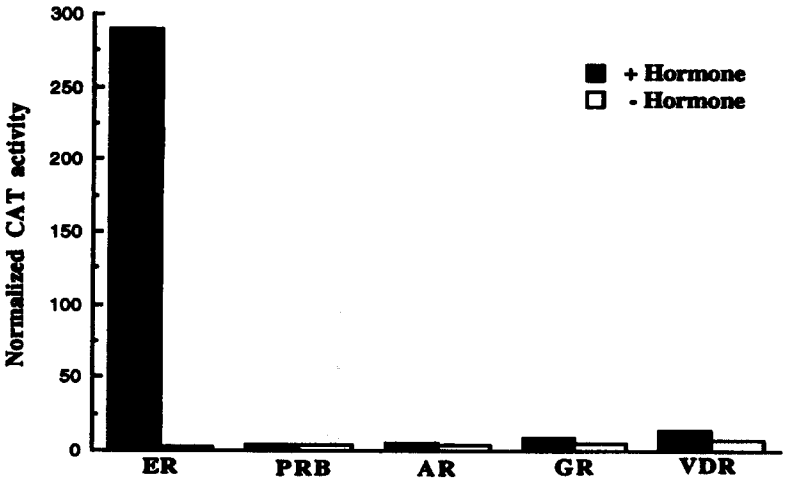


**Fig. 1. Activation of the CKB promoter by various receptors and their ligands. HeLa cells were transfected with different expression plasmids as described in Experimental. CKB gene activities were determined via CAT and $\beta$-gal assays. 17$\beta$-estradiol, progesterone, dihydrotestosterone, dexamethasone, or 1,25(OH)$_2$D$_3$, at final concentrations of $10^{-7}$ M, were added to cells transfected with the corresponding nuclear receptor expression plasmid (shown as ER, PRB, AR, GR, or VDR), respectively. Control plates received an equivalent amount of vehicle (ethanol). ER, estrogen receptor; PRB, progesterone receptor B form; AR, androgen receptor; GR, glucocorticoid receptor; VDR, vitamin D receptor.**

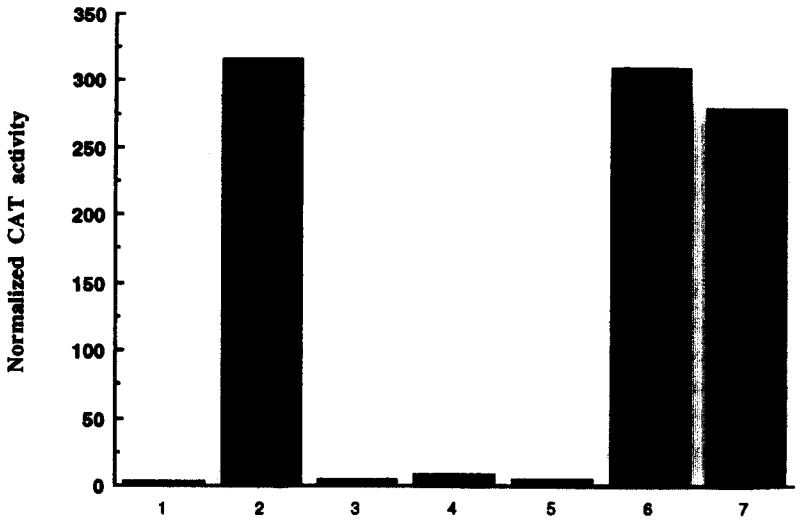


**Fig. 2. Specific activation of the CKB promoter by estrogen. HeLa cells were transfected with different nuclear receptor expression plasmids as described in Experimental. 17β-estradiol ($10^{-8}$ M), progesterone ($10^{-7}$ M), ICI 164,384 ($10^{-6}$ M) or RU486 ($10^{-6}$ M) were added to cells transfected with ER and/or PR expression constructs. Control plates received an equivalent amount of vehicle (ethanol). CKB gene activities were determined via CAT and β-gal assays. 1, ER alone; 2, ER plus 17β-estradiol; 3, ER plus ICI-164,384; 4, ER plus 17β-estradiol and ICI-164,384; 5, PRB plus progesterone; 6, ER and PRB plus 17β-estradiol and progesterone; 7, ER and PRB plus 17β-estradiol, progesterone and RU486.**

These results suggest that the hormonal specificity of the rat CKB gene is restricted to estrogen.

We further studied the possible role of progesterone receptor (such as direct competition at the CKB promoter) on estrogen-dependent CK activation. When transfecting estrogen and progesterone receptors simultaneously, neither progesterone nor its antagonist RU486 demonstrated any inhibitory or stimulatory effects on estrogen-induced CKB promoter activation (Fig. 2). This implies that in this assay system liganded progesterone receptors do not influence the activity of estrogen receptor.

*Effects of anti-progesterone treatment on decidualization*

As shown in Fig. 3 and Table 1, stimulated uterine horns displayed a dramatic increase in wet weight (over 10-fold) compared to non-stimulated horns ($P < 0.001$). Administration of DB3 or RU486 prior to oil stimulation completely blocked decidualization

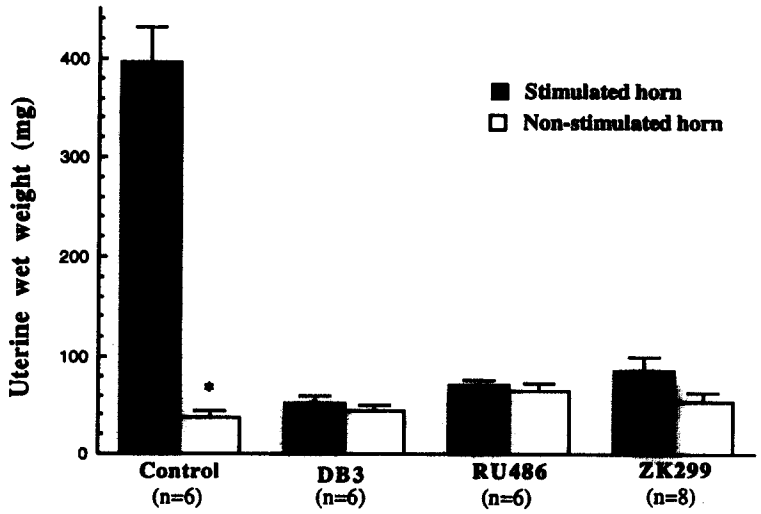


**Fig. 3. Effects of anti-progesterone treatment on oil-induced uterine decidualization. Female mice were injected with either progesterone antagonists (RU486 or ZK299; 0.1 mg s.c.) or monoclonal antibody to progesterone (DB3; 1.35 mg i.p.) on day 3 of pseudopregnancy. Control animals received 0.1 ml sesame oil (s.c.). On day 4, a small amount (10 μl) of oil was administered intraluminally to the right uterine horn (stimulated), while the left horn (non-stimulated) was undisturbed. At necropsy on day 7, the uterine horns were removed, trimmed, blotted and weighed. The asterisk represents a value (mean ± SEM) statistically different from that of the stimulated horn ($P < 0.001$).**

*Table 1. Effects of DB3, RU486 and ZK299 on oil-induced deciduomata formation*

| Treatment | No. of mice | Net uterine weight gain (mg) | % Inhibition | Ovary weight (mg) |
|---|---|---|---|---|
| Control | 6 | 359.2 ± 33.9 | 0 | 3.9 ± 0.3 |
| DB3 | 6 | 7.1 ± 2.2* | 98.0 | 4.5 ± 0.4 |
| RU486 | 6 | 5.7 ± 3.3* | 98.4 | 4.0 ± 0.2 |
| ZK299 | 8 | 32.5 ± 16.6* | 91.0 | 4.2 ± 0.2 |

Pseudopregnant BALB/c mice were treated with either progesterone antagonists (RU486 or ZK299; 0.1 mg s.c.) or monoclonal antibody to progesterone (DB3; 1.35 mg i.p.) on day 3 after mating. Control animals received 0.1 ml sesame oil (s.c.). Approximately 30 h after treatment, 10 μl of oil was injected into the lumen of the right uterine horn, and the opposite horn was left undisturbed to serve as an internal control. Necropsies were performed about 72 h following oil stimulation. Net uterine weight gain was calculated by subtracting the weight of the left horn from that of the right horn. Percent inhibition was estimated using the following formula:

$$\% \text{ Inhibition} = 100 - \frac{\text{Weight gain in treated group}}{\text{Weight gain in control group}} \times 100$$

*Values (mean ± SEM) differ significantly from the control group ($P < 0.001$).

($P < 0.001$) whereas treatment with ZK299 gave about 91% inhibition ($P < 0.001$).

*Effects of anti-progesterone treatment on uterine CK activity*

Figure 4 shows that in vehicle-treated animals the CK activity of stimulated uterine horns was about four times lower than that of the control horns (0.38 ± 0.03 vs 1.44 ± 0.10 IU/mg protein; $P < 0.001$); the difference between decidualized and control horns was abolished following treatment with DB3, RU486, or ZK299 ($P > 0.2$), and the levels of CK activity in both uterine horns were comparable to that measured in the non-stimulated horns of control females ($P > 0.1$).

## DISCUSSION

The present studies demonstrate that: (i) the rat CKB gene promoter was specifically stimulated by 17β-estradiol in HeLa cells transfected with the estrogen receptor whereas other nuclear receptors (i.e. progesterone, androgen, glucocorticoid and vitamin D) elicited little if any effect on this promoter; (ii) decidual cell reaction in pseudopregnant mice was inhibited by progesterone receptor antagonists (RU486 and ZK299) and a progesterone specific antibody (DB3) administered 1 day prior to oil stimulation; and (iii) uterine decidualization resulted in a significant reduction in CK activity which was reversed following anti-progesterone treatment.

Pentecost *et al.* [3] have previously reported that a 1.7 kb DNA fragment containing the rat CKB promoter is inducible by estrogen in HeLa cells transfected with the human estrogen receptor. Our studies not only confirm but also extend this observation by examining the effects of four additional hormones in parallel with estrogen in a similar system. The general conclusion reached from our cotransfection experiments, i.e. specific activation of the rat CKB gene promoter by estrogen, is in agreement with previous results obtained in rats, which showed that both CKB mRNA [3, 4] and protein [5–7] levels were greatly elevated upon estrogen stimulation.

During early pregnancy (days 3–6) in mice, there is a steep rise of progesterone concentrations in the circulation which peaks at about 30 ng/ml on day 6,

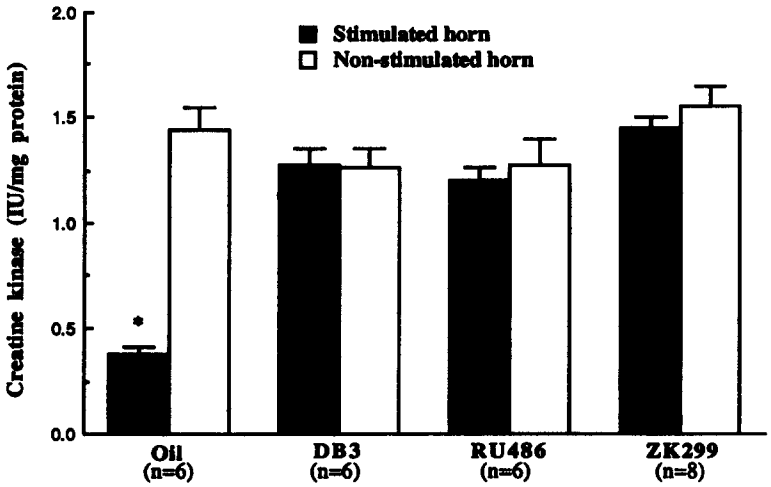


**Fig. 4. Effects of anti-progesterone treatment on CK activity in the uterine tissue. Mature mice were treated with either progesterone antagonists (RU486 or ZK299; 0.1 mg s.c.) or monoclonal antibody to progesterone (DB3; 1.35 mg i.p.) on day 3 of pseudopregnancy. Control females received 0.1 ml oil (s.c.). On day 4, 10 μl of sesame oil was injected into the lumen of the right uterine horn (stimulated), leaving the left horn (non-stimulated) undisturbed. Uterine samples collected at necropsy on day 7 were homogenized and assayed for CK activity (IU/mg protein). The asterisk indicates a value (mean ± SEM) significantly different from that of the non-stimulated horn ($P < 0.001$).**

whereas 17β-estradiol concentrations remain relatively constant during the same period [37]. This endocrine profile coincides with the window during which the uterus is undergoing an important transformation, i.e. decidualization, in order to provide a receptive and supportive environment for blastocyst implantation. Progesterone acts upon the estrogen-primed uterus to facilitate this process [19, 38]. The design of our *in vivo* experiments was therefore to utilize the high concentrations of endogenous progesterone present during early days of pseudopregnancy, and thereby induce uterine decidualization with an artificial stimulus (oil) [12]. Subsequent manipulation of the endocrine status was achieved by perturbing progesterone action with anti-progesterone treatment in an attempt to block oil-induced decidual cell reaction.

It is clear from our results that the progesterone antagonists RU486 and ZK299, and anti-progesterone antibody (DB3) were highly efficacious in blocking deciduomata formation, indicating that the endometrial sensitivity was greatly reduced. The ability of RU486 to prevent uterine responses to various stimuli has also been shown in rats [39], monkeys [40] and ewes [41]. Vinijsanun and Martin [13] have further demonstrated that the inhibitory effects of RU486 and another anti-progestin compound, ZK98734, on mouse uterus were restricted to the actions mediated by progesterone. It would appear that RU486 was slightly more potent than ZK299 in our model, which may have resulted from the higher receptor binding affinity of RU486 (D. Mais, unpublished observation). It should be noted that the mechanisms by which these progesterone antagonists and antibody exert their *in vivo* effects are distinct, although their effects upon decidualization are similar. The antagonists act directly on the progesterone receptor [42] whereas the antibody causes ligand sequestration in the circulation as well as in target tissues, leading to progesterone withdrawal [16].

A number of studies have indicated that the CK level is a reliable index of estrogenic activity in the rat uterus [4–6]. Using a pseudopregnant mouse model, we were able to demonstrate that CK activity was significantly decreased in stimulated vs non-stimulated uterine horns, suggesting that estrogen action was suppressed in the process of decidualization, presumably due to a predominant role of progesterone. Anti-progesterone treatment not only blocked decidual cell reaction but also reversed the progesterone-mediated CK decrease, implying that the estrogen effect on CK is restored, i.e. unopposed estrogen action. It appears that the effects of progesterone and its antagonist RU486 on uterine CK expression are indirect since HeLa cells, simultaneously transfected with progesterone and estrogen receptors and treated with both progesterone and 17β-estradiol, showed no change in stimulation of the CKB promoter. Furthermore, addition of RU486 into this system did not demonstrate any inhibitory or stimulatory effect.

As previously reported, estrogenic responses were found during anti-progesterone treatment, both in mice and rats, characterized by either morphological or biochemical means [16, 17]. Estrogen-inducible epithelial mitosis was seen only in day 3 pregnant mice treated with DB3 [43], but not in ovariectomized females supplemented with both estrogen and progesterone and treated with RU486 or ZK98734 [13]. However, a slightly different picture was presented in castrated monkeys undergoing hormonal therapy. While RU486 blocked progesterone action and allowed 17β-estradiol to act in a normal fashion in target tissues, it also had anti-proliferative effects that opposed estrogen action on the uterine epithelial and stromal cells [14, 15, 18]. It has been suggested that this anti-estrogenic action is mediated through a non-competitive mechanism [14]. Because the phenomenon was not observed in other species such as mice [13], it would be interesting to investigate whether the anti-proliferative property of RU486 is species-restricted (e.g. primates) and shared by other progesterone antagonists.

In conclusion, estrogen-specific CK activity in rodent uterus appears to be a reliable marker not only for determination of estrogenic activity but also for detection of unopposed estrogen action associated with progesterone antagonism. The approach has certain advantages over the classical histochemical methods as a fast and quantitative method. This may offer an alternative strategy to assess the relative potency of potential progesterone and/or estrogen antagonists *in vivo*.

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